Master's Thesis

FACS-based Phenotype Analysis of White Blood Cells: Interplay with Lynch Syndrome Related Circulating Micro-RNAs

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Lynch Syndrome (LS), caused by mutations in DNA mismatch repair genes, is an inherited cancer predisposition increasing the risk of developing cancer already at young age. Factors affecting the cancer risk in LS are still understudied. Epigenetic factors such as circulating micro-RNAs (cmiRs) – non-coding RNAs that epigenetically regulate gene expression and travel in blood – have been described to affect that risk. It has been demonstrated that cancer free LS carriers have distinct cmiR profiles from healthy individuals but share similarities with colorectal cancer patients. As white blood cells, also referred as leukocytes, participate in cancer prevention it could be possible that these differentially expressed cmiRs affect cancer risk and development through leukocytes. The aims of this thesis were to develop a method for studying miR expression in different leukocyte types and for examining whether miR-15a-5p would regulate gene expression of inflammation promoting and possibly cancer associated molecule interleukin-1β in leukocytes. For the first aim leukocytes were extracted from the human blood of healthy individuals, and different leukocyte types sorted with a flow cytometer. The miRs were then extracted from sorted subpopulations and expression levels of miR-339-5p, let-7e-5p, miR-451a, miR-320a and miR-15a-5p studied with qPCR. Results revealed that leukocytes expressed all of these cmiRs. miR-320a showed significant expression in all leukocyte subtypes. Especially neutrophils showed prominent miR-320a expression suggesting it could have significance in neutrophil functions related regulation. For the second aim, miR-15a-5p was transfected into human leukocyte culture and production of interleukin-1β triggered with lipopolysaccharides. Gene expression levels of interleukin-1β in miR transfected and non-transfected cultures were examined with qPCR, but clear difference was not detected. Results indicated that more repetitions are required for better evaluation. Nevertheless, the designed protocols proved functional for the purposes and this study strengthened the connection between cmiRs and leukocytes indicating importance for further investigations.

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Littunen Henna-Riikka	Valkosolutyyppien solulajitteluun pohjautuva fenotyyppianalyysi: Lynchin syndroomaan liittyvien verenkierron mikro-RNA:iden ja valkosolujen vuorovaikutus
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DNA:n korjausgeenien mutaatiosta aiheutuva Lynchin syndrooma (LS) on perinnöllinen syöpien kehittymiselle altistava sairaus. LS kantajien syöpäriskiin vaikuttavia tekijöitä ei vielä ole kunnolla tutkittu. Epigeneettisten tekijöiden, kuten ei-koodaavien verenkierron mikro-RNA:den (cmiR), jotka säätelevät geenien ilmenemistä, on kuitenkin todettu vaikuttavan syöpäriskiin. Tutkimuksissa on havaittu tiettyjen cmiR:iden ilmenemistasojen poikkeavan LS:n kantajilla verrattuna terveisiin ei-kantajiin. LS kantajien cmiR tasot olivat hyvin samankaltaiset kolorektaalisyöpäpotilailta tutkittujen cmiR tasojen kanssa, mikä viittaa siihen, että löydetyt miR:t voisivat vaikuttaa syövän kehittymiseen tai torjuntaan. Koska valkosolut toimivat syövän torjunnassa, on mahdollista, että nämä LS:ssa poikkeavissa määrin ilmenevät verenkierron miR:t (LS cmiR:t) vaikuttaisivatkin syöpäriskiin valkosolujen kautta. Tämän työn tarkoituksena oli kehittää menetelmä cmiR:iden ilmentymismäärien tutkimiseen erityyppisissä valkosoluissa, ja siten tarkastella ilmentävätkö valkosolut myös LS cmiR:ita. Tätä varten terveiltä ihmisiltä otetuista verinäytteistä eristettiin valkosolut ja ne lajiteltiin tyypeittäin erillisiksi solupopulaatioiksi virtaussytometrilla. Solujen miR:t eristettiin ja qPCR:n avulla tutkittiin sisälsivätkö näytteet seuraavia LS cmiR:ita: miR-339-5p, let-7e-5p, miR-451a, miR-320a ja miR-15a-5p. Tulokset osoittivat, että valkosolut ilmensivät kaikkia edellä mainituista cmiR:ista. miR-320a tasot olivat lisäksi huomattavia eri valkosolutyypeissä, erityisesti neutrofiileissä. Tämä voisi viitata siihen, että miR-320a säätelisi neutrofiilien toimintaa. Lisäksi työssä tutkittiin vaikuttaako miR-15a-5p syövän kehitykseen yhdistetyn ja tulehdusta aiheuttavan viestiaineen, interleukiini-1β:n (IL-1β) säätelyyn. miR-15a-5p:tä siirrettiin viljeltyihin ihmisen valkosoluihin, IL-1β:n tuotto käynnistettiin lisäämällä viljelmiin lipopolysakkaridia ja IL-1β:n geenien ilmentymistasoja valkosoluissa tutkittiin qPCR:lla. Selkeää muutosta IL-1β:n ilmenemisessä miR-15a-5p sisältävissä soluissa verrattuna kyseistä cmiR:ta sisältämättömiin soluihin ei kuitenkaan havaittu. Tutkimus osoitti kehitettyjen menetelmien kuitenkin olevan toimivia samankaltaisiin tutkimustarkoituksiin

osoittaen valksolujen ja LS cmiR:iden välisen yhteyden ja sen tarkemman tutkimisen tärkeyden tulevaisuudessa.

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TERMS AND ABBREVIATIONS

Terms

Term	Definition
Apoptosis	A programmed cell death.
Biomarker	A gene, molecule or other substance that can be utilized for recognizing a disease or other condition.
Circulating micro-RNA	A micro-RNA traveling in the bloodstream.
Cytokines	Small proteins released from cells. They act as signalling molecules between cells especially in inflammation related functions and immune responses.
Epigenetic changes	Alterations in gene regulation caused by non-genetic modifications thus affecting gene expression, but not changing the structure of DNA.
Interleukin	A cytokine secreted by one type of leukocyte but affecting to another type of leukocyte.
Lynch Syndrome	An inherited predisposition causing significantly increased risk for multiple cancers, especially colorectal cancer.
LS cmiRs	Circulating micro-RNAs that are differentially expressed in Lynch syndrome carriers.
Micro-RNA	A non-coding RNA molecule that regulates gene expression.
Microsatellites	Similar simple DNA sequences exhibiting repeatedly.
Microsatellite instability	Abnormal number of adjacent simple repetitive DNA sequences.
Neoplasm	A limited area of tissue consisting of abnormally growing cells, but which has not yet become cancerous.
Oncogene	A gene that enhances tumour development.
Tumour suppressive gene	A gene that suppresses tumour growth by regulating cellular growth.

Abbreviations

Abbreviation	Meaning
cDNA	complementary DNA
cmiR	circulating micro-RNA
CRC	colorectal cancer
DE miR	differentially expressed micro-RNA
FACS	fluorescence activated cell sorting
HPBL	human peripheral blood leukocytes

IL	interleukin
LS	Lynch syndrome
LS cmiR	Lynch syndrome related circulating micro-RNA
LPS	lipopolysaccharide
MHC	major histocompatibility complex
miR	micro-RNA
mRNA	messenger RNA
MMR genes	mismatch repair genes
NK cell	natural killer cell
NKT cell	natural killer T cell
pri-miR	primary micro-RNA
TNF	tumour necrosis factor

1 INTRODUCTION

Lynch syndrome (LS), an inherited predisposition causing a significantly increased risk for multiple cancers, is the most common inherited cause of colorectal cancer (CRC) (Lynch et al. 2015). LS is a result of mutations in DNA mismatch repair (MMR) genes. The disease is dominantly inherited in the germline and these pathogenic gene variants predispose their carriers to malignant tumour types already at in early age, and significantly increase the risk for multiple cancer formation (Lynch et al. 2015). CRC that is one of the most fatal cancers worldwide is also the most common cancer occurring among LS carriers (Olenius et al. 2022, Siegel et al. 2022, Lynch et al. 2015).

The clinically applied guidelines for testing LS conclude that LS is recommended to be tested in individuals with a high incidence of cancers in their family or with known LS carriers in their family when they apply to be tested themselves (Vasen et al. 2013). The guidelines are under constant development and newest ones have widened the older criteria and stated that they are not considered sufficient for identifying all LS cases (Seppälä et al. 2021). The diagnosis of the syndrome can be confirmed by immunohistochemical analysis for tumours or by genetic testing (Peltomäki et al. 2023). Genes, their regulation through epigenetic mechanisms and lifestyle factors can affect the cancer risk of LS carriers (Tiwari et al. 2016, Sievänen et al. 2021). However, suitable biomarkers for evaluating the individual cancer risk on LS carriers do not yet exist. The early detection of cancer in early state is crucial for better survival expectancy and therefore this kind of novel biomarkers are needed for distinguishing the individuals with greater risk of developing tumours.

Circulating micro-RNAs (cmiRs) and leukocytes in blood circulation have been studied and cmiRs considered potential biomarkers for cancers and other diseases. Leukocytes play key roles in immune defence also preventing tumour formation, whereas cmiRs can epigenetically regulate gene expression in cells. cmiRs can cause alterations in gene expression, which may promote, suppress or prevent tumour development. A concrete connection also between LS and cmiRs was discovered in a recent study that found several cmiRs with altered expression in LS carriers (Sievänen et al. 2022). The finding indicated the significance of further studies in the role of cmiRs in cancer prevention as many details in the action mechanisms and secretion of these cmiRs in LS are not yet well understood.

1.1 Lynch Syndrome

The MMR gene mutations causing LS are pathogenic and result defect in the function of these genes, which are responsible of repairing errors in DNA. This kind of impairment affects negatively to genomic stability as it exposes DNA

sequences to changes including insertions, deletions and single base switches leading to a mismatch (Lynch et al. 2015).

Repeat sequences of one, two or three base pairs in DNA are called microsatellites (Vilar and Gruber 2010). Microsatellite instability (MSI) referring to slight change in the number of microsatellites between a template DNA and newly synthetized DNA strand can be considered one of the hallmark features of LS (Geiersbach & Samowitz 2011, Tanakaya 2019). It is also one hallmark of cancer (Hanahan and Weinberg 2011).

The following genes have been recognized as LS related MMR genes: MLH1, MSH2, MSH6, and PMS2, and pathogenic mutations can occur in any of them (Møller et al. 2018). Additionally, a deletion mutation in epithelial cellular adhesion molecule (EPCAM) gene is linked to LS (Dominguez-Valentin et al. 2020). EPCAM is responsible of MSH2 promoter methylation, which plays crucial role in genetic regulation processes such as DNA repair and gene expression (Ligtenberg et al. 2013, Menezo et al. 2020). According to Win and colleagues (2017) the prevalence of PMS2 is the most common (1 in 714) and MSH6 second most common (1 in 758). In comparison, MLH1 and MSH2 were discovered to be clearly rarer prevalence being 1 in 1946 and 1 in 2841, respectively. Additionally, cancer risk is different between the mutated MMR genes MLH1 and MSH2 emphasizing much greater risk than the other two (Peltomäki et al. 2023). Besides CRC, including the cancers of colon and rectum, LS increases the risk of cancer in other organs encompassing endometrium, ovaries, stomach, small intestine, bile duct, upper urinary tract, pancreas, prostate and kidneys (Peltomäki et al. 2023).

Seppälä and colleagues (2016) have estimated that there are 10 000 LS carriers in Finland, whereas estimations of the overall prevalence of LS varies between 1 of 250-1000 (Tanakaya 2019). Amsterdam and Bethesda criteria have been used to assess the possibility and need to test LS. However, all the LS carriers do not meet either of these criteria, and there are also non-LS carriers who fall under these guidelines (Muller et al. 2019). New guidelines have been prepared, but the issue is still not completely solved (Seppälä et al. 2021).

Besides MMR gene mutations lifestyle factors and epigenetic regulation has an impact on the cancer risk of LS carriers (Tiwari et al. 2016, Sievänen et al. 2021). Recently, micro-RNAs (miRs) have been suggested as possible biomarkers for several diseases (Kang et al. 2022, Steinberg et al. 2023, Kumar et al. 2023). Differential expression of certain cmiRs between LS carriers and non-LS carriers has been reported and LS carriers cmiR profiles has been shown to match the profiles of CRC patients (Sievänen et al. 2022).

Even though interest in studying miRs has grown in recent decades, there is still a lot to discover. The function of many miRs is still not completely known and there is only a little evidence on the cellular origin of miRs. Whether and how they can inhibit tumour growth or impose carcinogenesis in LS carriers, is also understudied.

1.2 Human leukocytes

Human peripheral blood leukocytes (HPBL) play key roles in immune defence including the elimination of cancer cells, which makes them interesting for research in the context of cancer prevention. Interestingly, leukocyte based methods for detecting LS carriers have been under development in recent years (Gallon et al. 2021).

Human blood consists of erythrocytes (red blood cells), leukocytes (white blood cells), platelets and plasma. Blood composition is described as follows: About 55 % of blood composition is plasma, which has a role in transportation of substances and osmotic regulation, for example. Plasma is mostly water (95 %) but also includes blood electrolytes such as sodium and calcium, plasma proteins and substances like nutrients, hormones and cmiRs. The remaining 45 % of blood content is blood cells. One µl of human blood includes 5 – 6 million erythrocytes, 250 000 – 400 000 platelets and 5000 – 10 000 leukocytes (Campbell et al. 2018).

Leukocytes are typically divided into 5 following types: neutrophils, eosinophils, basophils, monocytes and lymphocytes, which can further be specified in subgroups of B , T and natural killer (NK) cells (LaRosa and Orange 2008, Saidani et al. 2024). The count of leukocyte types in blood of a healthy human is shown in Table 1. The differentiation of leukocytes happens in bone marrow, except for T cells that develop in thymus (Xing et al. 2016, Saidani et al. 2024). Neutrophils, eosinophils and basophils are referred as granulocytes due to their structure: They posses different kinds of granules in their cytoplasm and are easy to distinguish by the the shape of multi-lobed nucleus specific for each type (Skinner & Johnson, 2017).

TABLE 1. The number of different leukocyte types in healthy adults per one µl of whole blood (Campbell et al. 2018).

Leukocyte type	Number in the blood of a healthy individual per microliter
Neutrophils	2500–7000
Eosinophils	30–320
Basophils	0–300
Monocytes	200–800
T cells	680–4250
B cells	100–600
NK cells	50–1000

1.2.1 Leukocytes function in immune defence

The main function of leukocytes is to defend the body against infections and diseases by either exterminating phatogenetic organisms with phagocytosis or attacking them via antibodies and sensitized lymphocytes, which are capable of destroying or inactivating the phatogenes (Hall and Guyton 2011). Due to the immune defence functions, leukocytes can also be referred to as immune cells.

Besides bacteria and viruses, different leukocyte types also play part in immune defence against cancerous and otherwise defective cells (Turvey and Broide 2010).

Functions of granulocytes and NK cells are more related to innate immune system, which is responsible of the first responses on possible pathogens and foreign particles entering human body (Turvey and Broide 2010). Innate immune defence reacts in a same way to all new threats and responses are relatively fast (Turvey and Broide 2010). Often, the reactions of leukocytes of innate immune system result in inflammation, that in cellular level can be determined as continuous infiltration of leukocytes in certain area or tissue due to injury in a tissue or infection (Medzhitov 2008, Newton and Dixit 2012). Especially neutrophils, as well as monocytes and their mature form macrophages are linked to inflammation (Newton and Dixit 2012).

Lymphocytes, excluding NK cells, are specialised for producing and enhancing the adaptive immunity, which is described by Iwasaki and Medzhitov (2010) as follows. In contradiction to innate immune system the adaptive immune system activation takes much longer, but the response is specific for the exact pathogen or foreign particle identified. The function of adaptive immune system is a secondary response and thus takes place if the foreign object has been recognized at least once already before. Even though different leukocyte types in innate and adaptive immune system have various roles, their functions are not separate but rather complementary to each other (Iwasaki and Medzhitov 2010).

1.2.2 Leukocytes participate in cancer prevention

It has been shown that cancers cause clear alterations in infiltration of immune cells to tumor microenvironment but also in their circulation (Akinbami et al. 2013, Strati et al. 2018, Turri et al. 2023). In CRC, a higher eosinophil count in cancerous tissue and circulation has been connected to better prognosis and a low count is related to a higher mortality rate (Fernández-Aceñero et al. 2000, Wei et al. 2018). In contradiction, a higher count of some other leukocyte types has been suggested to promote the spreading of cancer. For example, some studies suggest that a subtype of neutrophils called tumour-associated neutrophils promote tumour growth, tumour angiogenesis, and metastasis (Mizuno et al. 2019). The number of neutrophils also rises according to tumour stage (Satomi et al. 1995). All leukocytes can be found in a tumour microenvironment and according to some estimates, up to 90 % of the mass of the tumour is immune cells (Fridman et al. 2012). Thus, the importance of understanding the functions of leukocytes in cancer development, including prevention, is apparent.

A tumour formation starts with a mutated cell that escapes all proof-reading and passes all checkpoints during and after cell division. The mutated cell further divides and forms a limited area of abnormally grown cells, referred as neoplasm. When mutations accumulate in these abnormally growing cells it can eventually lead to tumorigenesis due to enhanced activation of oncogenes that support tumour development and growth, or decreased activation of tumour-suppression genes which normally restrict tumour formation (Corthay

2014.). MSI predisposes cells to mutations and increases the probability of this kind of changes in genome making LS carriers more prone to neoplasm as explained before. When the neoplasm or tumour obtains malignant changes and starts to grow uncontrollably with the potential of spreading it becomes cancerous. Immune cells can recognize and destroy mutated cells which is why neither abnormal nor malignant changes in cells nor defects in signalling pathways usually lead to cancer (Schreiber et al. 2011).

Immune system participates in cancer prevention in three ways (Schreiber et al. 2011). I. Viral infections cause genomic changes that may induce cancer and the role of immune system in protecting the host against viruses is fully accepted. II. Inflamed tissue microenvironment generally is linked to cancer progression as a favourable environment for tumour development. Even though immune cells cause inflammation by eradicating the cause of it they can also resolve it quickly. III. The immune cells destroy neoplastic cells due to the recognition of tumour-cell-associated ligands expressed by neoplastic cells (Schreiber et al. 2011). This can also be referred to as immune surveillance (Swann and Smyth 2007).

Cancer immunoediting is a term used to describe the complex relationship between the immune system and the development of neoplastic cells/tumour, which consists of the following phases: elimination, equilibrium, and escape. O'Donnel and colleagues (2019) have well described these phases: During the elimination phase, both innate and adaptive immune systems aim to eradicate all mutated neoplastic cells, which the intracellular safeguarding mechanisms have failed to fix or demolish. If all these cells are not destroyed, immunoediting progresses to equilibrium during which the immune system still tries to target all malignancies, but the neoplasm can keep growing. Continuous targeting of neoplastic cells by immune defence may lead to modifications in the tumour genome that reduce their responsiveness to the attack of immune cells. This can restrict the recognition of the eradication of incipient tumour and lead to the escape phase in which the tumour growth becomes uncontrollable and leukocytes can no longer prevent the cancer.

Even though it has been long known that leukocytes play roles in cancer prevention, these action mechanisms are still not fully recognized (Uribe-Querol and Rosales 2015). Studies have perhaps focused more on the functions of leukocytes in late equilibrium and escape phases of cancer immunoediting as multiple studies have indicated the significance of different leukocytes in several cancers (Mizuno et al. 2019). Indeed, these studies have given valuable information. However, information of how tumour formation is naturally prevented by immune cells is equally important as I. it has been shown that the prognosis for surviving cancer is the better the earlier the incipient tumour is detected, II. knowing prevention mechanisms can boost the discovery of novel treatments since many current cancer therapies rely on enhancing the function of immune cells against cancer and III. cancer treatments are expensive, and this could help to save a lot of money.

Neutrophils and macrophages have been shown to have tumour promoting functions, but there is also evidence that their first aim is to prevent cancer and

suppress tumour formation. Both neutrophils and macrophages have polarization phenotypes of which one operates as anti-tumourigenic (type 1) and other as pro-tumourigenic (type 2) cells (Fridlender et al. 2009, Murray 2017). N1 type of neutrophils are cytotoxic towards tumour cells as they produce for example tumour necrosis factor (TNF)-α, and they also stimulate CD8+ T cell activation (Fridlender et al. 2009). In macrophages, the M1 phenotype expresses inflammation-related cytokines and chemokines that recruit NK cells to kill the potential tumour cells, for example (Mantovani 2002, Murray 2017).

NK cells have multiple tactics for eliminating tumour cells that has been reviewed by Mahasa and colleagues (2016): NK cells can cause the lysis of tumour cells by forming lytic pores or they can cause tumour cells to enter apoptosis, a programmed cell death, by binding to apoptosis receptors in the tumour cell. The lysis can also be done in antibody dependent way, as NK cells receptor FcγRIIIa (CD16), can bind to antibodies expressed by tumour cells. In addition, special type of NK cells called NKT cells are capable of inhibiting tumour growth by secreting pro-inflammatory cytokines like interferon-γ, for example.

T cells on the other hand, function as a part of adaptive immune system that is alerted by innate immune responses and has specialized actions against different types of cells or invading organisms. Major histocompatibility complex class I and II (MHCI and MCHII) proteins are associated with functions of T cells: Different types of T cells recognize epitopes expressed by MHCI and MHCII in tumour cells (O´Donnel et al. 2019). The CD8+ cytotoxic T cells that are activated through the protein epitope of MCHI can kill cancer cells by secreting cytotoxic substances or ligands signalling cell death for the cancerous cells (Mahasa et al. 2016). To prevent T cells for recognizing and killing the cancerous cells, they can develop mutations due to which they don't express MHC related peptide epitopes anymore. However, MHCI and molecules related to its function are known to inhibit NK cells, so their downregulation enables NK cells to target neoplastic cells instead, therefore covering for T cells (Mohme et al. 2017).

1.2.3 Importance of cytokines in leukocyte operations in cancer prevention

Cytokines are crucial signalling molecules that mediate several signalling pathways, especially in immune system. They are soluble proteins secreted primarily by different types of leukocytes and their actions can be auto-, endo- or paracrine, meaning that they can act in same cell that produces them, the neighbouring cells or remote cells (Zhang and An 2007, Arango Duque and Descoteaux 2014, Liu et al. 2021). Cytokines can be subgrouped according to the cell type they are originated or their ways of operation. For example, lymphokines and monokines are lymphocyte and monocyte originated cytokines, respectively (Zhang and An 2007). Chemokines are cytokines that promote chemotaxis, and cytokines originated in one leukocyte type but having functions in another type are referred to as interleukins (IL) (Zhang and An 2007).

Cytokines can also be divided in inflammation inhibiting (anti-inflammatory) and promoting ones (pro-inflammatory). IL-4 is an example of anti-inflammatory cytokine. It promotes T cell differentiation into type 2 T helper

cells characterized by secretion of cytokines, recruitment of other leukocytes and activation of M2 polarized macrophages and enhances the activity of type 2 T helper cells (Croft and Swain 1995, Ouyang et al. 1998, Gurram and Zhu 2019).

Pro-inflammatory cytokines include IL-1β, IL-12, interferon-γ, and TNF-α, for example (Liu et al. 2021). IL-1β is produced by immune cells of innate immune system, such as monocytes and macrophages (Lopez-Castejon and Brough 2011). It has been connected to several diseases and cancers. Nakao and colleagues (2005) have shown that IL-1β enhances vascularization. Their study indicated that expression of IL-1β in lung cancer cells supports tumour growth by macrophage infiltration and improves neovasculature. In addition, their research proved IL-1β to cause infiltration of neutrophils and macrophages on the site of IL-1β protein expression. IL-1β has also been reported to enhance invasiveness by promoting inflammation (Apte et al. 2006).

Two steps are required to produce IL-1β. First, priming signal representing damage-associated molecular pattern (DAMP) or pathogen-associated molecular patterns (PAMP) that can be lipopolysaccharide (LPS) or IL-1β itself activates a Toll-like receptor (TLR), through which the transcription of IL-1β gene is activated (Rébé and Ghiringhelli 2020). TNF-α can also trigger signalling pathway leading to the transcription by binding to corresponding TNF receptor. The end result is inactive pro-IL-1β that requires further cleaving to its functioning form (Rébé and Ghiringhelli 2020). The cleavage happens by cascades, usually caspase-1 (Lamkanfi and Dixit 2014).

However, caspase-1 enzyme, too, needs separate activation and it is induced by a protein-complex inflammasome located within cells. Similarly to IL-1β gene transcription, signalling pathway leading to assembly and active function of inflammasomes can also be triggered by LPS binding to TLR among some other DAMPs or PAMPs (Guo et al. 2015). Caspase-1 can bind to assembled inflammasome by adaptor apoptosis-associated speck-like protein (ASC) capable of activating the caspase with specific interaction pathways (Li et al. 2018, Hochheiser et al. 2022).

1.3 Micro-RNAs function as genetic and epigenetic regulators in cancer prevention

Micro-RNAs (miRs) are non-coding RNA molecules that consist of 19-25 nucleotides and regulate gene expression modification of partially complementary messenger RNAs (mRNAs) (Lu and Rothenberg 2018). In the genome, miRs often occur in clusters in chromosomes and are found almost everywhere throughout the genome except for chromosome Y (Filipów & Łaczmański 2019).

Mature miRs are produced from primary microRNAs (pri-miRs) in multistep processes. Pri-miRs are transcribed from either intergenic (between genes) or intragenic (within genes) parts of genome. The most common miR biogenesis method happens via a canonical pathway, which has been comprehensively reviewed by O'Brien and colleagues (2018): Transcribing of pri-

miRs is executed by RNA polymerase II or III in the nucleus. A complex called microprocessor formed by endonuclease enzyme Drosha and double-stranded-RNA-binding protein DGCR8 cleaves pri-miR to create a hairpin like structured precursor miR (pre-miR). Exportin-5 and RanGTP then transport pre-miR into the cytoplasm where it is once more cleaved this time by double-stranded RNA-specific ribonuclease (Dicer) to degrade the hair pin loop and obtain the final size of the miR (19-25 nucleotides). At this point, the miR consists of two strands of which one is disposed and the other attached into RNA-induced silencing complex (RISC). More specifically, miR attaches to one of the proteins of family argonaute (AGO 1-4) that is part of RISC. Thus, miR and RISC form a functioning unit (miRISC) that is ready to target mRNA.

miRs have been connected to important cellular processes such as apoptosis, cell proliferation and differentiation, and cellular signalling. One mRNA molecule is usually targeted by one miR (Filipów & Łaczmański 2019). Thus, altered expression of even one miR could already lead to critical changes in signalling pathways and significantly impact cellular functions. The miR bound to RISC guides the miRISC-complex to mRNA complementary to the miR (O'Brien et al. 2018). Usually, the miR has non-base paired tail in 5′ end as a probe for complementary mRNA. Therefore, the miRISC generally attaches to 3′ untranslated region of the complementary mRNA by base pairing (O'Brien et al. 2018). This enables the RISC to inhibit the translation by decaying the mRNA or repressing translation (Eichhorn et al. 2014). Mature miRs can also bind to TLR1, leading to activated downstream signalling pathways (Peng and Croce 2016).

Besides cells, free miRs can be found in extracellular body fluids including blood (Weber et al. 2010). Through blood, the cmiRs gain access everywhere in human system and may have functions specific to tissue types. miRs are secreted in circulation in extracellular vesicles, attached to RNA-binding proteins or lipoprotein complexes, of which the RNA-binding protein associated transportation is the most common method (Vickers et al. 2011, Turchinovich et al. 2011). Especially AGO2 protein has been linked to cmiRs but also other AGO protein family members have been suggested to be associated (Turchinovich et al 2011). cmiRs are not easily affected by temperature changes, extreme pH levels, or degradation by ribonucleases and are thus considered very stable molecules (Chen et al. 2008). Mitchell and colleagues (2008) have shown them to withstand multiple freezing and thawing cycles as well as at least 24h incubation at room temperature. As cmiRs are present in blood, obtaining a sample containing them is uncomplicated. These characteristics make cmiRs relatively easy to handle and thus good molecules for research and possible novel biomarker applications.

Indeed, cmiRs potential as novel biomarkers is already widely recognized. Several studies have shown deviant expression patterns of cmiRs to associate with diseases, such as cardiovascular and neurodegenerative diseases (Tijsen et al. 2010, van den Berg et al. 2020). Recent studies have also presented aberrant expression of cmiRs in Japanese encephalitis virus infection and non-small lung cancer, for example (Khandelwal et al. 2020, Baluni et al. 2022). In addition, cmiRs can function in multiple activation paths that affect tumour growth and

development (Acunzo et al. 2015). They have been linked to different cancers in many distinct organs and tissues (Calin and Croce 2006, Ullah et al. 2014).

The impacts of cmiRs on tumour development can be both suppressing or enhancing. If the target gene of miR regulation functions in the tumour suppressing pathway, up-regulation of the miR then inhibits the translation of this gene and can support tumour development (Wang and Luo 2015). This kind of miRs are referred as oncogenic. On the contrary, miRs that are downregulated in cancer, as they normally inhibit cancer formation and progression, are classified as tumour suppressive miRs (Wang and Luo 2015).

Besides leukocytes, miRs can also play a role in inflammation through their impact on inflammasome. Certain miRs have been considered epigenetic regulators of inflammasome activity due to their ability to either increase or decrease expression of genes of inflammasome complex. Poli and colleagues (2020) have listed 15 miRs that regulate NLRP3 inflammasome genes and are associated in diseases such as breast cancer, gastric cancer and renal inflammatory disease when expressed abnormally. This implies that miRs have crucial role in cancer prevention by regulating genes of substances related to inflammatory response.

1.3.1 Differential expression of circulating micro-RNAs in Lynch syndrome and colorectal cancer

Indications of cmiRs and miRs linkage to CRC have been presented in multiple studies (Zhang et al. 2014, Zhang et al. 2018, Buglyó et al. 2022, Farc et al. 2023). Sievänen and colleagues (2022) have studied cmiRs and recognized several differentially expressed (DE) cmiRs in cancer-free LS carriers compared to non-LS carriers (referred to as LS cmiRs from now on). They also showed that the expression levels of these LS cmiRs match the levels of these cmiRs in CRC patient's blood, which implies that the expression patterns of these cmiRs are associated with CRC. It remains uncovered whether the LS cmiRs could affect cancer risk by regulating immune responses through immune cells. In addition, there is still a lot to discover about the functions and roles of different LS cmiRs.

Due the significant effects cmiRs have on cellular processes including cell cycle control and apoptosis, it is important to gain basic knowledge of LS cmiRs, so that they can be better studied and possibly utilized in the future. The miR expression in the study of Sievänen and colleagues (2022) was measured from blood serum raising a question of the origin and target cells of these cmiRs in blood circulation. Information on the matter is important as understanding the exact function mechanisms of the miRs requires knowledge of their production and secretion pathways including information on their cellular origin and main target cells.

1.4 Leukocytes as potential cellular origin of circulating micro-RNAs

As leukocytes are responsible of immune responses and cmiRs of the gene regulation that affects the immune responses a connection between functions of leukocytes and cmiRs can be drawn. For example, both leukocyte and cmiR expression levels are often altered in cancer in a cancer specific manner. Zhang and colleagues (2017) studied expression levels of cmiRs in patients with colon adenoma and colon cancer and determined eight of the detected DE miRs to be associated with the adenomas and cancer. Lee and others (2006) have shown that number of leukocytes can increase in colon cancer and this increased count was associated with poorer prognosis. Sromek and others (2017) monitored changes in levels of three cmiRs in plasma of non-small cell lung carcinoma patients and observed their level to be increased compared to non-cancerous individuals. Similarly, numbers of leukocytes in lung cancers are known to be altered (Thomson et al. 1986). Likewise, alterations in levels of cmiRs in other cancers has been reported as well (Swellam et al. 2018, Buglyó et al. 2022, Farc et al. 2023). As cmiRs and leukocytes are present in the blood and both of their numbers vary depending on tumour type this could imply that leukocytes express and secrete cmiRs.

Only a limited number of studies exist on the cellular origin of cmiRs, but leukocytes can potentially be considered primary source. Pritchard and colleagues (2012) have studied the variations in the numbers of blood cells and cmiRs and reported corresponding changes between leukocytes and cmiRs. Uil and colleagues (2021) studied miR content and the origin of extracellular vesicles (EVs) showing that EVs carrying miRs can be originated from leukocytes among other studied cell types. In addition, expression patterns of cmiRs in leukocytes has been previously studied in Moyamoya disease (Kang et al. 2022) and several DE cmiRs between Moyamoya patients and healthy individuals were identified.

1.5 Aims of the study

Cancer free LS carriers express specific cmiR profile (Sievänen et al. 2022). Blood cells, including different types of leukocytes, have been indicated the major origin of cmiRs (Pritchard et al. 2012). Thus, leukocytes can be considered as a potential source for the LS cmiRs as well. LS cmiRs might also target functions of leukocytes, for example, by associating inflammasome activation and IL-1β gene expression regulations (Fernández-Aceñero et al. 2000, Haneklaus et al. 2012, Zamani et al. 2020). However, this topic remains understudied.

This study aimed to reveal whether LS cmiRs, previously identified from LS carriers' serum, can be found in leukocytes. Another aim was to study if some of LS cmiRs impact on cytokine expression in leukocytes. The hypotheses were that LS cmiRs are expressed at least in some of the leukocyte types and these LS cmiRs alter cytokine gene expression in leukocytes.

LS cmiRs examined in this work included miR-339-5p, let-7e-5p, miR-451a, miR-320a and miR-15a-5p. The research questions were: 1. Are these LS cmiRs expressed by circulating leukocytes and if so, in which leukocyte types are they expressed by the most. 2. Do the higher expression levels of miR-15a-5p affect the IL-1β cytokine gene expression in leukocyte subpopulations? 3. Are the chosen research methods suitable for studying these implications?

Flow cytometry was utilized for single cell analysis and sorting of different leukocyte subpopulations. The miR content in these leukocyte populations was estimated with quantitative polymerase chain reaction (qPCR) after extraction of the RNAs from the cells. miR-15a-5p has been associated with altered IL-1β expression levels in previous studies and was chosen for testing the designed method here as well (Ye et al. 2016, Lou and Huang 2020). HPBL were cultured and IL-1β expression levels in miR-15a-5p affected cells were studied with qPCR similarly as done with sorted leukocyte subpopulations. Among the above-mentioned research questions, this work aimed to optimize sufficient protocols for similar or related studies for future research.

2 MATERIALS AND METHODS

HPBL used in this study were sourced from whole blood samples from healthy, volunteered blood donors. Whole blood was collected in 9 ml EDTA K3 tubes (Pamark) and used immediately for the experiments. The blood cell compositions of the donated samples were measured with a Sysmex XP-300 hematology analyser (Roche Diagnostics Oy) right after donation. The same device was also used for determining the blood cell composition after purification of leukocytes, described in section 2.1.

CytoFLEX SRT (BE33091, Beckmann Coulter, Suzhou, China) flow cytometer was used for Fluorescence-Activated Cell Sorting (FACS) to identify and separate leukocyte subpopulations. The flow cytometry samples were analysed, and figures produced with CytExpert SRT (version 1.0.2.10002, Beckmann Coulter Inc.) software. The flow cytometer was equipped with three lasers: violet, blue and red. Filters used in the experiment are listed in Table 2 that also shows which laser–filter combinations were used to detect the fluorescent signals of each fluorochrome tagged antibodies. OMIP-023 antibody panel (Thermo Fischer Scienfitic, Bocsi et al. (2014)) was used for labelling the cells with antibodies to be able to distinguish the leukocyte types with flow cytometer. A few modifications were made to the antibody panel fluorochromes compared to the original publication in order to optimize the panel for the available CytoFLEX SRT instrumentation. Information of the antibodies in newly optimized panel are represented in Table 3.

TABLE 2. Laser and filter combinations used in FACS analysis to produce and detect the fluorescent signal of each fluorochrome tagged antibody.

Laser	Filter	Name of the filter channel in the software	Antibodies
violet	450/45	V450-PB	CD16, CD56, DAPI
violet	525/40	V525-KrO	CD45
blue	525/40	FITCH	CD19, CD14, CD8a
violet	610/20	Violet610	CD3
red	660/10	APC	HLA
red	780/60	APC-A750	CD4

All qPCR measurements were executed with C1000 Touch Thermal Cycler including CFX384 Optics Module (786BR04810, Bio-Rad, Singapore) using CFX Maestro (version 4.1.2433.1219, Bio-Rad) software. For incubations of reverse transcription reactions Mastercycler, *ep*gradient S (Eppendorf, Hamburg, Germany) was used. For spinning the thin-walled 384-well PCR plates (HSP3805, Bio-Rad) before executing qPCR C3i multifunction centrifuge (Thermo electron corporation) was used. All laboratory work was conducted in accordance with standard aseptic practices appropriate to the purpose.

TABLE 3. Summary table of the information of the antibodies produced by Invitrogen and equipped with fluorochromes of e-Biosciences™ (Carlsbad, USA).

Antibody	Fluorochrome	Antibody clone
Anti-Hu CD45	e-Fluor™ 506	Hl30
Anti-Hu CD56 (NCAM)	Super Bright™ 436	TULY56
Anti-Hu CD19	FITCH	HIB19
Anti-Hu CD16	Super Bright™ 436	eBioCB16 (CB16)
Anti-Hu CD14	FITCH	61D3
Anti-Hu CD8a	FITCH	OKT8 (OKT-8)
Anti-Hu CD4	APC-eFluor™ 780	SK3 (SK-3)
Anti-Hu CD3	Super Bright™ 600	OKT3
Anti-Hu HLA-DR	APC	LN3
Anti-Hu CD38	Pe-Cyanine5	HIT2

2.1 Purification of leukocytes from human peripheral blood

Leukocytes were extracted and purified from the whole blood by red blood cell (RBC) lysis. Blood was mixed with RBC lysis buffer (0,15 M NH_4Cl, 0,01 M $NaHCO_3$, 1 mM disodium EDTA, pH 7,4) in 1:10 and incubated at room temperature (RT) for 15 minutes for the buffer to lyse red blood cells. The mixture was centrifuged at 500 x *g* for 5 min (Mega Star 1.6, VWR, Germany) to separate

the intact cells and supernatant discarded. The cell pellet was washed twice with 25 ml of 1xPBS (Phosphate Buffered Saline, EuroClone, MI, Italy) and centrifuged as before after both washes. Because one round of lysis did not remove all RBCs a second round of lysis was performed, but with shorter incubation time (10 min) with the lysis buffer to avoid unnecessary stressing of the leukocytes that might cause more cell death. After the final wash with 1xPBS and discarding the supernatant, the cell pellet was suspended into 1 ml of 1xPBS, and the blood cell composition was measured again with Sysmex to confirm the leukocyte concentration and that the sample included non or only a minor amount of red blood cells (max 0,03 x 10^9/l) and platelets (max 1:9 ratio to leukocytes). Of this purified leukocyte sample 10^6 leukocytes were dissolved in 1xPBS in concentration of 10^6 cells/ml and saved as a control for sorting. This sample was stored at + 4 °C until the FACS experiment was finished and then treated as sorted leukocyte samples.

2.2 Analysis and sorting of leukocyte subpopulations

For FACS analysis leukocytes were labelled with fluorochrome tagged antibodies specific for different subpopulations. Altogether, 7 different leukocyte subpopulations were sorted. These populations were neutrophils, eosinophils, T cells, NKT cells, B cells, NK cells and monocytes. Because the flow cytometer was only capable of sorting 4 different cell types at once, chosen leukocyte types were analysed and sorted in two sets. The other set (set 1) was designed for sorting neutrophils, eosinophils, T cells and NKT cells. In the other (set 2) B cells, NK cells, T cells and monocytes were sorted. The samples with used antibodies and their target leukocyte types in both sorting sets are presented in Table 4. The two sorting sets had to be done on different days and thus the leukocytes of set 1 and 2 originated from different blood samples and separate total leukocyte samples were saved for both sets.

To prevent antibodies' non-specific binding the purified leukocyte samples were first treated with 0,5 % bovine serum albumin (BSA) in PBS pH 7,4 at RT as follows. First, the samples were centrifuged at 500 x *g* for 5 min and the supernatant discarded. The cell pellet was then suspended in 0,5 % BSA in PBS pH 7,4 so that the cell concentration was 1x10^7 cells/ml. The desired number of cells (1x10^6 - 3x10^6) was divided into FACS tubes (352235, Falcon) and 1 µl of antibody/antibodies were added towards 1x10^6 cells per sample according to Table 4.

After adding the antibodies, samples were vortexed and incubated in the dark at RT for an hour. Then the samples were washed twice with 2 ml of 1xPBS: After adding the PBS to the samples, the tubes were vortexed and centrifuged (Mega Star 1.6, VWR, Germany) at 500 x *g* for 5 min and the supernatant was discarded. Finally, the cells were suspended in the 1xPBS in concentration of 1x10^6 cells/ml and kept on ice in the dark until analysis and sorting with the flow cytometer.

TABLE 4. Samples with antibodies used in sorting sets and the leukocyte types the antibodies target.

Sorting set	Sample/antibodies	Target leukocyte type
1 and 2	Control (no antibodies)	-
1 and 2	CD45	All leukocytes
1 and 2	CD56	NK and NKT cells
2	CD19	B cells
1 and 2	CD16	Monocytes, NK cells, neutrophils
1 and 2	CD14	Monocytes
1 and 2	CD8	T cells, Cytotoxic T cells
1 and 2	CD4	T cells, Helper T cells
1 and 2	CD3	T cells
2	HLA	B cells, monocytes
1 and 2	Sorting sample (all antibodies above)	
1 and 2	DAPI	Dead cells

Prior to the sample analysis and sorting in FACS assay, the fluorescent signals of samples with only one of the used antibodies in every filter channel were recorded and possible small leakages into wrong filter channels compensated with the CytExpert SRT-software. The gains of the fluorescent signals were adjusted so that the intensity was ~10^5 in logarithmic scale. The intensity of the fluorescence from cells was analysed with an area (A) or height (H) setting. The intensity of the fluorescence from the cell is measured during the time the cell moves past the laser light in flow cytometer and recorded as the intensity as a function of time. The A setting uses the area of the peak and H the maximum height of the peak. In Figures 2–8 the A and H refer to these.

To ensure that all fluorochromes of the antibodies produced proper fluorescent signals, they were first tested individually and as "minus one"-samples, meaning that samples included all but one of the used antibodies. Additionally, as some of the antibodies had the same fluorochrome, these were also tested separately so that samples didn't consist any of these antibodies. These tests included the antibodies in Table 4 and CD38, which were needed for the identification and sorting of basophils. However, the fluorescent signal of CD38 leaked significantly to the other filter channel. Therefore, CD38, and also basophils, were excluded from the FACS experiment and T cells were sorted in both sets instead.

FACS experiments were executed at RT (batch 1) or + 4 °C (other sorting batches). The measurements for the analysis were done within 5 hours and sorting started within 6 hours after RBC lysis. In this quantitative analysis 10 000 events were recorded in each sample, except for the one that included all the antibodies. In this case, only 3000 – 4000 events were recorded to minimize the loss of the leukocytes from the sorting sample. The gating strategy for the analysis and sorting of different leukocyte subtypes is described in more detail in the OMIP-023 panel study by Bocsi and research fellows (2014). After gating the chosen leukocyte types were sorted in the FACS tubes filled with 2 ml of

medium (1x RPMI Medium 1640, A10491-01, Gibco). During sorting experiments the medium was maintained at + 4 °C. In total, three batches of both sets were sorted for monitoring the LS cmiR expression in leukocytes.

To test the accuracy of sorting additional batches of both staining sets were prepared and sorted. After sorting, the sorted cells were analysed again with the flow cytometer using the same gates as used prior to sorting. The accuracies were calculated as percentages from all recorded events by comparing how many cells were in the final target gate versus in the whole sample.

2.3 RNA extraction from sorted leukocyte subpopulations

Immediately after finishing the cell sorting, the sorted samples and the unsorted total leukocyte sample were transferred into Eppendorf tubes and centrifuged at 500 x *g* for 5 min to separate cells and the supernatant. Next, 3 µl of synthetic Spike-in cel-miR-39-3p (MS00019789, Qiagen) (miR-39-3p), which was used as a reference miR, was added to every sample. For RNA extraction, three different extraction kits or kit combinations were used. For the first two batches of both sorting sets RNEasy Plus Mini Kit (Qiagen) was used. The miRs from the third batch of set 1 were extracted with MiRNeasy Mini Kit (Qiagen). The same kit was also used for the third batch of set 2, but RNeasy UCP MinElute Spin columns (1105058, Qiagen) that extracted all the RNA instead of miRs were used instead of the Mini Spin columns of the kit.

The miR or total RNA extraction was done according to the instructions of the manufacturer. However, it should be noted that the first steps in the extraction protocol of all kits included adding of cell lysing buffer and vigorous shaking to break down the cells, and after executing these steps the samples were temporarily stored at – 80 °C. The extraction protocol was finished following the manufacturer's instructions and the concentrations of RNA in samples were measured with Nanodrop (ND-1000, Spectrophotometer).

2.4 Analysis of miR expression in leukocyte subpopulations with qPCR

The miR expression in leukocyte types was studied with qPCR. The following methods were executed for all extracted RNA samples. For qPCR, the RNAs needed to be reversely transcribed into complementary DNAs (cDNAs). The cDNA conversion was done with miRCURY LNA RT Kit (Qiagen). Reactions were prepared according to instructions of the kit manufacturer, adding 25 ng of RNA per reaction and diluting samples to 1:1 in water after finishing the protocol. Due limited yield of RNAs of T cells and NK cells in batch 2 of sort set 2 only 16,8 ng (T cells) and 22,4 ng (NK cells) of RNA was added in reverse transcription reactions instead of 25 ng.

The qPCR reactions were prepared using and according to instructions of miRCURY® LNA® SYBR Green PCR kit (Qiagen) but with small changes in qPCR reactions (Table 5). The miR expression of miR-339-5p, let-7e-5p, miR-451a,

miR-320a and miR-15a-5p was tested. In addition, the expression of miR-39-3p was tested as a reference miR. More specific information on these miR primers is represented in Appendix 1. Due vast number of samples the qPCR measurements were done in two separate sets, the first one including let-7e-5p, miR-451a and miR-39-3p and second one including miR-339-5p, miR-320a and miR-15a-5p. Three repetitions of every miR per leukocyte type cDNA sample were prepared according to Table 5. All primers had been tested beforehand for contaminations and proven free of any. To ensure no contaminations in other reaction substances occurred during handling three no template control (NTC) samples without any cDNA were prepared by replacing the volume of cDNA template with RNAse free water.

TABLE 5. qPCR reaction components and their volumes per reaction.

Component	Volume (µl)
2x miRCURY SYBR Green Master Mix	5
PCR Primer mix	1
RNAse free water	2
cDNA template (0.625 ng/µl)	2
Total 1x reaction volume	10

The qPCR reactions were added on 348-well plates that were sealed with a thin plastic sticker film. To ensure all the sample solutions were in the bottom of the wells and to remove possible air bubbles the plates were briefly centrifuged with a C3i multifunction centrifuge (Thermo electron corporation). The following conditions for qPCR were run for all samples: one cycle for 2 min at 95 °C, 40 cycles of 10 s at 95 °C for denaturation and 60 s at 56 °C for annealing and extension, finally followed by the melting curve analysis program starting from 60 °C the temperature increasing 0,5 °C every 5 s until reaching 95 °C.

The results were exported and further analysed in Microsoft Excel (version 16.81). The relative gene expression of the miRs in leukocyte subpopulations was determined with the $2^{-\Delta\Delta C_T}$ method often referred also as Livak-Schmittgen method (Livak and Schmittgen, 2001). First, the average cycle threshold (C_T) value for different miRs in every sample type was calculated. These results were normalized against the reference miR (miR-39a-3p) by determining ΔC_T values of the target miRs separately for every leukocyte type and the unsorted total leukocyte samples for both sorting sets (TL1 and TL2) according to the Equation 1:

$$\Delta C^T = C^{T\ Avg}(target\ gene) - C^{T\ Avg}(reference\ gene) \quad (1)$$

Next the $\Delta\Delta C_T$ values were obtained by comparing the ΔC_T values of the leukocyte types and TL2 to the ΔC_T value of TL1 which was used as a reference sample (Equation 2).

$$\Delta\Delta C^T = \Delta C^T(target\ sample) - \Delta C^T(reference\ sample) \quad (2)$$

Finally, the relative gene expression was calculated according to Equation 3:

$$RGE = 2^{-\Delta\Delta C_T}, \quad (3)$$

where RGE = relative gene expression. These results were transformed into a dot plot.

2.5 IL-1β gene expression in miR-15a-5p transfected primary leukocytes

For studying whether miR-15a-5p had impact on the expression of IL-1β on leukocytes, HPBL were purified as in chapter 2.1. As an uncultured control 10^6 leukocytes in the concentration of 10^6 cells/ml in 1xPBS were saved and RNAs extracted from them with RNEasy Plus Mini Kit (Qiagen) according to instructions of the kit. The rest of the primary leukocytes were then suspended in at + 37 °C prewarmed growth medium (RPMI-1640, R0883-1l, Sigma Aldrich), 10% FBS (10270-106, Gibco), 1% 100x GlutaMAX (35050-038, Gibco), 1% Penicillin Streptomycin (15140-122, Gibco) and cultured in the middle 8 wells of 24-well-plate in concentration of 0,5 x 10^6 cells/ml. The experiment set-up is illustrated in Figure 1. To let the primary leukocytes recover from the change of environment from whole blood into the growth medium, the plate was incubated for a day in + 37 °C at 5 % CO_2 (HERAcell 150i CO2 Incubator, Thermo Scientific).

On the second day, the miR transfection was done. miR-15a-5p was transfected into two of the well, and synthetic negative control miR into other two wells. Information of these miRs is listed in Appendix 1. Transfection was executed with lipofectamine 3000 -reagent (Invitrogen, CA, USA) that enabled the miRs to enter the cells. Mastermix of lipofectamine and opti-MEM (31985062, Gibco) in 1:32 was prepared. Both negative miR and miR-15a-5p were mixed with opti-MEM and then 1:1 with lipofectamine including mastermix so that the final concentration of the miRs in leukocyte culture suspensions was 8,9 nM. Similar volumes of both miR transfection solutions, lipofectamine–optiMEM mastermix only and optiMEM only were each added into two wells to control the effects of lipofectamine and miRs. The cells were again incubated overnight and on the third day of culturing, the IL-1β expression was triggered by treating the cells with lipopolysaccharide (LPS) (from Escheria coli 0111:B4, L2630-10MG, Sigma Aldrich, MO, USA) by adding 0,9 ng/µl of LPS in one of each two identical wells. The cells were then incubated as before for four hours. The viability of the leukocytes during culturing was monitored with a light microscope (Zeiss Axio) daily during the whole experiment.

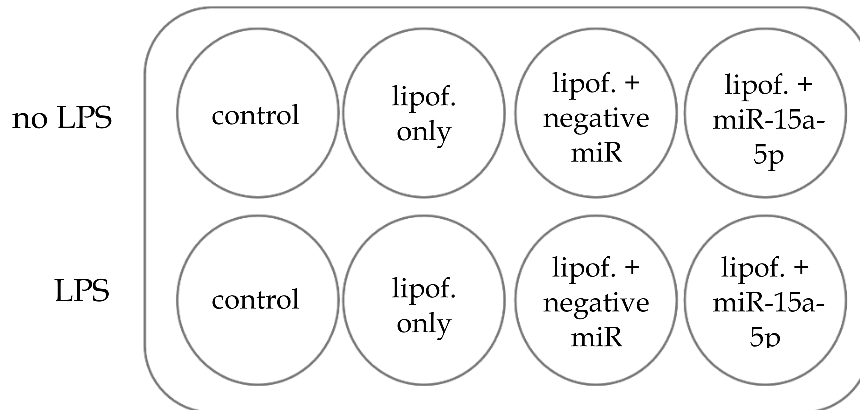

Figure 1. Illustration of the leukocyte cultures in on the last day of culturing. Negative miR refers to non-regulating synthetic miR that was used as a control for the effect of miR of interest, miR-15a-5p. Lipofectamine (lipof.) was used to assist the transfection of the miRs in the cells and it's impact alone was also observed (lipof. only).

IL-1β gene expression was studied via expression of RNAs in leukocytes. After the LPS treatment part of the cells were still free in the medium and part were attached to the bottom. The liquid was first collected, centrifuged at 600 x *g* for 5 min, and the supernatant was carefully discarded. Approximately 20 µl of supernatant was left in the sample tube to avoid accidental removal of the cells. The RNA of the attached cells was obtained by adding cell lysing reagent of the RNA extraction kit (RNeasy Mini Kit, Qiagen) into the wells in the volume instructed by the manufacturer. By mixing this reagent in wells with cells the cells were detached or partly lysed, and the RNA of these cells was freed into the solution. This solution was then combined with the liquid of the same well and RNAs extracted according to the instructions of the kit. This experiment was repeated two more times as described.

2.6 qPCR analysis of IL-1β gene expression

Extracted RNAs including samples of every well and the uncultured leukocyte sample were converted to cDNAs with RT^2 First strand Kit (330404, Qiagen) or QuantiTect Reverse transcription Kit (205313, Qiagen). The optional gDNA elimination protocol of the instructions was also executed. All reverse transcriptions were done according to instructions of the kits for 150 ng of RNA/sample. The qPCR reactions were prepared with RT^2 SYBR Green qPCR Mastermix (330502, Qiagen) for 1:6 diluted cDNAs following the instructions of the manufacturer. IL-1β primers (Hs IL1B 1 SG, QT00021385, QuantiTect® Primer Assay, Qiagen) were used for amplification of the target gene. GAPDH was used as a reference gene and the GAPDH primer mix (Hs GAPDH 2 SG, QT01192646, QuantiTect® Primer Assay, Qiagen) used for its amplification. Three repetitions with IL-1β primers and three with GAPDH primers of each sample were prepared. Conditions for qPCR run for all samples included one cycle for 10 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation and 60 s at 60 °C for annealing and extension, followed by the melting curve analysis

program starting from 65 °C the temperature increasing 0,5 °C every 5 s until reaching 95 °C. Results were further analysed in Microsoft Excel similarly as described in chapter 2.4, but using GAPDH as a reference gene in Equation 1 and cDNAs of unsorted leukocyte sample as a reference sample in Equation 2.

3 RESULTS

Here, first, the leukocyte viability assessments during the FACS assay are presented. Next, the results of HPBL phenotype analysis and sorting are displayed, followed by data on the amount of micro-RNAs in leukocyte subpopulations after each sorting. The qPCR analysis results of the micro-RNA content in these subpopulations is also presented. Results of the impact miR-15a-5p in expression of IL-1β are also presented. Last, the results show the impact of transfected miR-15a-5p in the expression of IL-1β in primary leukocyte culture.

3.1 Leukocytes remained viable in FACS sample preparations

The primary test of leukocyte viability after RBC lysis and after storing leukocytes at + 4 °C overnight showed that cell viability decreased significantly during these storing conditions. After RBC lysis the viability was 89 % and after the storing 75 %. Even though the time between RBC lysis and flow cytometer analysis was three hours longer in sorting experiments due to the labelling of the cells and analysis of multiple other samples the percentage of living cells remained the same as in the primary test (Table 6). This shows that procedures including red blood cell lysis, leukocyte purification, antibody attachment and washes affected only slightly to the viability of the cells. As all samples were treated similarly during the preparations it is safe to assume that the viability percentage remained the same in every sample.

TABLE 6. Viability of leukocytes after RBC lysis and labelling with antibodies in every sorting batch. Viability was calculated from gated "all leukocytes".

Sorting set	Batch	Cell viability (%)
set 1	1	93.32
	2	95.15
	3	90.21
	purity check	93.55
set 2	1	93.99
	2	89.46
	3	93.43
	purity check	91.25

3.2 Flow cytometer successfully sorted leukocyte subpopulations

The amount of sorted leukocyte types between sorting batches was mostly similar (Table 7). Nevertheless, as the batches were obtained from different individuals variation between batches was expected. T cells and neutrophils were the most abundantly represented cell types, whereas populations of B cells, NKT cells, eosinophils and monocytes were quite small. The quantitative FACS analysis of the purity test of sorted leukocyte subpopulations also shows the gating processes for distinguishing leukocyte types, which can be observed in Figures 2-8.

TABLE 7. Percentages of all leukocytes and leukocyte subpopulations in each sorting batch (1-3) of both sorting sets calculated from all recorded events.

	Leukocyte type	Amount in sorting sample (%)		
		Batch 1	Batch 2	Batch 3
set 1	all leukocytes	87.41	89.39	79.90
	neutrophils	46.16	45.06	29.41
	eosinophils	3.63	1.92	2.71
	T cells	12.93	22.43	-
	NKT cells	2.55	3.96	1.43
set 2	all leukocytes	85.39	89.56	91.13
	B cells	1.07	1.00	0.86
	NK cells	7.55	5.68	3.96
	T cells	20.12	14.38	21.02
	monocytes	3.86	3.51	1.87

Purity checks revealed the sorting process to be most accurate for T cells (Table 8). Purity levels of sorted NK cells, B cells and neutrophils were also good as they were over 80 %. However, the accuracies for the other three sorted leukocyte types were clearly smaller. Results suggest that eosinophil sorting was the least accurate.

Mostly, the impurities of the sorted samples were small particles, like debris or degraded cells, that appeared outside the "All leukocytes" gate in Figures 2-8 A. In sorted neutrophil and eosinophil samples ~9 % and ~5 % of the cells, respectively, formed clusters, whereas in other samples the tendency of the cells to cling to each other was clearly smaller (Figures 2-8 B).

TABLE 8. Percentages of the desired leukocyte types (purity) in sorted samples in both sorting sets. The percentages were calculated from all recorded events.

Sorting set	Sorted leukocyte type	Purity
set 1	Neutrophils	82.0 %
	Eosinophils	45.7 %
	T cells	92.4 %
	NKT cells	61.6 %
set 2	B cells	85.7 %
	NK cells	89.0 %
	T cells	90.7 %
	Monocytes	54.4 %

However, a part of the sorted samples also consisted of leukocyte types other than those desired. The sorted eosinophil sample included potentially a small number of basophils and neutrophils (Figure 3 C and E). The monocyte sample had some lymphocytes as well and results suggested that the sample might have also included basophils (Figure 8 C). In addition, approximately 1/5 of the cells in the sorted NKT cell sample fell into the gate of T cells (Figure 4 D).

Besides the main leukocyte types sorted in this work, the FACS analysis revealed additional information on phenotypes of the leukocytes. With the antibodies used in this study, cytotoxic and helper T cells could be distinguished as their own populations (Figure 7 E).

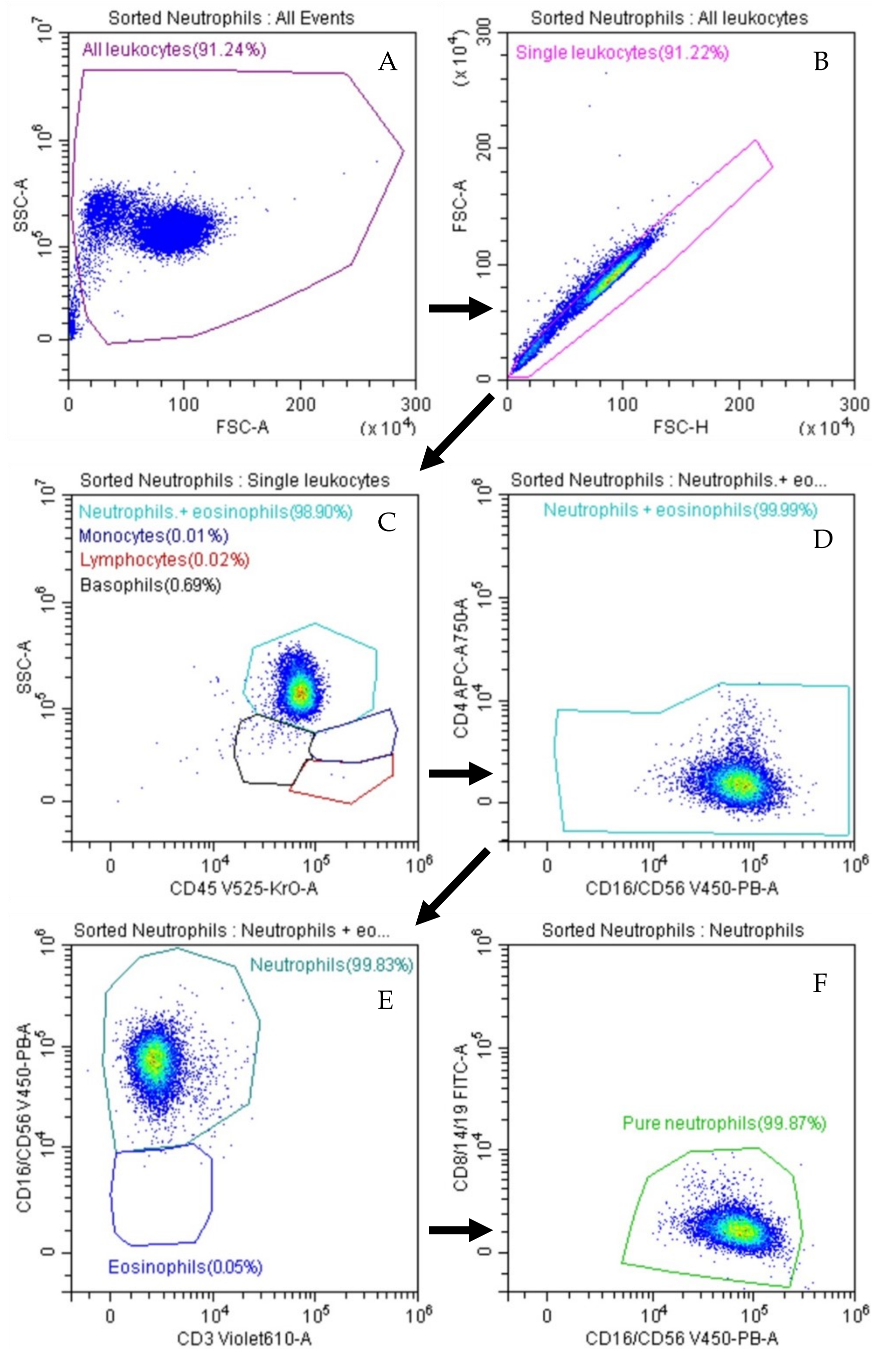

Figure 2. Pseudocolour plots of FACS analysis of the accuracy of neutrophil sorting (A, C-F) and of the tendency of the cells to cling to each other (B). One dot represents a single cell. Both x- and y-axes represent the intensity of fluorescence in logarithmic scale, except for SSC (side scatter) and FSC (forward scatter) that display the intensity of the scattered light. The intensity of FSC is represented in linear scale. Other titles of the axes describe antibodies and the filter channel used to detect the fluorescent signal. The name of the antibody is mentioned first followed by the name of the filter. Letter A in the end of name of the filter channel indicates the area setting and H the height setting. Arrows illustrate the gating process and percentages of the cells inside the gate are calculated from the cells of the previous gate.

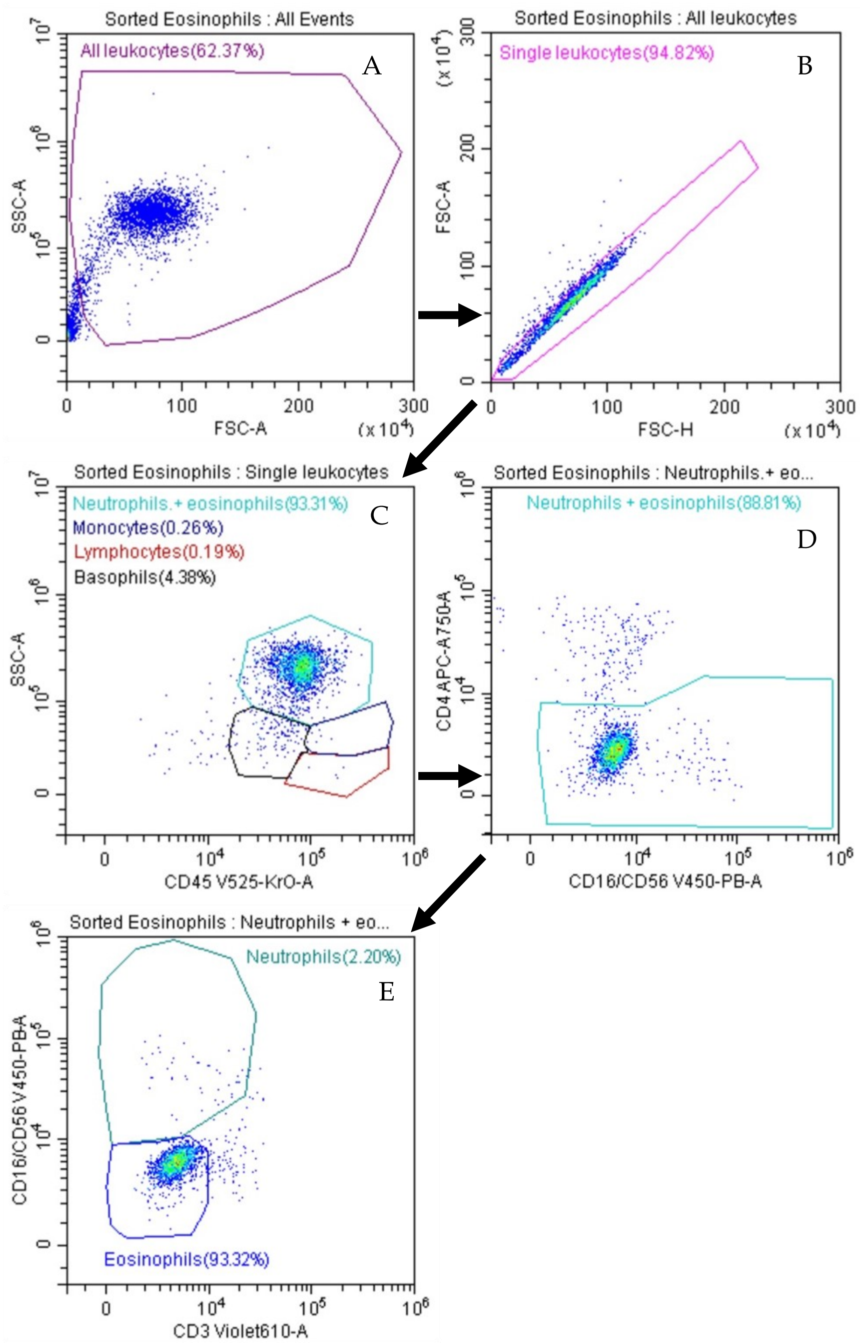

Figure 3. Pseudocolour plots of FACS analysis of the accuracy of eosinophil sorting (A, C-E) and tendency of the cells to cling on each other (B). Axis scales, and titles are same is in Figure 2. The percentages of the cells inside each gate are also determined in a similar manner as described in Figure 2.

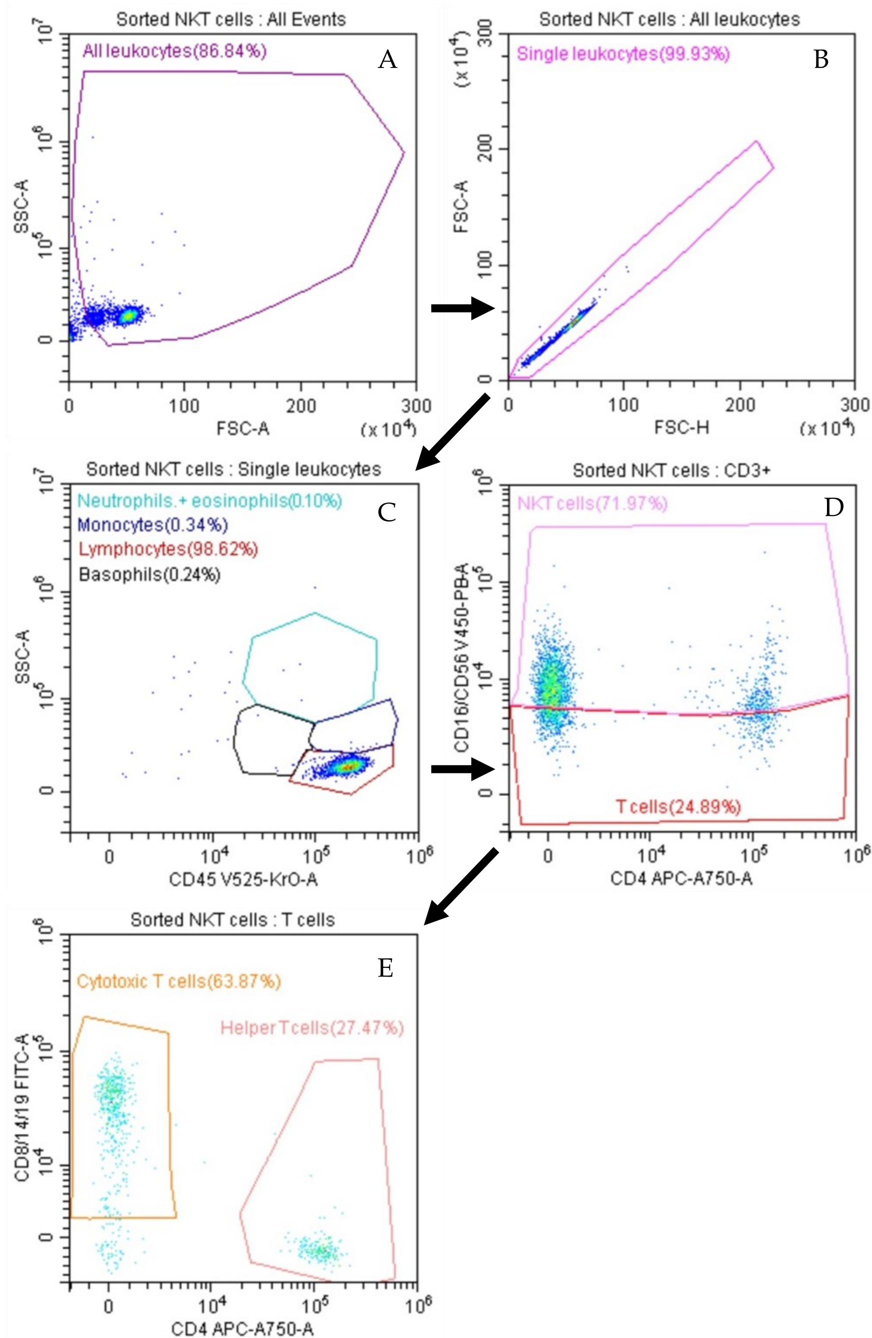

Figure 4. Pseudocolour plots of FACS analysis of the accuracy of NKT cell sorting (A, C-E) and tendency of the cells to cling on each other (B). Axis scales, and titles are same is in Figure 2. The percentages of the cells inside each gate are also determined in a similar manner as described in Figure 2.

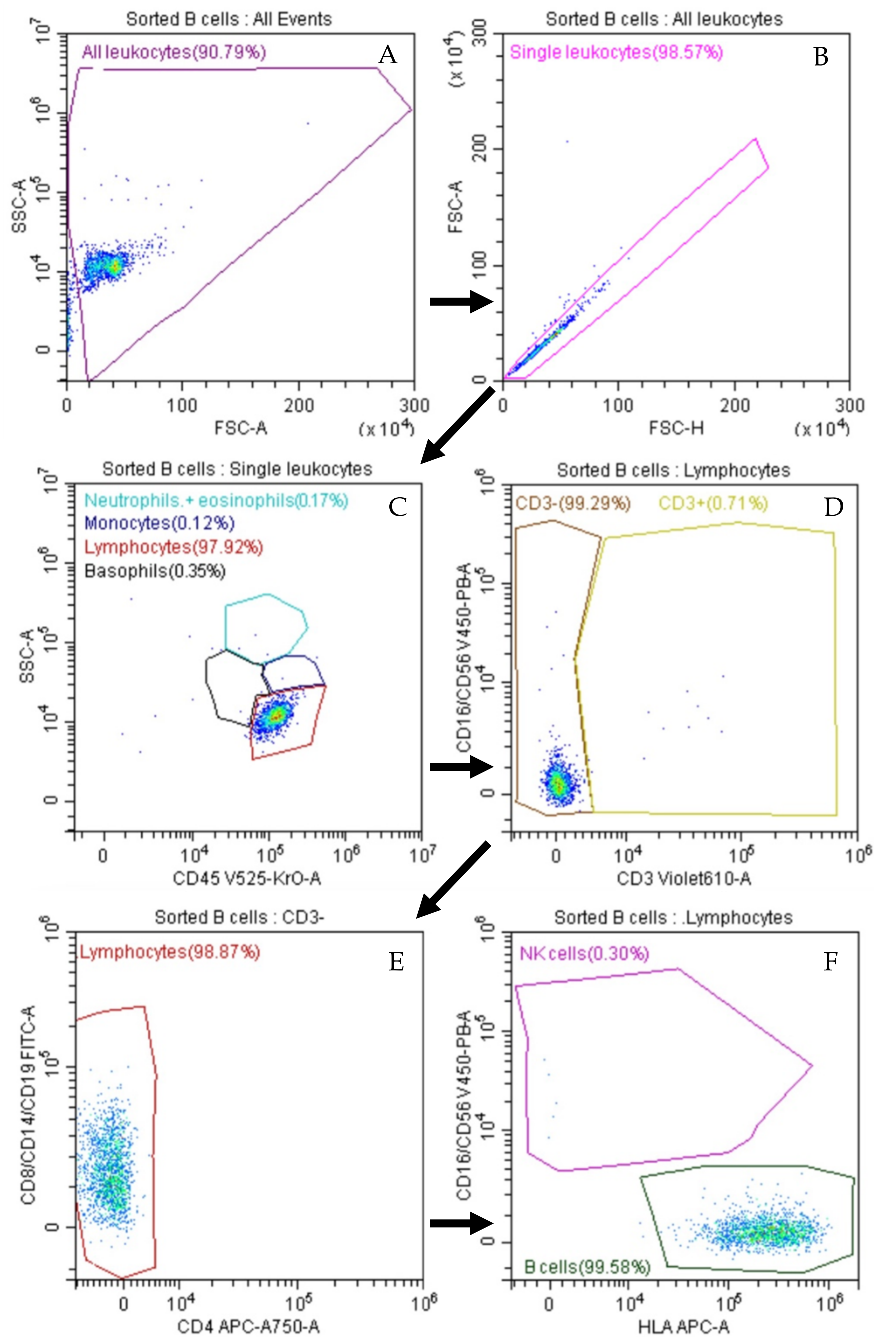

Figure 5. Pseudocolour plots of FACS analysis of the accuracy of B cell sorting (A, C-F) and tendency of the cells to cling on each other (B). Axis scales, and titles are same is in Figure 2. The percentages of the cells inside each gate are also determined in a similar manner as described in Figure 2.

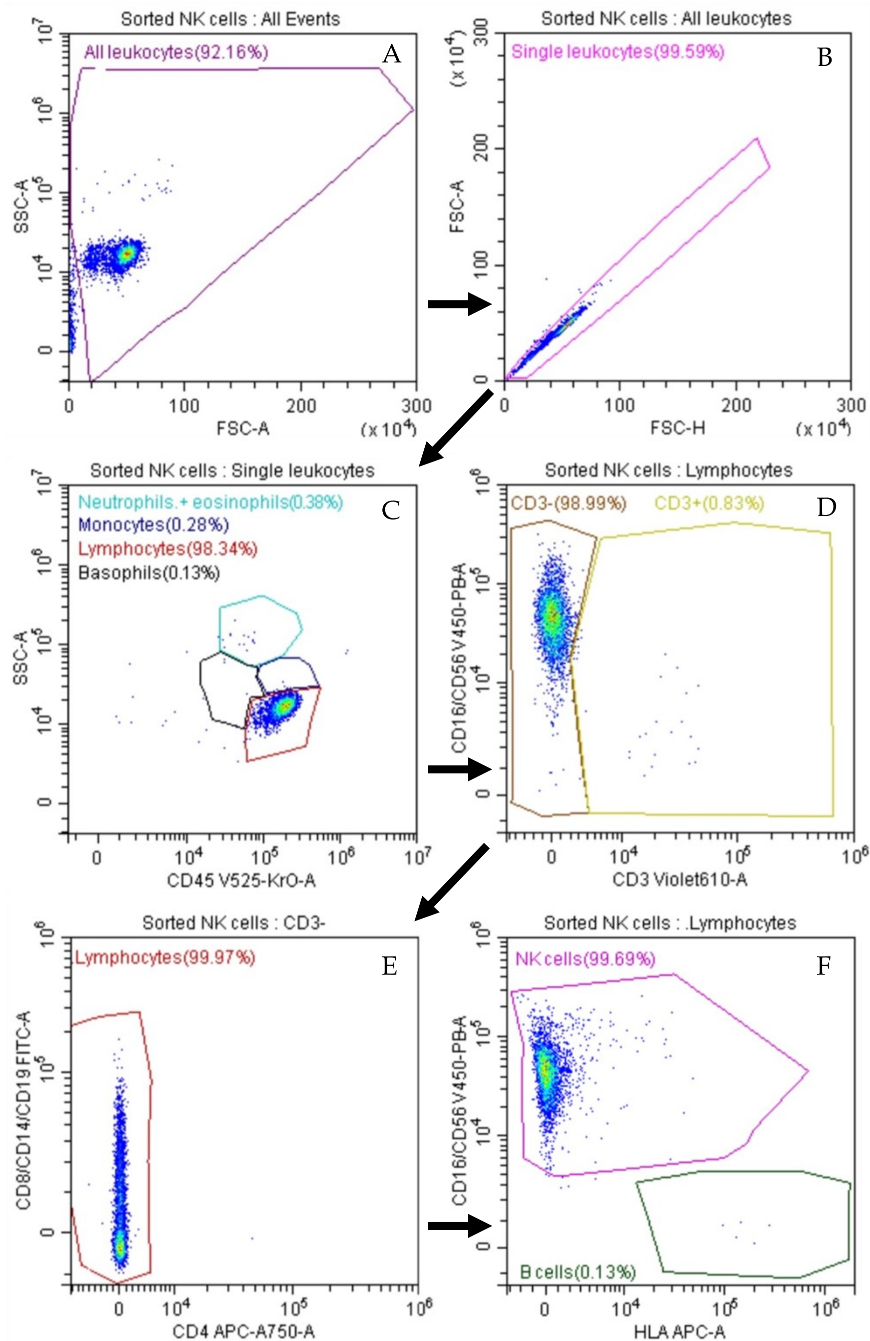

Figure 6. Pseudocolour plots of FACS analysis of the accuracy of NK cell sorting (A, C-F) and tendency of the cells to cling on each other (B). Axis scales, and titles are same is in Figure 2. The percentages of the cells inside each gate are also determined in a similar manner as described in Figure 2.

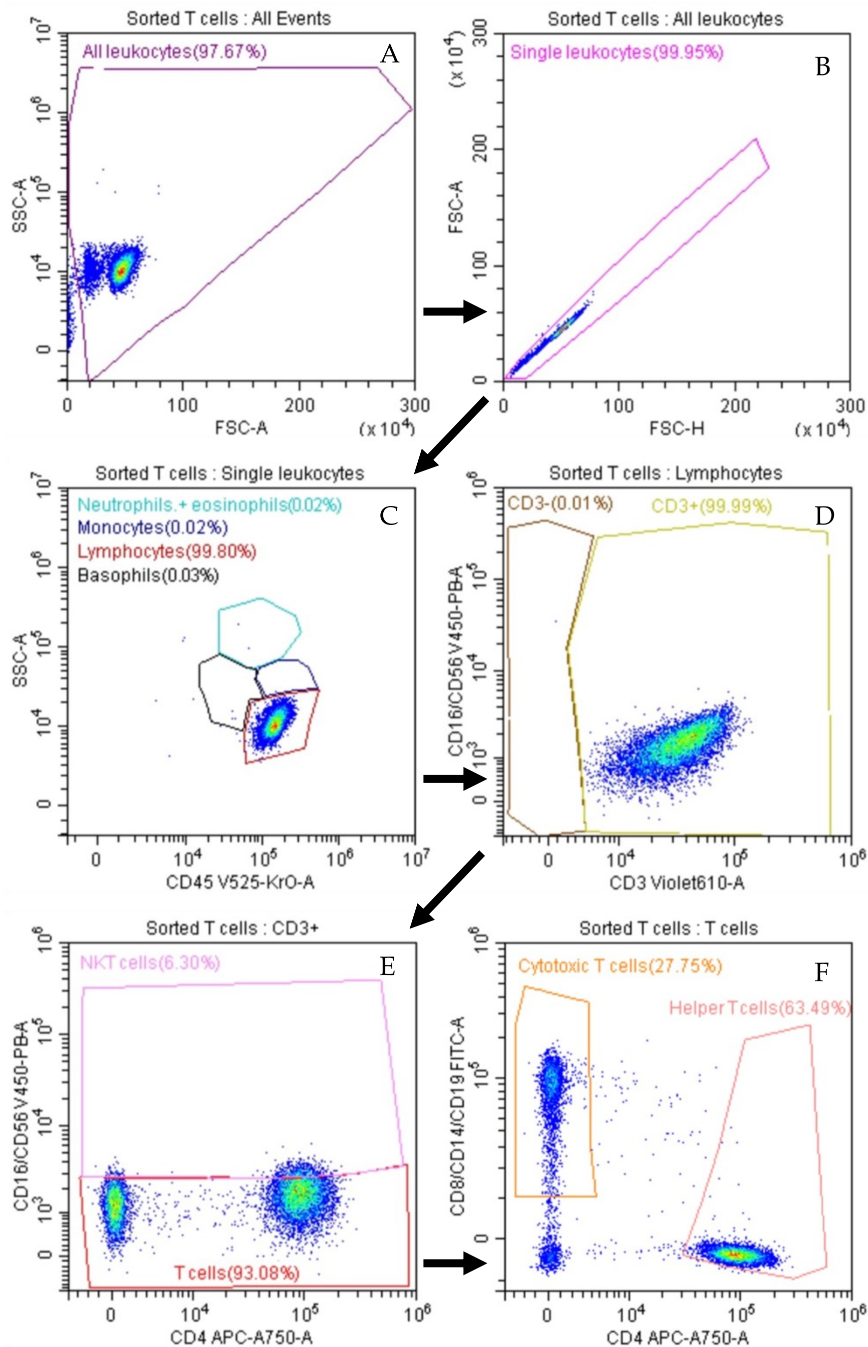

Figure 7. Pseudocolour plots of FACS analysis of the accuracy of T cell sorting (A, C-E) and tendency of the cells to cling on each other (B) in sorting set 2. In addition, proportion of T cell subtypes, cytotoxic and helper T cells of T cell gate could be analysed and is presented (F). Axis scales, and titles are same is in Figure 2. The percentages of the cells inside each gate are also determined in a similar manner as described in Figure 2.

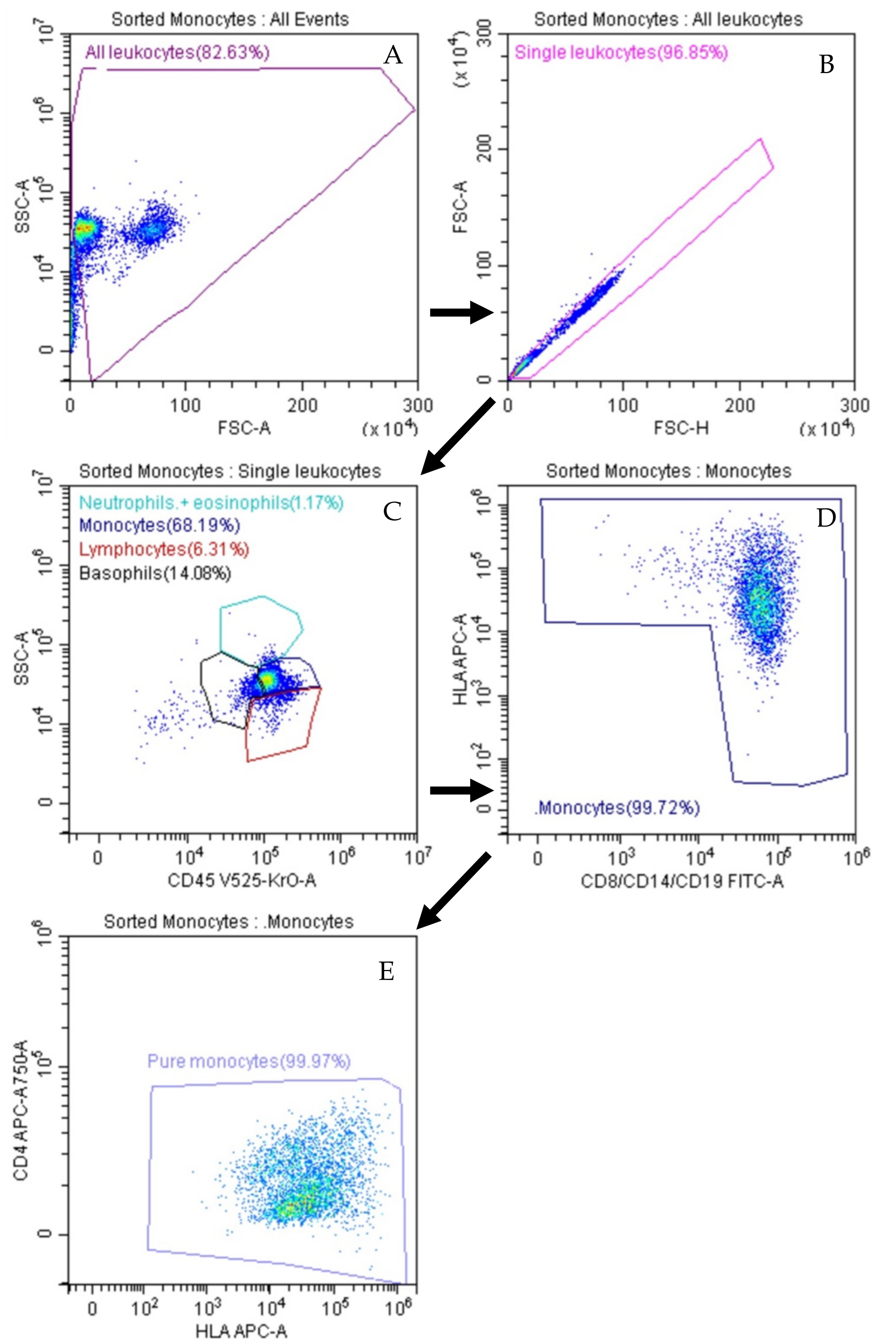

Figure 8. Pseudocolour plots of FACS analysis of the accuracy of monocyte sorting (A, C-E) and tendency of the cells to cling on each other (B). Axis scales, and titles are same is in Figure 2. The percentages of the cells inside each gate are also determined in a similar manner as described in Figure 2.

3.3 Yield variations of miRs in RNA extraction

Since challenges were encountered in isolating the required amount of miRNA from sorted subpopulations of leukocytes, it is deemed necessary to compare the yields of different isolation batches here, so that these findings can potentially aid in selecting the most efficient RNA isolation method in the future. The RNA

yields of leukocyte subpopulations and total leukocyte samples, and kits (1-3) used for extraction are represented in Table 9. The highest yields of miRs were obtained in batch 1 for neutrophils, eosinophils, and monocytes. The yield of miRs from NKT cells was two to three times higher with kit 2 than kit 1. The best kit for miR extraction from NKT cells was possibly kit 2 as the miR yield was two to three times higher than with kit 1. The last kit (3) used provided the highest RNA concentrations from B cells, NK cells and T cells. These concentrations were clearly higher than any of the previous ones for B and T cells. However, it should be further noted that the columns used in this kit combination extracted all RNA and not just miRs.

TABLE 9. Total RNA or micro-RNA/RNA yields (C) of different leukocyte subpopulations and total leukocyte sample, corresponding RNA extraction Kit (1-3 **) and information on whether only miRs or all total RNA was extracted.

Sample*	Batch 1			Batch 2			Batch 3		
	C (ng/ ul)	Total RNA/ miRs only	Kit	C (ng/ ul)	Total RNA/ miRs only	Kit	C (ng/ ul)	Total RNA/ miRs only	Kit
TL1	31,9	miRs only	1	11,4	miRs only	1	43,6	miRs only	2
N	9,9	miRs only	1	3,2	miRs only	1	5,2	miRs only	2
E	18,0	miRs only	1	2,4	miRs only	1	5,4	miRs only	2
T1	13,4	miRs only	1	1,2	miRs only	1	-	miRs only	2
NKT	2,2	miRs only	1	2,7	miRs only	1	6,0	miRs only	2
TL2	29,3	miRs only	1	20,7	miRs only	1	53,4	total RNA	3
B	3,2	miRs only	1	4,5	miRs only	1	30,3	total RNA	3
NK	19,3	miRs only	1	2,0	miRs only	1	20,8	total RNA	3
T2	8,9	miRs only	1	7,3	miRs only	1	24,8	total RNA	3
M	36,8	miRs only	1	5,1	miRs only	1	24,5	total RNA	3

*TL1 = unsorted total leukocyte sample from set 1 containing all leukocyte types, N = neutrophils, E = eosinophils, T1 = T cells from set 1, NKT = NKT cells, TL2 = unsorted total leukocyte sample from set 1 containing all leukocyte types, B = B cells, NK = NK cells, T2 = T cells, from set 2, M = monocytes. **1 = RNEasy Plus Mini Kit, Qiagen, 2 = MiRNeasy Mini Kit, Qiazol, 3 = MiRNeasy Mini Kit, Qiazol, but Mini Spin columns replaced with UCP MinElute Spin columns.

Drastic alteration in the obtained concentrations of miRs was detected between batches 1 and 2 despite no changes being made in the protocol. When comparing Tables 8 and 9, the miR concentrations did not seem to be dependent on the

number of sorted cells. Interestingly, in the first batch, the miR concentrations in eosinophils, NK cells, and monocytes were the highest (Table 9) even though the yields of these cells in sorting were among the lowest (Table 8). On the other hand, the yields of neutrophils were the greatest of leukocyte types (Table 8). Still, the concentration of miRs in them was half lower than in eosinophils in batch 1 for example, and relatively low also in other sorted batches (Table 9).

3.4 Leukocytes did express LS cmiRs

All studied LS cmiRs were expressed in total leukocyte samples collected after RBC lysis, which thus included all the leukocyte types present in that sample (Figure 9). The greatest relative expression levels were seen with miR-320a in the total leukocyte sample as well as in leukocyte subpopulations. However, the range of expression was wide and the higher expression values obtained from the other repetition were significantly higher, thus increasing the average. Other miRs were either not expressed in subpopulations or the expression level was very small.

Let-7e-5p was detectably expressed in neutrophils, eosinophils and NKT cells. Minor expression could be seen in NK and T cells. MiR-339-5p and miR-15a-5p were expressed only slightly in some leukocyte subpopulations: NK cells and eosinophils showed slight expression of miR-339-5p and miR-15a-5p expression was detectable only in NKT cells. B cells and monocytes did not seem to express any other miRs except for miR-320a. Monocytes even presented the second highest expression level of that miR compared to other leukocyte subpopulations.

Especially neutrophils seemed to express miR-320a (Figure 9). The average relative expression of this miR was even greater in the neutrophil population than in the total leukocyte sample used as a reference. Neutrophils were also the greatest source of miR-451a.

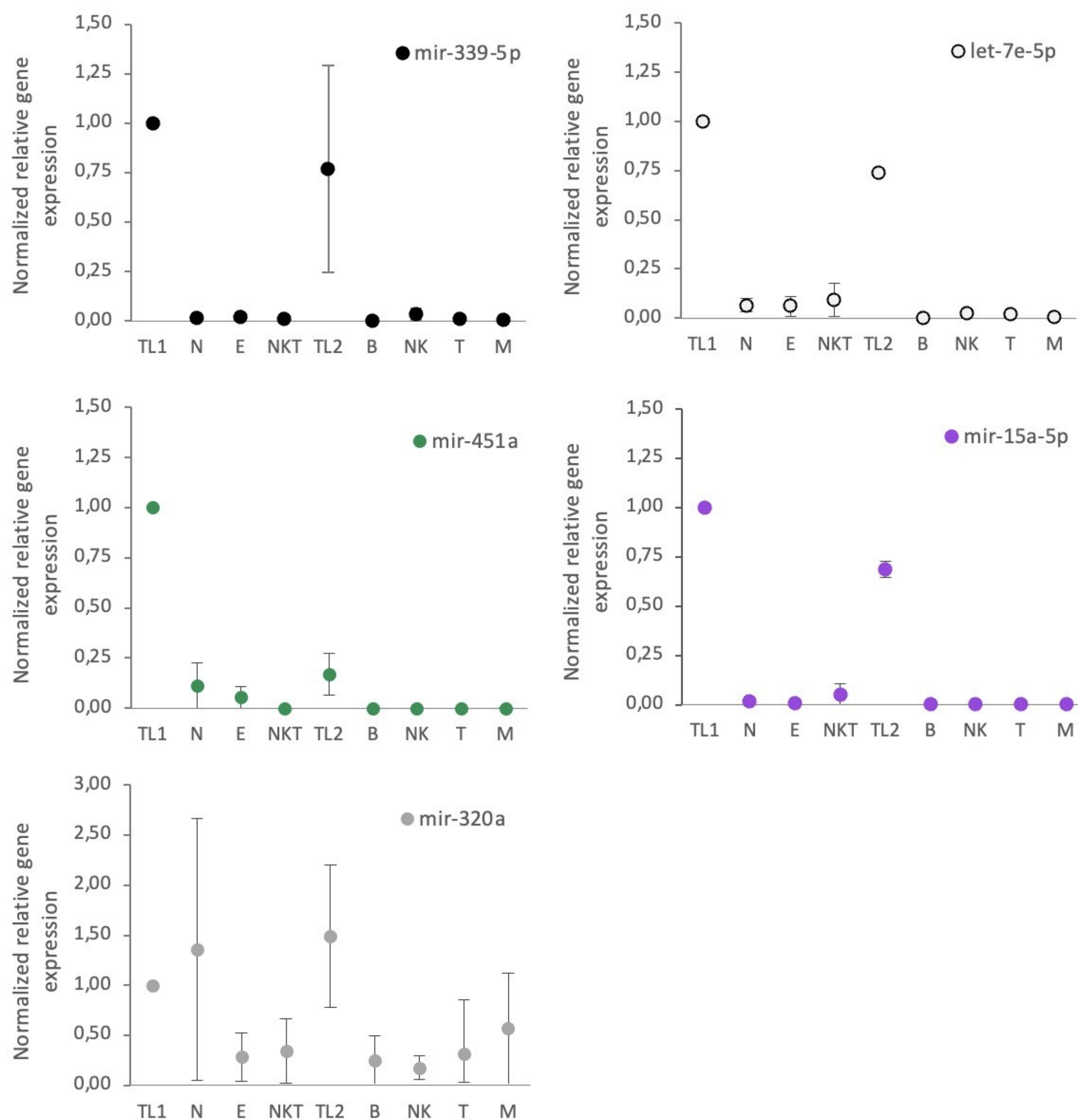

Figure 9. Average values and ranges of the normalized relative gene expressions of studied miRs in leukocyte subpopulations. Ranges are results from two separate qPCR analysis for all leukocyte types except for T cells. The average gene expressions of miRs in T cells includes in total of three separate measurements and the range represents the highest and the lowest values of these. TL1 = total leukocyte sample from sorting set 1, N = neutrophils, E = eosinophils, NKT = NKT cells, TL2 = total leukocyte sample from sorting set 2, B = B cells, NK = NK cells, T = T cells, M = monocytes.

3.5 miR-15a-5p did not cause significant change in IL-1β expression in LPS induced leukocytes

Relative gene expression of IL-1β in cultured human leukocytes is presented in Figure 10. As the qPCR did not give reliable results from the third repetitive test, the results are only shown from two of the repetitions. Figure 10 indicated clearly that LPS indeed successfully triggered the IL-1β gene expression. However,

lipofectamine inhibited the inducement of the expression. The variation between the two repetitions of this experiment was immense and partly contradictory: In the first test the miR-15a-5p increased the gene expression slightly in comparison to lipofectamine + LPS control but not as much as the negative control miR. In the second test, the expression level was lowest in lipofectamine + LPS treated leukocytes, and miR-15a-5p increased the gene expression more than the negative control miR or the LPS alone.

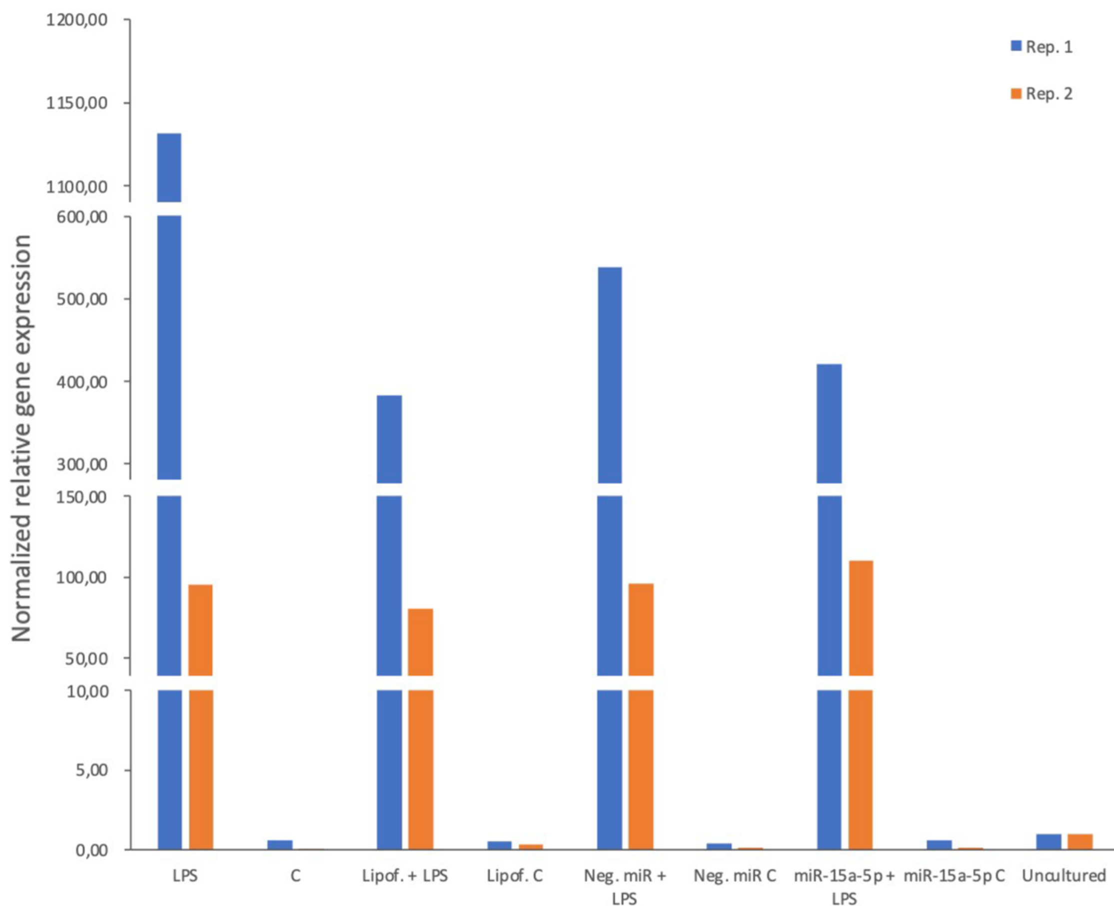

Figure 10. Relative gene expression of IL-1β in human peripheral blood leukocytes treated with LPS only; Lipofectamine and LPS; lipofectamine, negative miR and LPS; lipofectamine, miR-15a-5p and LPS and all previously mentioned without LPS as well as in uncultured leukocytes. Values of the two repetitive tests are presented of prepared leukocyte culture. Abbreviations: C = control (no LPS), lipof. = lipofectamine, neg. miR. = negative control miR, Rep. 1 = repetition 1, Rep 2 = repetition 2.

The visual estimation of the number of living cells with a light microscope showed each well still included a significant and approximately equal number of live cells. The microscopical observations done daily and after LPS treatment indicated no contaminations but showed a decrease in confluence towards the end of the culturing. Most of the cells were detected to attach to the bottom of the wells.

4 DISCUSSION

4.1 FACS assay set-up is sufficient for future experiments

This study provides the optimized protocol for leukocyte subpopulations sorting and the miR expression analysis in these populations, suitable and ready for use for future studies.

The aim of flow cytometry experiments was to distinguish and sort living HPBL, and results clearly demonstrated this aim was achieved. HPBL viability was shown remained high, and several leukocyte subpopulations were successfully separated with a purity rate of over 80 %. The current protocol and used antibody panel can thus be stated excellent for sorting these cell types including neutrophils, T cells, B cells, and NK cells. Successful separation of various leukocyte types enables further studies with pure populations. Additionally, in the future studies, variations in the numbers of leukocyte subpopulations between different study groups, such as between healthy and diseased individuals can be examined. Also, sorted cell populations could be cultured as the cells remain alive during the procedure and various properties of the specific leukocyte types studied *in vitro* cultures.

However, the purity of eosinophil, monocyte and NKT cell sample was inferior. A significant proportion of T cells detected during the purity test in the sorted NKT cell population was most likely caused by the closeness of NKT and T cell populations and gates drawn in flow cytometry analysis. To improve the purity of NKT cell population the gates could be drawn further away from each other by leaving space between the edges of the gates. As a drawback, this would cause a significant decrease in number of the sorted NKT cells. While NKT cells are quite rare in circulation, in this experimentation gates were drawn more broadly.

For the other leukocyte subpopulations, the conclusions for causes of poorer purity are not as straightforward. The purity was the smallest in the leukocyte subpopulations that were the rarest in the sorting sample, except for B cells, suggesting that the sorting accuracy might depend on sorted cell population size (Tables 7 and 8). The waiting time between the start of the first sorting and reanalysing the sorted samples in purity tests is also one possible error source. During the long waiting time, it is possible that the antibodies started to detach from the cells, or that receptor proteins which were targeted by antibodies were degraded by intracellular mechanisms, resulting in decreased purity upon reanalysis. Part of the eosinophils may also have gone through apoptosis following the breaking of the cells which could lead to an increased amount of debris and possibly dimmer fluorescence of the antibodies. Wen and colleagues (2013) have reported eosinophil half-life to be as short as only few hours in blood, and a relatively short lifespan has been suggested previously as well (Walsh et

al. 1998, Park and Bochner 2010). However, good viability results have also been reported in eosinophil cultures even after 24 h (Cao et al. 2020). Nevertheless, without further experiments it cannot be stated whether the reason for inferior accuracy in purity tests relies on the flow cytometer, detachment of the antibodies or apoptosis of these leukocyte types or somewhere else.

One more improvement for this assay would be increasing the time-efficiency. Currently, the designed protocol requires the cell sorting to be done within the same day of leukocyte purification resulting in long working hours. To further improve user-friendliness and applicability, it would be beneficial to optimize a step for freezing and storing the purified leukocyte samples. Thus, the FACS assay could be executed at a later date.

4.2 Leukocytes are a potential source of studied LS cmiRs

This study showed that all the studied cmiRs are expressed in human leukocytes. The expression of all studied cmiRs was well detectable in total leukocyte populations, providing evidence that these LS cmiRs are expressed in human leukocytes. Recently, an increasing number of studies have investigated cmiR expression in leukocytes in various contexts and shown leukocytes to have distinguishable cmiR profiles compared to other cell types (Kang et al. 2022, Kauschke et al. 2023, Steinberg et al. 2023, Chandler et al. 2023). In certain research conditions, miR levels are more appropriate to study directly from leukocyte extracts rather than blood plasma, for example (Kauschke et al. 2023).

The reason why most of the studied cmiRs were only slightly or not detected in leukocyte subpopulations yet showed well detectable expression in total leukocyte samples is uncertain. The total leukocyte samples included only a minor number of platelets. Therefore, they can be excluded as an origin of the detected expression. As all the leukocyte subpopulations were not sorted in this experiment it is also possible that these cmiRs are more highly expressed in those non-sorted populations, like basophils, for example.

Even though RT-qPCR is often used in similar experiments, it might not be sensitive enough to detect the expression levels from a small amount of the template miR. The expression levels of miR-451a, let-7e-5p, miR-339-3p, and miR-15a-5p could instead be analysed with digital droplet PCR that is more sensitive and sufficient to detect even small amounts of template in the samples (Mao et al. 2019).

4.3 miR-320a is expressed by different leukocyte types

The expression of miR-320a was predominantly detected in all leukocyte subpopulations in this study. In literature, miR-320a has been reported to be associated with different diseases and especially cancers. Researchers have indicated it to be downregulated, and thus tumour suppressive miR in cancers such as hepatocellular carcinoma, CRC, and non-small cell lung cancer (Hao et al. 2020, Zhang et al. 2021, Khandelwal et al. 2021). Zhang and colleagues (2021)

have linked it to signalling pathways restricting cell proliferation. Khandelwal and colleagues (2021) transfected mimic- and inhibitor-miR-320a into human lung cancer cells and reported decreased apoptosis levels in the inhibitor-transfected cells and increased apoptosis in mimic-transfected cells. They also indicated that the inhibition of miR-320a elevated cell migration and invasion potential, and that mimic-miR-320a caused opposite effects. In LS, circulating miR-320a-3p was shown to be downregulated in healthy LS carriers when compared to healthy non-LS-carriers demonstrating the potential role already in cancer predisposition (Sievänen et al. 2022).

Fortunato and colleagues (2019) have studied cmiR expression in epithelial and blood cells of lung cancer patients. They found several leukocyte types to express different cmiRs including miR-320a. Increased expression of miR-320a in granulocytes of high-risk lung cancer patients was also reported. Furthermore, Fortunato and research fellows (2019) studied the association of the miR-320a to leukocytes; they suggested neutrophils to secrete miR-320a and the secretion to contribute macrophage polarization into M2 type that supports cell proliferation. This is in line with the results of this study that suggested neutrophils to potentially be one of the main sources of miR-320a.

4.4 IL-1β expression not affected by miR-15a-5p in peripheral blood leukocyte culture

One aim of this work was also to investigate whether miR-15a-5p regulates IL-1β expression in human leukocytes. Currently, released research articles support the conclusion that miR-15a is associated with inflammatory signalling and could restrict or decrease the inflammatory reactions. For example, miR-15a has been linked to the inflammatory signalling pathway of nuclear factor kappa B and Wnt/β-catenin signalling pathway (Wang et al. 2017, Lou and Huang 2020, González-López et al. 2023). Inflammatory processes are regulated through both of these pathways individually and in cooperation (Ma and Hottiger 2016, Liu et al. 2017, Liu et al. 2022). In addition, miR-15a-5p has been shown to regulate CD8+ T cell and NK cell-mediated anti-tumour responses in neuroblastoma (Pathania et al. 2022). These evidences strongly suggest that miR-15a can be an important factor in inflammation related reactions.

The results of this study showed great variation and even contradiction between repetitions. Lou and Huang (2020) have studied the molecular mechanisms associated with the function of miR-15a-5p in sepsis in a very similar manner as used here. In their study mouse macrophage line RAW264.7 cells were cultured in similar conditions as the primary leukocytes in this study, but in a slightly different medium. Where in this thesis miR-15a-5p was transfected into the cells Lou and Huang (2020) inhibited the expression of miR-15a-5p. Transfected cells were cultured 48h after which similarly to this thesis work, the cells were stimulated with LPS for 4 h to trigger the expression and production of inflammatory response related substances including IL-1β. Enzyme-Linked Immunosorbent Assay (ELISA) kits were used to observe the expression level of

IL-1β in the culture supernatant. The expression of miR-15a-5p in macrophages was determined by extracting RNAs, conducting cDNA conversion and then qPCR.

The results of Lou and Huang (2020) indicated that LPS alone increased the IL-1β expression level significantly, which was also observed in this work. In addition, Lou and Huang (2020) reported that the inhibition of miR-15a-5p decreased the IL-1β expression. This could suggest that in contrast miR-15a-5p induces IL-1β expression which was partly detected in this work (Figure 10). In another study, Ye and colleagues (2016) transfected miR-15a into retinal endothelial cells and showed that miR-15a diminished IL-1β levels compared to negative miR control and non-miR-transfected control. A similar effect was also detected in IL-1β gene expression in other repetitions of this study but in clearly smaller scale. As Ye and colleagues used different cell type and cultured them in high glucose concentration, results are not straightforwardly comparable to the ones obtained in this work.

Both Lou and Huang (2020) and Ye and research fellows (2016) used higher concentrations of LPS and transfected miR than those used in this work, which suggests the miR concentration chosen for this study can be too low to induce regulatory effect. To test this hypothesis dilution series of the miR should be introduced to leukocyte cultures in conjunction with this assay.

Additionally, in both previous studies the scientists observed the IL-1β protein levels directly while in this experiment the expression of mRNA levels of IL-1β were examined. According to literature the correspondence of the levels of mRNA and protein product are mostly well correlated (Maier et al. 2009, Edfors et al. 2016). In LPS triggered protein synthesis, it has been shown that up-regulation of mRNA of immune response proteins leads to increased protein expression levels (Jovanovic et al. 2015). This indicates that an increase in the amount of mRNA and the cytokine itself should be detected. However, it has also been demonstrated that in an induced changing state of a cell the amount of protein product can increase slower than that of mRNA (Eichelbaum & Krijgsveld 2014, Jovanovic et al. 2015). Therefore, it could be possible that the transcription activity and levels of RNA corresponding to IL-1β were higher earlier than at a timepoint measured here. In the light of the results miR-15a-5p cannot be confirmed to have impact on IL-1β gene expression under these experimental conditions.

4.4.1 Sensitivity of IL-1β and lack of neutrophils and eosinophils as main challenges

While observing the primary leukocyte cultures with a light microscope a shock caused by lipofectamine and transfection of miRs into the leukocyte cultures, similarly to LPS treatment, seemed to cause decrease in cell confluence. A significant part of the dead cells were likely neutrophils and eosinophils. The commonly accepted estimations of the life spans of these cell types in human blood are less than a day (Park and Bochner 2010, Farahi et al. 2012, Lahoz-Beneytez et al. 2016). Some findings have opposingly suggested neutrophils to

live even several days as demonstrated by Pillay and research fellows (2010) and comprehensively reviewed by Hidalgo and colleagues (2019). However, it is likely that most neutrophils or eosinophils did not remain alive longer than ~24 h in culturing circumstances. Therefore, it is possible that miR-15a-5p affects the regulation of IL-1β expression in neutrophils and eosinophils. Other leukocyte types have a minimum lifespan longer than the culturing experiment in total and it is safe assume that they were represented in the culture during the whole experiment.

A challenge with studying the expression of IL-1β is the sensitivity of it for any changes in cell culture. As the leukocytes for each repetitive tests were obtained from different individuals the relative sizes of the leukocyte subpopulations were indeed different. As can be detected from FACS experiment (Table 7) and the reference values of human peripheral blood leukocytes (Table 1), individual variation can be relatively great. In addition, secretion pathways of IL-1β are still not fully understood, but several different pathways are described to associate (Rébé and Ghiringhelli 2020). The response time for stimuli in different pathways may vary causing more variables affecting the outcome reported here.

4.4.2 Future improvement suggestions for IL-1β expression assay

In the light of previously mentioned weaknesses of the LPS triggered IL-1β expression experiment more repetitions are required to better take into account the natural variation of relative sizes of leukocyte subpopulations in cultures. Preparing several similar cultures on the same time from the same blood sample could also reduce variation detected in this study. Additionally, as suggested before, different concentrations of the transfected miR between the previously reported and the concentration used in this study should be examined to reveal the dependence in regulatory effect.

Whether miR-15a-5p could regulate expression of IL-1β in neutrophils and eosinophils remained unstudied were they absent in the culture. A laboratory cell line of neutrophils is likely required for repeating the experiment for neutrophils as they can be cultured for longer time periods. By combining this assay and the FACS assay optimized here for leukocyte sorting this study could be repeated to peripheral blood leukocyte subpopulations separately. Even though under these experimental conditions miR-15a-5p did not seem to regulate IL-1β gene expression in primary leukocyte cultures, other LS cmiRs could possess regulatory abilities. This assay can be used to reveal the potential regulatory relations between peripheral blood leukocytes or leukocyte subpopulations and IL-1β. In addition, it is applicable for other cytokines and regulatory proteins as a relatively easy and accessible method.

5 CONCLUSIONS

This study successfully optimized assay tools for identifying and sorting HPBL subpopulations. The study also demonstrated that miR-320a, miR-451a, let-7e-5p, miR-339-5p and miR-15a-5p, previously identified in LS serum samples are expressed by leukocytes. Among these, miR-320 was notably the highest level expressed. miR-320a may have a functional role in cancer regulation, as suggested by the literature. In addition, the study investigated whether miR-15a-5p could regulate IL-1β expression in HPBL cultures, as it has also been linked to inflammatory response pathways in previous studies. However, the results suggested that, under these experimental conditions, miR-15a-5p did not participate in the regulation of this cytokine during LPS-induced stress. The experiment set-up used here proved to be functional for analysing IL-1β expression through mRNA levels. However, more studies are required to confirm the miR-15a-5p association in IL-1β expression regulation in human leukocytes, especially in neutrophils that were not represented in the experiment.

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Jyväskylä April 20, 2024
Henna-Riikka Littunen

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APPENDIX 1. MIR PRIMERS AND MIRS

Information of miR primers used in qPCR assays:

miR-339-3p: hsa-miR-339-3p, YP00206007, miRCURY miRNA Assay, Qiagen

miR-451a: hsa-miR-451a, YP02119305, miRCURY miRNA Assay, Qiagen

miR-320a: hsa-miR-320a, YP00206042, miRCURY miRNA Assay, Qiagen

let-7e-5p: hsa-let-7e-5p, YP00205711, miRCURY miRNA Assay, Qiagen

miR-15a-5p: hsa-miR-15a-5p, YP00204066, miRCURY miRNA Assay, Qiagen

miR-39-3p: cel-miR-39-3p, YP00203952, miRCURY miRNA Assay, Qiagen

Information of the miRs transfected in leukocytes in IL-1β expression assay:

miR-15a-5p:
miRIDIAN microRNA Human hsa-miR-15a-5p - mimic, 2 nmol, C-300482-03-0002, Horizondiscovery

Negative control miR:
miRIDIAN microRNA Mimic Negative Control #1, 5 nmol, CN-001000-01-05, Horizondiscovery

APPENDIX 2. REAGENTS

Information of the reagents used for RBC lysis buffer:

disodium EDTA:	EDTA disodium salt dihydrate, cat: 34549-100G, Honeywell, Germany
$NaHCO_3$:	cas: 31437, 500g, Riedel-de Haën, Sigma Aldrich Laborchemikalien
NH_4Cl:	cas: 31107, 1kg, Riedel-de Haën, Sigma Aldrich Laborchemikalien

Information of other reagents

Bovine serum albumin (BSA):	cat: 0332-100G, Amresco, Ohio, USA