MICRORNA SPECIES REGULATE IMMUNE PROCESSES: A FOCUS ON NK CELLS IN CHRONIC ARTHRITIS

by

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DEDICATION PAGE

This thesis is dedicated to my grandfather, Stafford Yearwood, a man who embraced life, who never hesitated to help others and was always there to support and encourage me; my biggest cheerleader. I will always cherish the time we had together.

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ABSTRACT

Rheumatoid arthritis (RA) is a common chronic autoimmune/inflammatory disease involving innate/adaptive lymphocytes and soluble mediators. Natural killer (NK) cells are innate lymphocytes that direct immunity through cytotoxicity and cytokine production. The contributions of NK cells are ill-defined in RA, although their function(s) may be regulated by microRNAs (miRNAs). MiRNAs are non-coding RNAs that inhibit mRNA. To investigate the miRNA-NK cell interplay, we optimized highly efficient transfection techniques in serum-free media for enhancing or interrupting NK cell miRNA signaling. We targeted miRNAs that are expressed differently in patients with RA compared to healthy individuals. Our findings suggest that miR-146a-5p and miR-155-5p, two miRNAs upregulated in RA, reduce NKG2C expression, a hallmark of adaptive NK cells. We expect chronic overexpression of these miRNAs inhibits adaptive NK cells and their ability to eliminate auto-reactive B/T cells through antibody dependent cellular cytotoxicity (ADCC), thereby reducing treatment efficacy for therapies that rely on effective ADCC.

LIST OF ABBREVIATIONS AND SYMBOLS USED

3p	3 prime
5p	5 prime
ADCC	Antibody dependent cellular cytotoxicity
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
BRM	Biologic response modifiers
cDNA	Complementary DNA
CTV	Cell tracer violet
ddPCR	Droplet digital PCR
DMARD	Disease modifying antirheumatic drugs
FBS	Fetal bovine serum
FCS	Flow cytometry standard
FLS	Fibroblast-like synoviocytes
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
IKK	IκB kinase
IL	Interleukin
IMF	Immunofluorescence buffer
IL-R	Interleukin receptor
IRAK1	Interferon regulatory-associated kinase 1
IRF4	Interferon regulatory factor 4
ITIM	Immunoreceptor tyrosine-based inhibiting motif
IWK	Izaak Walton Killam
JIA	Juvenile idiopathic arthritis
KIR	Killer immunoglobulin-like receptor
LIR1	Leukocyte Ig-like receptor 1
LNA	Locked nucleic acid
mAb	Monoclonal antibody
MCMV	Murine cytomegalovirus
MICA/B	Major histocompatibility complex class 1 polypeptide-related sequence A/B
miRNA	MicroRNA
MFI	Mean fluorescent intensity
NCR	Natural cytotoxicity receptor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NKG2	Natural killer group 2
NSAIDS	Non-steroid anti-inflammatory drugs
OA	Osteoarthritis
P/S	Penicillin and streptomycin
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffer saline
PLZF	Promyelocytic leukaemia zinc finger protein
pp150	Phosphoprotein 150
pri-miRNA	Primary miRNA
RA	Rheumatoid arthritis
REACH	Restitution and Enhancement in Arthritis and Chronic Heart disease
RISC	RNA induced silencing complex
RTqPCR	Real-time quantitative polymerase chain reaction
RTX	Rituximab (anti-CD20)
SHIP-1	SH-2 containing inositol 5’ polyphosphatase 1
SOCS1	Suppressor of cytokine signaling 1
STAT	Signal transducer and activator of transcription
TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor
TRAF6	TNF receptor associated factor 6
UTR	Untranslated region
α	Alpha
β	Beta
κ	Kappa
γ	Gamma
x*g*	Gravity
ζ	Zeta
°C	Degrees Celsius
g	Grams
rpm	Rotations per minute
ng	Nanogram
nM	Nanomolar
μg	Microgram
μL	Microliter
μM	Micromolar
mL	Milliliter
mM	Millimolar
IU	International units

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CHAPTER 1: INTRODUCTION

1.1 Chronic Inflammation

In healthy individuals, the inflammatory response is essential for proper immune functioning and maintenance of homeostasis. The recognisable, classical signs of inflammation include swelling, increased temperature, pain, redness, and loss of function[1]. This response is aimed at recruiting immune cells, removing pathogens, and promoting tissue regeneration[2]. Despite its vital role in maintaining health, unwarranted and prolonged inflammation, originating from either pathogenic or non-pathogenic triggers, can lead to a variety of chronic inflammatory diseases and increase the risk of several systemic comorbidities, including but not limited to cardiovascular diseases, autoimmunity, and cancer[3,4]. Our research focuses on chronic autoimmune joint inflammation, herein simplified as chronic/autoimmune arthritis. Specifically, the chronic arthritis disease of interest in our research is rheumatoid arthritis (RA).

1.2 Autoimmune arthritis

1.2.1 Clinical features of rheumatoid arthritis

RA is one of the most common chronic heterogeneous inflammatory diseases, with overlapping pathophysiology but variable immunobiology. It affects approximately 1% of the worldwide population[5]. It is characterized by local inflammation of the joint synovium and contributes to the deterioration of both bone and cartilage tissue, causing pain, stiffness, restricted range of motions, and joint deformities. Approximately 40% of patients experience systemic symptoms which include low grade fever, fatigue, and

stiffness[3]. Patients suffering from RA are also at an increased risk for developing atherosclerosis and cardiovascular complications[6,7]. Taken together, patients with RA experience a range of pathological symptoms and medical complications.

Apart from the far-ranging symptoms and systemic organ involvement, RA also impacts the quality of life in patients. Compared to healthy individuals, patients with chronic RA reported having decreased quality of physical functioning, general health, vitality, social functioning, and mental health[8]. These, along with the physical symptoms, are indicative of the reduced quality of life experienced by patients as a result of chronic RA disease.

1.2.2 Arthritis immunopathology

The immune mechanisms responsible for the initiation and maintenance of arthritis diseases are heterogeneous and not necessarily consistent between patients. It is expected that protein modifications by citrullination in pre-RA stages and the production of antibodies targeted against these proteins permits the emergence of autoimmunity[7,9]. Additionally, rheumatoid factors, autoantibodies that bind the Fc region of IgG, form immune complexes with IgG and contribute to disease pathology in seropositive patients[10]. Current evidence suggests that these processes are at least partly responsible for joint specific symptoms such as bone loss and arthralgia (**Figure 1.1**)[11]. Other immunological mediators of inflammation include the increased production of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor (TNF), and IL-18, as well as infiltration of autoreactive B and T cells and innate immune cells to the sites of inflammation, including natural killer (NK) cells (**Figure 1.1**)[10,12–14]. In the early

phases of RA and other forms of arthritis, stromal cells, including fibroblast-like synoviocytes (FLS), play crucial roles to the onset of disease including cytokine and chemokine release as well as cartilage destruction[6,13]. In fact, FLS cells may play a role in antigen presentation and contribute to the local adaptive immune response[7]. Meanwhile, osteoclasts, stimulated by the local inflammatory environment, erode the bone[6]. Innate (macrophages, mast cells, dendritic cells, neutrophils, and NK cells) and adaptive immune cells (B and T lymphocytes) also infiltrate the joint[6]. These cells cause further damage and swelling mainly through cytokine production (TNF, IL-1, and IL-6)[12,15] and auto-antigen driven immune reactions, respectively. High levels of circulating cytokines are thought to drive arthritis co-morbidities including increased cardiovascular risk, metabolic syndrome, osteoporosis, and increased cancer risk[7]. The inflammation leading to RA is multifactorial and only partially understood. Hence, strategies to understand and mitigate inflammation – strategies that will be identified through research – may enable better control of RA and its systemic pathology.

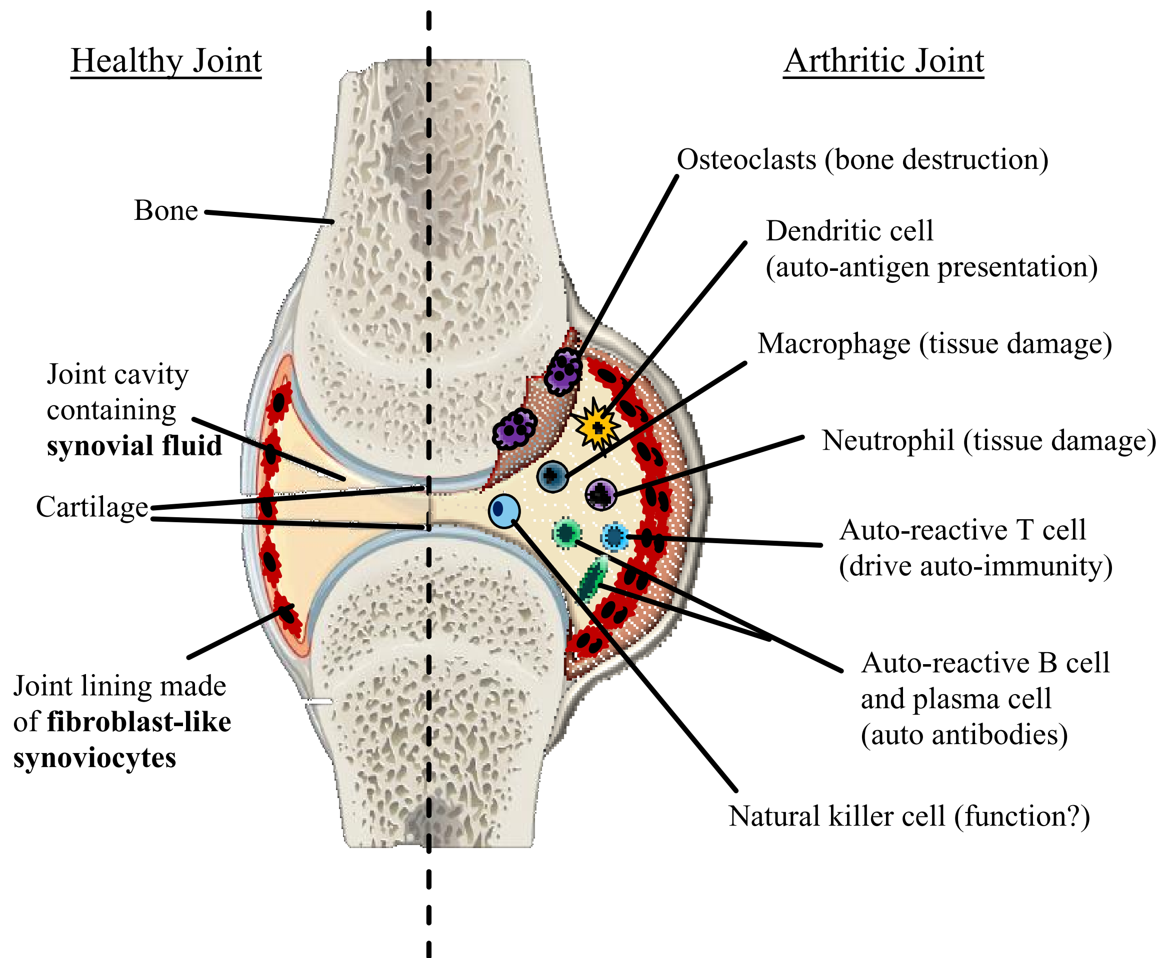

Figure 1.1 Healthy joint compared to an arthritic joint. Normal joint anatomical features are shown in the healthy joint (left) while infiltrating immune cells are shown in the arthritic joint (right).

1.2.3 Treatment approaches

Current treatment options for autoimmune arthritis have a broad range of effectiveness and specificity. Non-specific mediators include non-steroidal anti-inflammatory drugs (NSAIDS) and corticosteroids. NSAIDS, such as Ibuprofen, typically target pain and inflammation, while other NSAIDS, such as COX-2 inhibitors, are anti-inflammatory with fewer side effects. Corticosteroids broadly target T cell and macrophage function and decrease the production of pro-inflammatory cytokines. The obvious pitfall of these treatments is that they are not specific and therefore cannot directly target the immune cells or processes responsible for the inflammation specific to an individual patient. More specific treatments include disease-modifying antirheumatic drugs (DMARD) which are comprised of immunosuppressive drugs and biological response modifiers (BRM). The combination of these therapies has shown to be more effective than DMARD monotherapy when treating RA[16]. Conventional DMARD therapy, usually with methotrexate, functions by generally suppressing the immune system, thereby alleviating pain and limiting join damage[17]. BRMs have more specific immune targets and function to inhibit cytokines, cytokine receptors, or cellular activation[18]. BRMs are prescribed in combination with DMARD therapies such as methotrexate when DMARD monotherapy fails. Despite the various therapeutic options, and due to the heterogeneity nature of arthritis, many patients do not respond to therapy and some may have dangerous immune inhibiting side effects[16]. Furthermore, BRM treatments are expensive, thereby contributing significant financial burden on patients and health care systems alike. Annual direct and indirect health care costs for RA are estimated to be $12.6 and $39 billion, respectively[19,20]. The inability to confidently predict patient-specific treatments and the

high cost associated with treatments reinforces the requirement for further investigation of the cellular and genetic contributors of arthritis pathology.

1.2.4 Genetic and environmental risks of chronic arthritis

The risk of developing chronic arthritis, especially as an adult, is determined by complex interactions between both environmental and genetic factors[21–23]. A major environmental risk factor for RA is smoking, which has been proposed to contribute to protein citrullination, a form of post-transcriptional modification and a common target for auto-antibodies in the early stages of RA[11]. Other RA environmental risk factors include prolonged stress, obesity, and increased age[7,24].

In addition to environmental factors, genetic risk factors such as female biological sex and immunogenetics can also affect the likelihood of developing arthritis/inflammatory disease. Specific human leukocyte antigens (HLA), receptors necessary for the immune system to differentiate between the body's cells and foreign invaders, are common risk factors that allow for the prediction of arthritis disease outcomes[25]. The specific allelic variations in the class I and II HLAs, *HLA-B*08-Asp* and *HLA-DRB1(04)*, respectively have been linked to an increase in disease development, severity, and treatment response [21,23,25–28]. Of note, some variants, such as *HLA-DRB1(13)*, are more commonly found in healthy individuals and may play a protective role against RA and other autoimmune diseases[29]. Likewise, the class II HLA gene, *HLA-DRB1(11)*, is a strong risk factor for systemic juvenile idiopathic arthritis (JIA)[30,31]. Genetic variants in other receptors, such as the Natural Killer Group 2A, C, and D (NKG2A/C/D) receptors, have also been shown to influence arthritis susceptibility,

severity, and the ability to respond to treatments[5,32,33]. In Korean patients the combination of the NKG2 activating gene variants, *NKG2C102*Ser/*Ser* and *NKG2D72*Ala/*Ala*, were determined to increase the risk of RA 12-fold. Meanwhile, *NKG2C102*Phe/*Phe* in association with the same *NKG2D* variant was hypothesised to contribute to RA protection[32]. Moreover, genetic variants of *NKG2D* that disrupt NKG2D-dependent pathways have been correlated to increase autoimmune pathology and worsened anti-TNF treatment efficacy[5]. These findings imply that genetic variants in cellular receptors have a role in increasing the risk of RA, as well as providing tools to better predict patient responses to RA treatments.

Aside from receptors, genetic variants in CD3ξ, an intracellular membrane-bound signal transducer involved in NK cell activation, has also been associated with increased risk of RA[34]. In fact, patients with RA typically express lower CD3ξ than healthy individuals[34,35]. Thus, an individual's immunogenetics, through their allelic variation of *HLA*s and *NKG2A/C/D*, influences the risk, severity, and type of arthritis in a patient.

1.3 Natural Killer Cells

1.3.1 Introduction to NK cells

The roles and contributions of stromal cells, innate and adaptive immune cells in the pathology of chronic arthritis have been extensively reviewed[36]. However, the function of both local and circulating NK cells have not been thoroughly investigated, and their roles and interactions with signaling molecules abundant in arthritis remain to be fully elucidated. A better mechanistic understanding of NK cells in arthritis may enable

researchers and clinicians to precisely apply existing therapies and design new effective treatments.

Natural killer cells are innate immune lymphocytes that direct inflammation through the release of soluble mediators and targeted cellular cytotoxicity[37]. Traditionally, NK cells have been most studied for their roles as mediators of anti-viral and anti-cancer immunity[38,39]. NK cells have also demonstrated the ability to mediate and promote tolerogenic immunity in the context of pregnancy[37,40] while, paradoxically, permitting successful fetal growth by implantation and trophoblast invasion[41]. The ability of NK cells to mediate and coordinate pro-inflammatory and tolerogenic immune reactions suggests dual functions which could drive either inflammatory and/or tolerogenic reactions in arthritis and other inflammatory/autoimmune diseases. This dual functionality of NK cells may be a feature of their highly diverse subsets.

For the most part, human NK cells are phenotypically divided into $CD56^{dim}$ and relatively immature $CD56^{bright}$ subsets, which are mostly located in the periphery and secondary lymphoid tissues respectively[42–44]. $CD56^{dim}$ NK cells express high levels of CD16 (FcγRIIIA) and are highly cytotoxic, whereas $CD56^{bright}$ NK cells express low levels of CD16 and are primarily responsible for the release of cytokines and chemokines. In the context of RA, circulating $CD56^{dim}$ NK cells are positively correlated with better treatment response and reduced disease flares[45]. $CD56^{bright}$ NK cells are thought to contribute to disease mediating pro-inflammatory cytokines such as interferon gamma (IFNγ) and TNF in inflamed joints[44]. Thus, NK cells appear to have roles in both the promotion of autoimmune reactions in RA, as well as a protective role through tolerogenic immune mechanisms that remain to be fully elucidated.

1.3.2 NK cell immunogenetics and education

The functional capacities of NK cells differ between and within individuals because of variable expression of both activating and inhibiting receptors. Killer immunoglobulin-like receptors (KIRs) represent a family of surface NK cell transmembrane glycoproteins that are mostly responsible for inhibitory signaling when interacting with self-cells. The highly polygenic and polymorphic assortment of KIRs and their HLA ligands establishes an individual's immunogenetics which in turn governs NK cell education[46]. In this process, NK cells are rendered self-tolerant to avoid autoimmunity. NK cells found in the synovial fluid of patients with RA demonstrated a decrease in overall KIR expression suggesting that educated NK cells may play a protective role in RA[44,47].

Natural cytotoxicity receptors (NCRs) are type I membrane immunoglobulin superfamily proteins that stimulate NK cell effector functions. All NCRs are activating receptors that may detect internal changes, including stress and viral proteins, in damaged or infected cells[48,49]. The expression of these receptors may be reduced by NK cell stimulation with transforming growth factor (TGFβ), thereby reducing overall NCR-mediated cellular lysis[50,51]. The three NCRs of interest in our research are NCR1 (NKp46), NCR2 (NKp44), and NCR3 (NKp30). Of these receptors, NKp46 and NKp30 are expressed on both resting and activated cells, while NKp44 is particularly expressed on activated cells[51,52]. Due to the fact that NCRs can respond to cellular stress by activating the NK cells, it is possible that NCR activation could contribute to RA pathology.

The NK cell receptors and available ligands for those receptors shape the functional capacities of NK cells between and within donors, thereby contributing to high

inter donor functional and phenotypic variability. In the context of arthritis, the specific contributions of NK cell KIRs and NCRs in orchestrating or maintaining disease are poorly understood and require further investigation to determine their relevance in disease.

1.3.4 Adaptive NK cells

Despite their extensive roles in innate immunity, NK cells have recently demonstrated the ability to bind and use antibodies[37,52]. The NK cells best known for this function are referred to as adaptive NK cells (**Figure 1.2**). Adaptive NK cells may be expanded when exposed to cytokines (IL-12, IL-15, and IL-18)[53] or human cytomegalovirus (HCMV). HCMV seropositive individuals exhibit further increases in adaptive NK cell frequencies when infected with other viruses, including hantavirus, HIV, and chikungunya virus[54,55].

Adaptive NK cells were first identified as responders to murine cytomegalovirus (MCMV) in mice and shortly thereafter, an analogous population was identified in humans[38]. HCMV is highly prevalent, but relatively benign, in the healthy human population. Nevertheless, lifelong HCMV may have multifactorial impacts on human health including contributing to the manifestation and pathogenesis of chronic inflammatory diseases and the provision of heterologous innate immune responses against pathogens[56]. In the context of RA, HCMV infection may induce an expansion of autoreactive CD4+/CD28- T cells[57,58]. The HCMV assembly/egress protein, phosphoprotein 150 (pp150), has also been shown to induce autoantibody production. Anti-pp150 mediates depletion of circulating $CD56^{bright}$ NK cells by binding to the surface protein cancerous inhibitor of protein phosphatase 2A. As previously mentioned,

$CD56^{bright}$ NK cells are inversely correlated with disease flares so their anti-pp150-mediated depletion may contribute to autoimmune disease onset[59]. Given HCMV's role in promoting the expansion of adaptive NK cells, as well as its induction of autoantibodies and autoreactive T cells, lifelong HCMV infection may contribute to manifestation and pathogenesis of chronic arthritis in some individuals.

The specific roles of adaptive NK cells in RA remains to be thoroughly investigated. A hallmark receptor of adaptive NK cells is a high surface expression of the activating receptor NKG2C and its dimerization partner, CD94. The binding of NKG2C/CD94 and its ligand, HLA-E, promotes the activation of NK cells. To regulate this activation and prevent auto-immunity, the inhibitory receptor NKG2A/CD94 can override the activation response by inhibiting NKG2C signaling and sequestering HLA-E through its higher affinity for ligand binding[60]. It should be noted that NKG2C expression is not necessary for adaptive NK cell differentiation, since people deficient in NKG2C can still mount a functional adaptive NK cell response[61]. $NKG2C_{null}$ individuals compensate for this by expressing higher levels of CD16 and its co-receptor, CD2, on their adaptive NK cells. Other phenotypic features of adaptive NK cells include higher surface expression of the differentiation marker CD57[62] and CD2[54]. Adaptive NK cells have a lower expression of NKG2A/CD94 and NCRs (NKp46 and NKp30)[54] compared to other NK cells. These NK cell populations are also negative for the transcription factor, promyelocytic leukaemia zinc finger protein (PLZF)[63], which normally binds to the promoter regions of FcεRγ, spleen tyrosine kinase, and Ewing's sarcoma's/FLI1-activated transcript 2 (EAT-2)[64]. The extent of phenotypic and molecular changes support that adaptive NK cells are more phenotypically differentiated than conventional NK cells[54].

In addition to phenotypic differences, adaptive NK cells have distinct functions compared to regular NK cells. Adaptive NK cells rapidly respond to re-challenge by producing large amounts of cytokines (IFNγ and TNF) and targeted cellular killing through antibody dependent cellular cytotoxicity (ADCC)[37,56,65,66]. Briefly, CD16 on adaptive NK cells bind the Fc region of target bound antibodies. Cross-linking of the CD16 and Fc region initiates rapid NK cell degranulation in a lytic synapse thereby killing the target cell[65]. Given the requirement of antibodies in ADCC, adaptive NK cells may contribute to arthritis pathogenesis or mediate the clearance of auto-reactive B/T cells depending on the antibody reactivity.

Although adaptive NK cells have the potential to mediate auto-reactivity when bound to autoantibodies, it is unlikely that they contribute significantly to the pathogenesis in arthritis. This is because patients with RA typically have lower ADCC reactivity than healthy individuals due a decrease in CD3ζ expression[35]. CD3ζ regulates CD16 surface expression on NK cells and is phosphorylated following Fc cross-linking thereby conveying CD16 signal transduction[67,68]. A decrease in ADCC reactivity may protect patients from ADCC-mediated auto-reactivity but may also interfere with the efficacy of antibody-based treatment for patients with RA.

ADCC, and adaptive NK cells more generally, are important mediators of monoclonal antibody (mAb) therapy aimed at eliminating auto-reactive B/T cells. Rituximab (RTX) is an anti-CD20 mAb that interferes with the activation and differentiation of B cells and drives complement and ADCC-mediated B cell cytotoxicity. As a result, there is a reduction in B cell production of cytokines, chemokines, and autoantibodies such as rheumatoid factor[69,70]. RTX has been adopted for treatment of

patients with diseases of B cell dysregulation, including RA, where B cells pathologically produce antibodies against “self” proteins. In patients with B cell lymphoma, those with stronger ADCC functionality exhibit superior NK cell-mediated cytotoxicity against IgG-bound targets, resulting in improved response to and overall survival with RTX[71]. We expect that a similar phenomenon occurs in patients with RA when treated with RTX and other mAbs therapies compatible with ADCC. The strength and extent of a patient’s adaptive NK cell-mediated ADCC reactivity may therefore influence treatment efficacy with mAb therapies that use ADCC.

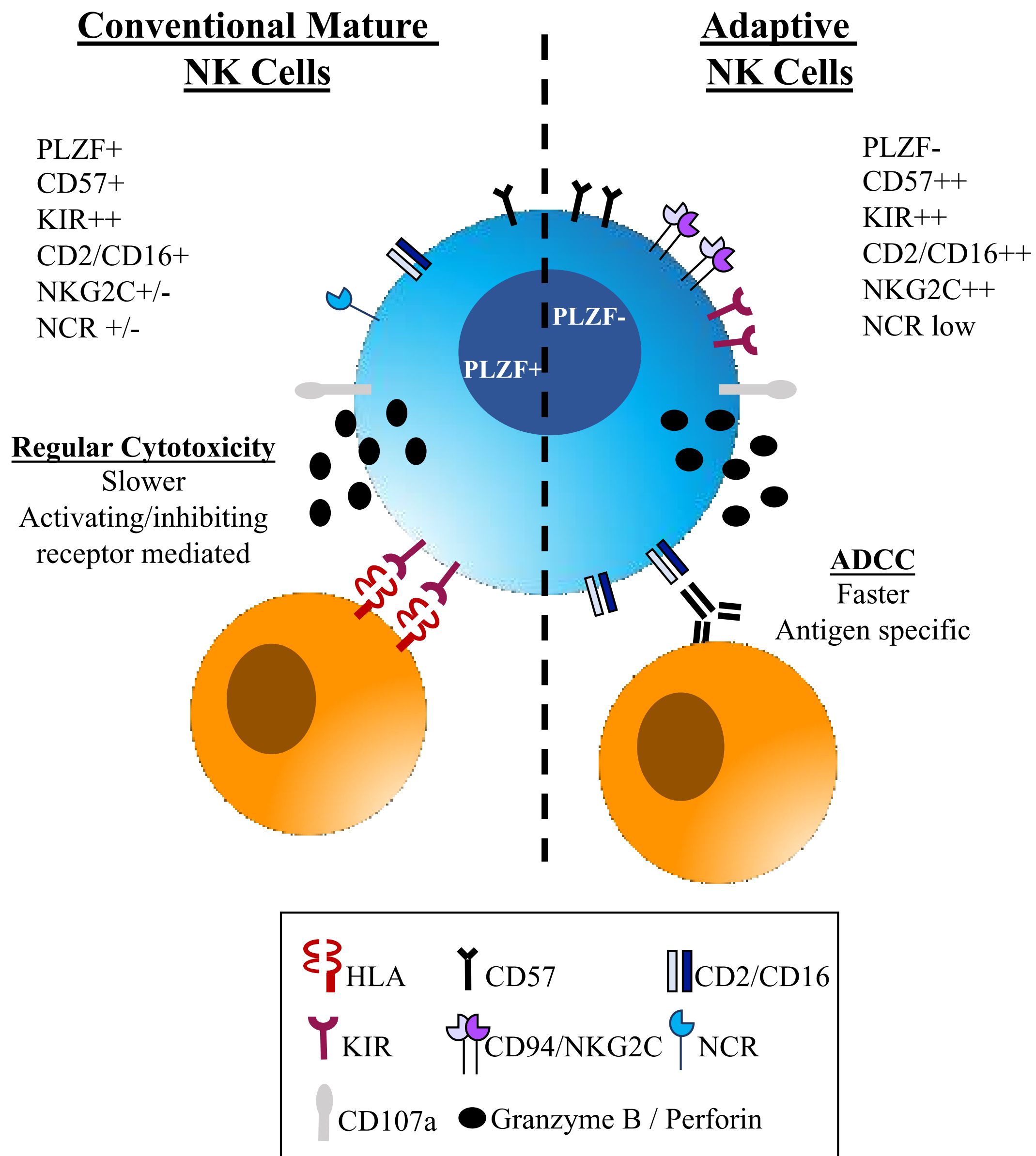

Figure 1.2. Conventional versus adaptive NK cell. Conventional NK cells (left) are PLZF^{+} and mediate cellular cytotoxicity through inhibitory and activating signals. Adaptive NK cells are PLZF^{-} (right), express lower NCRs, and have higher expression of CD57, CD2/CD16, and NKG2C. They mediate cellular cytotoxicity through ADCC which is faster and has antigen specificity.

1.3.5 NK cells in chronic arthritis and autoimmune diseases

The role of NK cells in chronic arthritis and autoimmune disease is controversial and relatively unclear. NK cells have both detrimental and protective roles in several inflammatory and autoimmune diseases, dictated by genetic variation and specific NK cell subsets[4,41,44,45,72,73].

CD56^{dim} NK cells

It is generally accepted that CD56^{dim} NK subsets have a protective role in RA and other inflammatory diseases. The quantity of circulating CD56^{dim} CD16^{+} NK cells is inversely correlated with disease flares in several autoimmune diseases including RA, Kawasaki disease, multiple sclerosis, systemic lupus erythematosus, and type 1 diabetes[41]. CD56^{dim}, but not CD56^{bright} NK cells, have also been inversely correlated with worsened disease activity score in RA[45]. Patients with autoimmune disease typically display a decrease in cytotoxic function and absolute circulating CD56^{dim} CD16^{+} NK cells[44,74–76]. It is unclear whether the decrease of circulating CD56^{dim} NK cells reflects an overall reduction in absolute circulating NK cells (90% of which are CD56^{dim}) or is a result of tissue infiltration by these NK cells. It is expected that CD56^{dim} CD16^{+} NK cells help eliminate auto-reactive T and B cells by mediating cell lysis and limiting cellular proliferation by targeting the cyclin-dependent kinase inhibitor responsible for regulating the cell cycle, p21[4,41,44,45,74]. In fact, depletion of total NK cells in mice enhanced the development of auto-antibody secreting B cells and exacerbated overall inflammation[44,73] Together, these findings imply that CD56^{dim} NK cells contribute to RA protection.

CD56^{bright} NK cells

CD56^{bright} NK cells are speculated to have a detrimental role in autoimmunity by infiltrating to sites of inflammation, including skin lesions in psoriasis and synovium in RA[44]. At these sites they provided immune-stimulatory signals, mainly IFNγ and TNF, which contribute to the inflammatory microenvironment[44,45,73,74]. NK cell derived IFNγ promotes monocyte to dendritic cell differentiation, Th1 differentiation of naïve T cells, and activates macrophages to amplify the inflammatory response[44,45,73]. It is possible that CD56 ^{bright} NK cells have a role in initiation and maintenance of site-specific inflammation.

NK cell immunogenetics

Due to the ability of NK cells to recognise 'self HLA' through KIR-HLA receptor-ligand pairs, it is not surprising the NK immunogenetics and education may have a role in inflammatory and autoimmune disease. Although no combination of KIR and HLA can be classified as beneficial or detrimental to overall health, some forms of education have been correlated with various autoimmune and inflammatory diseases[41]. For instance, *KIR2DL2* paired with the *HLA-C* encoding *Asp-77* (*HLA-C1* group) is enriched in patients with Kawasaki disease and Crohn's disease, while *KIR2DL2* paired with the *HLA-C* encoding *Lys-77* (*HLA-C2*) increases a patients susceptibility to develop type 1 diabetes[41,73]. Some immunogenetics may also be protective against autoimmune diseases. *KIR2DS1* without a pair to *HLA-C2* may have a protective role against development of RA[41]. A better understanding of the implications of education on human health and the

associated risks for developing autoimmune or inflammatory diseases will contribute to their use as clinical biomarkers.

Aside from identifying RA risk, immunogenetics could help improve patient prognosis and patient specific precision therapy. For instance, RA patients positive for *KIR2DL2* and *KIR2DS2* were able to respond better to methotrexate than *KIR2DL2/S2* negative individuals[77]. Moreover, higher levels of total circulating NKs has been associated with response to Tocilizumab, a mAb that inhibits IL-6R[45].

The overall function of NK cells, and their subsets, remains to be fully elucidated in the context of autoimmune inflammatory disease. However, NK cells provide a valuable opportunity for new genetic and phenotypic biomarkers of disease as well as new potential therapeutic targets for treating patients with autoimmune and inflammatory diseases.

1.3.6 Targeting NK cells for immunotherapeutic approaches

The vast majority of NK cell mediated immunotherapeutic techniques currently under investigation are targeted for anti-cancer therapy. NK cells have a crucial role in tumor immune-surveillance and many patients with cancer have functional deficiencies or reduced NK cells[43,78]. In addition to this, NK cells can target tumors through perforin and granzyme B mediated lysis, ADCC, and TNF related apoptosis inducing ligand (TRAIL) and FAS activation[43,79]. Several strategies have been employed to improve NK immunotherapy including genetic modification of NK cells aimed at increasing NK survival, transducing NK cells with chimeric antigen receptors to increase tumor specificity, and reducing inhibitory NK cell receptors[80,81]. Current genetic modifications

of NK cells for their use in immunotherapy is mainly accomplished through viral mediated transfer of genes[43]. This technique is called transduction. To avoid the complications associated with viral transduction, especially insertional mutagenesis, and to improve immunotherapeutic potential, researchers need to optimise highly efficient, non-viral mediated genetic modification of NK cells.

1.3.7 NK cell transfections

Despite the growing use of NK cell genetic manipulations for immunotherapy, strategies to effectively modify the cells without inducing cellular damage, excessive differentiation or activation are lacking. Efficient NK cell transfection would provide a novel opportunity for NK cell research and genetic manipulation for NK cell immunotherapy. Current techniques for NK cell genetic manipulation include transduction and transfection.

Transduction involves the delivery of genetic material through a genetic vector. However, compared to adaptive B and T cells, NK cell viral transduction has lower efficiency due to their pattern recognition receptors that trigger apoptosis and cellular activation after transduction[82]. Successful NK transduction often requires the use of NK cell lines or primary NK cells that have undergone *ex vivo* expansion. A disadvantage of this is that expanded NK cells become more phenotypically similar, displaying an upregulation of specific activating (NKG2D, CD16, NKp30) and inhibiting (KIR2DL1, and NKG2A) receptors[83]. To best represent human circulating NK cells in our research, we wanted to avoid losing any NK cell diversity within our individual study participants. Typical efficiencies of lentiviral transduction range from 15-40% and multiple rounds of

expansion and transduction may be required to achieve desired transgene expression[82]. Finally, virally transduced NK cells are also at the risk of insertional mutagenesis, which may lead to a variety of complications in both research and clinical use of transduction[82]. The major benefit of transient transfections is that they avoid insertional mutagenesis.

The other commonly used method of genetic manipulation is transfection which includes nucleofection/electroporation and chemical transfection, including lipofectamine. The advantage of transfection is that it is independent of cellular division and directly inserts the genetic material into the cell. However, transient gene expression may not be sufficient to induce long-term clinical responsiveness because the genes are not integrated into the recipient cell[80,82]. Electroporation or nucleofection involves the delivery of genetic material into the cells following a short electric shock to permeabilize the cellular membrane. Although electroporation may achieve high cellular efficiencies, the electric shock may cause damage to the cells that is not fully understood; this may include disruptions of cell phenotype, function, and/or proliferation[82]. Meanwhile, chemical transfection such as lipofectamine, delivers genetic material into the cell via a lipid vesicle delivery system[84]. This method has recently been shown to alter type I interferon signaling in macrophages[85] and may have other undesirable effects on cellular activation, phenotype, and/or proliferation. Other proprietary chemical transfection techniques also exist, including TransIT-TKO from Mirus that was optimized in this project for primary NK cell transfection. The TransIT-TKO technique is advantageous because it can efficiently transfect freshly isolated, primary NK cells without, to our knowledge, affecting their function, phenotype, or viability.

There are several obstacles involved in genetic manipulation of primary NK cells but overcoming these challenges can provide new opportunities for NK cell-based research, immunotherapies, and other clinically based therapeutics.

1.4 MicroRNA

1.4.1 General characteristics of miRNAs

MicroRNAs (miRNAs) are short (~22 base long) non-coding RNAs that function in post-transcriptional regulation of mRNAs to prevent expression of the proteins they encode. Briefly, miRNAs are produced in the nucleus, exported to the cytoplasm where they are processed by Dicer, a helicase with an RNase motif [86,87]. Mature miRNAs then complex with RNA-induced silencing complex (RISC) and act as a template for binding to the 3' untranslated region (UTR) on their mRNA target(s)[88–90]. Together, the RISC-miRNA complex inhibits translation by either promoting the degradation of the mRNA or blocking the translational machinery (**Figure 1.3**)[42,91,92]. The mRNA targets of miRNAs overlap extensively and thus can regulate multiple pathways and biological processes simultaneously. There are more than 2000 miRNAs in the human genome[87], and they regulate approximately 30-60% of all human genes[91], including genes necessary for cellular activation, proliferation, and/or apoptosis. In addition to regulating mRNA within a cell, miRNAs demonstrate significant stability within human biofluids, including plasma and synovial fluid, due to an association with the stabilizing Argonaut protein, Ago2[93,94]. Thus, miRNAs may be released to alter the expression of proteins of surrounding cells. MiRNAs endow central regulatory roles both within a cell and to the

surrounding microenvironment. As a results, we expect they contribute, and regulate, the pathology of several diseases, including RA.

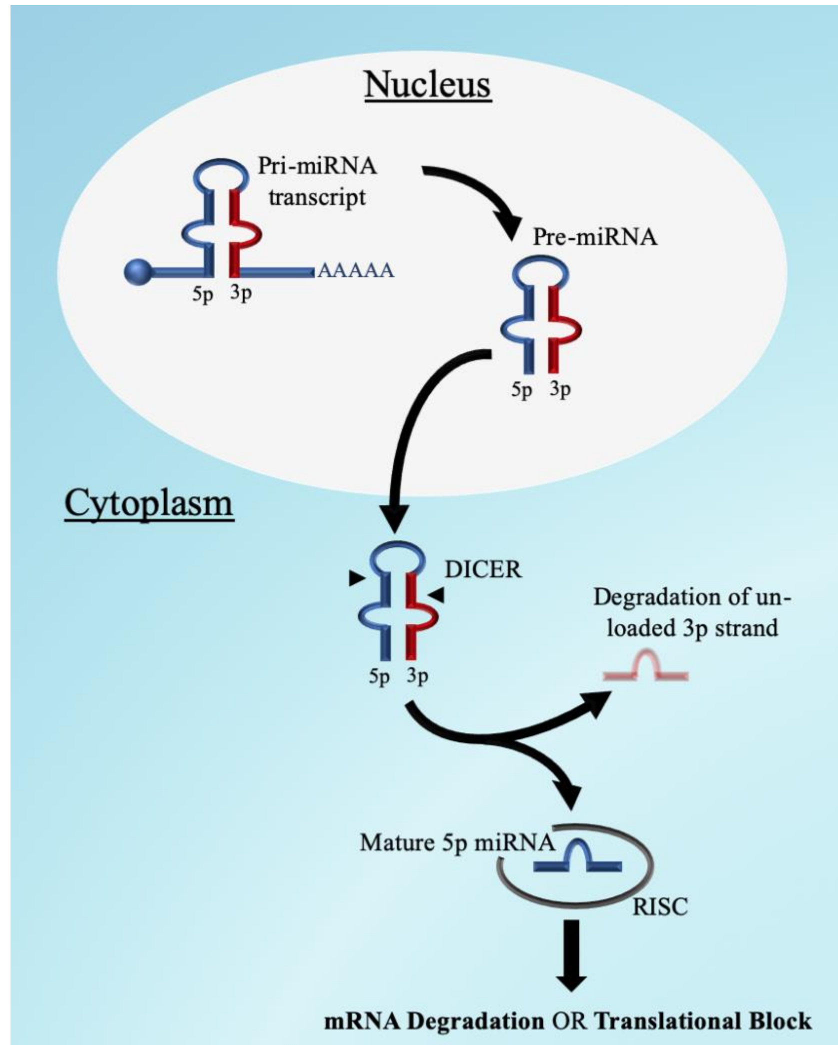

Figure 1.3. MiRNA processing and mRNA inhibition. MiRNAs are transcribed in the nucleus intro a primary miRNA (pri-miRNA) transcript. The pri-miRNA transcript is processed in the nucleus to create pre-miRNA. The pre-miRNA is exported to the cytoplasm where Dicer cleaves the double stranded molecule and RISC incorporates a single stranded miRNA to act as a template for translation inhibition via mRNA degradation or translational block. In this figure, the 5p strand of the miRNA transcript is loaded onto RISC while the 3p strand is degraded. Each miRNA molecule targets several different mRNAs and results in regulation of various cellular mechanisms.

1.4.2 Role of miRNAs in inflammatory disease

Recently, miRNAs have gained significant momentum as biomarkers and therapeutic targets in research for several inflammatory diseases, including RA. This is largely due to their role(s) in regulating various inflammatory processes including cellular activation, cytokine synthesis, expression of adhesion molecules, and angiogenesis[95,96]. For instance, miR-146a may regulate nucleotide-binding oligomerization domain-containing protein 2 (NOD2) derived gut inflammation in inflammatory bowel disease. In the context of arthritis, we and others have identified numerous miRNAs that are significantly up or down regulated in the plasma and/or synovial fluid of RA and JIA patients compared to age and sex matched healthy donors (**Figure 1.4**)[88,97–99]. Many miRNAs are undergoing investigations to determine their relevance in disease pathology and potential use as biomarkers or therapeutic options. A non-exhaustive list of miRNAs, their functions in NK cells and relevance in various inflammatory and autoimmune diseases is highlighted in **Table 1.1.** We expect that miRNAs have a role in both arthritis pathogenesis and treatment response.

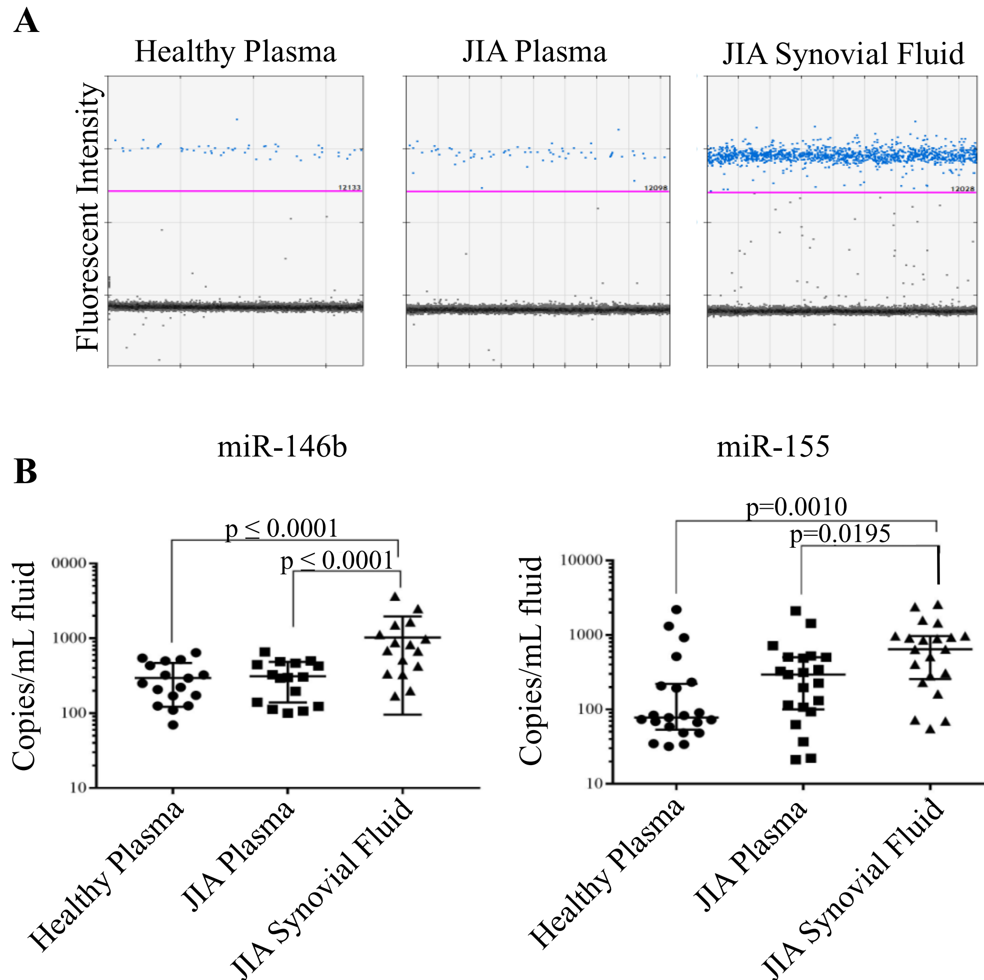

Figure 1.4. MiR-146b-5p and miR-155-5p are overexpressed in JIA synovial fluid. A) Representative figure analysis of miR-146b-5p in plasma and synovial fluid by ddPCR. RNA was isolated from JIA patient plasma and synovial fluid and normal donor plasma. The figure shows the number of analysed droplets (x-axis) and the intensity of fluorescence (y-axis). The grey dots represent negative droplets and the blue dots represent positive droplets. The threshold for positive droplets is represented as a pink line. B) MiR-146b-5p and miR-155-5p are overexpressed systemically in JIA. The expression as analyzed by ddPCR (A) and normalized to reference miRNAs, miR-93-5p and miR-191-5p. The median and interquartile range is shown in the graph (n=21). Statistical significance was determined by non-parametric two tailed Mann-Whitney test. This figure was adapted from a preliminary figure presented in my honours project.

Table 1.1 Non-exhaustive list of miRNAs in inflammatory disease.

miRNA	Expression in disease	Disease	Function(s) in disease	Function in NKs	Ref.
miR-16	↑	RA	Increase Th17 ratio Correlates with active disease	Regulate maturation	97,100–103
miR-21	↑	RA, OA, psoriasis, systemic sclerosis	Increases TNF Increase STAT3 (Th17) Decrease STAT5 and Foxp3 (Treg)	Downregulate AKT signalling	98,100,104
miR-27a-3p	↓	**RA, OA**	**Inhibit FLS migration/invasion** **Negatively regulate NFκB** **Inhibit T cell proliferation**	**Inhibit cytotoxicity**	42,98,105–108
miR-30	↓	RA	Regulate autophagy and apoptosis	Unknown	97,109
miR-34a	↓	RA	Inhibit synovial cell proliferation	Unknown	98,110,111
miR-124	↓	RA	Regulate synoviocyte proliferation Inhibits TNF and IL-6 signaling	Inhibit AKT signaling	97,112
miR-125b-5p	↑	**RA, OA**	**Regulate pro-inflammatory signaling, B cell differentiation**	**Regulate maturation**	25,45,92,108–111
miR-132	↓	RA, OA	Potential diagnostic marker Impair production of IL-1b, IL-6 and IFNβ	Regulate IL-12 mediated IFNy production	110,117,118
miR-143/145	↑	RA, psoriasis	Increase FLS responsiveness to TNF and vascular endothelial growth factor Increase chondrocyte IL-1β responsiveness Regulates osteogenesis & chondrogenesis	Unknown	98,100,119
miR-146a-5p	↑	**RA, JIA, OA (early)**	**Inhibit pro-inflammatory responses** **(IL-1β, TNF, type I IFN)**	**Regulate maturation & function**	86,88,91,101,102,119–128
miR-155-5p	↑	**RA, JIA, psoriasis**	**Promotes pro-inflammatory signaling** **Positively correlated with DAS28**	**Interfere with KIR-HLA signaling** **Increase IFNy and TNF production**	83,96,97,114,125–132
miR-181	↑	JIA, RA	Regulates T cell sensitivity to antigens Regulates B cell antibody class switch Inhibit CD163 (innate pattern recognition receptor) expression on macrophages	Regulate maturation	45,93,96,133–135
miR-223-3p	↑	**RA, IBD**	**Inhibits IKK in NFκB signaling** **Regulate myeloid precursor differentiation into osteoclasts; abnormal bone erosion**	**Regulate maturation**	**1,92–94,101,105,117,128,136–138**
miR-363	↓	RA	Regulate DC integrin αv expression	Regulate growth	100,111

Bold indicates miRNAs studied in this project.

1.4.3 Candidate miRNAs

Candidate miRNAs were selected based on preliminary experimental evidence of differential expression in RA patients as well as from extensive literature review. This project was initiated with five miRNA candidates: miR-27a-3p, miR-125a-5p, miR-146a-5p, miR-155-5p, and miR-223-3p. A summary of their roles in NK cell signaling and function can be found in **Figure 1.5.**

miR-27a-3p

In RA, miR-27a-3p is significantly decreased in the serum, synovial fluid, and FLS relative to age and sex matched healthy individuals[105]. One of its mRNA targets, follistatin-like protein 1, an extracellular protein that promotes the expression of TNF, IL-1β, IL-6 and IL-8, is correlated with RA disease severity. MiR-27a-3p also has a role in the inhibition of FLS migration and invasion[105]. Furthermore, miR-27a-3p is decreased in osteoarthritis (OA) chondrocytes[98] and negatively regulates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling[105]. In NK cells, miR-27a-3p can inhibit cytotoxicity by targeting the mRNAs of granzyme B and perforin[42,106,107]. Altogether, miR-27a-3p may play a protective role in RA by inhibiting pro-inflammatory cytokine release and invasion of both FLS and NK cells to the sites of inflammation. However, it is still not clear whether the miR-27a-3p mediated inhibition of NK cytotoxicity is beneficial in RA.

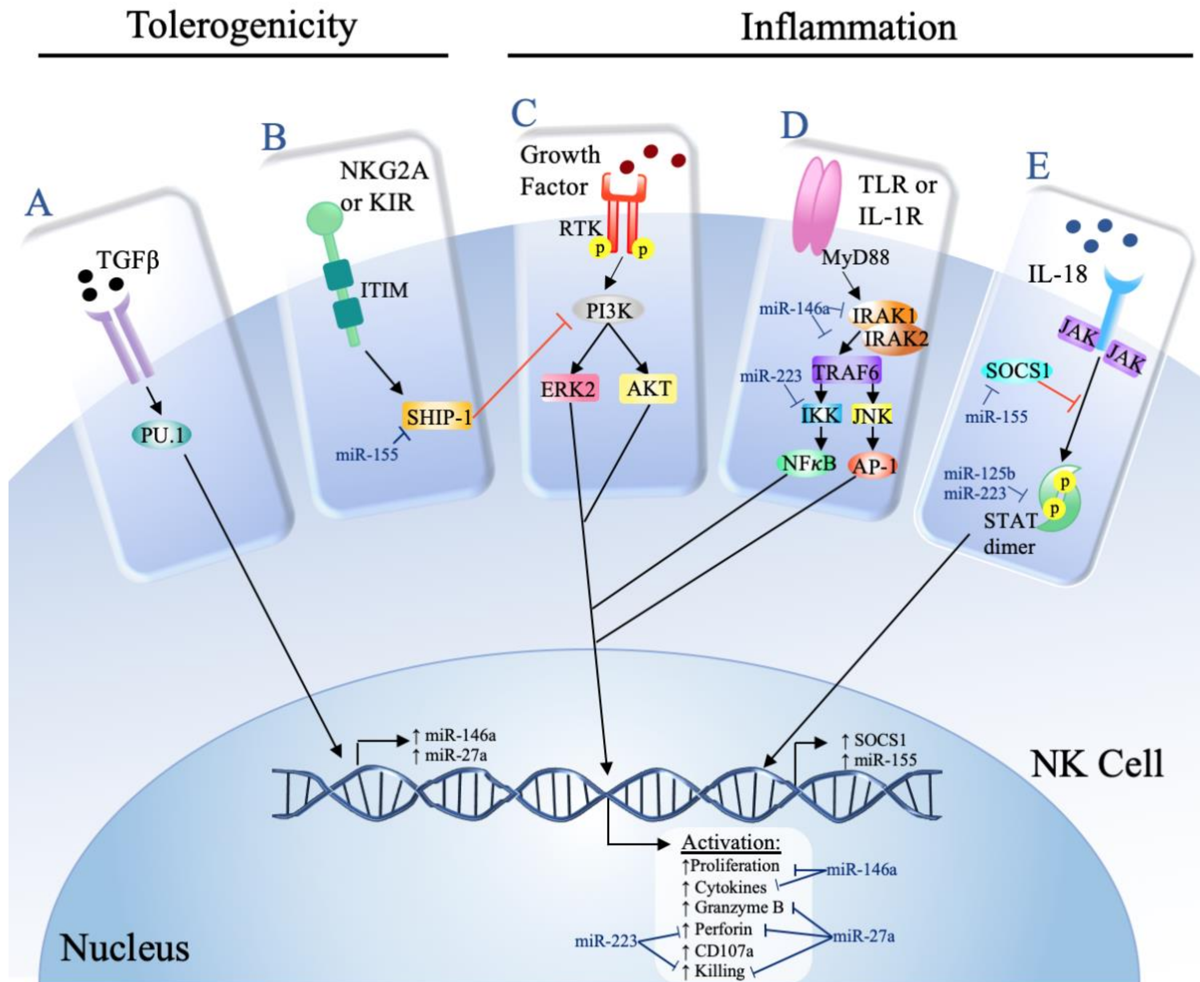

Figure 1.5. NK miRNA signaling pathways. Natural killer cell tolerogenic signaling may be induced by cytokines, such as TGFβ (A), or activation of inhibitory receptors KIR and/or NKG2A (B). **A)** By means of PU.1, TGFβ increases the production of miR-146a-5p and miR-27a-3p. These miRNAs are responsible for the downregulation of various inflammatory NK functions. **B)** KIR and NKG2A ITIM signaling results in downstream activation of the SHIP-1 phosphatase, which inhibits the phosphoinositide-3 kinase (PI3K) pro-inflammatory protein. SHIP-1 is inhibited by miR-155-5p. Inflammatory signalers such as growth factors (C), toll like receptors (TLRs), interleukin receptor (IL-R) 1 (D), or IL-18 (E) promote NK cell activation. **C)** Growth factors bind to receptor tyrosine kinases (RTK) which dimerize and autophosphorylate to subsequently activate the transcription factors extracellular signal regulatory kinase 2 (ERK2) and AKT via PI3K. **D)** Activation of TLRs and IL-R1 leads to the up-regulation of NFκB and acid-phosphatase 1 (AP-1). These transcription factors, along with ERK2 and AKT, promote NK cell activation. Upstream proteins, IRAK1/2 and TRAF6 are inhibited by miR-146a-5p, while IκB kinase (IKK) is inhibited by miR-223-3p. **E)** IL-18 signaling promotes the production of miR-155-5p and SOCS1 through the JAK-STAT pathway. SOCS1 is a negative inhibitor of the JAK-STAT signaling and miR-155-5p targets and inhibits SOCS1. STAT is targeted by miR-223-3p and miR-125b-5p.

miR-125b-5p

Patients with RA and OA display elevated levels of miR-125b-5p compared to healthy controls[97]. Intriguingly, patients with low levels of miR-125b-5p at the time of disease flares are significantly less likely to respond to RTX treatment. Thus, the overexpression of miR-125b-5p can potentially function as a biomarker of RTX treatment efficacy[97]. MiR-125b-5p functions in the regulation of signaling pathways during inflammation, B cell differentiation, TNF production, and apoptosis[97,113–115]. It can specifically interfere with granulocyte-colony stimulation factor, and signal transducer and activator of transcription 3 (STAT3)[113]. MiR-125b-5p expression can be induced by anti-inflammatory bromodomains and extra-terminal motif (BET) protein inhibitors (BETi), which prevent BET proteins from interacting with transcription factors[33]. In multiple myeloma cells, BETi treatment induces miR-125b-5p mediated inhibition interferon regulatory factor 4 (IRF4). IRF4 is a transcriptional repressor of major histocompatibility class 1 polypeptide-related sequence A (MICA), so its miR-125b-5p mediated inhibition results in increased MICA expression[33]. This makes the cells more sensitive to NK mediated cytotoxicity through the NK activating MICA receptor, NKG2D[33,116]. Finally, miR-125b-5p is upregulated in CD56^{bright} NK cells and may have a role in NK maturation[42]. The role of miR-125b-5p is not entirely clear in chronic arthritis and NK cell function, making it an interesting new avenue for research.

miR-146a-5p

MiR-146a-5p is commonly overexpressed in a variety of inflammatory diseases and often correlates with disease severity in RA, JIA, and OA[125–127]. In fact, one group of researchers found patients with RA have significant polymorphisms and overexpression

of miR-146a-5p[128]. Interestingly, miR-146a-5p has an anti-inflammatory role and targets the 3' UTR of mRNAs such interleukin-1 receptor-associated kinase (IRAK1), TNF receptor associated factor 6 (TRAF6), IL-1β, IL-6, IL-8, and STAT1[91,143–148]. Polymorphisms in the 3' UTR miRNA binding site on IRAK1 has been linked with increased susceptibility to RA and psoriatic arthritis[127]. MiR-146a-5p has been identified to inhibit pro-inflammatory responses including IL-1β mediated inflammation, type 1 IFN pathways, TNF signaling and others[120,121]. Because miR-146a-5p is regulated by NFκB pro-inflammatory signaling pathways, it is understood that miR-146a-5p functions as a regulator of inflammation in a negative feedback manner[119,122,123,128]. Mice deficient in miR-146a-5p have been identified to develop autoimmune disease, have exaggerated pro-inflammatory responses, develop tumors, and undergo myeoproliferation[86,91]. Finally, miR-146a-5p may be used as a biomarker to monitor inflammatory disease severity and treatment efficacy[88]. In fact, some researchers have suggested the use of miR-146a-5p as a candidate for anti-inflammatory treatment for asthma[121]. In the context of arthritis, many clinical studies have identified an increase in plasma miR-146a-5p (approximately 4.3 fold increase[86]) in patients with RA who respond to anti-TNF treatments[88,128].

In addition to exhibiting an anti-inflammatory function, miR-146a-5p has been identified as an important regulator of both NK cell differentiation and function[124]. Specifically, miR-146a-5p may promote the maturation from $CD56^{bright}$ to $CD56^{dim}$ NK cells and may regulate KIR expression[42]. Some potential KIR mRNA targets of miR-146a-5p include KIR2DL1 and KIR2DL2[42]. *In vitro* miR-146a-5p transfection of the NK cell line, NK-92, conferred lower expression of STAT1 as well as decreased NK-92 cytotoxicity[124].

MiR-146a-5p displays high relevance in both RA, as a clinical biomarker and mediator of inflammation, as well as NK cell maturation and regulation of activation; thereby making it an ideal candidate for our research.

miR-155-5p

We and others have identified elevated levels of miR-155-5p in chronic arthritis including RA and JIA[86,131–135]. It is also prominent in patients diagnosed with psoriasis[119]. MiR-155-5p plays a crucial role in pro-inflammatory activation and contributes to the development of chronic inflammation, auto-immunity, cancer, and fibrosis[86,131,135]. Many researchers have identified miR-155-5p as a potential therapeutic target for inflammatory and autoimmune diseases[86,134]. Expression of miR-155-5p is promoted by cellular activation through cytokines: IL-12, IL-15, and IL-21[129,136]. Downstream signaling of these cytokines is mediated by Janus kinase (JAK) / STAT1 which directly promotes the expression of miR-155-5p. Consequently, miR-155-5p functions as a positive feedback loop by inhibiting suppressor of cytokine signaling 1 (SOCS1), which would normally inhibit STAT1[119]. In chronic arthritis, miR-155-5p is expressed by both innate and adaptive immune cells and promotes chronic immune activation and inflammation[86]. Furthermore, miR-155-5p is positively correlated with worsened disease severity as well as TNF and IL-1β expression in patients with RA[131,132,134]. In NK cells, immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling from inhibitory KIR and other receptors activates SH-2 containing inositol 5' polyphosphatase 1 (SHIP-1)[39,149]. SHIP-1 can be inhibited by miR-155-5p and therefore interfere with NK cell KIR/HLA-mediated cellular recognition [86,130–132]. Moreover, miR-155-5p increases NK expression of IFNy and

TNF[135,136]. MiR-155-5p mediated inflammation can be controlled through anti-inflammatory cytokines (IL-10), resolvins, glucocorticoids, post-transcriptional negative regulators (Tristetraprolin), and miR-146a-5p[86]. In sum, miR-155-5p is an important mediator of inflammation and autoimmunity making it an ideal candidate for research in NK cells and chronic arthritis.

miR-223-3p

A major function of miR-223-3p involves the regulation of granulopoiesis and differentiation of myeloid innate immune cells, such as neutrophils and macrophages[1,91]. In fact, miR-223-3p is upregulated in myeloid progenitor cells and downregulated in lymphoid progenitor cells including T, B, and NK cells[1]. MiR-223-3p normally functions as a negative feedback to control excessive innate immune responses by myeloid cells but displays increased expression in patients with RA and inflammatory bowel disease[1,99,132,141,142]. MiR-223-3p is a specific RA biomarker when compared to other forms of chronic arthritis, and is positively correlated with disease severity[99,132]. Moreover, patients with RA who express relatively lower levels of miR-223-3p are predicted to respond to anti-TNF/DMARD combination treatments and display an increase in miR-233-3p post therapy[88]. Despite its significant role in regulating neutrophils and macrophages, miR-223-3p also regulates the activation of lymphoid cells at baseline. For example, at baseline, miR-223-3p inhibits IKK within NFκB signaling, STAT3, and granzyme B in NK cells[1,106,122,140,141]. Upon NK cell activation, especially with IL-15, miR-223-3p expression is decreased thereby allowing the NK cell to become activated[122,140,141]. Furthermore, miR-223-3p, like miR-146a-5p, is predicted to have a

role in NK cell maturation from CD56^{bright} CD16^{-} cells to CD56^{dim} CD16^{+} cells[42]. Lentivirus induced silencing of miR-223-3p in mice has been shown to prevent collagen induced arthritis[1]. In addition to this, miR-223-3p could prevent osteoclast mediated joint destruction[142]. Overall, miR-223-3p appears to have a relatively protective function in RA. Its combined roles in NK cells and RA make miR-223-3p relevant to our research.

1.5 Rationale & Hypothesis

1.5.1 Rationale

It is well established that NK cells have critical roles in surveillance and response against virally infected and cancerous cells. However, their role in orchestrating or preventing autoimmune reactions is not well established. These functions, along with their functionally diverse activating and inhibiting receptors, make NK cells ideal candidates for novel immunotherapeutic approaches, including treatments aimed at controlling inflammatory arthritis. Establishment of efficient NK cell transfection techniques further expands their therapeutic potential.

A crucial step toward NK-immunotherapy for inflammatory arthritis is to understand how NK cells may function in patients with inflammatory disease and how the inflammatory microenvironment (i.e. miRNAs) influence NK cells. NK cells appear to have dual complexity in RA and may induce cytokine mediated-inflammation, or help eliminate auto-reactive T and/or B cells to protect against adaptive immunopathology[73]. RA specific differentially expressed miRNAs may contribute to the regulation of NK cells and determine the balance between destructive and protective functions in patients with RA. We set out to investigate how miRNAs regulate conventional and adaptive NK

cells in the context of autoimmune disease. To accomplish this, we developed and optimized novel transfection techniques for primary human NK cells that have widespread applications. We expect that miRNAs may control the phenotype and function of adaptive NK cells, thereby playing a pivotal role in ADCC function and, by extension, mAb treatment efficacy in patients with RA. A better understanding of this interplay will help us determine precise patient therapies with current clinically available treatments (**Figure 1.6**).

1.5.2 Hypothesis

We hypothesize that NK cell immunogenetics and miRNA signatures define NK cell activation, inflammation regulation, and treatment efficacy in patients with RA and other inflammatory diseases. Specifically, we predict that miR-146a-5p, and potentially miR-155-5p, inhibit the differentiation of adaptive NK cells and results in decreased ADCC-mediated responsiveness to mAb therapy in patients with RA. The applications of this research will allow for precise patient therapies and provide insight to the miRNA-regulated contributions of adaptive NK cells in RA.

1.5.3 Objectives

To further develop our understanding of these miRNA-NK cell interactions, we divided our investigation into five aims:

1. Identify the miRNAs produced by and impacting NK cells under pro-inflammatory (IL-18) and tolerogenic (TGFβ) stimulations.

2. Develop a novel transfection technique to efficiently deliver candidate miRNAs into primary NK cells.
3. Determine the functional/phenotypic response of NK cells to the up- and down-regulation of candidate miRNAs.
4. Determine the role of NK cell education in miRNA response.
5. Determine the how miRNAs alter the phenotype and function of adaptive NK cells.

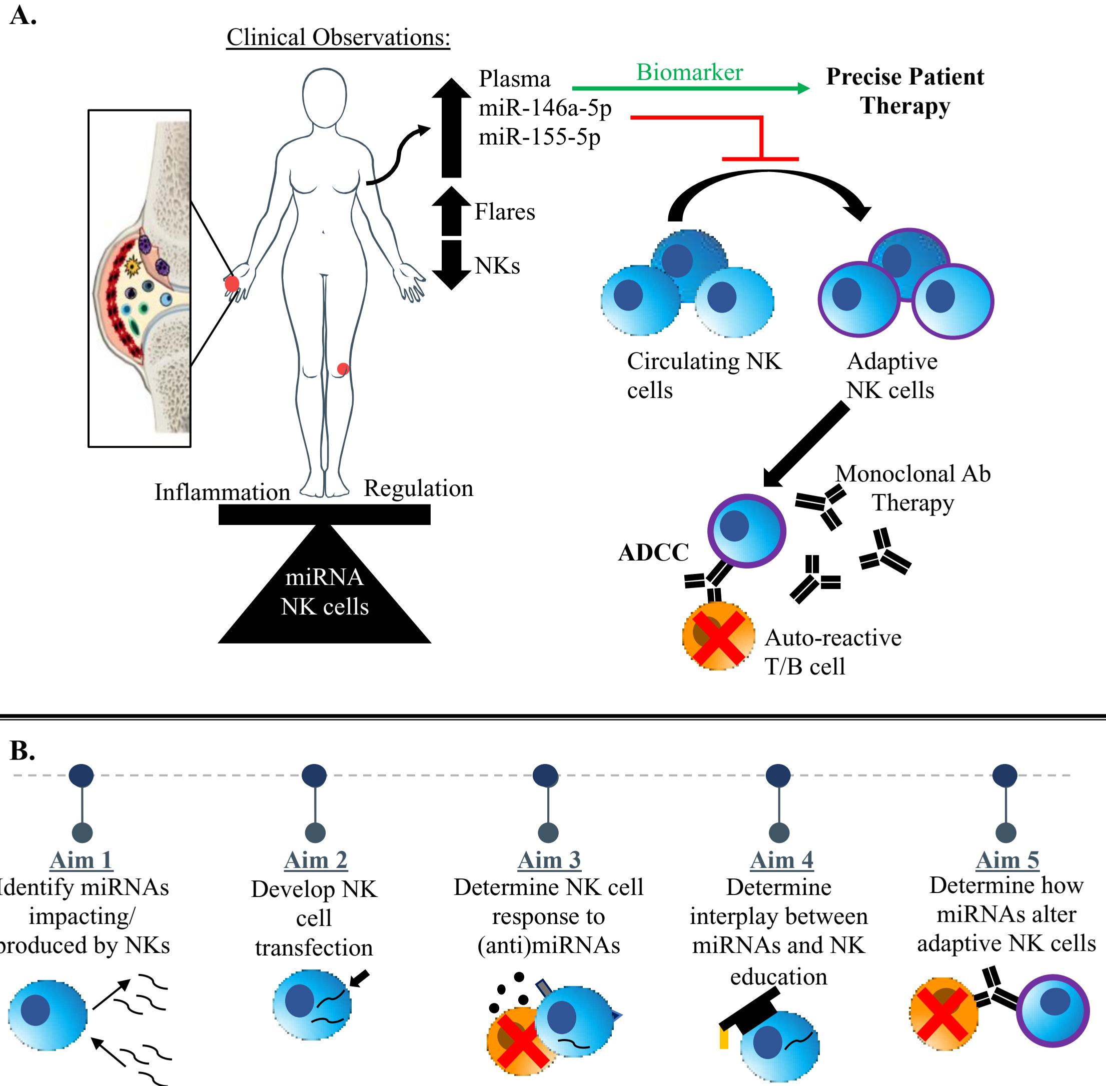

Figure 1.6. Project overview. A) Patients with arthritis have an upregulation of plasma miR-146a-5p and miR-155-5p and decreased circulating NK cells associated with disease flares. We **hypothesize** that NK cell immunogenetics and miRNA signatures define NK cell activation, inflammation regulation, and treatment efficacy in patients with RA and other inflammatory diseases. We predict circulating NK cells adopt an adaptive phenotype and eliminate auto-reactive T and/or B cells through ADCC with mAb therapy. We propose miR-146a-5p inhibits adaptive NK phenotype (NKG2C) and, potentially, function. Therefore, miR-146a-5p may be measured as a biomarker of monoclonal treatment efficacy and help identify precise patient therapies. B) Outline of aims 1-5 as described in objectives.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell isolations and culturing

2.1.1 Peripheral blood mononuclear cells

Healthy donor peripheral blood, designated for peripheral blood mononuclear cell (PBMC) isolation, was collected with informed consent from donors under Research Ethic Board protocols (Izaak Walton Killam (IWK), Project # 1005110, Dalhousie REB #2016-3842, and Canadian Blood Services REB project nc00024). Briefly, blood acquired from healthy donors or Canadian blood services were washed with 1x phosphate buffer saline (PBS) (Biological Industries) and layered onto 15 mL of Ficoll-Paque PLUS® (VWR). Layered blood was centrifuged at 2,200 rpm with the brake off to separate lymphocytes from plasma and remaining blood cells. The lymphocytes were collected, resuspended in 50 mL 1x PBS, and centrifuged at 1,500 rpm for 5 minutes. The cells were resuspended in 5-10 mL of red blood cell Lysis Buffer (VWR) for 3-5 minutes and washed once more with 1x PBS. Although most experiments were conducted on fresh cells, those that were not were frozen in 90% fetal bovine serum (FBS) (Sigma-Aldrich) and 10% dimethyl sulfoxide (Sigma-Aldrich), and stored in liquid nitrogen vapour phase. Prior to experiments with frozen PBMCs, the cells were thawed and allowed to recover at 37°C and 5% CO_2 overnight in RPMI media (Gibco) containing 1x penicillin and streptomycin (P/S) (Wisent), 10% FBS, and 1,000 IU/mL IL-2 (Peprotech). To avoid introduction of exogenous mammalian miRNAs during experimentation, PBMCs were cultured in X-vivo™10 Serum-free Hematopoietic Media (Lonza) containing L-

glutamine (3 mM) (Gibco), L-serine (1.8 mM) (Alfa Aesar), L-asparagine (0.6 mM) (Multicell), and 100 IU/mL IL-2 after overnight recovery.

2.1.2 Natural killer cells

Primary healthy human NK cells were isolated from whole blood using the RosetteSep™ Human NK Cell Enrichment Cocktail (Stemcell Technologies) as per manufacturer's instructions. In short, equivalents of 15 mL of whole blood was incubated with 250 µL RosetteSep™ cocktail for 20 minutes at room temperature. The blood was diluted 1:1 with 1x PBS containing 2% FBS and layered over 15 mL of Ficoll. The tubes were centrifuged at 1,200 x*g* for 20 minutes at room temperature with the brake off. The enriched NK cell layer was collected and washed twice with 1x PBS 2% FBS and centrifuged at 300 x*g* for 10 minutes at room temperature with low brakes. Primary NK cells were cultured in X-vivo media with 100 IU/mL IL-2 at 1-2x10^6 cells/mL and rested for a minimum of 2 hours at 37°C and 5% CO_2 before any experiments proceeded.

2.1.3 NK-92 cell line

The NK cell line, NK-92® (ATCC® CRL-2407™), was maintained in American type culture collection (ATCC) recommended media (αMEM media without ribonucleosides and deoxyribonucleosides, but with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum, and 12.5% FBS) at a concentration of 2-3x10^5 cells/mL with 100 IU/mL IL-2. Media was replaced every 2-3 days as needed.

2.1.4 K562 cell line

The K562 lymphoblast cell line (ATCC® CCL-243™) was maintained in RPMI 1640 (Gibco) containing 10% FBS, 1x P/S, 2 mM L-glutamine (Gibco) and 50 μM 2-mercaptoethanol (Sigma). The cells were split every 3-4 days to a concentration of $1.5x10^5$ cells/mL as required.

2.1.5 Fibroblast-like synoviocytes

All research completed with FLS cells was approved by the IWK Research Ethics Board (IWK, Project # 1005378) and, because all FLS patients are minors, the guardians of all study participants signed informed consent documents. Whole synovial fluid was collected from treatment naïve polyarticular and oligoarticular JIA patients and pelleted at 1,000 x*g* for 15 minutes at room temperature. The synovial fluid was removed from the cellular pellet and stored at -80°C. The cellular pellet was resuspended in 10 mL of RPMI with 10% FBS and layered over 3 mL of Ficoll. After a 25-minute centrifugation at 540 x*g* with slow acceleration and brakes off, the mononuclear cell layer was collected and transferred to a new tube where the cells were washed twice with RPMI media. The cells were plated at $1\text{-}10x10^6$ cells / mL in RPMI media containing 10% FBS, 100 IU/mL P/S (Gibco), 2 μM L-glutamine (Gibco), and 50 μM 2-mercaptoethanol (Sigma-Aldrich)) and cultured for 24 hours at 5% CO_2 and 37°C. This allowed for adherence of the FLS cells such that the mononuclear cells could be collected and stored at -80°C for later use. The remaining adherent FLS cells were maintained in complete α-MEM (Gibco), containing 20% FBS, 1 mM sodium pyruvate (Gibco), 2 μM L-glutamine, 100 IU/mL P/S, 50 μM 2-mercaptoethanol, and 1x non-essential amino acids (Gibco), with media changes every 2-

3 days. Once the cells reached 80% confluency, they were trypsinized and split. Aliquots of passages 3-4 were set aside for long term cryo-storage in RPMI containing 10% dimethyl sulfoxide and 50% FBS[150].

2.1.6 Optimization of serum free media

Mammal derived serum may contain conserved miRNAs that could misled our results. Therefore, X-vivo serum free media was tested against RPMI (containing 10% FBS) and ATCC NK-92 recommended media (containing 12.5% FBS and 12.5% horse serum) on NK-92, PBMCs, and primary NK cells. The NK-92 cells were resuspended at $2x10^5$ cells/mL, while the PBMCs and NK cells were resuspended at $1\text{-}2x10^6$ cells/mL in all three medias. The cells were cultured for 4 days with 100 IU/mL IL-2 and the viabilities of the cells were assessed by trypan blue and flow cytometry on either all days or days 1 and 4. There were no additional media changes or replacement of IL-2 during this experiment.

2.2 Selection of candidate miRNAs

Candidate miRNAs were identified based on experimental findings and extensive literature review. The Restitution and Enhancement in Arthritis and Chronic Heart disease (REACH) collaboration at Dalhousie University assisted in candidate selection through preliminary assessment of miRNAs in an Exiqon microarray using droplet digital PCR (ddPCR). Here, differential miRNA expression was measured in pooled plasma from patients with RA, and pooled plasma from healthy age and sex matched donors was used as a control. Additionally, some miRNAs were preliminarily measured in JIA synovial

fluid and plasma samples compared to age- and sex-matched healthy donor plasma. The miRNA measurements in JIA patient biofluids were accomplished prior to my graduate degree as part of an IWK summer studentship and presented as preliminary data for my honours project (**Figure 1.4**). Five candidate miRNAs were selected for this investigation: miR-27a-3p, miR-125b-5p, miR-146a-5p, miR-155-5p, and miR-223-3p.

2.3 Optimization of NK cytokine treatments

2.3.1 IL-18 and TGFβ stimulation of NK cells

To assess NK cell production of miRNAs under pro-inflammatory and tolerogenic stimulation, we treated primary NK cells with IL-18 and TGFβ. Primary NK cells at 1-$2x10^6$ cells/mL in serum free X-vivo media with 100 IU/mL IL-2 were treated for 6, 12, and 18 hours with 50 ng/mL IL-18 (R&D) or 5 ng/mL recombinant human TGFβ1 (Peprotech) to determine the optimal time for NK cell activation or inhibition, respectively. After each time point, the cells were pelleted, supernatants were removed and stored at -80°C for later use, and the pellets were lysed with 700 μL Qiazol (Qiagen). The pellets were vortexed for 30 seconds and allowed to sit at room temperature for 5 minutes prior to being stored at -80°C. Cellular mRNA expression of IFNγ and TNF were measured by real time quantitative polymerase chain reaction (RTqPCR), as described in Chapter 2.7 Molecular biology. We determined a 12 hour treatment would be optimal to functionally activate or inhibit the NK cells.

Due to the possibility of additional post-transcriptional regulation of IFNγ and TNF, we also assessed the IFNγ and TNF protein production and function of NK cells after a 12 hour pre-treatment with IL-18 or TGFβ. Cytokine pre-treated NK cells and no

treatment controls were co-cultured with K562s for five hours. Intracellular protein and NK cell function was assessed by flow cytometry, as described in Chapter 2.6. This experiment confirmed that 12 hours was sufficient to activate or inhibit the cells.

Finally, miRNA cDNA was created from the RNA of cells treated for 12 hours and all five candidate miRNAs (miR-27a-3p, miR-125b-5p, miR-146a-5p, miR-155-5p, and miR-223-3p) were assessed by RTqPCR. Of note, miR-125b-5p was below RTqPCR limit of detection and was omitted from this experiment as a result.

2.4 Optimization of primary NK cell transfections

2.4.1 MiRNA mimic and inhibitor technology

The miRNA mimics and inhibitors used for this project were Qiagen's miRCURY® locked nucleic acid (LNA)® miRNA mimics and inhibitors. The Qiagen LNA technology allows the miRNA mimic/inhibitor to have increased stability due to ribose ring Watson-Crick conformational binding. The miRNA mimics were designed to include three RNA strands, one miRNA guide strand bound to two LNA modified passenger strands. When incorporated into a cell, the two LNA passenger strands, which are too short to be used as miRNAs, are rapidly degraded while the guide miRNA strand is incorporated by RISC, thereby effectively transfecting cells with a mature miRNA mimic. Meanwhile, miRNA inhibitors are comprised of an antisense oligonucleotide complimentary to the miRNA target. Once introduced into the cytoplasm, the inhibitor binds their target miRNA and forms a highly stable heteroduplex, thus sequestering the target miRNA. As a negative control of transfection, a scrambled miRNA negative control sequence was used (Qiagen). The scrambled miRNA was identified by Qiagen as having no hits with >70%

homology with any sequence in any organism according to NCBI and miRbase databases. The scrambled miRNA was used to optimise transfection viability and efficiency as described in Chapter 2.4 sections 2.4.2-5.

To easily assess transfection efficiency by flow cytometry, all miRNA mimics and inhibitors, with the exception of the miR-223-3p mimic, were fluorescently labeled with FAM. The miR-223-3p mimic sequence was not compatible with the company's technique for FAM tagging. To assess miR-223-3p transfection efficiency by flow cytometry, the mimic was co-transfected with FAM-labeled scrambled control to a final concentration of 25 nM. Transfection efficiencies were also assessed by RTqPCR after 18 hours and 2.5 days using methods described in Chapter 2.7 Molecular biology. To assess phenotypic and functional changes in miR-223-3p mimic transfected NK cells, only miR-223-3p mimic was transfected into the cells. All mimic and inhibitor sequences can be found in **Table 2.1.**

Table 2.1. Qiagen miRCURY® LNA® miRNA mimic and inhibitor sequences.

Qiagen miRCURY® LNA® miRNA mimics and inhibitors	Sequence
miR-27a-3p mimic	UUCACAGUGGCUAAGUUCCGC
miR-27-3p inhibitor	AAGUGUCACCGAUUGAAGGCG
miR-125b-5p mimic	UCCCUGAGACCCUAACUUGUGA
miR-125b-5p inhibitor	AGGGACUCUGGGAUUGAACACU
miR-146a-5p mimic	UGAGAACUGAAUUCCAUAGGCUG
miR-146a-5p inhibitor	ACUCUUGACUUAAGGUAUCCGAC
miR-155-5p mimic	UUAAUGCUAAUCGUGAUAGGGGUU
miR-155-5p inhibitor	AAUUACGAUUAGCACUAUCCCCAA
miR-223-3p mimic	UGUCAGUUUGUCAAAUACCCCA
miR-223-3p inhibitor	ACAGUCAAACAGUUUAUGGGGU

2.4.2 NK transfections – Lipofectamine

Before attempting to transfect primary NK cells, we optimized lipofectamine transfection of the NK-92 cell line in X-vivo serum free media using the Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific). NK-92 cells were plated at $5x10^5$ cells/mL in X-vivo serum free media with 100 IU/mL IL-2 in a 96 well U-bottom plate. Meanwhile, OptiMEM serum reduced media (Thermo Fisher) was mixed with 3, 4.5, 6, or 7.5 μL of lipofectamine reagent such that the final solution volume was 25 μL. In a separate tube, OptiMEM media was mixed with 25 nM FAM-labeled scrambled mimic to a final volume of 25 μL. These solutions were combined, gently mixed, and incubated for 20 minutes at room temperature prior to addition to the cells. The cells were incubated for 24 hours prior to flow cytometry assessment of transfection efficiency and viability. The optimal dilution of the lipofectamine reagent on NK-92 cells was 7.5 μL with a viability of 95.5% and an efficiency of 37.4%.

Unfortunately, this protocol could not be translated for use with primary NK cells as it resulted in poor cellular viability. Several strategies and protocol modifications were attempted to optimize primary NK cell transfection with lipofectamine. These strategies included: serial dilutions of lipofectamine reagent to a minimum of 1 μL/well, increasing the concentration of IL-2, adding 10-100 ng/mL IL-15 (ProSpec) and IL-15R (ProSpec), spinning the cells in a U bottom plate at 150 x*g* for 3 minutes, and increasing total cellular concentration to $1x10^6$ cells/mL. Cellular viability was assessed by trypan blue and flow cytometry while transfection efficiency was assessed by flow cytometry.

2.4.3 NK transfections – Nucleofection

Transfection of primary NK cells by nucleofection was attempted by using Amaxa® Human NK Cell Nucleofector® Kit (Lonza) with the manufacturer's protocols. A 12-well plate was pre-incubated with 2 mL/well supplemental X-vivo media at 37°C. Pre-equilibrating the media was a manufacturer suggestion to improve cellular viability post nucleofection. The NK cells (2-3x10^6 cells), were suspended in 100 μL room temperature Nucleofector® solution and were combined with 5 μg of FAM-labelled miRNA scrambled mimic. The sample was transferred to a certified cuvette, with caution to avoid introduction of bubbles at the bottom of the cuvette. The cuvette was placed into an Amaxa™ Nucleofector™ II (Lonza) and subjected to program U-008. Approximately 500 μL of pre-equilibrized X-vivo media was applied to the sample and the sample was then transferred to the 12-well plate. The transfection efficiency was measured only twice by detection of FAM+ NK cells by flow cytometry because cellular viability was extremely low and other attempts of this transfection technique were followed by viability assessment by trypan blue. Due to continued low viability, this method was not used for NK cell transfection for subsequent experiments.

2.4.4 NK transfections – TransIT-SiQuest

Isolated primary human NK cells were attempted to be transfected with FAM-labeled scrambled mimic (Qiagen) using the TransIT-SiQuest® Transfection reagent (Mirus). NK cells were resuspended in X-vivo media with 100 IU/mL IL-2 at 1x10^6 cells/mL in 510 μL and rested for at least two hours after isolation. Meanwhile, 50 μL OptiMEM serum reduced media (Thermo Fisher) was gently mixed with 0.5, 1.5, or 3 μL TransIT-siQuest

transfection reagent. Scrambled mimic was added to the OptiMEM-siQuest solution to a final concentration of 25 nM and mixed gently. This solution was incubated at room temperature for 20 minutes before the solution was added dropwise to the cells. The cells were assessed by trypan blue and stained with a fixable viability dye 24 hours post transfection. The transfection efficiency, as determined by percentage of FAM positive NK cells, and cellular viability was assessed 24 hours post transfection by flow cytometry.

2.4.5 NK transfections – TransIT-TKO

Primary human NK cells, FLS, or PBMCs were transfected with 25 nM of mimic or inhibitor miRNAs (Qiagen) using the TransIT-TKO® Transfection reagent (Mirus) per manufacturer's directions. Briefly, 1-2x10^6 primary human NK cells or PBMCs were resuspended in 510 μL of X-vivo serum free media with 110 IU/mL IL-2. Alternatively, 80% confluent FLS (passage 4) cells had their regular growth media replaced with X-vivo media 2 hours prior to cellular transfection. In a separate sterile microfuge tube, 50 μL of Opti-MEM™ Reduced Serum free media (Thermo Fisher) was combined with 1 μL of the TKO reagent and 25 nM of a FAM-labeled mimic/inhibitor miRNA. This solution was incubated in the dark at room temperature for 20 minutes. Subsequently, the Opti-MEM-TKO-mimic solution was added dropwise to the cells. The addition of this solution made the final concentration of IL-2 100 IU/mL in the NK and PBMC wells. Transfected cells were rocked gently and incubated at 37°C under 5% CO_2 conditions for either 18 hours or 2.5 days.

To optimize this technique, we attempted transfections with 0.5, 1, 2.5, and 4 μL of TransIT-TKO reagent, and tested the transfection efficiency by flow cytometry after 18 hours and 2.5 days. Being the most successful transfection technique employed, TransIT-TKO was used for all subsequent NK cell transfections. Transfection controls included a FAM-labeled scrambled (non-targeting) oligonucleotide transfected control as well as a no transfection control.

2.5 Measurement of NK cell cytotoxicity

2.5.1 Cell trace violet staining of target cells

Target cells (K562s or autologous PBMCs) were stained with a cell trace violet (CTV) to better distinguish effector and target cells on the flow cytometer. This was accomplished using the CellTrace™ Violet Cell Proliferation Kit (Thermofisher) immediately before co-culturing. The target cells were resuspended up to $20x10^6$ cells/mL in 1 mL of 1.25 μM CTV in 1x PBS with 2% FBS and incubated at 37°C, 5% CO_2 for 30 minutes. The cells were washed twice with 30 mL 1x PBS 2% FBS and centrifuged at 300 x*g* for 10 minutes. Once the cells were stained, they were resuspended in X-vivo media in preparation for co-culturing.

2.5.2 K562 co-culturing assay

The supernatants of all effector cells (PBMCs or primary NKs) were collected prior to any additional manipulation of the cells and stored at -80°C for later use. Prior to co-culture, the target cells were stained with CTV as previously described. The effector cells were cultured for an additional 5 hours with or without K562s in X-vivo serum free

media with 100 IU/mL IL-2 and anti-LAMP1 (CD107a) antibody (1/200). PBMCs were co-cultured at a 3:1 effector to target ratio and primary NK cells were co-cultured at a 1:1 ratio. After the first hour, 1x Brefeldin A (eBioscience) was added to all cells designated for intracellular staining. Surface and intracellular staining was completed once the incubation was concluded as described in Chapter 2.6 Flow cytometry.

2.5.3 Antibody dependent cellular cytotoxicity assay

To assess adaptive NK cell function in the context of autoimmunity, NK cell ADCC was assessed against autologous B cells. Upon blood donation from healthy individuals, the blood was separated to isolate NK cells (3/4 of donation) and whole PBMCs (1/4 of donation) at the same time. Whole PBMC and NK cell isolations were completed as described in Chapter 2.1 Cell isolations and culturing. After isolation, whole PBMCs were resuspended in X-vivo media with 100 IU/mL IL-2 at $2x10^6$ cells/mL. The isolated NK cells were plated in a 24 well plate at $1\text{-}2x10^6$ cells/mL in 510 μL X-vivo media and incubated for 2 hours. After a 2 hour rest period, the cells were transfected with miRNA mimics or inhibitors as described in Chapter 2.4.5 NK transfections – TransIT-TKO. Transfected NKs and whole PBMCs were incubated at 37°C and 5% CO_2 for 2.5 days.

Two and a half days later, whole PBMCs were stained with CTV and resuspended at $2.5x10^6$ cells/mL in X-vivo serum free media. Transfected NK cells were resuspended in X-vivo serum free media to a concentration of $5.2x10^5$ cells/mL. The percentage of B cells in PBMCs is 3-10% according to BioRad and 5-10% according to Miltenyi. Therefore, transfected NK cells were co-cultured with CTV+ whole PBMCs by adding 50 μL NKs to 150 μL PBMCs. This provided a 1:1 ratio of transfected NK cells to B cells,

assuming approximately 7% of PBMCs were B cells. A concentration of 5 μg/mL of anti-CD20 RTX (Cedarlane) chimeric antibody and 1/200 anti-LAMP1 (CD107a) antibody was added to the co-cultures, the cells were spun at 200 x*g* for 3 minutes and incubated at 37°C under 5% CO_2 conditions for 2 hours. After the incubation was complete the cells were stained with the ADCC panel as described in Chapter 2.6 Flow cytometry.

2.6 Flow cytometry

2.6.1 Flow cytometry staining protocol

All spins were conducted at 800 x*g* for 5 minutes. In microcentrifuge tubes, the cells were pelleted, washed with immunofluorescence buffer (IMF) (1x PBS, 0.5% bovine serum albumin (BSA), 0.1% sodium azide, pH=7.38) buffer, and incubated with 1x Fc block (1/8 solution of 0.1g/mL 50% human IgG and 50% heat aggregated human IgG, pH=8.5) at 4°C for 15 minutes. Brilliant stain buffer (BD Biosciences) containing predetermined mAbs was added directly to the Fc incubated cells to a final volume of 100 μL. The cells were incubated at room temperature in the dark for 20 minutes. The cells were washed with 1x PBS, resuspended in 1x PBS containing 1/1000 eFluor506 fixable viability dye (Thermo Fisher) and incubated for 30 minutes at 4°C in the dark. Following another IMF wash, the cells were either resuspended in 1% paraformaldehyde, stained for intracellular antigens with the Fixation/Permeabilization Solution kit (BD Bioscience) as per manufacturer's instructions, or stained for annexin V. To stain intracellularly, the cells were washed with 1x BD Perm/Wash™ buffer, resuspended in Fixation/Permeabilization solution, and incubated at 4°C for 20 minutes. Proceeding another wash with 1x BD Perm/Wash, the cells were resuspended in BD Perm/Wash containing predetermined

intracellular mAb at their respected concentrations. Finally, the cells were washed once more with 1x BD Perm/Wash and resuspended with IMF buffer. To assess the apoptotic state of the cells, non-fixed surface stained cells were resuspended in 50 μL 1x annexin V binding buffer (BD) with 0.625 μL FITC-annexin V (BD) and incubated for 15 minutes at room temperature in the dark. The cells were directly transferred to polystyrene flow cytometry standard (FCS) tubes containing an additional 400 μL 1x annexin V binding buffer. Annexin V stained cells were acquired live. Data collection was completed at Dalhousie University's CORE flow cytometry facilities: BD FACS Canto II or BD Fortessa. A complete table of mAbs, fluorochromes, dilutions and manufacturers can be found in **Table 2.2.**

2.6.2 Cell sorting

To assess baseline expression of miRNAs in specific NK cell populations, whole PBMCs or primary fresh NK cells were sorted using the BD FCS Aria III at Dalhousie University's Core flow cytometry facility. Donors were pre-selected, based on the presence of a bimodal NKG2C phenotype, were rested overnight in X-vivo media with 1000 IU/mL IL-2 for PBMCs and 100 IU/mL IL-2 for primary NK cells. Collections tubes were coated with 4% BSA in 1x PBS at 4°C overnight. The next morning, the cells were stained as previously described using sorting buffer (1x PBS, pH=7-7.4, 25 mM HEPES (Gibco), 0.5% BSA (Sigma), 1mM ethylenediamine tetraacetic acid (Sigma)) instead of regular IMF buffer, filtered with a 40 micron cell strainer, and resuspended in 500 μL sorting buffer. The cells were sorted into two viable populations: $CD56^{+}CD3^{-}NKG2C^{+}$ and $CD56^{+}CD3^{-}NKG2C^{-}$. Once the sort was complete, primary sorted

populations were assessed for total purity by re-running a small sample on the FCS Aria III. MiRNA expression of each population was assessed by RTqPCR as described in Chapter 2.7 Molecular Biology.

2.6.3 Flow cytometry staining panels

The following flow cytometry panels were created to assess phenotypic and function change of NK cells post miRNA mimic and inhibitor transfections (**Table 2.2**). All antibodies were originally titrated at 1/20, 1/60, and 1/200 to choose an appropriate dilution and then adjusted as needed.

Table 2.2 Flow cytometry antibodies and panels

Laser (excitation)	Fluoro-chrome	Marker	Biological Function	Clone	Dilution	Supplier	Panel
Violet (405nm)	BV786	KIR3DL1 (CD158e)	NK cell KIR	DX9	1/200	BD	1, 2
	BV711	IFN-γ	Cytokine	B27	1/60	BD	2
	BV711	NKp46	Activating receptor	9E2	1/100	BD	3
	BV711	NKG2D	Activating receptor	1D11	43485	BD	1
	BV605	CD16	Fc Ab receptor	3G8	1/50	BD	4
	BV650	CD3	T and NKT cell marker	UCHT1	1/200	BD	1, 2, 4
	eFluor506	Viability	/	/	1/1000	ThermoFisher	1, 2, 4
	BV421	CTV	Marker for target cells	/	1.25 uM	ThermoFisher	1, 2, 4
Blue (488nm)	FITC	Annexin V	Marker of apoptosis	/	1/80	BD	4
	FITC	FAM-miRNA	miRNA mimic/inhibitor	/	25 nM	Qiagen	1, 2, 4
Green (532nm)	PeVio770	KIR2DL1 (CD158a)	NK cell KIR	143211	1/200	Miltenyi	1, 2
	PeCy7	CD19	B cell marker	HIB19	1/100	BioLegend	4
	PE-Cy5.5	KIR2DL2 /S2/L3	NK cell KIR	GL183	1/200	BC	1, 2
	PeVio615	CD56	NK cell marker	REA196	1/200	Miltenyi	1, 2, 4
	PE	NKp30	Activating receptor	REA832	1/100	Miltenyi	3
	PE	STAT1	Signaling molecule	REA272	1/20	Miltenyi	4
	PE	Perforin	Cytotoxic granule	REA1061	1/200	Miltenyi	2
	PE	NKG2C	Activating receptor	134591	1/200	R&D	1, 4
Red (628nm)	APC-H7	CD107a	Degranulation marker	H4A3	1/200	BD	1, 4
	APC-H7	TNF	Cytokine	cA2	1/20	Miltenyi	2
	AlexaFluor 700	CD2	CD16 co-receptor	TS1/8	1/200	BioLegend	4
	AlexaFluor 700	Granzyme B	Cytotoxic granule	GB11	1/200	BD	2
	APC	CD57	Differentiation marker	REA769	1/200	Miltenyi	4
	APC	NKp44	Activating receptor	P44-8	1/100	BD	3
	APC	TRAF6	Signaling molecule	EP591Y	1/200	Abcam	2
	APC	NKG2A	Inhibiting receptor	Z199	1/60	Miltenyi	1, 2
	APC	LIR1 (ILT2, CD85)	Inhibiting receptor	GHI/75	1/60	BioLegend	1, 2

1 - surface K562 degranulation panel; 2 - intracellular K562 degranulation panel; 3 - NCR Abs;
4 - ADCC / adaptive NK cell panel

2.6.4 Flow cytometry analysis

Stained cells were read using a BD LSR Fortessa SORP or BD Canto II with FACS Diva Software (BD) and analysed with FlowJo (10.5.2) Software (Treestar, Ashland, OR). To ensure consistent geometric means, all experiments read on the Fortessa were preceded by Sphero™ 8 peak Rainbow Calibration Beads (Biolegend) and voltages were adjusted such that all experiments matched. A new baseline was set with every panel prior to running experiments. Briefly, UltraComp beads (eBioscience) were stained with 0.5-2 μL of mAb and incubated for 20 minutes at room temperature. The beads were washed with 1x PBS (800 x*g* for 5 minutes) and resuspended in IMF buffer. Additionally, the cells designated for each experiment, NK cells, PBMCs, or a combination of target and effector cells, were stained with the appropriate panel of antibodies as described in Chapter 2.6.1. The UltraComp beads and cells were read on the appropriate flow cytometer and compensation was calculated to ensure all mAbs and their conjugated fluorophores were compatible. Finally, 8 peak Rainbow Beads were run, without compensation but with the final voltages, and the output mean fluorescent intensity for each fluorophore was set for that panel.

2.7 Molecular biology

2.7.1 RNA lysis and isolation

Total RNA, including microRNA, was isolated using the miRNeasy RNA Isolation kit (Qiagen) as described by the manufacturer. NK cells were lysed with 700 μL of Qiazol, vortexed vigorously, and rested at room temperature for 5 minutes before long term storage at -80°C. At time of RNA isolation, 140 μL of chloroform was added to room

temperature lysates followed by vigorous shaking for 15 seconds. The cells were centrifuged at 12,000 x*g* at 4°C for 15 minutes to separate the homogenate into three distinct phases: upper aqueous, white protein rich interphase, and a lower organic phase. After centrifugation, the upper RNA containing aqueous phase was removed, mixed with 1.5 equivalents of absolute ethanol, added to the provided column, and centrifuged at 12,000 x*g* for 15 seconds. The cells were washed with 350 μL RWT buffer and an on-column DNase digestion was performed. DNase I stock (10 μL) was added to 70 μL of RDD buffer. The RDD-DNase I solution was added to the column and incubated for 15 minutes at room temperature. The cells were washed once more with 350 μL RWT buffer and once with 700 μL with RPE buffer. Following this, the column was spun at 16,000 x*g* for 1 minute to dry the column. The isolated RNA was eluted from the column with 30 μL RNase-free sterile water. RNA was stored at -80°C. RNA was tested for quantity and quality using a NanoDrop spectrophotometer, however, the RNA concentration was too low to accurately measure RNA quantity.

2.7.2 cDNA synthesis

To assess molecular changes in both miRNA and mRNA expression, the miScript II RT kit (Qiagen) and iScript cDNA Synthesis kit (Bio-Rad) were used as per manufacturer's instructions. To make miRNA-cDNA, 2 μL of HiSpec buffer, 1 μL of 10x miScript nucleic mix, 3.5 μL of RNase-free water, 1 μL of reverse transcriptase, and 2.5 μL of RNA were mixed and incubated for 75 minutes at 37°C. This was followed by a 5 minute incubation at 95°C to inactivate the reverse transcriptase. The 10 μL reaction was diluted to a final volume of 100 μL and stored at -20°C. Alternatively, cDNA from mRNA was

created in a PCR strip by mixing 2 μL of 5x iScript buffer, 0.5 μL of reverse transcriptase, 5 μL of RNase-free water, and 2.5 μL of RNA template. The PCR strip containing the cDNA reactions was placed into a CFX Connect™ real-time PCR detection system thermocycler (Bio-Rad) where it was primed at 25°C for 5 minutes, reverse transcribed at 46°C for 20 minutes, inactivated at 95°C for 1 minute, and held at 4°C. Once the reaction was complete, the cDNA was diluted 1/10 and stored at -20°C. For both miRNA and mRNA derived cDNA, and no-reverse transcriptase controls, where reverse transcriptase was omitted from the reaction mixture, was included to ensure there was no DNA contamination.

2.7.3 Real time quantitative PCR

To determine the expression of various miRNAs and mRNAs, 20 μL RTqPCR reactions were created containing molecular grade sterile water, 1x SsoAdvancedTM Universal SYBR® Green Supermix, 0.25 mM forward and reverse primers, and 5 μL of the appropriate cDNA. Reference genes for miRNA cDNA expression were selected by comparing stable expression of *miR-93-5p, miR-103a-3p, miR-191-5p, RNUB6*, and *RNU48* on non-stimulated, IL-18, and TGFβ pooled cells using the GeNorm algorithm[151,152]. Of these reference genes, *miR-191-5p* and *miR-103a-3p* were the most stable.

The references used to assess mRNA expression were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine-guanine phosphoribosyl transferase (*HPRT*). A temperature gradient was run on each primer to select an appropriate annealing temperature. All thermocyclers were conducted in the CFX

Connect™ real-time PCR detection system (BioRad) and analysed with CFX Maestro™ software (**Table 2.3-2.4**). All miRNA primers are supplied by Qiagen and all mRNA primers are designed by BioRad. Each PCR plate contained technical duplicates of the reactions as well as a no template control and no-reverse transcriptase control(s).

Table 2.3. RTqPCR cycling for miRNA derived cDNA.

<table>
<tr><th></th><th>Cycle Step</th><th>Degrees (°C)</th><th>Time (seconds)</th></tr>
<tr><td></td><td>Activation</td><td>95</td><td>30</td></tr>
<tr><td rowspan="3">Repeat 40 times</td><td>Denaturation</td><td>95</td><td>10</td></tr>
<tr><td>Annealing</td><td>Primer specific</td><td>15</td></tr>
<tr><td>Extension
& Plate Read</td><td>70</td><td>20</td></tr>
<tr><td></td><td>Melt Curve</td><td>4-95</td><td>5 / degree</td></tr>
</table>

Table 2.4. RTqPCR cycling for mRNA derived cDNA.

<table>
<tr><th></th><th>Cycle Step</th><th>Degrees (°C)</th><th>Time (seconds)</th></tr>
<tr><td></td><td>Activation</td><td>95</td><td>30</td></tr>
<tr><td rowspan="3">Repeat 40 times</td><td>Denaturation</td><td>95</td><td>10</td></tr>
<tr><td>Annealing</td><td>60</td><td>15</td></tr>
<tr><td>Extension
& Plate Read</td><td>72</td><td>15</td></tr>
<tr><td></td><td>Melt Curve</td><td>4-95</td><td>5 / degree</td></tr>
</table>

2.7.4 Droplet digital PCR

DdPCR was used to assess the expression of miRNA of low quantity samples. Briefly, 22 µL ddPCR reactions were prepared using 1x QX200 EvaGreen Supermix (BioRad), 0.1 µM forward and reverse primers, 5 µL cDNA template, and water. A DGE cartridge (BioRad) was placed in its holder and 20 µL of well mixed ddPCR reactions were added to the center wells with caution to avoid introduction of introduce bubbles. A volume of 65 µL of QX200™ Droplet Oil for EvaGreen (BioRad) was added to the appropriate well

and the cartridge was covered with a rubber Droplet Generator Gasket DG8 gasket. Droplets were generated using QX200™ Droplet Generator (BioRad). Droplets were transferred to a 96-well PCR plate and the plate was sealed with a PX1 PCR Plate Sealer (BioRad) at 180°C for 6 seconds. The sealed plate was placed in a BioRad C1000 Touch™ thermocycler and our standard ddPCR protocol for EvaGreen miRNA was used (**Table 2.5**). The droplets were read by the QX200™ Droplet Reader (BioRad) and data was assessed using QuantaSoft™ software version 1.7. All primer efficiencies and annealing temperatures, as well as in-house designed sequences, can be found in **Table 2.6.**

Table 2.5. ddPCR cycling.

<table>
<tr><th></th><th>Cycle Step</th><th>Degrees (°C)</th><th>Time (minutes)</th><th>Ramp Speed (°C/second)</th></tr>
<tr><td></td><td>Activation</td><td>95</td><td>5</td><td>2</td></tr>
<tr><td rowspan="3">Repeat 50 times</td><td>Denaturation</td><td>95</td><td>0.5</td><td>2</td></tr>
<tr><td>Annealing</td><td>Primer specific</td><td>1</td><td>2</td></tr>
<tr><td>Extension</td><td>70</td><td>1</td><td>2</td></tr>
<tr><td></td><td rowspan="2">Melt Curve</td><td>4</td><td>5</td><td>2</td></tr>
<tr><td></td><td>90</td><td>5</td><td>2</td></tr>
<tr><td></td><td>Infinite hold</td><td>12</td><td>∞</td><td>N/A</td></tr>
</table>

Table 2.6. Primer sequences, efficiencies, and annealing temperatures.

Primer	Sequence	Efficiency (%)	Annealing Temp. (°C)
miR-103a-3p(Ref)	AGC AGC ATT GTA CAG GGC TAT GA	106.1	56
miR-191-5p (Ref)	CGC GCA ACG GAA TCC CA	101.3	56
RNU48 (Ref)	Manufacturer's proprietary sequence	99.9	56
RNUB6 (Ref)	Manufacturer's proprietary sequence	91.7	56
miR-93-5p (Ref)	Manufacturer's proprietary sequence	100.5	56
HPRT (Ref)	Manufacturer's proprietary sequence	109.3	60
GAPDH (Ref)	Manufacturer's proprietary sequence	99.6	60
miR-27a-3p	TTC ACA GTG GCT AAG TTC CGC	103	56
miR-125b-5p	Manufacturer's proprietary sequence	94.4	56
miR-146a-5p	Manufacturer's proprietary sequence	98.9	56
miR-155-5p	GGT TAA TGC TAA TCG TGA TAG GGA AA	108.5	60
miR-223-3p	Manufacturer's proprietary sequence	96.2	56
IFNγ	Manufacturer's proprietary sequence	97	60
TNF	Manufacturer's proprietary sequence	96.9	60

* Ref indicates reference gene

2.8 Donor genotyping

2.8.1 DNA extraction

To assess the educational status and immunogenetics of each individual, DNA genotyping of *KIR* and *HLA* was completed for each NK and PBMC donor. Because NK cell reactivity is calibrated through education and differs between individuals, genotyping will help analyse phenotypic results and assess the role of NK education in each donor. DNA was extracted from whole blood or PBMCs using the gSYNC^{TM} DNA Extraction Kit (Froggabio) as per manufacturer's instructions. The samples were resuspended in 1x PBS such that the final volume was 200 μL and incubated with 20 μL Proteinase K at 60°C for 5 minutes. The cells were subsequently lysed with the addition of 200 μL GSB buffer and incubated at 60°C for 5 minutes, shaking the solution after every 2 minutes. To bind the DNA to the membrane in the provided column, 200 μL absolute ethanol was added to the

sample solution, the lysates were mixed immediately by vortex for 10 seconds, transferred to the column, and centrifuged at 14-16,000 x*g* for 3 minutes. The membrane was washed twice, once with 400 µL W1 buffer and once with 600 µL Wash buffer and dried with centrifugations of 14-16,000 x*g* for 3 minutes. Finally, the extracted DNA was eluted with 100 µL of DNase-free water into a 1.5 mL microcentrifuge tube.

2.8.2 KIR and HLA genotyping

KIR and *HLA* typing is routinely completed by the Boudreau laboratory technician, Fang Liu. Protocols for typing were derived from publications by Hsu, *et al*. Vilches, *et al.* and Hong, *et al*[153–155]. Cellular DNA (100 ng) was combined with 10 µL REdExtract-N-Amp™ PCR ReadyMix™ (Sigma Aldrich), 0.1 µM 595 and 360 internal controls, 2.5 µM forward and reverse primers (*KIR2DS2, KIR2DL2, KIR2DL3, KIR2DL1, KIR2DL4, KIR3DL1, KIR3DS1, KIR2DL5, KIR2DS3, KIR2DS3, KIR2DS5, KIR2DS4, HLA-C1, HLA-C2, HLA Bw4*), and water. The Eppendorf AG 22331 Hamburg thermocycler was used with the appropriate program (**Table 2.7** and **2.8**). End point PCR results were run on 1.5% agarose (Sigma Aldrich) gel electrophoresis and visualised with Red™ Imaging System (Alpha Innotech).

Table 2.7 *KIR* PCR program

	Degrees (°C)	Time (seconds)
Repeat 10 times	95	120
	94	10
	65	40
Repeat 20 times	94	20
	61	30
	72	30

Table 2.8 *KIR* Ligand (*HLA*) PCR program

<table>
<tr><th></th><th>Degrees (°C)</th><th>Time (seconds)</th></tr>
<tr><td rowspan="4">Repeat 37 times</td><td>95</td><td>300</td></tr>
<tr><td>95</td><td>15</td></tr>
<tr><td>63</td><td>20</td></tr>
<tr><td>72</td><td>60</td></tr>
<tr><td></td><td>72</td><td>300</td></tr>
<tr><td></td><td>4</td><td>∞</td></tr>
</table>

2.9 Statistical analysis

All statistical analyses were conducted on either normalised qPCR relative gene expression or flow cytometry geometric means as appropriate. Samples were tested for normality with the Shapiro-Wilk normality test, and passed normality if $\alpha=0.05$. If the data passed normality, analysis of variance (ANOVA) and parametric matched ratio paired *t* tests were completed. If the data did not pass normality, paired non-parametric Wilcoxon *t* tests were performed. Data for all statistical tests was deemed significant if $p<0.005$. All donors have been assigned specific colours and plots represent individual values or mean ± standard deviation.

CHAPTER 3: RESULTS

3.1 Optimal serum free media for miRNA studies on NK cells

MiRNAs are extremely conserved, often having the exact same or highly overlapping sequences in mammals, including: humans, mice, and cows. **Table 3.1** demonstrates a miRbase sequence blast of miR-146a-5p and miR-155-5p in these three species, clearly demonstrating the conservation of these miRNAs across mammalian species. Additionally, miRNAs are highly stable: they can be measured in human biofluids, but are also maintained in bovine or other animal-derived serums[156]. Both of these features make the study of miRNAs *in vitro* extremely difficult. Therefore, to limit the introduction of exogenous miRNAs, we optimized serum free media that supports the growth of the NK-92 cell line as well as primary NK cells *in vitro*.

Table 3.1 MiRBase sequence blast. miRBase sequence of human, mouse, and cow miR-146a-5p and miR-155-5p.

miRNA	Species	Sequence
miR-146a-5p	Human	UGAGAACUGAAUUCCAUGGGUU
	Mouse	UGAGAACUGAAUUCCAUGGGUU
	Cow	UGAGAACUGAAUUCCAUAGGUUGU
miR-155-5p	Human	UUAAUGCUAAUCGUGAUAGGGGUU
	Mouse	UUAAUGCUAAUUGUGAUAGGGGU
	Cow	UUAAUGCUAAUCGUGAUAGGGGU

Highlighting indicates sequence differences

Serum free X-vivo media was tested against RPMI, containing 10% FBS, and the ATCC recommended media, containing 12.5% horse serum and 12.5% FBS. NK-92 cells and primary NK cells were cultured in X-vivo, RPMI, and/or ATCC media with 100 IU/mL IL-2 for four days. The viability of the cells was assessed on days 1, 2, 3, and 4 or days 1 and 4 by trypan blue and/or flow cytometry (**Figure 3.1**). While growing NK-92 cells, X-vivo and RPMI media performed similarly and slightly out-performed the ATCC recommended media. NK-92 cells grown in serum free and RPMI conditions maintained a viability of 95%, while NK-92 cells in ATCC media decreased to a viability of 85% after four days in culture. The higher viability observed with X-Vivo and RPMI media was not significant by one-way ANOVA (**Figure 3.1B**). Similar to NK-92 results, primary NK cells grown in X-vivo serum free media maintained a higher average viability (92 ± 2%) than cells in ATCC media (87 ± 7%) after four days, although results were not significant by one-way ANOVA (**Figure 3.1D**). Nevertheless, these findings indicate that X-vivo serum free media can maintain primary NK cells *in vitro* and was therefore used in all subsequent experiments.

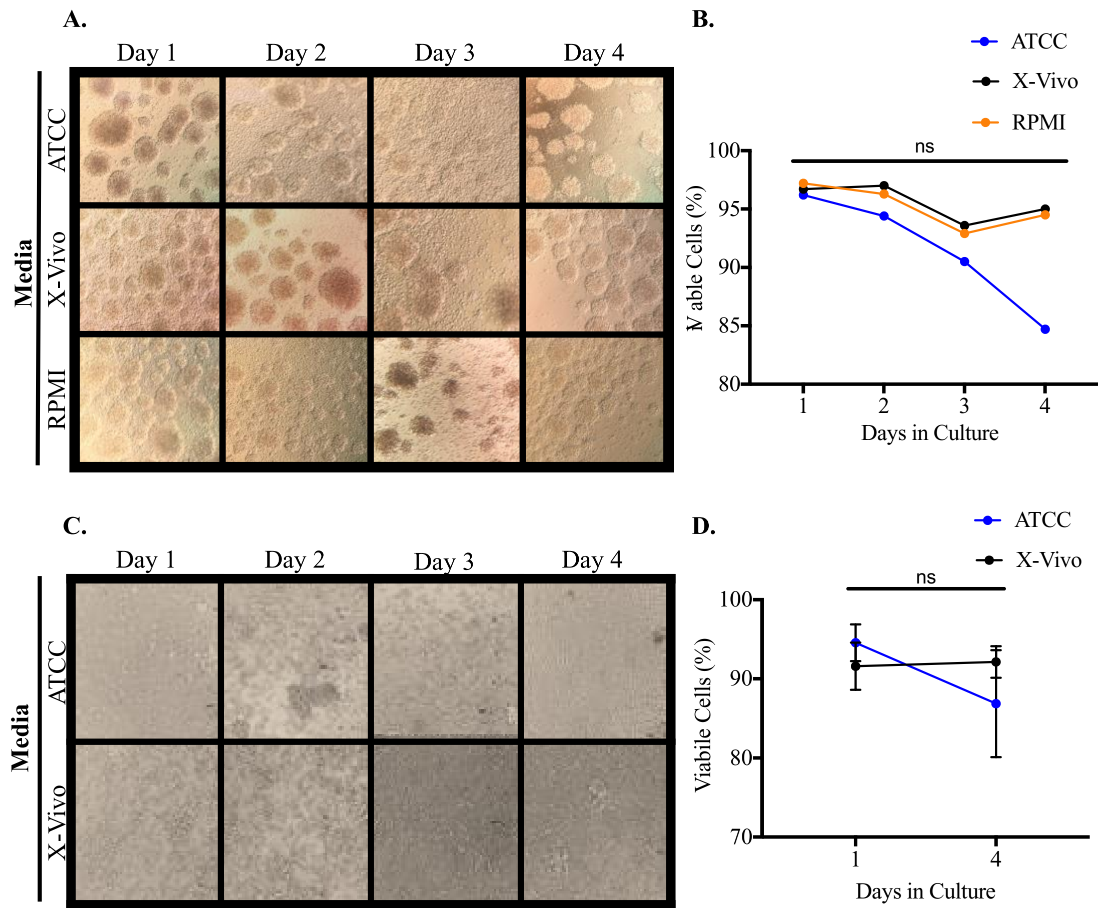

Figure 3.1 Primary NK cells can be maintained in serum free media for miRNA studies. NK-92 cells (A-B) and primary NK cells (C-D) were grown in ATCC recommended (25% serum), RPMI (10% serum), and X-vivo media with 100 IU/mL IL-2 up to four days and viability was assessed by trypan blue and flow cytometry. Images represent magnification with 20x objective lens. Data represented as individual or mean values ± standard deviation, n=1 NK-92 cells and n=3 primary NK cells. All data passed Shapiro-Wilk normality test. ns indicates no significant difference between medias by one-way ANOVA (B, D).

3.2 Pro-inflammatory and tolerogenic stimulation alter NK miRNA expression

MiRNAs are often dysregulated in various diseases, including arthritis, where inflammation may drive miRNA production to condition the local microenvironment[102,126]. To measure miRNA produced by NK cells, we exposed NK cells to inflammatory (IL-18) and regulatory (TGF-β) stimulation *in vitro*.

Originally identified as a cytokine that increases IFNγ production by Th1 cells[157], IL-18 can also activate NK cells to increase autocrine IFN-γ production, increase cytotoxicity, and proliferation[158,159]. IL-18 has also been identified to stimulate the expression of miR-155-5p[106], although it is unclear if this is a direct result of IL-18 signaling or an indirect result due to the increase of a miR-155-5p mRNA target, SOCS1. TGFβ was chosen for its inhibitory effects on NK cells, including reduced cytotoxicity and cytokine (IFNγ and TNF) production[50], as well as its ability to induce the expression of miRNAs, miR-146a-5p[124] and miR-27a-3p[108].

To determine the peak of NK cell miRNA expression under inflammatory and regulatory signaling, we performed an IL-18 and TGFβ stimulation time course analysis of NK, respectively. Primary human NK cells, grown in X-vivo media with 100 IU/mL IL-2, were stimulated with 50 ng/mL IL-18 or 5 ng/mL TGFβ for 6, 12, and 18 hours (**Figure 3.2 A**). The treatment concentrations were selected based on their common use in previous published literatures[53,108,142]. MiRNA expression was assessed by RTqPCR. Compared to a no stimulation control, IL-18 increased IFNγ and TNF mRNA at all time points, with that the exception that TNF mRNA expression was decreased after 18 hours. TGFβ treatment decreased the mRNA expression of IFNγ and TNF at all time points. For both treatments, the changes in IFNγ mRNA expression were larger than those observed

in TNF mRNA expression. The stimulation time selected from this experiment was 12 hours for three reasons: 1) the expression of IFNγ and TNF was still increased with IL-18 stimulation; 2) the largest decreased expression of these mRNAs was observed at 12 hours after TGFβ stimulation; and 3) it simulates a longer and therefore more chronic state of stimulation compared to 6 hours.

The expression of mRNA transcripts does not necessarily reflect the cytokine production by a cell. NK cells also have pre-existing acetylation and histone DNA modifications that support rapid production of cytokine transcripts to allow for the preparation for and quick reactions against biological threat[160]. In fact, NK cells are poised to rapidly respond to potential threats and contain pre-formed cytoplasmic granules. The concentration of cytokines largely relies on post-translational mechanisms. To verify that changes in gene expression observed by RTqPCR reflect differences in cytokine production, we assessed the protein level of these cytokines (**Figure 3.2 B, C**). To do this, human NK cells were pre-stimulated with IL-18 or TGFβ for 12 hours before being co-cultured at a 1:1 ratio with K562 cells for 5 additional hours. These conditions were compared to cells that were not pre-stimulated with cytokines but were co-cultured with K562s for 5 hours. The intracellular cytokine production was assessed by flow cytometry. As expected, IL-18 pre-stimulation increased the production of IFNγ and TNF, while TGFβ decreased the production of these cytokines compared to cells without pre-stimulation (**Figure 3.2 B, C**).

Finally, the expression of all five candidate miRNAs (miR-27a-3p, miR-125b-5p, miR-146a-5p, miR-155-5p, miR223-3p) in NK cells were assessed (**Figure 3.2 D, E**). These candidate miRNAs were all differentially expressed in patients with RA compared

to healthy donors and were identified through collaboration with REACH and literature reviews (**Table 1.1**). Human NK cells were stimulated for 12 hours with or without IL-18 or TGFβ. Of note, miR-125b-5p was below limit of detection by RTqPCR and was excluded from this experiment. As expected, the expression of miR-155-5p was significantly increased after a 12-hour IL-18 stimulation. In addition, the expression of miR-146a-5p was significantly increased in NK cells stimulated with IL-18. Stimulation with TGFβ significantly decreased NK cell expression of miR-155-5p and caused a non-significant decrease trend of miR-146a-5p. The expression of miR-27a-3p and miR-223-3p were unchanged by either cytokine stimulation. In sum, the activation of primary human NK cells with IL-18 stimulated an increased production of IFNγ and TNF as well as miR-155-5p and miR-146a-5p; TGFβ induced the reverse effect by reducing IFNγ, TNF, miR-155-5p and miR-146a-5p production.

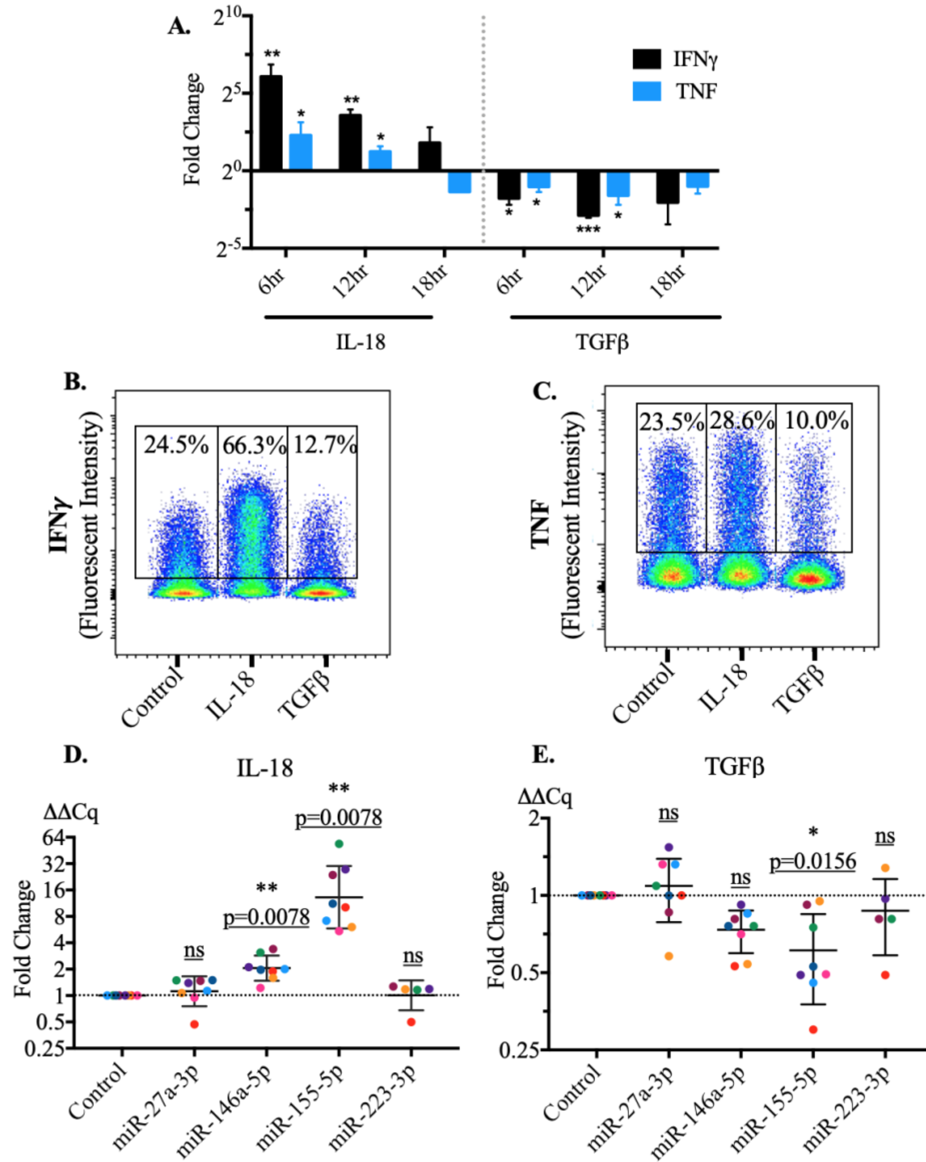

Figure 3.2 Cytokine stimulation alters miRNA expression by primary NK cells *in vitro*. (A) Primary NK cells in X-vivo media with 100 IU/mL IL-2 were stimulated with 50 ng/mL IL-18 or 5 ng/mL TGFβ for 6, 12, or 18 hours. NK cell activation/inhibition was assessed by IFNγ and TNF mRNA expression was assessed by RTqPCR, shown as fold change ± standard deviation. Viability was >90% for all conditions. Data passed Shapiro-Wilk normality test and significance was assessed by ratio paired *t* tests, n=3, *denotes p<0.05, **denotes p<0.01, and ***denotes p<0.001. Intracellular protein levels of IFNγ (B) and TNF (C) were assessed by flow cytometry after 12h with no stimulation, IL-18, or TGFβ followed by 5h K562 co-culture (1:1). Data are represented as mean fluorescent intensity (MFI) in a concatenated graph. MiRNA expression was assessed in primary NK cells after a 12-hour stimulation with IL-18 (D) or TGFβ (E) by RTqPCR. Data is represented as fold change ± standard deviation compared to scrambled transfection. Significance was assessed by ratio paired nonparametric Wilcoxon tests, n=5-8.

3.3 High miRNA transfection efficiency achieved with TransIT-TKO

3.3.1 TransIT-TKO outcompetes other transfection methods

Primary NK cell transfections are notoriously difficult due to low efficiencies and poor post-transfection viabilities. To assess the effect of individual miRNAs on NK cell phenotype and function *in vitro,* we optimized a new approach to transfecting NK cells. NK-92 cells were easily transfected, and we identified that 7.5 μL lipofectamine RNAiMAX transfection reagent with 25 nM FAM-labeled scrambled miRNA for 24 hours gave the optimal transfection efficiency in our experiment, with consistent high viabilities >90% and efficiencies of ~40% (**Figure 3.3 A**).

Transfection of primary NK cells was substantially more challenging than transfection of NK-92 cells. RNAiMAX, as well as several other transfection methods including nucleofection, TransIT-SiQuest, and TransIT-TKO, were attempted several times on primary NK cells from multiple healthy donors (**Figure 3.3 C, D, E, F**). Various technical modifications were attempted including decreasing transfection reagent quantity, increasing IL-2, adding other cytokines to improve viability, using different well plates, adding gentle centrifugation to concentrate the cells and transfection reagent, as well as increasing cellular density. NK cell transfection with lipofectamine, nucleofection, and TransIT-SiQuest consistently failed to achieve workable transfection and resulted in low viabilities and efficiencies. Ultimately, TransIT-TKO methodology, as described in Chapter 2.4.5, achieved the highest transfection efficiency by flow cytometry (**Figure 3.3 B, D, F**) and maintained the best cellular viability (**Figure 3.3 E, D**). TransIT-TKO was used for all future transfection experiments.

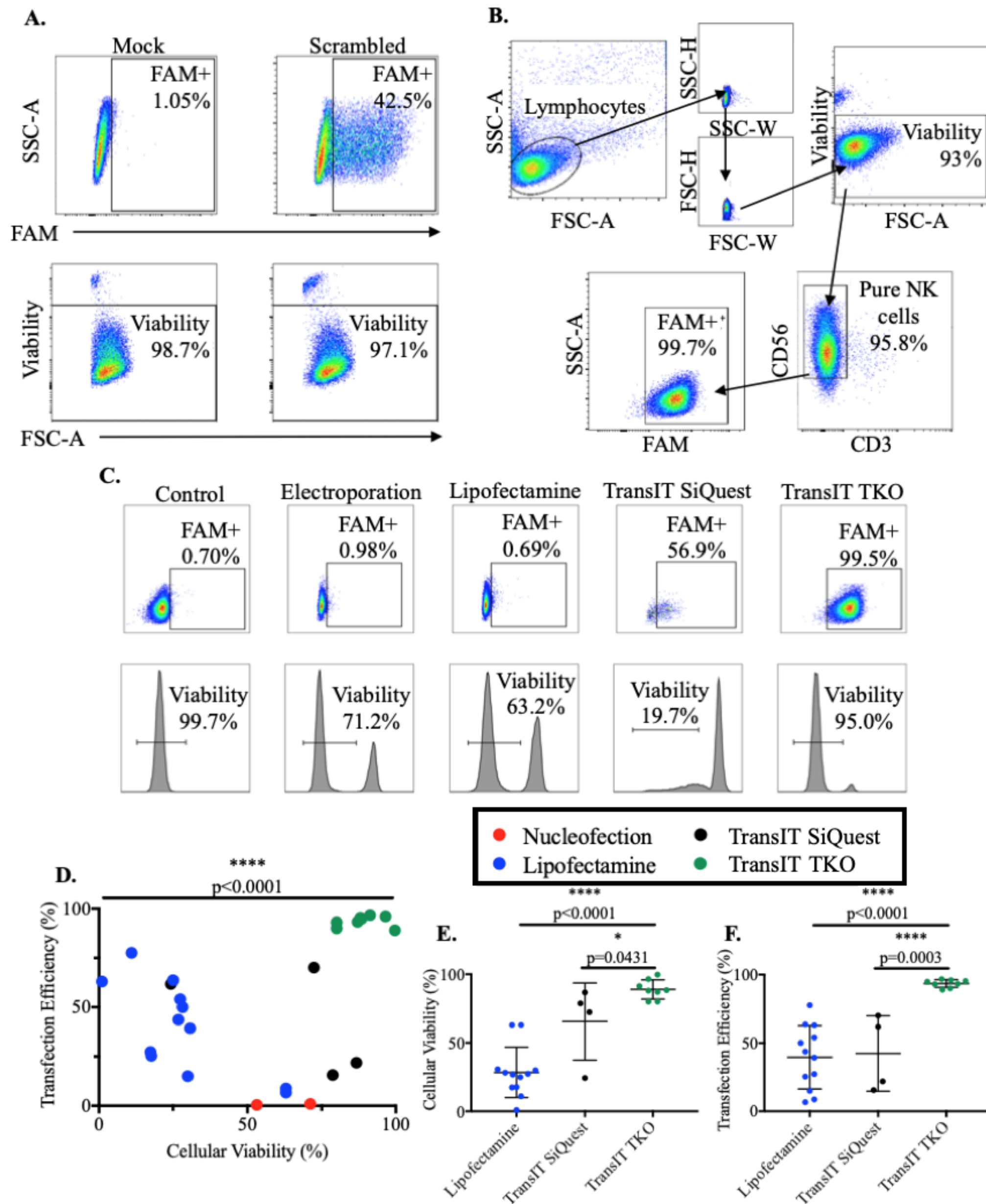

Figure 3.3 TransIT-TKO outcompetes other transfection methods. (A) NK-92 cells were efficiently transfected with FAM-labeled scrambled miRNA using Lipofectamine RNAiMAX transfection reagent (representative flow cytometry figure). (B) Gating strategy of primary NK cells to determine transfection efficiency, representative figure of TransIT-TKO scrambled transfected primary human NK cells. (C) Representative flow cytometry figure of all attempted transfection techniques of primary human NK cells. (D) X-Y plot of transfection efficiency (%) versus cellular viability (%) of individual attempts of primary NK cell transfection. Colours represent transfection technique. Data passed Shaprio-Wilk normality test and was assessed by one-way ANOVA. (E) Cellular viability post transfection. (F) Transfection efficiency of each technique. Plots represent individual viability/efficiency percentages ± standard deviation. Data passed Shapiro-Wilk normality test and was assessed by unpaired *t* tests.

3.3.2 Efficient NK delivery of miRNA mimic and inhibitors by TransIT-TKO

To assess functional delivery of mimic and inhibitors after 18 hours and 2.5 days of TransIT-TKO transfection, transfected NK cells were lysed and the miRNA was measured by RTqPCR (**Figure 3.4 A, C**). Transfection efficiencies were also determined by flow cytometry (**Figure 3.4 B**). Transfections with miRNA mimics efficiently delivered mature miRNA into the cells resulting in an average of over 1000 fold increase and 93±5% efficiency after 18 hours of transfection (**Figure 3.4 A, B**). Similarly, miR-146a-5p mimic and inhibitor transfection significantly increased mature miRNA delivery (fold increase of 785±220) and decreased cellular miRNA (fold decrease of 0.3±0.08), respectively. Therefore, TransIT-TKO can efficiently and functionally deliver sufficient quantities of miRNA mimic / inhibitor in primary human NK cells for downstream analyses.

3.3.3 TransIT-TKO can effectively transfect several cell types

We were surprised that TransIT-TKO, a reagent intended for siRNA delivery, had such high efficiency and viability when transfecting miRNA into primary NK cells. We wanted to determine whether this reagent was universally effective for other kinds of primary human cells, including PBMCs and JIA FLS (**Figure 3.5**). Between all three cell types, we achieved an average viability of 94.2±5% and efficiency of 98.4±0.3%. The fold increase (640±255) or decrease (0.27±0.2), as determined by RTqPCR, was comparable in all three cell types (**Figure 3.5 D**). Thus we determined TransIT-TKO is capable of transfecting a wide variety of primary human cells with extremely and consistently high efficiency without negatively affecting cellular viability.

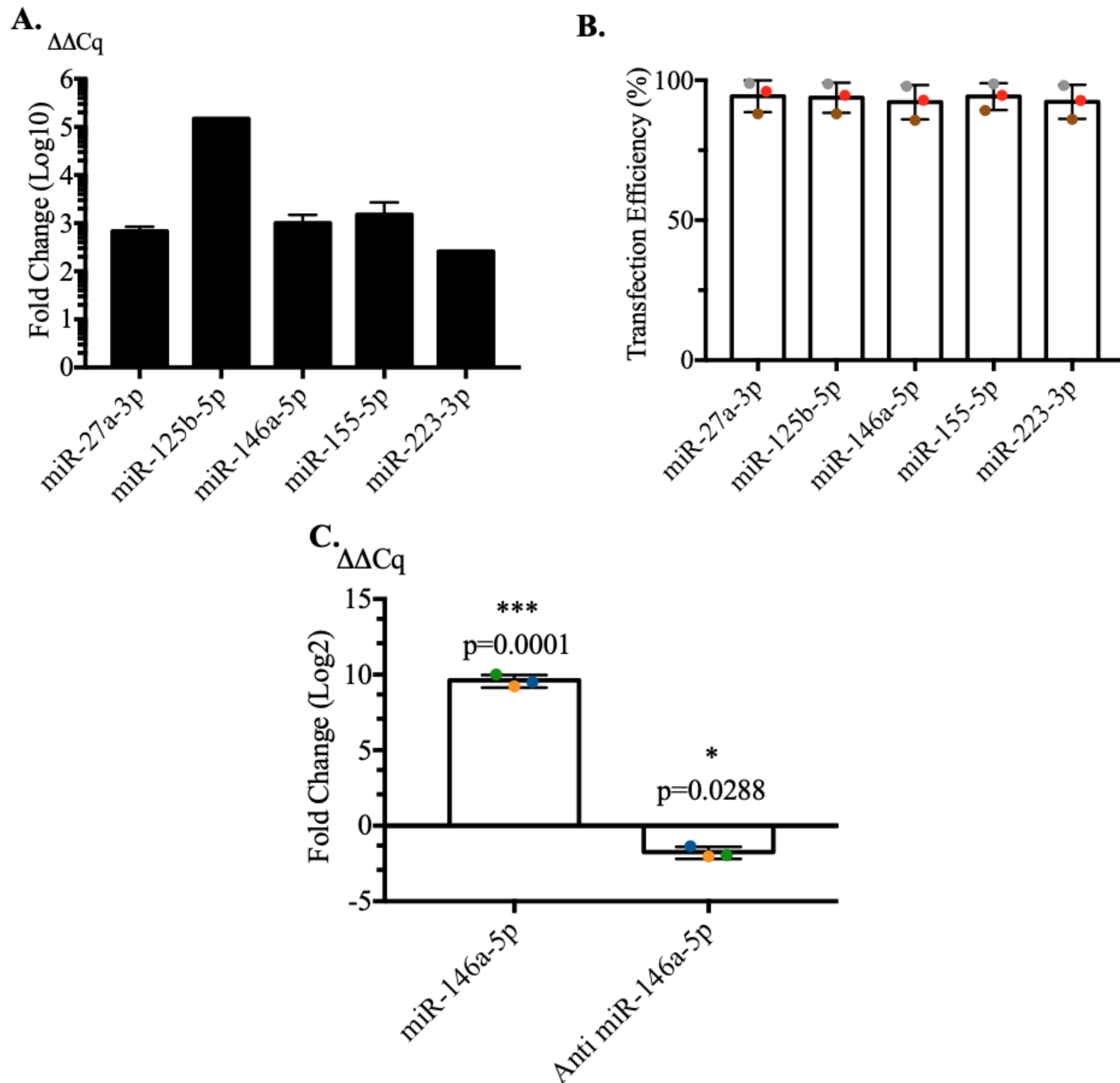

Figure 3.4 Efficient NK delivery of miRNA mimic and inhibitors by TransIT-TKO. (A) Primary NK cells were transfected with all five candidate miRNA mimics using TransIT-TKO for 18 hours. The cells were lysed and assessed for fold increase of miRNAs compared to the scrambled transfection, by RTqPCR n=1-3. (B) Transfection efficiency in primary human NK cells was assessed by flow cytometry after 18 hours of mimic transfection with TransIT-TKO, n=3. (C) Up- and down-regulation of miR-146a-5p was assessed by RTqPCR 2.5 days post mimic and inhibitor transfection, respectively. Average NK cell recoveries were 47±15% post transfection. RTqPCR fold change plots represents Log10 or Log2 fold change compared to scrambled transfected NK cells. All data passed Shapiro-Wilk normality test and a one-tailed ratio paired *t* test was run on relative expression data for miR-146a-5p after 2.5 days, n=3.

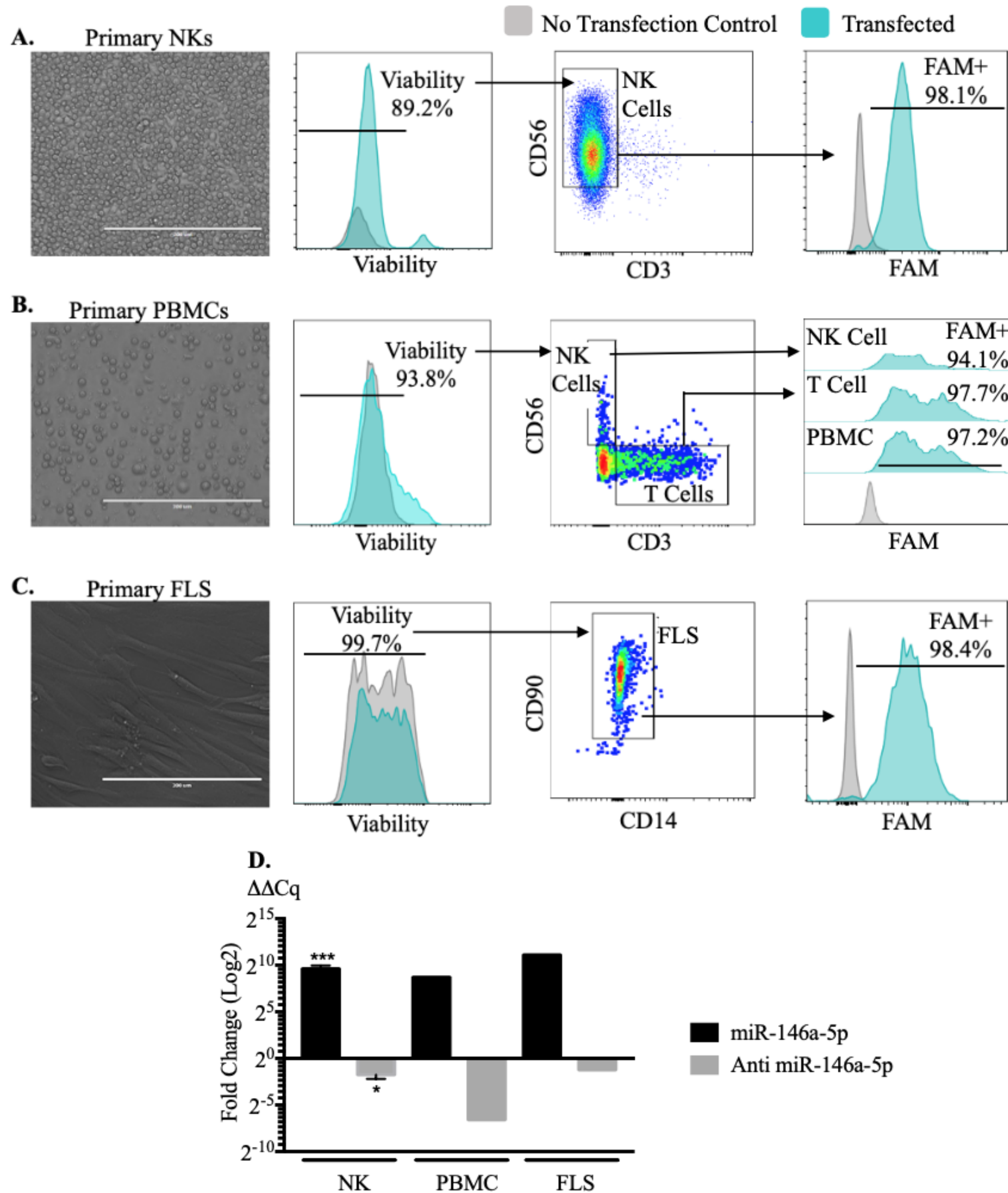

Figure 3.5 TransIT-TKO can effectively transfect several cell types. Primary NK cells (A), PBMCs (B), and JIA FLS (C) were transfected with TransIT-TKO for 18 hours. Images of each cell type, as well as flow cytometry cellular purity, viability, and efficiency are displayed. D) Transfection efficiency was also determined by up and down regulation of miR-146a-5p post mimic and inhibitor transfection compared to scrambled transfected cells, determined by RTqPCR, n=1-3. Data passed Shapiro-Wilk normality test and represented as fold change. Ratio paired one-tailed *t* test were run on relative expression of transfected primary NK cells, n=3. *** denotes p=0.0001 and * denotes p=0.0288.

3.4 MiRNAs do not alter NK cell degranulation, cytotoxicity, or cytokine production

Once our transfection technique was established, we could assess the *in vitro* functional changes in NK cells after miRNA mimic and inhibitor transfections, and thereby determine the role of each individual candidate miRNA.

Healthy donor NK cells were transfected with all five candidate miRNA mimics (miR-27a-3p, miR-125b-5p, miR-146a-5p, miR-155-5p, and miR-223-3p) for 18 hours in serum free media with 100 IU/mL IL-2 prior to 5-hour K562 co-culture (1:1) (**Figure 3.6.1**). The cells were assessed by surface and intracellular flow cytometry for changes in cytotoxicity (**Figure 3.6.1 A**), degranulation (**Figure 3.6.1 B-D**), cytokine production (**Figure 3.6.1 E-F**), as well as phenotypic markers (**Figure 3.7**). Specific cytotoxicity was calculated to correct for errors in NK to K562 ratios and thereby asses the amount of K562 cell death per NK cell. Baseline percentages of NK cells positive for CD107a (8.46±2.11%), granzyme B (98.6±0.8%), perforin (97.3±1.49%), IFNγ (1.03±0.5%) and TNF (2.13±1.17%) were roughly equivalent for all donors (n=3) with all mimic transfections and therefore not shown. There was also no observed difference in cytotoxicity, degranulation or cytokine production after miRNA mimic transfection when the cells were co-cultured with K562s (**Figure 3.6.1**). Nevertheless, we did observe some inter donor functional variability, which, as previously described, is to be expected due to variability in donor immunogenetics and NK cell education, which resulted in differences in NK cell function.

After observing no functional changes in transfected NK cells after 18 hours, we expected that alternations in cellular function could occur in the days following miRNA manipulation. To assess these longer term effects of miRNA transfection on NK cell

function, the experiment was repeated with miR-146a-5p mimic and inhibitor after a 2.5 day transfection (**Figure 3.6.2**). The time point 2.5 days was selected based on previous literature and because primary human NK cells could not be maintained *in vitro* longer than a few days without stimulating NK cell expansion, which could unintentionally introduce changes in NK cell phenotype and/or function and complicate experimental interpretations. The other miRNAs were not included so that the experiment may be repeated on more donors. MiR-146a-5p was chosen because its expression was increased in response to IL-18 stimulation in our previous experiment and because we were interested in its proposed anti-inflammatory functions in the context of RA. Inter donor variability was observed, and we again observed increases in CD107a, TNF, and IFNγ as well as a decrease in granzyme B and perforin when the cells were co-cultured with K562s compared to unstimulated cells. However, there was no observed difference in miR-146a-5p mimic or inhibitor transfected cells in terms of cytotoxicity (**Figure 3.6.2 A**), degranulation (**Figure 3.6.2 B-D**), or cytokine production (**Figure 3.6.2 E-F**). Therefore, miRNA mimic and inhibitor transfections had no effect on NK cell activation after 18 hour or 2.5 days of transfection.

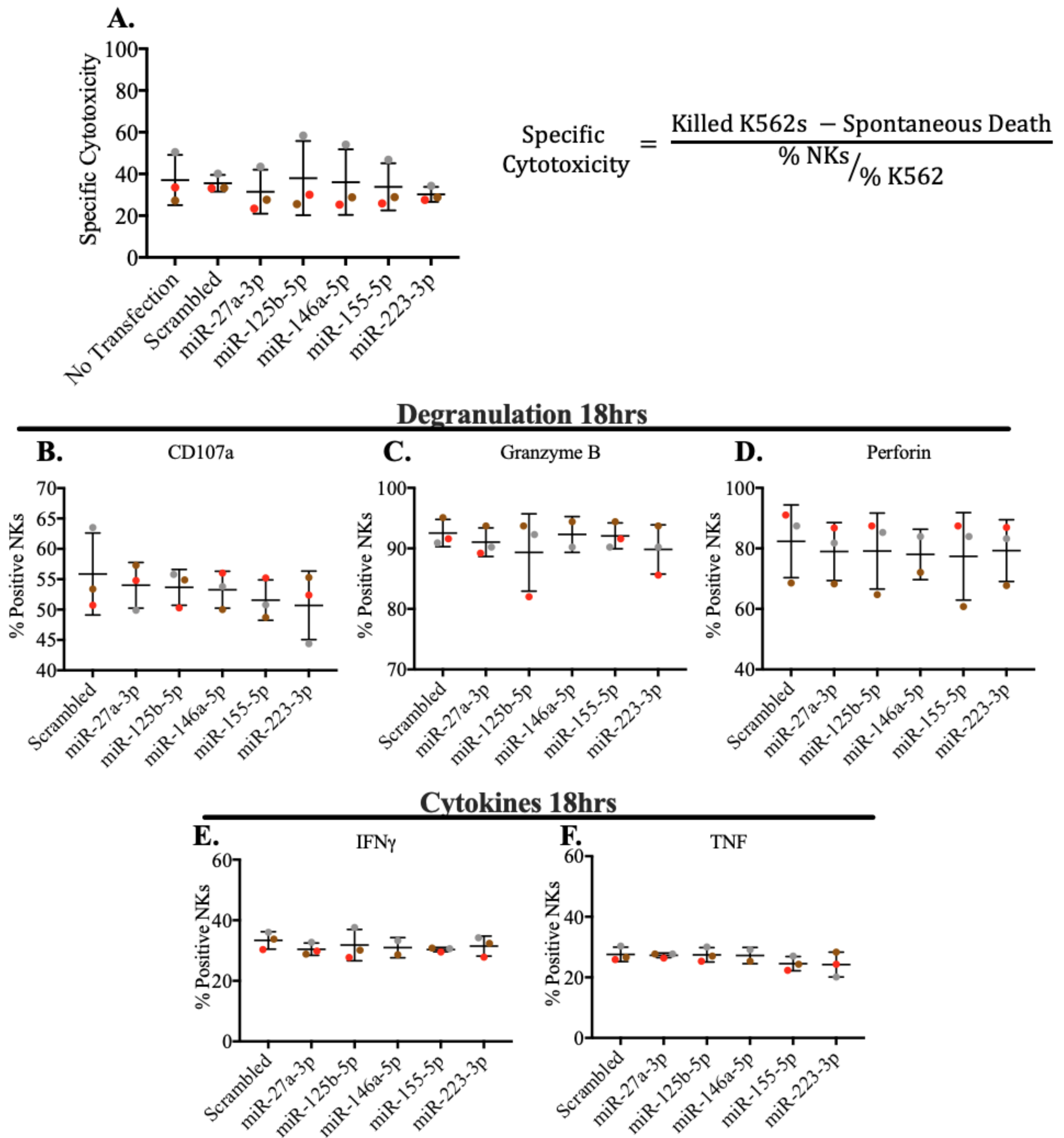

Figure 3.6.1. NK cell transfections do not alter NK degranulation, cytotoxicity, or cytokine production after 18 hours of transfection. Healthy donor NK cells were transfected with five miRNA mimics for 18 hours and co-cultured with K562s (1:1) for five hours. Samples were compared before and after K562 stimulation (baseline responses not shown). Scrambled transfected cells acted as a control. The cells were analysed using surface and intracellular flow cytometry. The cells were assessed for cytotoxicity (A), degranulation (B: CD107a, C: Granzyme B, D: Perforin), and cytokine production (E: IFNγ, F: TNF). Individual donors are represented by matched colours.

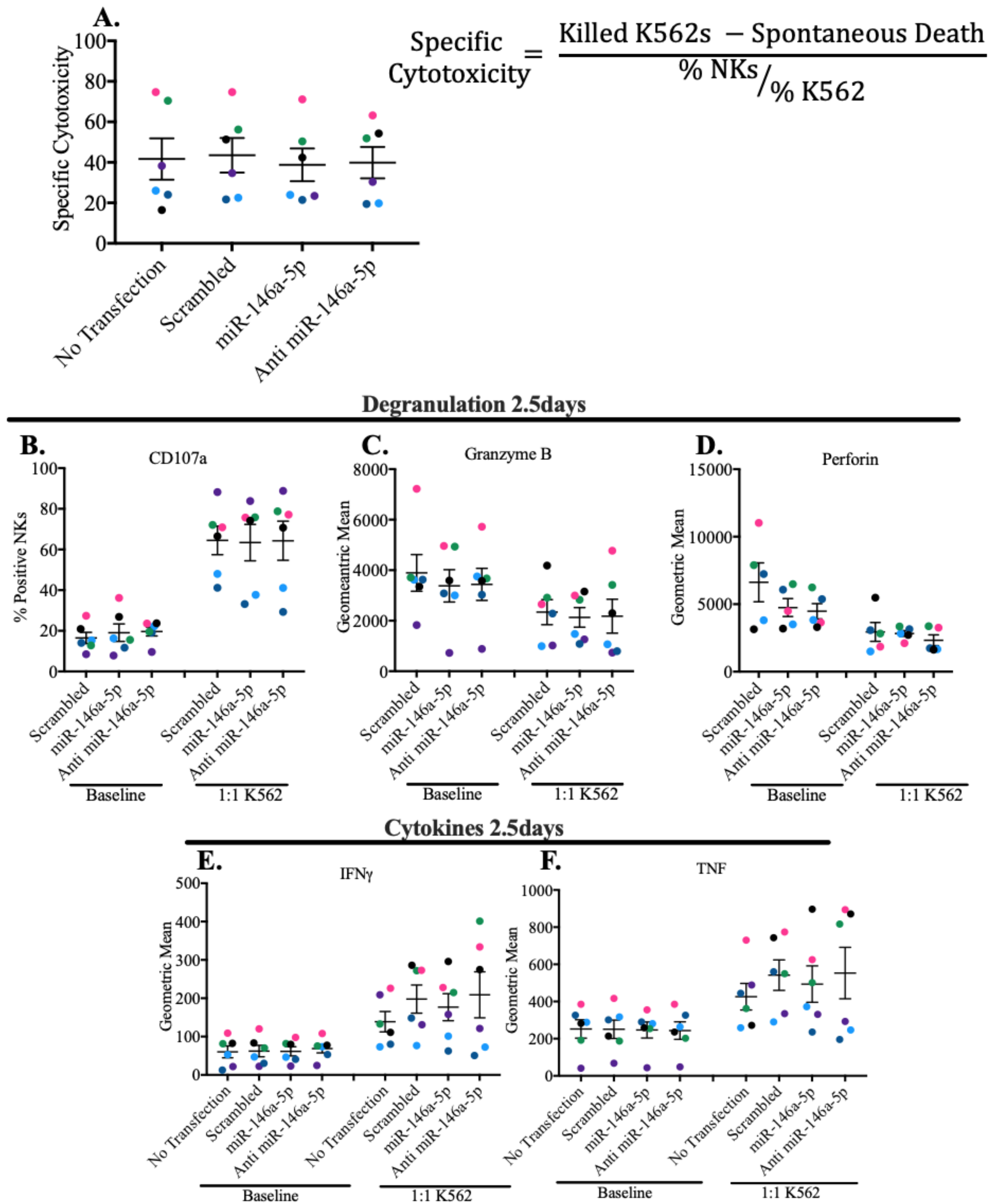

Figure 3.6.2. NK cell transfections do not alter NK degranulation, cytotoxicity, or cytokine production after 2.5 days of transfection. Healthy donor NK cells were transfected with miR-146a-5p mimic or inhibitor for 2.5 days with or without K562 co-culture (1:1) and were assessed by surface and intracellular flow cytometry. The cells were assessed for cytotoxicity (A), degranulation (B: CD107a, C: Granzyme B, D: Perforin), and cytokine production (E: IFNγ, F: TNF). Individual donors are represented by colour. Significance was assessed by multiple comparisons one-way ANOVA and deemed not significant.

3.5 NK cell transfections do not alter NK CD56, NKG2A, or NCR expression

In addition to functional changes, miRNAs have the potential to alter phenotypic markers on NK cells. The majority of markers measured are receptors that engage putative target cells or respond to environmental stimuli, therefore phenotypic changes in NK cells may reflect changes in the distribution of circulating NK cell subsets and have long term functional effects in chronic disease.

Primary NK cells were transfected with miRNA mimics for each of the five selected miRNA for 18 hours or with miR-146a-5p mimic and inhibitor for 2.5 days (**Figure 3.7**). Changes in cellular phenotypic markers, NKG2A/ leukocyte Ig-like receptor 1 (LIR1), NKG2D, CD56, and NCRs were assessed by flow cytometry. The NK cell activation marker, NKG2D, was not measured in cells transfected for 2.5 days due to a high requirement of NKG2D antibody for sufficient cellular staining. We instead measured NCR markers, NKp44, NKp46, and NKp30, after 2.5 days of transfection because Rady *et al*, suggested that their 3'UTR may be a target of miR-146a-5p[49]. There were no changes observed in NKG2D after 18 hours nor in CD56 and NKG2A/LIR1 expression after either time point with any miRNA mimic or inhibitor used (**Figure 3.7 A-E**). Additionally, there was no change in NCRs, NKp44, NKp46, or NKp30, after 2.5 days of miR-146a-5p mimic or inhibitor transfections (**Figure 3.7 F-H**). Although we did observe inter donor variability in all phenotypic markers, the miRNA mimic and inhibitor transfections had no overall effect on CD56, NKG2A/LIR1, NKG2D, or NCR expression.

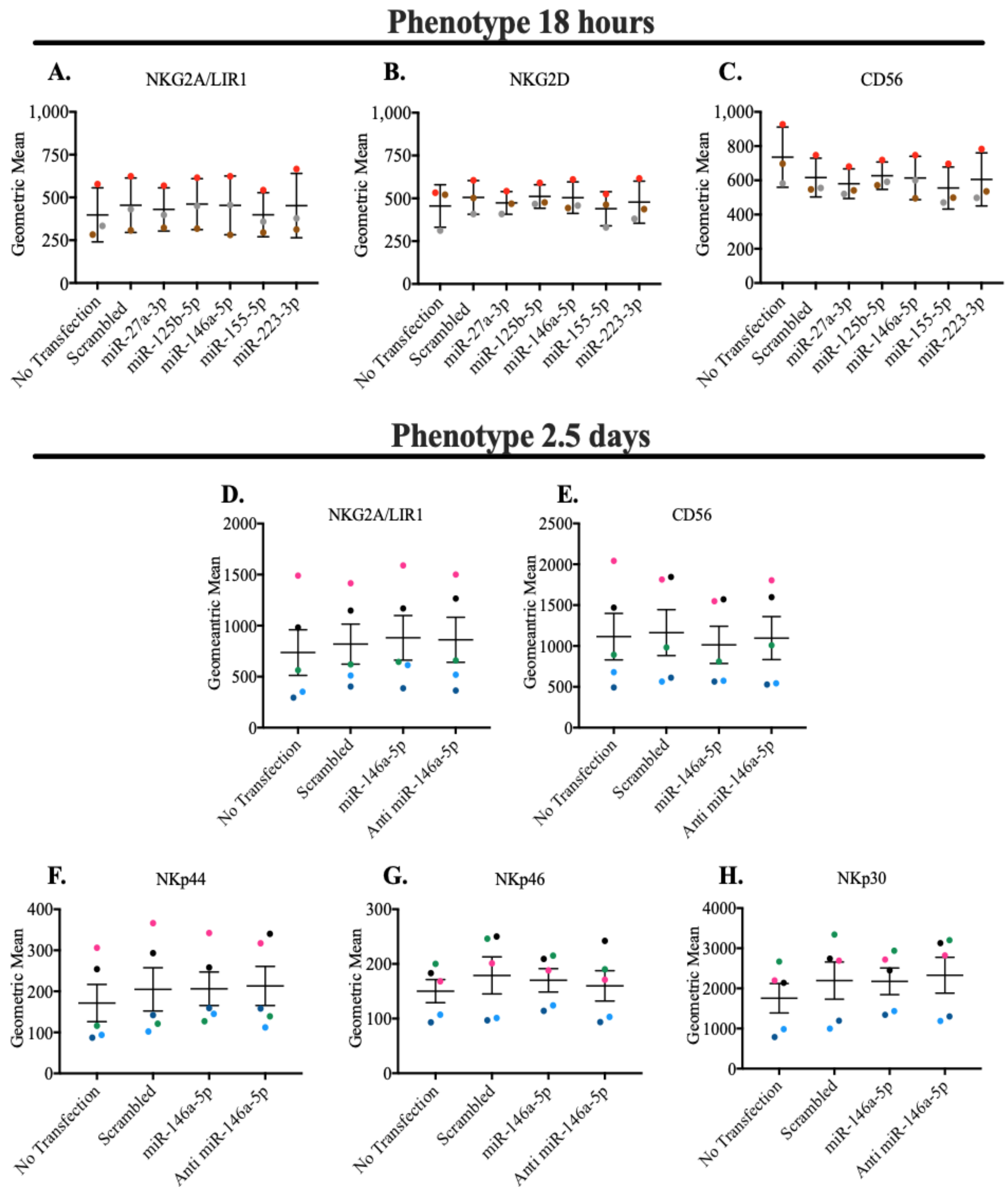

Figure 3.7 NK cell transfections do not alter NK CD56, NKG2A, or NCR expression. Healthy human NK cells were transfected for 18 hours with all mimics (miR-27a-3p, miR-125b-5p, miR-146a-5p, miR-155-5p, and miR-223-3p; A-C) or 2.5 days with miR-146a-5p mimic and inhibitor (D-H). Scrambled transfected cells and no transfected cells were used for controls. Changes in cellular phenotype and NCR expression were assessed by flow cytometry geometric means. Colours are assigned to each individual. Significance was assessed by multiple comparisons one-way ANOVA and deemed not significant.

3.6 MiRNAs have no effect on KIR expression or NK cell education

Aside from theoretically altering NK cell function and the phenotypic markers described above, miRNAs have the potential to affect KIR signaling. For instance, miR-155-5p can inhibit SHIP-1 signaling downstream of the ITIM intracellular domain of the KIR receptors, ultimately reducing KIR inhibitory signaling within a cell[161]. Others have also suggested the miRNAs, including miR-146a-5p and miR-223-3p, may have a role in regulating NK KIR expression as the cells mature from $CD56^{bright}$ to $CD56^{dim}$ subsets[42]. We assessed the overall NK cell KIR expression as well as the functional response of single positive KIR NK cell populations.

All donors were genotyped for *KIR* and *HLA* (**Table 3.2 A-B**), thereby providing a genotypic prediction for functional education (**Table 3.2 B-C**). Healthy donor NK cells were transfected with all mimics for 18 hours or miR-146a-5p mimic and inhibitor for 2.5 days as previously described. Total KIR expression was assessed after 18 hours or 2.5 days by flow cytometry (**Figure 3.8 A-F**). KIR expression was not affected by miRNA mimic or inhibitor transfection after 18 hours or 2.5 days (**Figure 3.8 A-F**). We determined the functional response (CD107a, IFNγ, TNF, granzyme B, and perforin) of NK cells expressing specific KIRs by gating down to the single positive KIR NK cell populations, excluding NKG2A and LIR1 molecules that can contribute to education. This allowed us to evaluate how each population of KIR^+ NK cell responds to miRNA manipulation. MiRNA transfections had no effect on single positive KIR NK cells at 18 hours or 2.5 days. Only CD107a single positive KIR expression after 2.5 days of transfection with miR-146a-5p mimic and inhibitor is displayed for simplification

(**Figure 3.8 G-I**) Altogether, candidate miRNAs had no effect on KIR expression or single positive KIR NK function after 18 hours and 2.5 days transfection *in vitro*.

Table 3.2 Donor *KIR* and *HLA* genotyping. DNA was extracted from whole donor blood and genotyped for *KIR* (A) and *HLA* (B). Educated *KIR*s represent *KIR*s that are co-expressed with their matched *HLA* (C).

A.

Donor	KIR Receptor											
	2DS2	*2DL2*	*2DL3*	*2DL1*	*2DL4*	*3DL1*	*3DS1*	*2DL5*	*2DS3*	*2DS5*	*2DS1*	*2DS4*
			0					0	0			0
	0	0	1	1	1	1	0	1	0	1	1	1
	0	0	1	1	1	1	0	0	0	1	0	0
	1	1	1	1	1	1	1	1	1	0	1	1
	0	0	1	1	1	1	0	0	0	0	0	1
	1	1	0	1	1	1	1	0	0	1	1	0
	0	0	1	1	1	1	1	1	0	1	1	1
	0	0	1	1	1	1	0	0	0	1	0	0
	1	1	0	1	1	1	1	0	0	1	0	0
	0	0	1	1	1	1	1	1	0	1	1	0
	0	0	1	1	1	1	1	1	0	0	1	1
	1	1	0	1	1	1	0	0	0	1	0	0

B.

Donor	KIR Ligand (HLA)		
	HLA-C1	*HLA-C2*	*HLA-B Bw4*
	1	0	1
	1	1	0
	1	0	0
	1	1	1
	1	0	1
	1	0	1
	1	1	1
	0	1	1
	1	1	1
	1	1	1
	1	1	0

C.

Donor	Educated KIRs
	2DL1, 3DL1
	2DL3, 3DL1
	2DL3, 2DL1
	2DL2, 2DL3, 3DL1
	2DL3, 2DL1, 3DL1
	2DL2, 3DL1
	2DL3, 2DL1, 3DL1
	2DL3, 2DL1, 3DL1
	2DL1, 3DL1
	2DL3, 2DL1, 3DL1, 2DS1, 3DS1
	2DL3, 2DL1, 3DL1
	2DL2

Education Pairs	
KIR	*HLA*
2DL1 *2DS1*	*HLA-C2*
2DL2 *2DL3*	*HLA-C1*
3DL1 *3DS1*	*HLA-B Bw4*

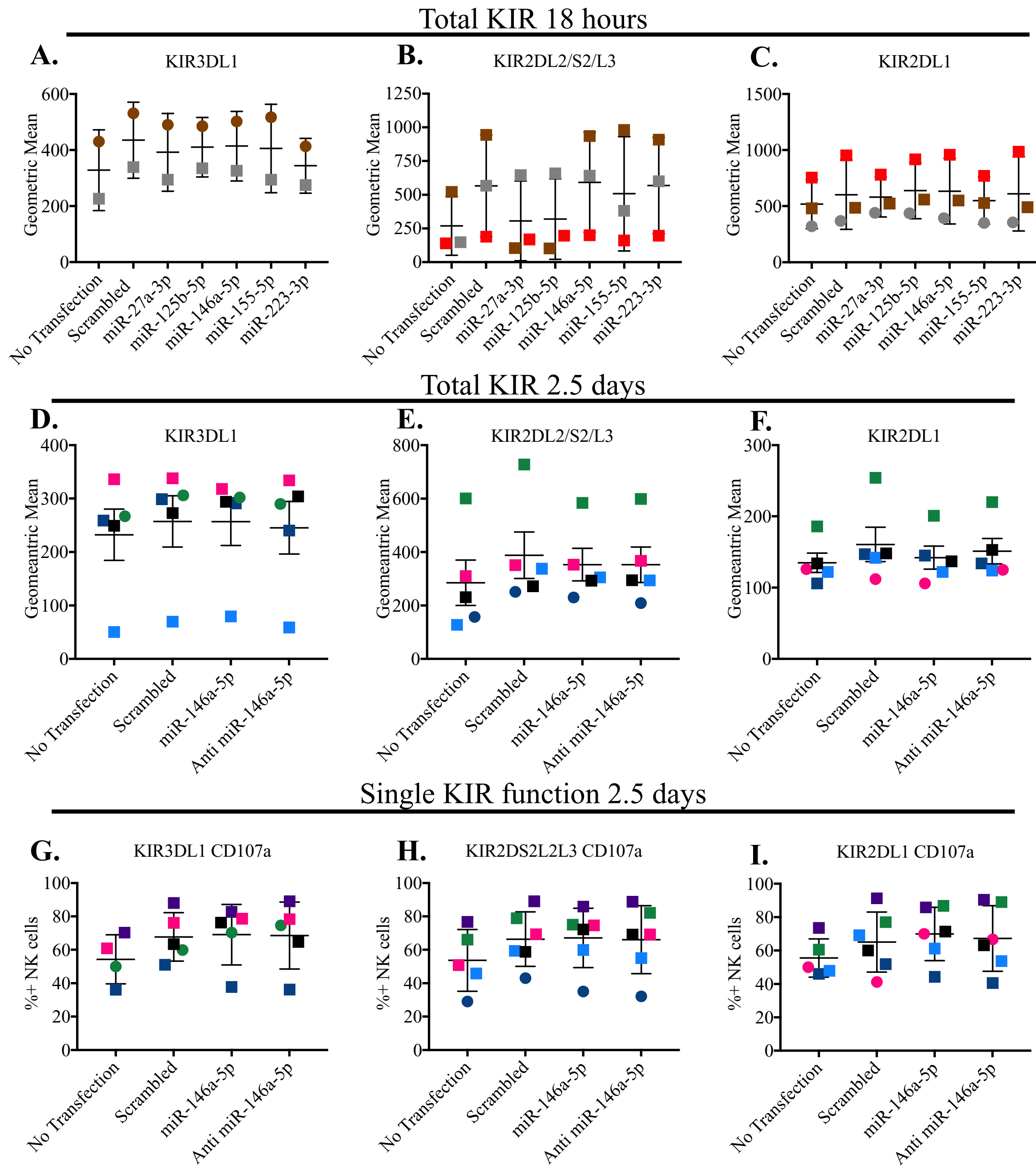

Figure 3.8 NK cell transfections have no effect on KIR expression or NK education. Healthy NK cells were transfected for 18 hours with all mimics (miR-27a-3p, miR-125b-5p, miR-146a-5p, miR-155-5p, and miR-223-3p; A-C) or miR-146a-5p mimic and inhibitor for 2.5 days (D-I). Total baseline KIR expression was assessed by flow cytometry, n=2-5 (A-F). Cellular function (CD107a) of single positive KIR populations was assessed by co-culturing 2.5 day transfected NK cells with K562s (1:1), n=6 (G-I). Individuals are represented by assigned colours. Squares denote populations expected to be educated based on *KIR* and *HLA* genotyping. Data was not significant according to a multiple comparisons one-way ANOVA.

3.7 MiR-146a-5p did not alter NK expression of TRAF6 or STAT1

We were surprised when we thus far observed no functional or phenotypic changes in the NK cells post any miRNA mimic/inhibitor transfection. To determine their relative impact on direct targets, we measured two proteins whose mRNA is directly targeted by miR-146a-5p: TRAF6 and STAT1. MiR-146a-5p was chosen for this experiment because of the availability of TRAF6 and STAT1 intracellular flow cytometry antibodies.

Primary NK cells were transfected with miR-146a-5p mimic and inhibitor for 2.5 days and compared to untransfected and scramble-transfected controls. To assess the effect of miR-146a-5p mimic and inhibitor on mRNA targets, we measured total intracellular TRAF6 and STAT1 protein. There was no observed change in STAT1 or TRAF6 intracellular protein (**Figure 3.9 A, B**). The lack of changes in these proteins may explain why we did not observe phenotypic or functional changes described above.

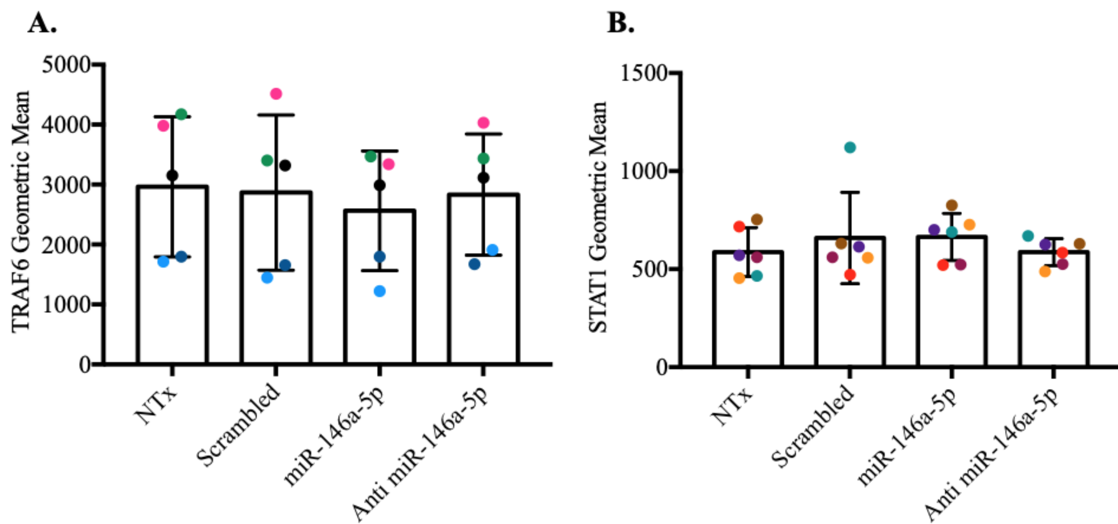

Figure 3.9. MiR-146a-5p mimic and inhibitor transfection did not alter NK expression of TRAF6 or STAT1. Primary NK cells were transfected for 2.5 days and assessed for intracellular total TRAF6 (A) and STAT1 (B) by flow cytometry. Bar graphs represent geometric mean of each protein ± standard deviation. Individual donors are represented by colour, n=5-6. Data was assessed by a multiple comparisons one-way ANOVA statistical test, and was determined to be non-significant.

3.8 MiRNAs regulate NK adaptive phenotype but not function

3.8.1 MiR-146a-5p and miR-155-5p regulate NK cell NKG2C expression

Although we observed no changes in TRAF6, STAT1, NK cell function, or previously described phenotypic markers, we did observe significant changes in the surface expression of NKG2C.

Primary NK cells were transfected with miR-146a-5p and miR-155-5p mimic and inhibitors for 2.5 days and/or 18 hours with or without K562 co-culture (**Figure 3.10 A-C**). NKG2C expression was assessed by flow cytometry and compared to a scrambled control. Transfection with miR-146a-5p mimic had no effect on NKG2C expression after 18 hours or 2.5 days. The inhibition of miR-146a-5p significantly increased the surface expression of NKG2C after 2.5 days of transfection in both baseline and K562 co-cultured NK cells. Surprisingly, mimic transfection with miR-155-5p significantly reduced NKG2C expression compared to the scrambled control both at baseline and with K562 co-culture (**Figure 3.10 C**). Due to limited number of NK cells available for this experiment, miR-155-5p inhibitor transfections was completed in only two donors, who did not show any difference in NKG2C expression according to multiple comparisons one-way ANOVA. Further analysis to include more donors and enable statistical tests is underway. Since increased surface expression of NKG2C is a hallmark of adaptive NK cells[162], it is possible that miR-146a-5p and miR-155-5p has a role in inhibiting NKG2C expression, and potentially, the generation of adaptive NK cell phenotypes.

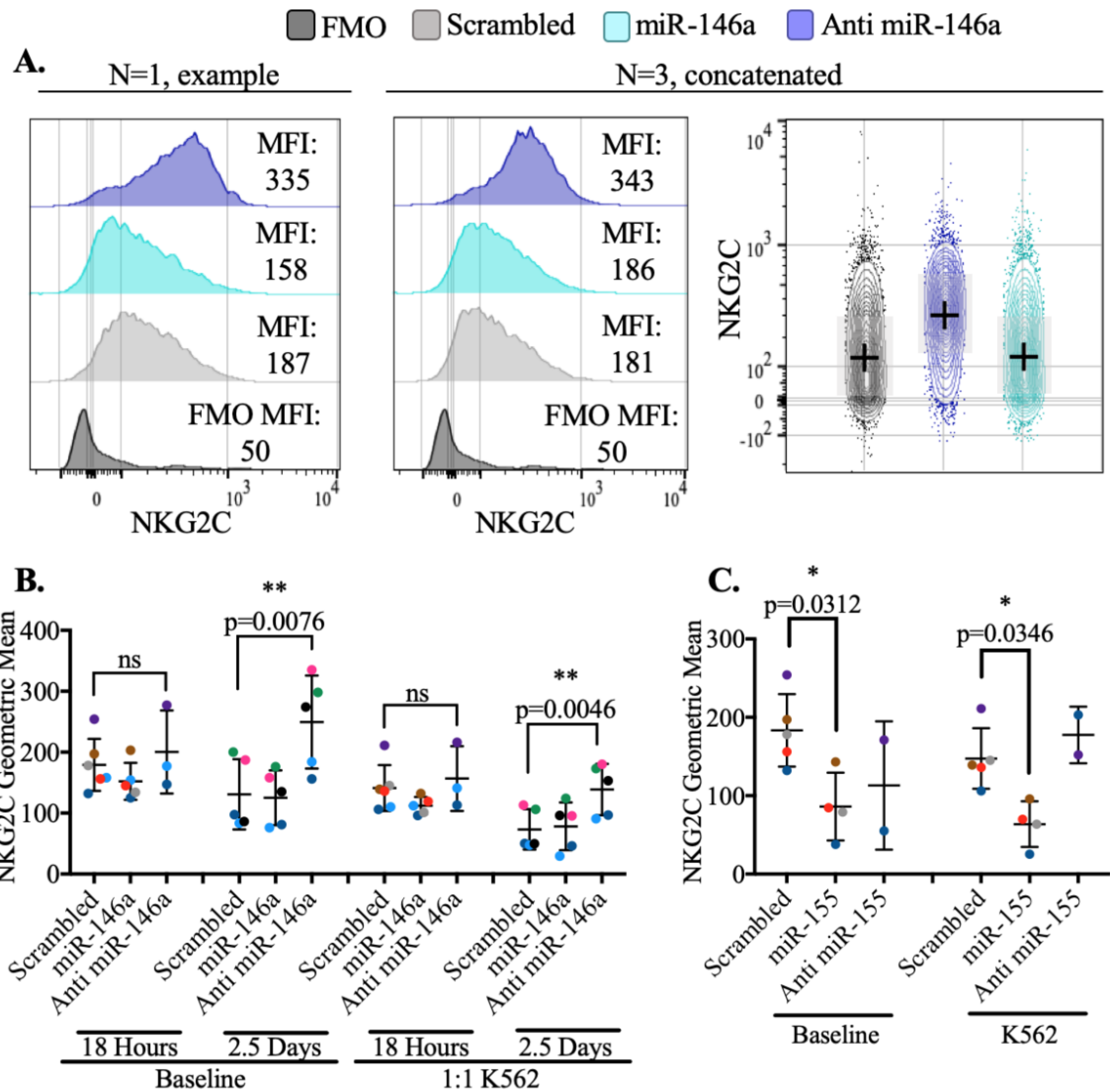

Figure 3.10 MiRNAs significantly alter NKG2C expression. Primary healthy NK cells were transfected with scrambled, miR-146a-5p, anti-miR-146a-5p, miR-155-5p, and anti-miR-155-5p in serum free X-vivo media with 100 IU/mL IL-2 for 18 hours or 2.5 days with or without an additional 5 hour K562 co-culture. All conditions were assessed by flow cytometry. A) Representative histogram overlays and contour plot of scrambled, miR-146a-5p, and anti-miR-146a-5p after 2.5 days in one or three donors. "+" indicates the geometric mean of each contour plot. B&C) Total primary NK cell NKG2C geometric mean after 146a mimic/inhibitor (B) and 155 mimic/inhibitor (C, 18 hours only) transfections. Colours represent individual healthy donors, n=2-5. All samples passed the Shapiro-Wilk normality test. All transfections passed Shapiro-Wilk normality test and were tested against respected scrambled controls using multiple comparisons one-way ANOVA and ratio paired *t* tests.

3.8.2 The role of miR-146a-5p in adaptive NK cell maturation is unclear

To assess the potential role of miR-146a-5p and miR-155-5p in adaptive NK cell maturation, the expression of these miRNAs was assessed by ddPCR or RTqPCR in NKG2C^{+} and NKG2C^{-} NK cells. Banked PBMCs and healthy donors were screened for high NKG2C expression using flow cytometry. Three banked PBMC and two healthy donors were identified as having a bimodal expression of NKG2C. Viable cells were sorted into two populations, CD3^{-}CD56^{+}NKG2C^{+} and CD3^{-}CD56^{+}NKG2C^{-} (**Figure 3.11 A**). Thereafter, ddPCR was used to assess miR-146a-5p in banked PBMCs (these samples yielded fewer NK cells compared with fresh donors), while RTqPCR was used to analyse miR-146a-5p and miR-155-5p in fresh donor populations.

Two of three banked PBMC donors had a higher expression of miR-146a-5p in NKG2C^{+} cells relative to NKG2C^{-} cells (**Figure 3.11 B**). It should be noted that the copies per μL are very low in this experiment, resulting in decreased reliability. In the fresh donors, one donor had higher relative expression of miR-146-5p in NKG2C^{+} cells, while the other donor had lower miR-146a-5p expression compared to NKG2C^{-} cells. Taken together, there is no consensus between miR-146a-5p expression in banked PBMC or fresh NK cell donors. Nevertheless, there are three possible roles of miR-146a-5p in adaptive NK cell maturation: 1) miR-146a-5p expression does not change and has no role in adaptive NK cell maturation, 2) miR-146a-5p changes dynamically throughout adaptive NK cell maturation but cannot be measured at end point, or 3) miR-146a-5p expression is different in adaptive and conventional NK cells, but a higher number of donors is required to appreciate this difference. Further experimentation is required to make a conclusion on the role of miR-146a-5p in the maturation of adaptive NK cells.

Surprisingly, NKG2C$^+$ NK cells had slightly higher relative expression of miR-155-5p than NKG2C$^-$ cells in both healthy donors (**Figure 3.11 D**). MiR-155-5p expression was not assessed in banked PBMC sorted cells because the expression was too low to accurately measured miR-155-5p. Higher expression of miR-155-5p in NKG2C$^+$ cells suggests that miR-155-5p supports adaptive NK cell maturation. This result is paradoxical to our previous results demonstrating a decrease in NKG2C expression after miR-155-5p mimic transfection. Therefore, miRNA regulation of NKG2C expression may be dynamic, time dependant and/or multifactorial.

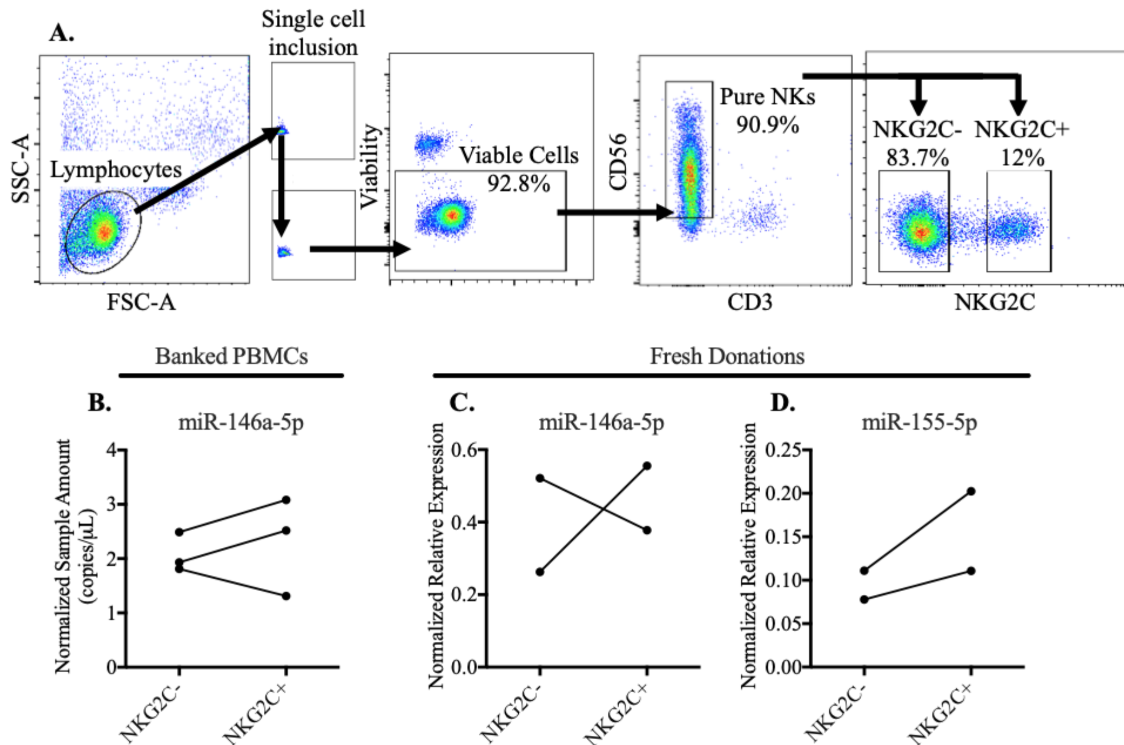

Figure 3.11 The role of miR-146a-5p and miR-155-5p in adaptive NK cell maturation is unclear. Banked PBMCs and healthy NK donors were assessed for bimodal expression of NKG2C prior to sorting. A) The cells were sorted into two populations, CD3$^-$CD56$^+$NKG2C$^+$ and CD3$^-$CD56$^+$NKG2C$^-$. B) The expression of miR-146a-5p of sorted banked PBMCs was assessed by ddPCR, represented as copies/µL. C-D) MiR-146a-5p and miR-155-5p expression in sorted fresh NK donor cells were assessed by RTqPCR, represented as normalized relative expression. Plots represent individual points normalised to reference genes, n=2-3. Reference genes included miR-103a-3p and miR-191-5p.

3.8.4 Adaptive NK cell function is unaffected by miR-146a-5p or miR-155-5p transfection

To assess the potential inhibitory role of miR-146a-5p and miR-155-5p on adaptive NK cell function, we optimised an NK cell ADCC assay against autologous B cells. Purified NK cells and PBMCs were isolated and rested overnight in serum free X-vivo media with 100 IU/mL IL-2. Primary NK cells were co-cultured with autologous PBMCs such that the final ratio of NK cells to target B cells was approximately 1:1. The cells were co-cultured in X-vivo serum free media, without additional IL-2, for 2 hours with anti-LAMP1 (CD107a) antibody and 0 μg/mL, 0.1 μg/mL, 1 μg/mL, 5 μg/mL, or 10 μg/mL RTX (**Figure 3.12**). B cell death and apoptosis, as well as NK surface expression of CD107a, CD56, CD16, CD57, and CD2, were analysed by flow cytometry. There was no change in surface expression of CD56, CD57, or CD2 with increasing concentrations of RTX (data not shown). Nevertheless, the externalisation of CD107a degranulation marker was increased with RTX concentration, plateauing around 1 μg/mL RTX (**Figure 3.12 A**). The opposite was observed with CD16, which displayed reduced expression with increasing RTX concentrations, once again plateauing around 1 μg/mL RTX (**Figure 3.12 B**). The reduced expression of CD16 is likely due to receptor-mediated endocytosis or RTX interference of the anti-CD16 antibody during the staining process. Specific B cell death and apoptosis both increased with RTX concentrations, peaking at 5 μg/mL RTX with 48±9.5% B cell death and 57.8±18% pre-apoptotic B cells (**Figure 3.12 C-D**). Total B cell death and apoptosis decreased when RTX concentrations reached 10 μg/mL, likely due to antibody saturation and interference. As a result of this experiment, future ADCC assays employed a concentration of 5 μg/mL RTX.

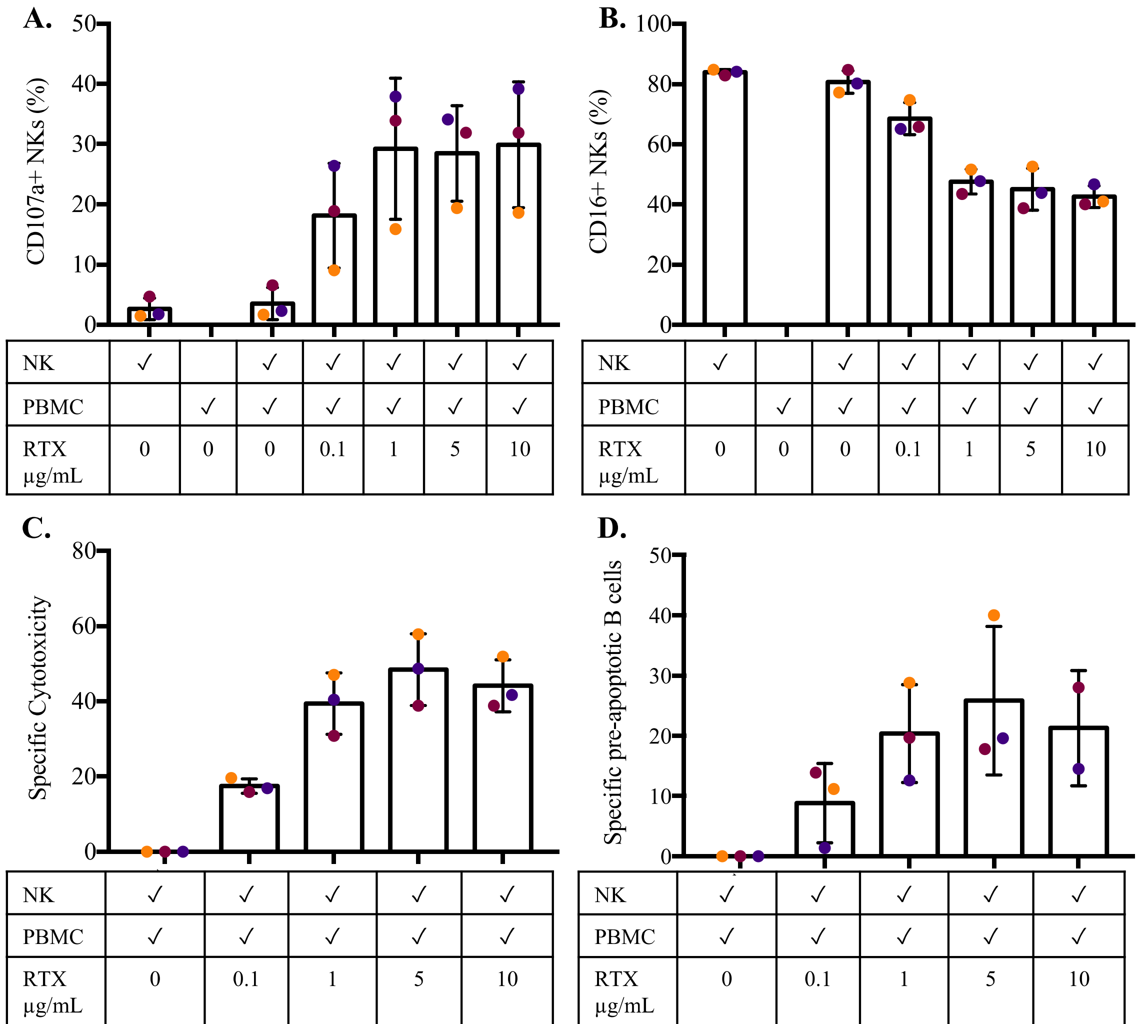

Figure 3.12 RTX titration for ADCC optimization. Primary NK cells were co-cultured for 2 hours with autologous PBMCs such that the final concentration of B cells and purified NK cells was 1:1. Varying concentrations of RTX, 0 µg/mL, 0.1 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL, were added to the co-cultures. NK cell activation (A, B) and specific B cell death and apoptosis (C, D) was assessed by flow cytometry. Specific death and apoptosis was calculated by dividing total B cell death by actual NK cell to B cell ratios. Individuals are represented by assigned colours.

The role of miR-146a-5p and miR-155-5p in adaptive NK cell function was determined by co-culturing 2.5 day mimic and inhibitor transfected NK cells with autologous PBMCs with or without RTX. NK ADCC-mediated B cells death was determined by flow cytometry and apoptosis was measured via annexin V staining. Adaptive NK cell function (CD107a) and phenotype (CD2, CD16, and CD57) were assessed by flow cytometry. Although B cell death (data not shown) and apoptosis (**Figure 3.13.1 A**) were increased when RTX was present, miR-146a-5p and miR-155-5p mimic/inhibitor had no effect on B cell viability. CD107a surface expression was also increased when RTX was present but did not change with miR-146a-5p/miR-155-5p mimic/inhibitor transfection (**Figure 3.13.1 B**). NK cell transfection had no effect on the phenotypic markers CD2, CD16, or CD57. Similar to previous results, CD16 and CD2 expression was reduced with RTX relative to co-cultures without RTX and NK cells alone (**Figure 3.13.2 A, B**). The expression of CD57 was unchanged throughout the experiment (**Figure 3.13.2 C**). Taken together, with the exception of NKG2C, miR-146a-5p and miR-155-5p mimic/inhibitor NK cell transfection had no observed effect on NK cell adaptive phenotype or ADCC function.

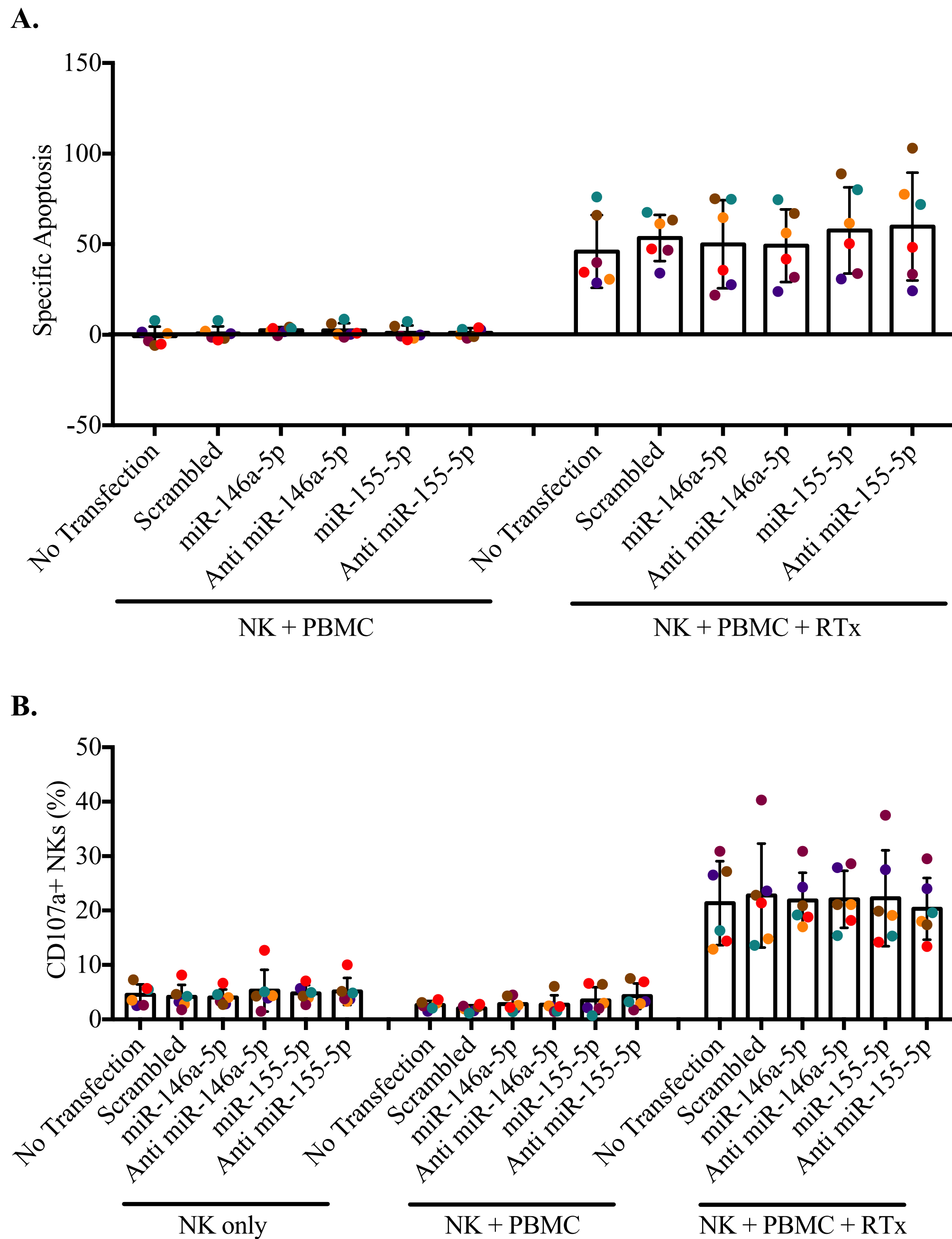

Figure 3.13.1 Adaptive NK cell function is unaffected by miR-146a-5p and miR-155-5p transfection. Primary NK cells were transfected with miR-146a-5p or miR-155-5p mimic and inhibitor for 2.5 days. Post transfection, the cells were co-cultured for 2 hours with autologous PBMCs with or without RTX such that transfected NK cells were at a 1:1 ratio with autologous B cells. A) Specific B cells apoptosis was assessed by annexin V staining. NK cells were assessed for CD107a(B) by flow cytometry. Bar graphs represent mean ± standard deviation. Colours represent individual donors, n=6. No statistical significance was observed between transfections with multiple comparisons one-way ANOVAs while cultured as NK cells only, with PBMCs, or with PBMCs and RTX.

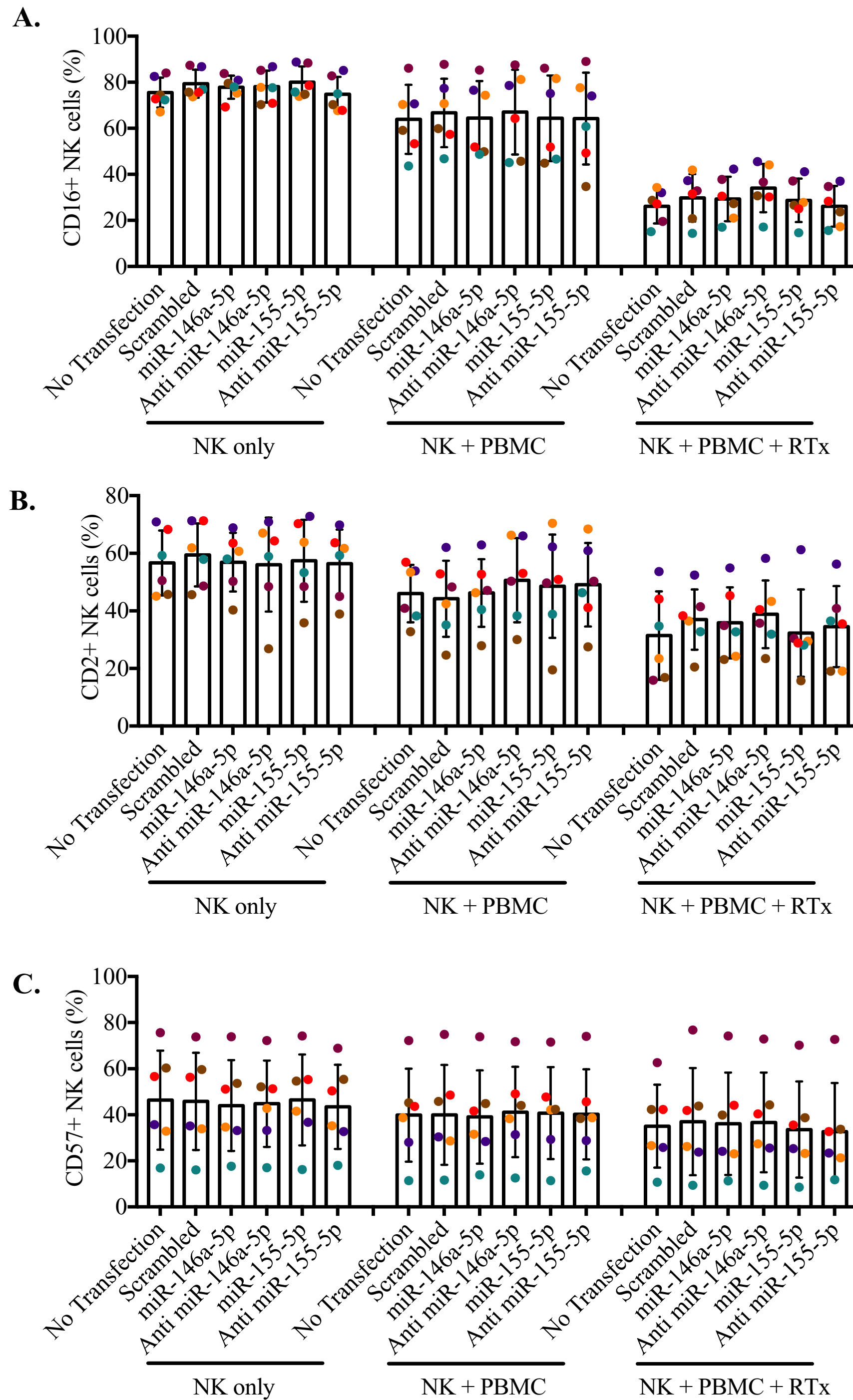

Figure 3.13.2 Adaptive NK cell phenotype is unaffected by miR-146a-5p and miR-155-5p transfection. Primary NK cells were transfected for 2.5 days with miR-146a-5p or miR-155-5p mimic and inhibitor. Transfected cells were co-cultured for 2 hours with autologous PBMCs with or without RTX such that transfected NK cells were at a 1:1 ratio with autologous B cells. NK cells were assessed for CD16(A), CD2(B), and CD57(C) by flow cytometry. Bar graphs represent mean ± standard deviation. Colours represent individual donors, n=6. Multiple comparisons ANOVA revealed no significance between transfections when cultured as NK cells only, with PBMCs, or with PBMCs and RTX.

CHAPTER 4: DISCUSSION

RA remains as one of the most common chronic, heterogenous inflammatory disease worldwide[5]. Although the pathology caused by several immune cells has already been established[6,12,15], little work has been done to identify the contributions of the NK cell. Furthermore, miRNAs have recently emerged as potential biomarkers, and have even been suggested as novel options for therapeutics[163]. The collective roles of miRNAs and their interactions with NK cells remain largely unclear. We set out to investigate the interplay between five candidate miRNAs (miR-27a-3p, miR-125b-5p, miR-146a-5p, miR-155-5p, miR-223-3p) and NK cells in the context of arthritis. We hypothesised that NK cell immunogenetics and miRNA signatures define the inflammation and regulation of NK cells and treatment efficacy in patients with RA. To investigate individual roles of miRNAs on NK cell phenotype and function, we developed a novel and versatile transfection technique that greatly out competes current established methods in terms of both efficiency and viability. Using this technique we found that two miRNAs upregulated by chronic inflammation and IL-18 stimulation impact expression of NKG2C on NK cells: miR-146a-5p and miR-155-5p. Under chronic conditions, this may regulate the differentiation of adaptive NK cells and determine the efficacy of mAb therapies that mediate therapeutic effects through ADCC.

4.1 Results in the context of established literature and autoimmune disease

4.1.1 NK cell miRNAs expression regulation and contribution to RA

To date, there has been little research investigating the production of miRNAs by NK cells, especially after stimulation with IL-18 and TGFβ. Nevertheless, a study conducted by Regis *et al*. found that stimulation with TGFβ1 lead to miR-27a-5p induction and subsequent downregulation of CX3CR1 in human NK cells[108]. In this investigation, we observed no change in NK expression of miR-27a-3p after stimulation with TGFβ. It is important to note that miR-27a-3p and miR-27a-5p, although derived from the same miRNA hairpin, are not the same miRNA. In fact, they are derived from the 3 prime and 5 prime ends of a primary miRNA (pri-miRNA) stem-loop transcript. This pri-miRNA is exported from the nucleus prior to the final cytoplasmic Dicer-mediated maturation[87]. In the cytoplasm the Dicer-processed, single stranded mature miRNA is loaded onto RISC. This loading may occur at similar 3p and 5p frequencies or may preferentially load one strand and degrade the other depending on the cellular type or biological state[87,164]. Therefore, it is likely that the NK cell stimulation with TGFβ represents results in the preferential loading of the 5p miRNA onto RISC while the 3p strand is degraded in the cytoplasm. The preferential loading of one miRNA strand over another may help explain why specific 3p and 5p miRNAs are upregulated in RA and other diseases.

Another investigation, by Xu *et al*., found that TGFβ stimulation of NK cells induced miR-146a-5p expression, leading to reduced STAT1 protein and therefore reduced effector NK function[124]. In contrast to this, we observed a slight, although not significant, decrease in miR-146a-5p expression after stimulation with TGFβ. However, our study used 5 ng/mL TGFβ for 12 hours while Xu *et al*. used 30 ng/mL for 4 hours. As

previously described, we chose a dose of 5 ng/mL from other established literature and 12 hours because we observed the largest decrease in IFNγ and TNF mRNA expression. The goal of the TGFβ stimulation in our study was to reduce NK cell cytokine production and K562 cytotoxicity such that the miRNA expression may be measured under tolerogenic stimuli. The differences in our observations and those made by Xu *et al.* implies two possible explanations: 1) that miR-146a-5p may be acutely increased by TGFβ stimulation following negative regulation or 2) that miR-146a-5p expression is different depending on the strength and timing of the TGFβ stimulation.

Finally, we observed a significant decrease in miR-155-5p expression in TGFβ stimulated NK cells. Previous research suggests that TGFβ stimulation may increase or decrease miR-155-5p signaling depending on the cell type and biological context[86]. Furthermore, the ultimate function and biological role of miR-155-5p may be different between various cell types and contexts. For instance, TGFβ stimulation of intestinal αCD3/CD28 activated T cells increased miR-155-5p compared to αCD3/CD28 activated T cells without TGFβ stimulation. This increase of miR-155-5p was thought to contribute to mucosal tolerance[165]. In the context of NK cells however, miR-155-5p has primarily been identified as a positive regulator of effector NK cell function, especially IFNγ[161]. Therefore, the decrease of miR-155-5p in TGFβ stimulated NK cells may be a result of general TGFβ-mediated inhibition of the cell. Since miR-155-5p is increased in the biofluids of RA patients, its decrease under tolerogenic stimulation suggests miR-155-5p has a role in promoting pro-inflammatory reactions in NK cells in RA patients.

Stimulation of primary human NK cells with IL-18 proved to have the opposite effect to TGFβ, inducing significant increased expression of both miR-155-5p and miR-

146a-5p. We did not observe a difference in the expression of miR-223-3p, miR-125b-5p, or miR-27a-3p. Previous studies often stimulated NK cells with IL-18 in conjunction with other cytokines, especially IL-12. This is because IL-12 induces upregulation of the IL-18 receptor[166]. Stimulation with IL-12 and IL-18 has been reported to significantly increase the expression of miR-155-5p and miR-146a-5p in NK cells[49,136]. However, the stimulation with IL-18 alone and subsequent expression of miR-155-5p and miR-146a-5p has not been previously reported. We observed that IL-18 stimulation, with low levels of IL-2, can significantly increase miR-155-5p and miR-146a-5p expression in NK cells. Therefore, IL-12 is not required for upregulation of miR-155-5p or miR-146a-5p in IL-18 stimulated NK cells.

In the context of autoimmune, inflammatory, and chronic arthritis, miR-155-5p is understood to have inflammatory/pathogenic roles while miR-146a-5p mediates tolerogenicity and immune inhibition[101]. This is despite their upregulation in the biofluids and PBMCs of patients with autoimmune/inflammatory diseases[97,102]. In fact, an investigation by Bogunia-Kubik *et al.* identified polymorphisms in miR-146a in patients with RA[128]. Therefore, it is plausible that miR-155-5p acts as a mediator of inflammation and pathogenesis while miR-146a-5p attempts to regulate, but fails to control, excess inflammation in patients with chronic inflammatory diseases. This is supported by our results because pro-inflammatory IL-18 NK stimulation significantly increased both miR-155-5p, to positively regulate NK activation, as well as miR-146a-5p, to subsequently control NK cell activity. Alternatively, TGFβ stimulation significantly decreased miR-155-5p expression to prevent NK cell activation and only slightly decreased miR-146a-5p expression because it is not required when the cell is inactive. These results may also

indicate that circulating NK cells may be contributing both miR-155-5p and miR-146a-5p to patient biofluids under chronic pro-inflammatory biological context. However, further investigation would be required to confirm this possibility.

4.1.2 Role of miR-146a-5p and miR-155-5p in adaptive NK cells

Although previous literature suggests a role for various miRNAs in the maturation of NK and other immune cells[100], to our knowledge, none have investigated their potential function in adaptive NK cell maturation. Investigations by Lam *et al*. suggest that miR-146a-5p has a role in regulating the $CD56^{bright}$ to $CD56^{dim}$ maturation of NK cells. In fact, the baseline expression of miR-146a-5p appears to be downregulated as the cells mature from $CD56^{bright}CD16^{-}$ to $CD56^{dim}CD16^{+}$ cells[42]. We predicted that further downregulation of miR-146a-5p may be involved/ required for adaptive NK cell maturation. Indeed, when the inhibitor miR-146a-5p was transfected into primary human NK cells we observed an increase in NKG2C surface expression, which was suggestive of an adaptive phenotype. However, there was no consistent up or down regulation of the baseline expression of miR-146a-5p in $CD3^{-}CD56^{+}NKG2C^{+}$ and $CD3^{-}CD56^{+}NKG2C^{-}$ NK cells. This may indicate that miR-146a-5p downregulation occurs as one of many possible biological triggers for adaptive NK cell maturation but returns to a baseline expression comparable to $CD56^{dim}CD16^{+}$ NK cells. Wagner *et al*, demonstrated that memory-like differentiation of NK cells may be achieved by stimulation with IL-12, IL-18, and IL-15, supporting the possibility that several biological signals are required for adaptive NK cell differentiation[53]. Future research should investigate the possible changes in miR-146a-5p throughout adaptive NK cell maturation.

In murine CMV infected mice, sorted, splenic Ly49H$^+$ 'memory' NK cells demonstrated an increased expression of miR-146a-5p 1.5 days post infection, which returned to baseline by day 7[122]. These results indicate a possible role for miR-146a-5p in early adaptive NK cellular responses and alludes to the necessity of other biological events. Therefore, miR-146a-5p may be necessary but not alone sufficient for adaptive NK cell maturation and function. We did not observe any functional difference between mimic or inhibitor miR-146a-5p transfected primary human NK cells *in vitro*. It is possible miR-146a-5p is increased as a result of adaptive NK cell activity but does not, in itself, promote/inhibit further adaptive and/or conventional NK cell function. It is likely that the purpose of miR-146a-5p upregulation post NK cell activation is to negatively regulate pro-inflammatory signaling in both conventional and adaptive NK cells. Therefore, the up- or down- regulation of miR-146a-5p prior to activation or challenge has no functional effect on NK cells because there is no pro-inflammatory processes to regulate prior to stimulation or challenge. Nevertheless, previous literature suggests that long term, chronic overexpression of miR-146a-5p may inhibit overall NK cell activity in a biological context[124]. The MCMV challenged Ly49H$^+$ mice only upregulated miR-146a-5p 1.5 days post infection, indicating that inhibitory effects of miR-146a-5p may only take effect after the NK cells have been activated for longer periods of time than we may achieve *in vitro*. Chronic overexpression of miR-146a-5p in RA and other autoimmune/inflammatory diseases[98] may have slow acting but long term inhibitory effects on conventional and/or adaptive NK cell function. It is likely that miR-146a-5p overexpression is not the only factor causing long term effects on NK cell phenotype and activity.

MiR-155-5p is also upregulated in plasma and other biofluids in patients with RA[99]. Despite being described as a "master-regulator of the immune response"[86], miR-155-5p has rarely been identified as a regulator of cellular maturation. A major function of miR-155-5p has instead been identified as the promotion of pro-inflammatory immune reactions through inhibition of SHIP-1 and SOCS1[131,135,167]. In the context of NK cells, this has usually been described as an increased production of IFNγ[161] and increased cytotoxicity[129]. We did not observe any change in either of these functions when miR-155-5p mimic and inhibitor transfected NK cells were co-cultured with K562s or autologous B cells. In fact, we observed a significant decrease in NKG2C expression when primary human NK cells were transfected with miR-155-5p mimic. The regulation of NKG2C is relatively unknown, although it is known to be overexpressed on adaptive NK cells, especially in CMV^+ individuals[168]. Additionally, $NKG2C^{null}$ individuals can still mount an adaptive immune response[61], so the exact function of NKG2C in adaptive immunity – beyond that of a phenotypic marker – is unclear. What is understood is that NKG2C is an activating NK cell receptor that binds to HLA-E on target cells[60]. Therefore, we were surprised that overexpression of miR-155-5p, which is traditionally describe to promote NK cell activity, decreased the expression of an activating receptor. It is possible that miR-155-5p overexpression inhibits the overall adaptive phenotype but current mechanisms are unknown.

The roles of both miR-146a-5p and miR-155-5p remain to be fully elucidated. However, our research suggests that these miRNAs have an inhibitory function in adaptive NK cells. Chronic overexpression of these miRNAs, as observed in arthritic diseases such as RA, may also inhibit adaptive NK cell function.

4.1.3 Role of adaptive NK cells in chronic arthritis and autoimmune disease

The function of adaptive NK cells in autoimmune and inflammatory diseases are not currently understood. However, the role of ADCC on adaptive NK cells has been associated with mAb treatment efficacy in a variety of diseases, including RA[169]. As previously described, ADCC is mediated though NK CD16 binding of the Fc region of the antibody, whose variable region is bound to a target cell. This brings the NK cell within close proximity to its target. Cartron *et al.* identified that follicular lymphoma patients with the 158V allotype of CD16 had better ADCC and responded better to RTX than patients with the 158F allotype[71], thereby alluding to the importance of adaptive NK cell ADCC for effective mAb therapy. We expect that a similar phenomenon occurs in patients with RA treated with RTX and that overall improved ADCC increases treatment efficacy. This is due to the imperative role of adaptive NK cells in eliminating auto-reactive B and T cells with mAb therapies through ADCC. Our results indicate that NK cells can target autologous B cells when co-cultured with RTX, although we did not observe functional differences with miR-155-5p or miR-146a-5p mimic/inhibitor transfections. Nevertheless, previous reports have identified a positive correlation between circulating NK cells and treatment efficacy[45]. Reduced circulating NK cells have also been positively correlated with worsened disease flares[41]. Therefore it is likely that adaptive NK cells mediate auto-reactive B and T cell elimination during mAb therapy. Assays determining the strength of an patient's NK ADCC may help determine treatment efficacy prior to treatment prescription[170].

Alternatively, adaptive NK cells may promote disease pathogenesis through ADCC when bound to auto-antibodies. Chronic autoimmune and inflammatory diseases are associated with high levels of auto-antibodies. In fact, auto-antibody production is considered a hallmark of RA pathogenesis[7]. Therefore, adaptive NK cells may drive auto-reactivity through ADCC when bound to auto-antibodies. Whole NK cell populations may also contribute to autoimmune pathogenesis through upregulation of MICA/B on stressed, inflamed tissues thereby activating NK cell through NKG2D[41]. Additionally, the production of pro-inflammatory cytokines can activate other cells and indirectly contribute to pathogenesis.

Overall, the function of conventional and adaptive NK cells is complex and likely contributes both damaging and protective roles in autoimmune and inflammatory diseases. Although adaptive NK cells may mediate auto-reactivity through interactions with auto-antibodies, it is still likely that they also have an important role in mAb therapy to eliminate auto-reactive B and T cells. Furthermore, the reduction of auto-reactive B cells will further reduce production of auto-antibodies. The exact functions of NK cells in mediating and/or protecting against arthritis pathology requires further investigation.

4.2 Applications of TransIT-TKO transfection

Genetic manipulation of cells is a powerful tool that can be used to significantly improve our understanding of cellular processes including cellular development, maturation, regulation, and function. The transfection of suspension cells, including NK cells, has proven to be a difficult task due to low cellular transfection efficiencies and viabilities. In this project, we successfully developed a new, extremely efficient, transfection technique and serum free media tailored toward miRNA manipulation that supports high primary

NK cell viability. Moreover, we have shown that our transfection technique is capable of transfecting several primary human cells, including whole PBMCs and FLS cells. Optimization of various transfection techniques provides a huge potential for research in both basic sciences and in the clinics.

The most common strategy for genetic manipulation of NK cells is viral-mediated transduction. The use of viruses increases the risk of insertional mutagenesis and often requires multiple rounds of transduction before an efficiency of 15-40% may be acquired[82]. NK cells are often required to be expanded prior to transduction which causes them to lose their subset diversity[83]. Our transfection technique, independent of cellular division, consistently achieves an efficiency of >90% after one round of transfection. Because of this, TransIT-TKO NK cell transfection may allow for genetic manipulation with minimal unwanted changes to the cell. Nevertheless, NK cells of the adaptive lineage can survive for months to years *in vivo*, which suggests that this non-insertional, highly efficacious protocol for NK cell manipulation could be clinically useful to reprogram NK cells. This technique provides novel opportunities for researchers and clinicians to genetically manipulate NK cells, and other cells, without losing clinical and individual relevance.

4.3 Critique and limitations

Although the use of human tissues provides major benefits ranging from direct human applications to smoother translation of knowledge, its exclusive use in this project contributed to a major limitation: all experiments were conducted *in vitro*. Specifically, the *in vitro* limitations that directly affected this research included: 1) limited time the

cells could be grown in culture and 2) the cells were grown out of biological context and 3) inter-donor variability. Long-term treatments and transfections would provide better insight to the interplay between NK cells and miRNAs. For instance, we observed no change in the expression of STAT1 or TRAF6 after the cells were transfected with miR-146a-5p mimic/inhibitor. This may be a result of the cell using unknown mechanisms of TRAF6 and STAT1 regulation to counteract the effects of miRNA transfection or slow turn-over rates of these target proteins. Regardless of the biological mechanisms, longer transfections would help determine chronic effects of inducing and inhibiting miRNA signaling. Primary cells can only be maintained in culture for short periods of time, especially in serum free conditions. Therefore, the establishment of long term, chronic cellular stimulations and/or transfections was, regrettably, not feasible *in vitro* under our serum free media constraints. Feeder cells lines would allow for long-term NK cell culturing in serum free media, however, because the feeder cells could produce and potentially release their own miRNAs, we could not use this method for our studies. Alternatively, survival cytokines could be added to culture media, but this is expensive over long periods of time. To transfect NK cells and investigate their phenotypic/functional changes, the cells had to be extracted from the circulation and isolated from other PBMCs, thereby preventing any normal cell-cell interactions. The NK cellular concentration was also significantly higher than anything found in a biological context, having unknown effects on the NK cells. Under *in vitro* conditions, the NK cell exposure to oxygen is significantly higher than *in vivo*, potentially affecting the NK cell metabolism. Finally, the highly variable nature of the NK cell provides further complications when attempting to interpret human-based experimental data. These limitations complicate the interpretations of this research because the changes we

observed in the highly diverse human NK cells in isolation may be different from those in a biological context.

Another major limitation of this project is the sole use of healthy donor peripheral blood NK cells. Healthy donor peripheral NK cells are not necessarily representative of circulating NK cells in patients with chronic arthritis, especially if a portion of the circulating NK cells mediate an important role in the resolution of inflammation and/or response to treatment. Compared to healthy individuals, Aramki *et al.* identified a significantly impaired NK cell activity in RA patients likely due to a decrease in various activating receptors on circulating NK cells[75]. Due to this, NK cells in a patient with chronic arthritis may respond differently to stimulation or transfection than healthy donor NK cells. Furthermore, the exclusive use of healthy human NK cells meant that we were limited to peripheral blood NK cells and could not investigate the role of joint-infiltrating $CD56^{bright}$ NK cells. Enrolment of volunteers with chronic arthritis participants to the project would allow for isolation and investigation of both circulating as well as synovial infiltrating NK cells. This would allow for a better appreciation of the suspected dual complexity of NK cells in chronic arthritis and inflammatory diseases.

Despite these limitations, the techniques and knowledge gained from this project can be used to foster future investigations that aim to better understand the interplay of both miRNAs and NK cells in chronic arthritis and inflammatory diseases. Future directions should address limitations in the availability of patient samples and attempt *in vivo* studies to better investigate miRNAs and NK cells in biologically relevant chronic-inflammatory models.

4.4 Future directions of research

Characterization of the integrated role(s) of miRNAs and NK cells in chronic arthritis disease is not complete. Chronic models are required to better understand the contributions of miRNAs, adaptive and conventional NK cells in RA.

To improve our understanding of adaptive NK cell maturation, and the potential role(s) of miR-146a-5p and miR-155-5p, future work should measure the expression of these miRNAs throughout adaptive NK cell maturation. Measurement of miR-146a-5p and miR-155-5p during a primary and secondary HCMV exposure in primary human CMV naïve NK cells may reveal a role for these miRNAs during NK cell adaptive maturation. Additionally, future research should include measurements of the transcription factor, PLZF, to better identify adaptive NK cells. We were unable to include PLZF in our analysis due to delayed manufacturer delivery of anti-PLZF flow cytometry antibodies. Measurement of miR-146a-5p and miR-155-5p throughout adaptive NK cell maturation and analysis of PLZF would confirm the potential inhibitory roles of miR-146-5p and miR-155-5p in adaptive NK cellular phenotype.

As previously discussed, a major limitation of this project was the exclusive use of *in vitro* experimentation. Unfortunately, due to time constraints, we were unable to establish and prepare an *in vivo* model of chronic arthritis. Although several murine models of arthritis exist, collagen induced arthritis (CIA) has been the most widely used for research in RA[171]. Under normal circumstances, type II collagen (CII) is exclusively found in articular cartilage joints. However, CII is one of the major auto-antigens in human RA[172]. Immunization of CII with complete Freund's adjuvant (CFA) elicits a high incidence of autoimmune murine arthritis[171,172]. Most often DBA/1 mice are used for CIA but HLA-DR mice, who express transgenic HLA-DR1 or DR4 to increase RA

susceptibility, may also be used[171]. For our purposes, NK cell deficient mice may also provide insight to overall NK cell function in arthritis disease. The use of an *in vivo* arthritis model would allow for assessment of circulating and joint infiltrating NK cell functions and NK cell / biofluid (plasma and synovial) miRNAs. Additionally, the role(s) of miRNAs and adaptive NK cells may be assessed under various monoclonal treatments. For instance, miRNA transfected NK cells may be adoptively transferred into the circulation of an arthritis mouse treated with RTX. RTX treatment would likely be in combination with MTX to imitate clinical practices. Using this technique, we may be able to directly identify how miR-146a-5p and miR-155-5p expression in circulating NK cells might affect treatment efficacy.

Addressing these future experiments would build on our current understanding of the interactions between miRNAs and NK cells in chronic arthritis disease.

4.5 Concluding remarks

With this project, we successfully developed new techniques for genetic manipulation of NK cells and explored the role(s) of miRNAs and NK cells in the context of arthritis.

Optimal transfection of NK cells has been historically very difficult. Requiring extensive work involving several manipulations of the cells to achieve relatively low efficiencies. Our new transfection technique can efficiently and reliably transfect NK cells in serum free conditions for up to 2.5 days without impairing cellular viability. Moreover, our transfection technique can reliably transfect other human cells, including PBMCs and FLS. TransIT-TKO transfection provides novel opportunities for NK cell genetic manipulation for clinical immunotherapies and basic research investigations.

Chronic arthritis diseases are extremely heterogenous and involve complex immunological reactions. Our current knowledge of miRNAs and NK cells in these diseases is poor. However, a better understanding of NK cells and miRNAs will allow for their use as clinical biomarkers. We have identified that primary human NK cells can upregulate both miR-146-5p and miR-155-5p with pro-inflammatory IL-18 stimulation. We expect that miR-155-5p upregulation functions to promote pro-inflammatory activity while miR-146a-5p acts as a negative regulator of cellular activity. It is possible that NK cells may contribute to the chronic overexpression of these two miRNAs in arthritic patient biofluids. Moreover, through our observations of NKG2C, miR-146a-5p and miR-155-5p may both have a role in inhibiting adaptive NK cell phenotype. Inhibition of the adaptive phenotype may reduce overall numbers of adaptive cells in chronic conditions, thereby reducing total adaptive NK cellular functions. Although this may reduce auto-antibody driven ADCC reactions, chronic reduction of adaptive NK cells and/or function may decrease treatment efficacy of mAb therapies. Therefore, miR-146a-5p, miR-155-5p, and baseline patient ADCC function may be potential biomarkers for patients with chronic arthritis and other inflammatory diseases (**Figure 4.1**). Further investigation is warranted to corroborate the potential interactions between miR-146a-5p, miR-155-5p, and adaptive NK cells, as well as the complex dual functionality of NK cells in arthritis.

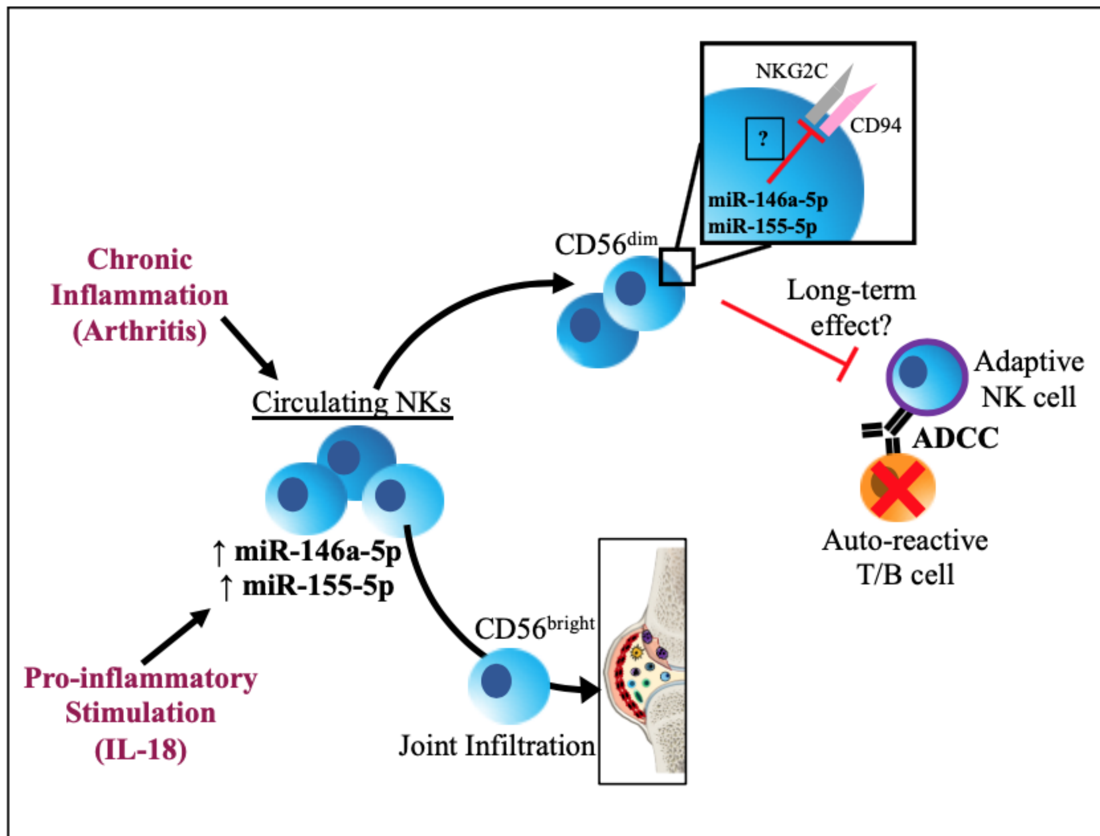

Figure 4.1 Compressed model of miR-146a-5p/miR-155-5p-NK cell interplay in RA. Pro-inflammatory conditions, including chronic inflammation and IL-18 stimulation, induces an increase in miR-146a-5p and miR-155-5p expression by circulating NK cells. Our findings suggest that miR-146a-5p and miR-155-5p inhibit NKG2C expression on $CD56^{dim}$ NK cells through unknown mechanisms. Under chronic conditions we predict this may inhibit overall adaptive NK cell differentiation and function thereby reducing their ability to eliminate auto-reactive T and B cells through ADCC abetted by mAb therapy. Meanwhile, $CD56^{bright}$ NK cells infiltrate into the joint and promote pro-inflammatory signaling through the release of IFNγ and TNF.

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