MicroRNA Regulation of CD16 in Human Natural Killer Cells

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Abstract

Natural killer (NK) cells are lymphocytes capable of killing virus-infected and tumor cells while leaving normal tissue unharmed. CD16 is an Fc receptor that allows NK cells to perform antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor cells. We hypothesize that miRNAs (miRs) have a regulatory role for CD16 expression in NK cells. We compiled a list of 69 miRs predicted in silico to target CD16 mRNA. This list was cross-referenced with a gene expression data set of 400 miRs measured in peripheral blood (PB) from 4 healthy donors. Of the 69 miRs candidates, 3 were found to be downregulated in the CD16+ NK cell populations compared to CD16- populations. RT-qPCR showed that CD16- human NK cells in PB had approximately 25 fold higher mir-218 expression than CD16+ populations ($p=0.008$, $n=4$). RT-qPCR was also used to measure miR-218 expression during NK cell maturation stages 3, 4a, 4b, and 5 NK cells from 5 donors. The higher expression of miR-218 in stage 4a and 4b developing NK cells correlated to their low expression of CD16. Using 293T cells infected with miR-218 pCDH luciferase construct, we were able to demonstrate a 35% reduction ($p=0.12$, $n=3$) in luciferase activity with overexpression of miR-218. Thus, we have preliminary evidence that miR-218 is an intrinsic negative regulator of CD16 expression in NK cells. Ongoing work will determine the effect of overexpression and knockdown of miR-218 on CD16 expression, ADCC killing, and cytokine production in a CD16+ cell line and primary NK cells.

Introduction

Natural killer cells are large granular lymphocytes capable of killing target tumor cells and leaving normal tissue unharmed.[1] CD16 is an Fc receptor that allows NK cells to perform antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor cells.[2-3] Currently, no factors—transcription factors, epigenetic factors, or microRNAs (miRs)—have been shown to regulate CD16 expression in NK cells. An understanding of CD16 regulation in NK cells is required to increased the availability of $CD16^+$ NK cells and enhance NK cell-mediated ADCC. Thus, we propose that a better understanding of regulatory mechanisms behind CD16 expression in human NK cells will allow for improvement in monoclonal antibody immune therapies.

The purpose of this research project is to identify and validate miR regulators of CD16 in NK cells. Five stages of NK cell development have been identified in the secondary lymphoid tissue.[4] Stages 1-3 are classified as immature NK cells while stages 4 and 5 are considered mature NK cells.[5] Several markers are used to distinguish NK cell stages (see Table 1). Stage 4 NK cells lack CD16 expression, while stage 5 NK cells acquire and highly express CD16. To date, little is known about the mechanism of transition from stage 4 NK cells to stage 5 NK cells. This research project aims to understand how CD16 is expressed and regulated in mature NK cells. We hypothesize that the miRs, identified in the preliminary studies, intrinsically regulate CD16 in NK cells.

Table 1: Minimal Definition of NK cell Development
Stage 3: CD34+, CD117-, CD94-, CD16-
Stage 4a: CD94+, CD34-, CD117-, NKp80-, CD16-
Stage 4b: CD94+, NKp80+, CD34-, CD117-, CD16-
Stage 5: CD94+/-, NKp80+, CD16+, CD34-, CD117-

Preliminary Studies

Utilizing the miR prediction strategy described in Witkos *et al*[6], we compiled a list of 69 miRs predicted *in silico* to target CD16 mRNA. This list was cross-referenced with a nanostring data set of 800 miRs measured in peripheral blood (PB) from 4 healthy donors. The first population, $CD56^{bright}/CD94^{high}$, lacks or has low expression of CD16, while the two other populations, $CD56^{dim}/CD94^{high}$ and $CD56^{dim}/CD94^{low}$, highly express CD16. Of the 69 miR candidates, 3 were found to be up-regulated in the stage 4 population compared to the stage 5 populations in all donor samples (Figure 1).

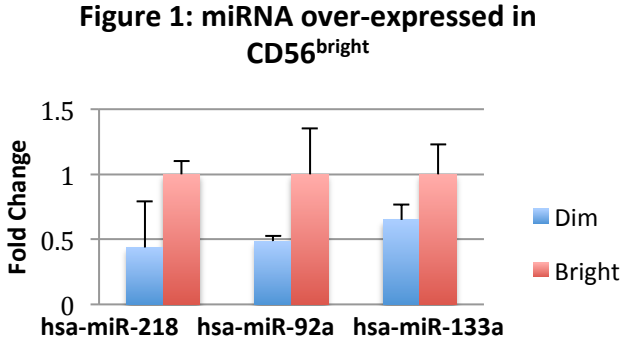
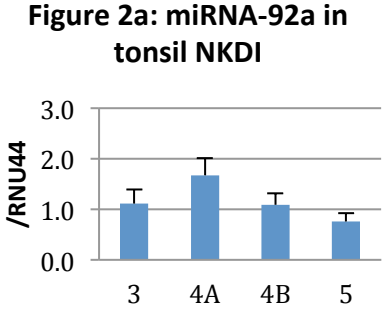
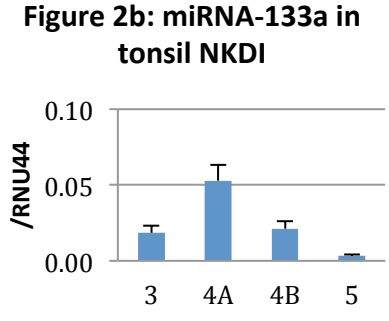


Figure 1: Peripheral blood was collected from four healthy human donors and three populations were sorted: $CD56^{bright}$ and $CD56^{dim}$, which are abbreviated bright and dim. MicroRNA expression was measured by nanostring gene expression assay. Fold change is reported as a ratio of miR expression in the $CD56^{bright}$ population compared to the corresponding $CD56^{dim}$ population.

NK cell development has traditionally been described as having 5 developmental stages, which can be found in the secondary lymphoid tissue (SLT), such as the tonsil. However, recent research has attempted to clarify subtle differences in NK cell developmental progression by defining stages 4a, 4b, 5a, and 5b.[4] Stage 4a NK cells can





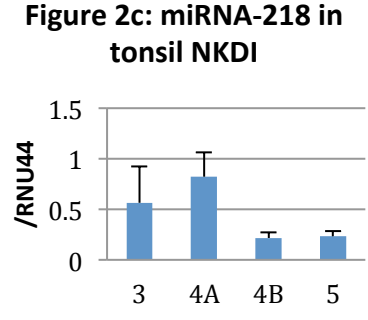


Figure 2: Tonsil tissue was collected from four healthy human donors and three populations were sorted: Stage 3, 4A, 4B, and 5 NK cells. MicroRNA expression was measured by qPCR. Fold change is reported as a ratio of miRNA expression in the $CD56^{bright}$ population compared to the internal control.

revert to stage 3, while 4b are committed as mature NK cells (unpublished). Stage 5a are less mature than stage 5b NK cells.[7] MicroRNA expression in tonsil stage 3, 4a, 4b, and 5 NK cells from 5 donors was measured by qPCR (Figure 2). The higher expression of miR in stage 4a and 4b NK cells correlates to a low expression of CD16 in stage 4 NK cells. Thus, this data suggests that miR serves as a possible regulatory repressor of CD16 in mature human NK cells. This proposed research project will further investigate the expression of these identified miRs in human CD16+ and CD16- NK cells to validate these preliminary findings found via nanostring in PB and qPCR in tonsil.

Research Methodology

To validate the preliminary nanostring data found in PB, expression of the 3 identified miRs were measured by qPCR in human CD16+ and CD16- NK cells from PB. After validation, we confirmed that miR targets the binding site, which is the 3' untranslated region (UTR) of CD16. This purpose was achieved via luciferase assay of transfected human NK-like cell line 293T with identified miRs from the preliminary data. First, we cloned the 3' UTR of CD16 mRNA and miR candidates to create two plasmids: a luciferase vector containing the CD16 3'UTR immediately after the luciferase gene and an expression vector that was empty or contained the candidate miR. These plasmids were used to transfect the 293T cell line creating a miR condition and a control. After transfection, the 293T cells were lysed and utilized in the luciferase assay to determine if the candidate miR targets the 3' UTR of the CD16 gene. The luciferase assay quantifies the extent to which miR reduces mRNA available for translation. The amount of CD16 expression with miR present was compared to CD16 expression in the control (empty

expression vector which does not contain the miR sequence). The expected outcome was to see a decrease in luciferase activity.

Results

We cloned the 3' UTR of CD16 mRNA and miR candidates to create two plasmids: a luciferase vector containing the CD16 3'UTR immediately after the luciferase gene and an expression vector which was empty or contained the candidate miRs: mir-92a, mir-133a or mir-218. These plasmids were used to transfect the 293T cell line creating a miR condition and a control. After transfection, the 293T cells were lysed and utilized in the luciferase assay to determine if the candidate miR targets the 3' UTR of the CD16 gene. The luciferase assay quantifies the extent to which miR reduces mRNA available for translation. The amount of CD16 expression with miR present was compared to CD16 expression in the control. We were able to demonstrate a 35% reduction ($p=0.12$, $n=3$) in luciferase activity with overexpression of miR-218 (Figure 3).

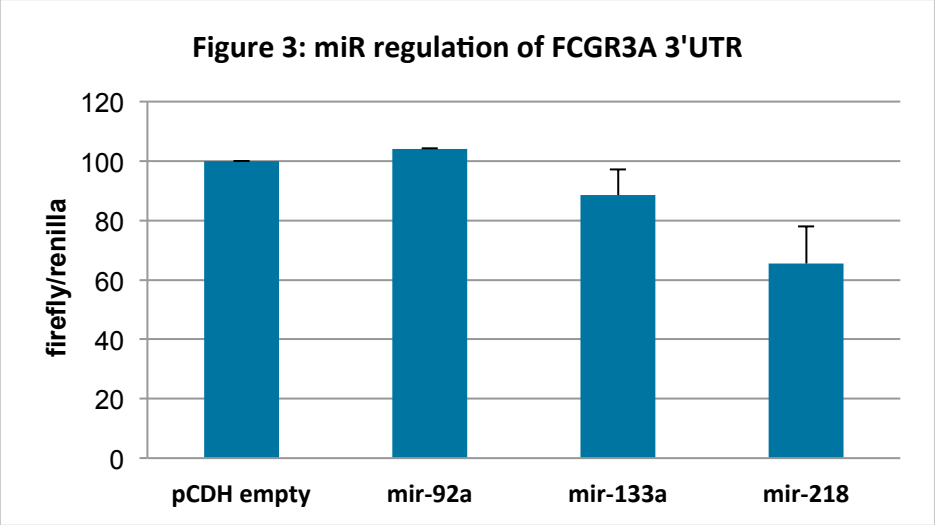


Figure 3: Confirmation that miR targets the 3' untranslated region (UTR) of CD16

Conclusion

Human $CD16^+$ natural killer cells are important mediators of ADCC. A better understanding of CD16 regulation in NK cells will create opportunities to improve anti-tumor antibody therapies. Increased surface expression of CD16 in mature human NK cells will offer greater potential to engage in ADCC to kill target tumor cells. Through this research project we obtained preliminary evidence that miR-218 is an intrinsic negative regulator of CD16 expression in NK cells. Ongoing work will determine the effect of overexpression and knockdown of miR-218 on CD16 expression, ADCC killing, and cytokine production in a CD16+ cell line and primary NK cells. Overexpression of miR-218 would be expected to decrease CD16 expression, while knockdown of mir-218 would be expected to increase CD16 expression. Additionally, to determine the developmental role of mir-218, stage 1 CD34+ pro-NK cells will be expanded and differentiated in an established NK cell expansion assay system. These future directions will help identify specific miRNA pathways that can be exploited to increase NK cell-mediated ADCC.

Citations

1. Benson DM Jr, *et al*. IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood*. 118(24):6387-91 (2011).
2. Clynes RA, *et al*. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumors. *Nature Medicine*. 6(4):443-6 (2000).
3. Weng WK and Levy R. Two Immunoglobulin G Fragment C Receptor Polymorphisms Independently Predict Response to Rituximab in Patients With Follicular Lymphoma. *J Clin Oncol*. 21(21):3940-7 (2003).
4. Freud AG, *et al*. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med*. 17;203(4):1033-43 (2006).
5. Caligiuri MA. Human natural killer cells. *Blood*. 112(3):461-9 (2008).
6. Witkos TM, *et al*. Practical Aspects of microRNA target prediction. *Curr Mol Med*. 11:93-109 (2011).
7. Yu J, *et al*. CD94 surface density identifies a functional intermediary between the CD56bright and CD56dim human NK-cell subsets. 115(2):274-81 *Blood*. (2010).
8. Cichocki F and Miller JS. In Vitro Development of Human Killer-Immunoglobulin Receptor-Positive NK Cells. *Natural Killer Cell Protocols: Cellular Molecular Methods*. (2010).