Assessing Natural Killer Cell Cytotoxicity Against Multiple Myeloma Cells with IL-6 and sIL-6R

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ABSTRACT

Multiple myeloma (MM) is a blood cancer affecting plasma cells. Proliferation of abnormal plasma cells in the bone marrow leads to reduced production of healthy antibodies that recognize infections. Malignant plasma cells in MM produce unusable antibodies that accumulate in the body. MM can result in complications including bone fragility, anemia, kidney failure, higher susceptibility to infection, etc. While MM remains to be an incurable form of cancer, research into cellular immunotherapy like the use of natural killer (NK) cells suggest the possibility for a treatment that can cure multiple myeloma. NK cells are lymphocytes of the innate immune system which can recognize and attack malignant cells without prior activation or recognition, making their functions highly attractive to study. Cytokines like interleukin-6 (IL-6) are inflammatory response messengers that are suspected to hinder cytotoxic activity for NK cells. Here we studied interactions between NK and MM cells through cultures of NK-92MI and MM.1s cells to assess the cytotoxicity of NK-92 MI cell with and without added IL-6 and soluble IL-6 receptor (sIL-6R). Our results suggest that the viability of MM.1s cells was significantly increased in the presence of IL-6 and sIL-6R, suggesting that IL-6 and sIL-6R play a critical role in the survival of MM cells from cytotoxic NK-92 MI cells. Our findings provide an interesting insight into therapeutic implications of NK cells in treating MM, particularly in the bone marrow tumor microenvironment enriched with IL-6 and sIl-6R.

Introduction

Multiple myeloma (MM) is a complex blood cancer which affects plasma cells, a type of white blood cell. Lymphocytes play an integral role in fighting infection and disease in the body. The B-cell lymphocyte class differentiates into plasma cells, which protect the body through the production of immunoglobulins. However, in the case of multiple myeloma, plasma cells can become malignant and cancerous. This in turn leads to the production of abnormal monoclonal immunoglobulins. These antibodies can be harmful or unable to be used by the body. While healthy plasma cells make antibodies to recognize and fight infections, malignant plasma cells in MM are unable to produce the same response, and instead produce unusable antibodies that accumulate in the body. For this reason, multiple myeloma is often characterized as a B-cell malignancy. The proliferation of abnormal plasma cells in the bone marrow leads to the formation of tumors which hinder healthy blood cell production and lead to weakening of the bone in their interactions with bone-tissue forming osteoblasts. Therefore, MM can result in complications including fragility of the bones, anemia, kidney failure, higher susceptibility to infection, and various other effects to the body systems. (American Cancer Society, 2018)

While MM remains to be an incurable form of cancer, research into therapies such as the use of natural killer (NK) cells suggest their possibility for a treatment for multiple myeloma. Current treatments include drug therapies, with proteasome inhibitors and steroids serving as the clinical standard for the treatment of MM. Proteasome inhibitors are used to block the function of proteasomes, which would prevent cancer cells from disposing of their proteins, therefore hindering them from properly growing and surviving in their environment. Proteasome inhibitors are used

throughout the entire disease progression. Additionally, high-dose steroids can be used to kill MM cells and decrease inflammation. (MMRF, 2021) However, novel approaches to the treatment of blood cancers have the potential to have better effects on minimizing the symptoms of the cancer and lessening the number of cancerous cells. NK cells are naturally produced lymphocytes which are able to recognize and attack malignant cells without prior activation or recognition, making their functions highly valuable to study. Cytokines produced by the body, such as interleukin-6 (IL6), are natural inflammatory responses that can affect cytotoxic activity of NK cells. Additionally, since MM primarily progresses in the bone marrow, previous research has shown that the interactions of bone forming cells, osteoblasts, may promote the survival of MM cells and hinder the activity of NK cells, as well as induce increased IL-6 production by osteoblasts. (Uhl et al., 2022). For this reason, the goal of this study is to better understand the interactions between NK cells, osteoblasts, and multiple myeloma cells in the environment IL-6. Additionally, the mechanisms of cytokines including IL-6 are of interest in understanding the optimal conditions which maximize NK cell cytotoxicity for its use as an effective multiple myeloma treatment. It is known that IL-6 follows the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway. The STAT pathway is a critical signaling cascade used by cytokines to transmit signals from the cell membrane to the nucleus, where they regulate gene expression. The process unfolds in several steps: first, IL-6 binds to its receptor, IL-6R. Subsequently, this binding event leads to the formation of the IL-6/IL-6R/gp130 complex, where IL-6 associates with its receptor and interacts with glycoprotein 130 (gp130) on the cell surface. This complex activation triggers the Janus kinases (JAKs), primarily JAK1 and JAK2, associated with the intracellular domain of gp130, leading to the phosphorylation of STAT proteins, mainly STAT1 and STAT3, linked with the cytoplasmic domain of the receptor complex. Phosphorylated STAT proteins then form dimers and translocate into the cell nucleus, where they regulate gene expression by binding to specific DNA sequences in the promoters of target genes, known as STAT response elements (SREs). This binding modulates the transcription of specific genes, either promoting or inhibiting their expression. Moreover, this signaling pathway can initiate a positive feedback loop of inflammation, particularly in MM, where it results in the upregulation of anti-apoptotic proteins, as evidenced by recent research (Huang et al., 2022).

Understanding the intricacies of IL-6/STAT signaling in NK cells is thus essential for deciphering its role in immune function and its potential implications for diseases like MM. Another point of interest is the effect of the soluble interleukin-6 receptor (sIL-6R), which is naturally present throughout the body. The receptor binds to IL-6 and forms a complex that can still activate cells expressing the transmembrane glycoprotein-130 (gp130) receptor, thereby modulating the IL-6 signaling pathway. To note, classic signaling of IL-6 through the membrane bound IL-6 receptor is restricted to certain cell types that express this receptor. However, IL-6 trans-signaling through sIL-6R can occur in a large number of cell types, due to the widespread expression of gp130 in almost all cells except granulocytes. This interaction between IL-6 and sIL-6R can have complex effects on immune cell function, including potentially influencing the cytotoxic activity of NK cells. Therefore, investigating the role of sIL-6R in the context of NK cell interactions with multiple myeloma cells and osteoblasts in environments with elevated IL-6 levels is crucial for understanding the intricate mechanisms underlying multiple myeloma progression and potential therapeutic intervention.

To better understand the interactions of NK cells with multiple myeloma and osteoblasts, it is planned to assess the cytotoxic potential of these cells with one another in environments of elevated levels of IL-6 and sIL-6R. To accomplish this, co-cultures and triple cultures of these cells were assessed to determine the optimal environment for NK cell function. Specific effector-to target (E:T) ratios were assessed to further understand the number of NK cells needed, which helps provide data for clinical application and effective dosages. Cytokine IL-6 and sIL-6R were added to each culture externally. Control groups without the addition of cytokines were also analyzed to understand the baseline function of each culture. From each culture, various information can be collected. Multiple myeloma transduced with td-tomato, which produces a quantifiable luminescence upon addition of luciferin, was used to track multiple myeloma viability. MM cell death was characterized by a decrease in luminescence.

Overall, the goal of this research is to better understand the mechanisms of natural killer cells in their role as possible immune therapies for multiple myeloma and to provide a thorough analysis of the effects of interleukin-6.

Methods

Cell Preparation

In this experimental setting, the natural killer cells, specifically the NK-92 MI interleukin-2 independent cell line, underwent cultivation in 6-well plates using StemCell Technologies MyeloCult H5100 culture medium supplemented with 1% penicillin-streptomycin. This process lasted for approximately 3 days post-thawing. Concurrently, the multiple myeloma cells enlisted for this study were derived from the MM.1s cell line, which was cultured in a 75 mL cell culture flask utilizing RPMI 1640 culture medium. The human fetal osteoblastic cell line, hFOB, was used and cultured with human osteoblast media in a 75 mL flask. cells were afforded the necessary time for proliferation prior to subsequent passaging and counting, ensuring their optimal contribution to the respective wells.

Experimental Design

Cells were seeded in a 96 well plate following the experimental design highlighted in *Figure 1*. In *Figure 1a*, cells were counted prior to seeding, with 50,000 of each cell type in each well in 500 µL of media each. To create the co-culture, MM.1s cells were seeded first, followed by the addition of NK-92 MI cells. The amount of media added was kept consistent in all wells. In *Figure 1b*, cells that are 'pre-treated' were exposed to IL-6 or IL-6 and sIL-6R for 24 hours prior to seeding with non-treated cells. Cells in *Figure 1b* and *1c* were cultured in 1:1 ratio with 10,000 of each cell type per well with 200 µL of the appropriate media.

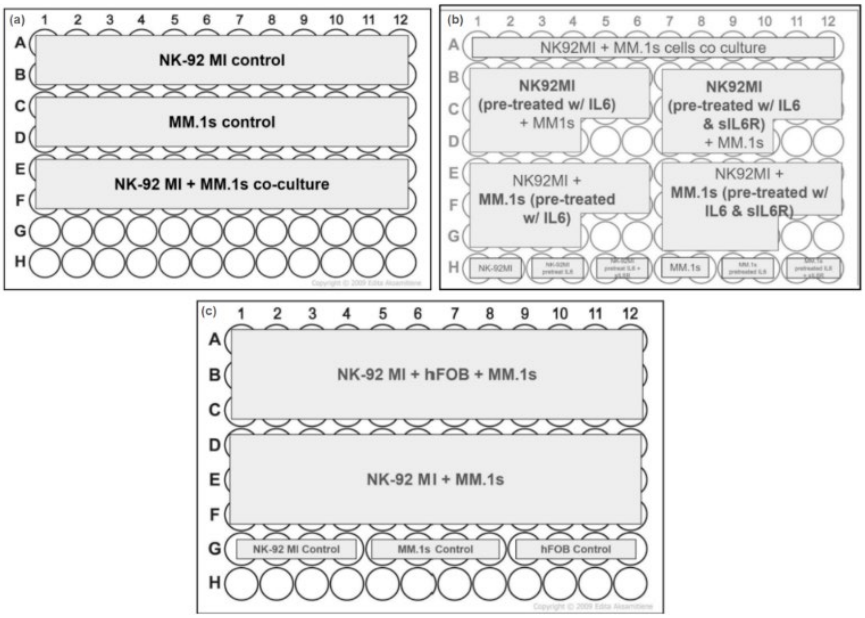

Figure 1. (a) Experimental design with NK-92 MI in monoculture, MM.1s cells in monoculture, and NK-92 MI and MM.1s in co-culture. (b) Experimental design with MM.1s and NK-92 MI control, MM.1s and NK-92 MI pre-treated with IL-6, MM.1s and NK-92 MI pre-treated with IL-6 and sIL-6R. Co-cultures include NK-92 pre-treated with IL-6 or IL-6 and sIL-6R with non-treated MM.1s cells, and MM.1s pre-treated with IL-6 or IL-6 and sIL-6R with untreated NK-92 MI cells, and NK-92 MI with MM.1s, non-treated control. (c) Experimental design with triple culture of NK-92 MI, MM.1s, and hFOB, co-culture of NK-92 MI and MM.1s, and monoculture controls of NK-92 MI, MM.1s and hFOB.

Cell Imaging & Data Collection

The cells were imaged using brightfield and TRITC channels on a Nikon Ti-E fluorescence microscope. The multiple myeloma cells used in this experiment are luciferase td-Tomato positive MM cells to visualize the progression of multiple myeloma viability using luminescence data. Additionally, multiple myeloma cells and co-cultures were imaged via TRITC red staining for better representation of the cell morphology as well as to distinguish between live and dead cells. Images were taken and adjusted under Nikon NIS-Elements. A duplicate well plate was created for imaging purposes.

Luminescence data was collected to measure multiple myeloma cell viability. A higher luminescence unit value indicates greater multiple myeloma viability. Since cells are transduced with td-Tomato, upon addition of the compound luciferin, the luciferase enzyme allows for luminescence expression, which was recorded using a plate reader. The SoftMax Pro software was used with plate reader settings as follows, plate type: 96 well standard opaque, height: 17.6 mm, integration: 1000 ms, and read height: 1.00 mm. Statistical analyses were performed with GraphPad Prism 9.0 (Dotmatics) and significance was determined using unpaired t-test with statistically significant results at $p \leq 0.05$.

Results

To visualize the viability of multiple myeloma cells in co-culture with natural killer cells, as well as the proliferation of the individual monoculture wells, microscopic images were taken shown in *Figure 2*. These images were taken 4 hours, 24 hours, and 48 hours following seeding of the cells. The monoculture of multiple myeloma showed high cell proliferation as well as viability, indicated by the bright red color highlighted through the TRITC channel of the microscope. The monoculture of natural killer cells shows enlarged clusters of the cells over time. In the co-culture, there is a clear decrease of multiple myeloma cells when they are exposed to the cytotoxic effects of natural killer cells.

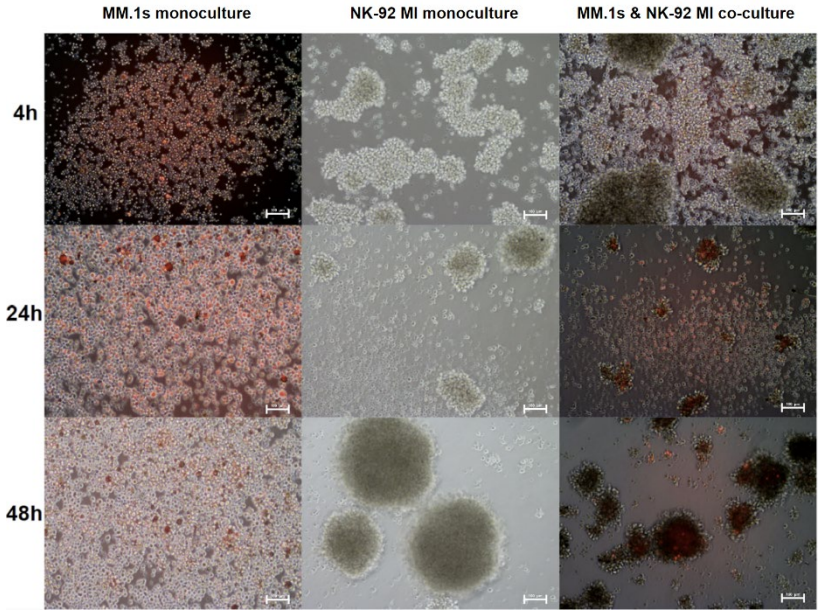

Figure 2. Monocultures of MM.1s and NK-92 MI cells and MM1.s/NK-92 MI coculture, taken at 4 hours, 24 hours, and 48 hours at 10x magnification with a Nikon Ti-E fluorescence microscope. Images including multiple myeloma are merged images with brightfield and TRITC channels to show fluorescence.

As mentioned, the MM.1s cell line used is transduced with td-Tomato, allowing for luminescence data to be collected through the addition of luciferin. The data is shown in *Figure 3*. Data was normalized to multiple myeloma control values. This demonstrates that over time, there is a decrease in multiple myeloma viability as compared to the control wells. This relationship is most significantly shown in the 48-hour data point.

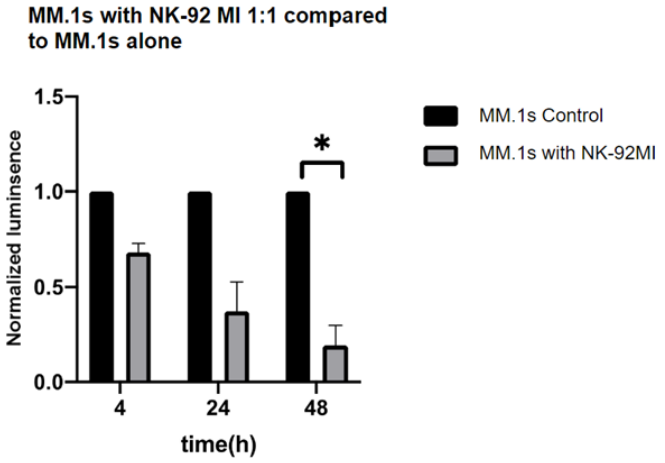

Figure 3. MM.1s with NK-92 MI in a 1:1 ratio compared to MM.1s in monoculture. The black bars represent MM.1s in monoculture, gray bars represent co-culture of MM.1s and NK-92 MI normalized to MM.1s control. X-axis represents the time in hours, with three time points at 4 hours, 24 hours, and 48 hours. Y-axis represents multiple myeloma viability in terms of normalized luminescence. Asterisk symbol represents statistical significance at p-value < 0.05.

To test the effect of IL-6 in different concentrations, MM.1s was placed in monoculture with external IL-6 or IL-6 and sIL-6R added. As shown in *Figure 4*, cultures of MM.1s with both IL-6 and sIL-6R had greater luminescence (MM viability) at 4 hours, compared to those with IL-6 only, which were comparable to the control at this time point. At the 200 ng/mL concentration within 24 hours, the IL-6 condition was closest to the IL6 and sIL6R condition. Overall, in all conditions at 48 hours, the IL-6 condition was the most viable compared to both the control and the condition with IL6 and sIL-6R.

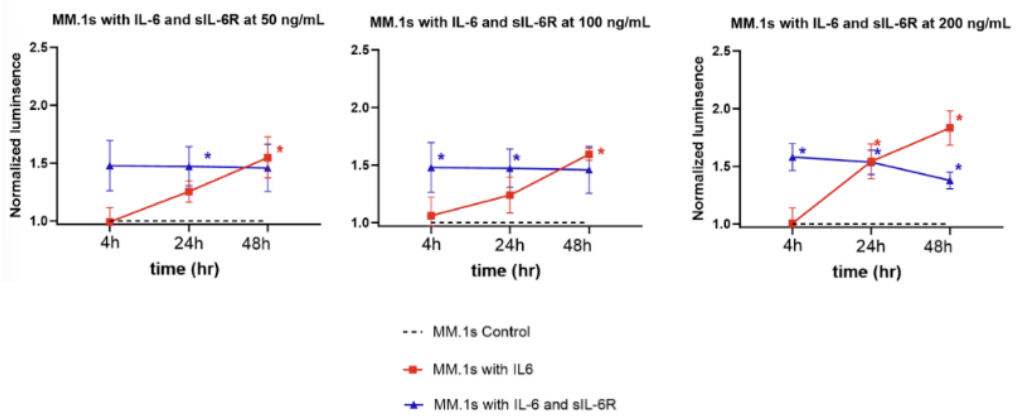

Figure 4. MM.1s with IL-6 or IL-6 and sIL-6R at 50 ng/mL (left graph), 100 ng/mL (center graph), and 200 ng/mL (right graph) concentrations of each cytokine or receptor. The red line represents treatment with IL-6, and the blue line represents treatment with IL-6 and sIL-6R. Dashed line represents MM.1s control. Asterisk symbol represents statistical significance at p-value < 0.05.

In *Figure 5*, MM.1s cells pre-treated with IL-6 for 24 hours showed greater viability as compared to with both IL-6 and sIL-6R at time points 0 hours to 24 hours. Overall, both conditions show greater viability than the control at 4 hour and 24-hour timepoints.

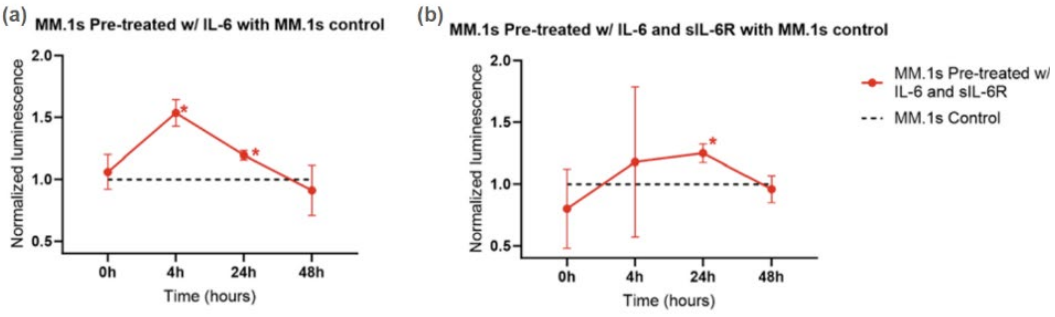

Figure 5. MM.1s with IL-6 or IL-6 and sIL-6R at 200 ng/mL concentrations of each cytokine or receptor. (a) The red line represents treatment with IL-6, and (b) the red line represents treatment with IL-6 and sIL-6R. The dashed line represents MM.1s control. Asterisk symbol represents statistical significance at p-value < 0.05.

The imaging data for the results obtained in *Figure 5* are shown in *Figure 6*. Microscopic imaging is significant in showing that addition of IL-6 or IL-6 and sIL-6R result in an increase in multiple myeloma proliferation and are not shown to negatively impact its growth.

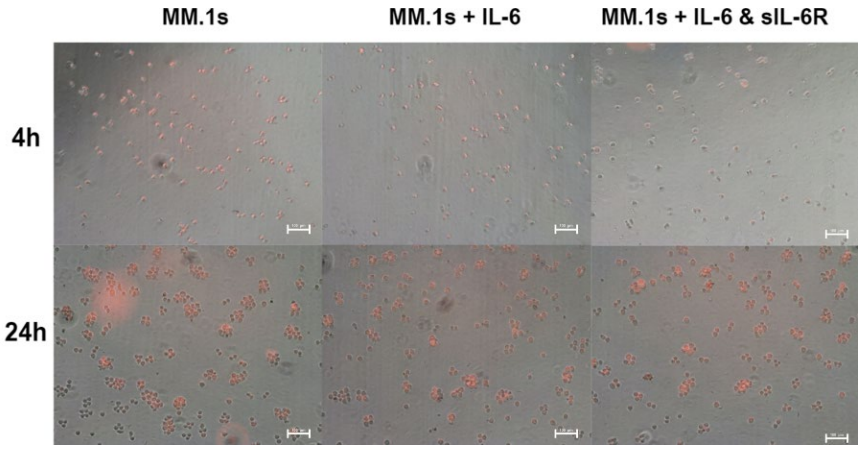

Figure 6. Imaging data for MM.1s control and with IL-6 and IL-6 & sIL6-R at 4 hour and 24-hour time points following pre-treatment period of 24 hours, merged images with brightfield and TRITC at 10x.

To incorporate natural killer cells and observe their cytotoxic effects in an IL-6 rich environment, cells were co-cultured in four different conditions as shown in *Figure 7*. NK-92 MI cells were pre-treated (exposed to IL-6 or IL-6 and sIL-6R for 24 hours) and co-cultured with non-treated MM.1s cells. Similarly, MM.1s cells were pre-treated and separately co-cultured with non-treated NK-92 MI cells. The results indicate that overall, pre-treatment of both NK-92MI and MM.1s cells with IL-6 or IL-6 and sIL-6R in a co-culture with non-treated cells were more viable than the control, which was a co-culture of NK-92MI and MM.1s with no IL-6 added, at time points 4 through 48 hours. However, MM.1s cells pretreated with either IL-6 or IL-6 and sIL-6R both were more viable than the pretreated NK-92MI cells, indicating that IL-6 has a significant effect on multiple myeloma viability, even in the presence of natural killer cells.

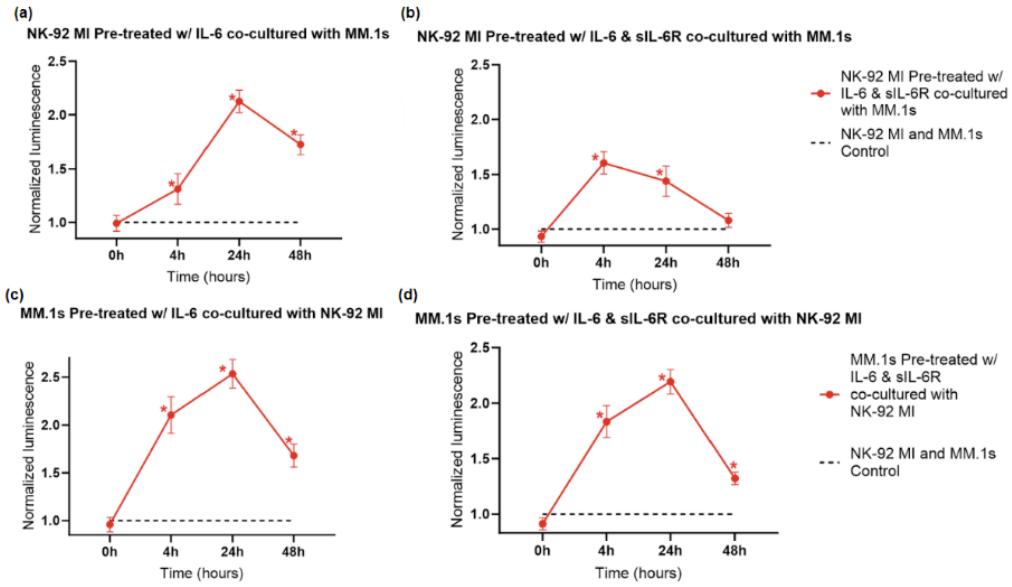

Figure 7. Graphs showing luminescence data normalized to control of (a) co-culture of NK-92 MI pre-treated with IL-6 with MM.1s, (b) NK-92 MI pre-treated with IL-6 and sIL-6R with MM.1s, (c) MM.1s pre-treated with IL-6 with NK-92 MI, and (d) MM.1s pre-treated with IL-6 and sIL-6R with NK-92 MI. The red line indicates co-culture treated with respective cytokine or cytokine and receptor. The black dashed line indicates the NK-92 MI co-culture control.

The imaging data for the graphs shown in *Figure 7* are displayed below in *Figure 8*. The images show a greater presence of multiple myeloma cells after 24 hours as compared to the 4-hour time point. Additionally, these images confirm the multiple myeloma cells are alive due to the presence of red luminescence exposed by the fluorescent imaging. Unlike *Figure 2*, there is minimal clustering of NK cells, indicating that the MM cells can proliferate in environments with high IL-6 and sIL-6R.

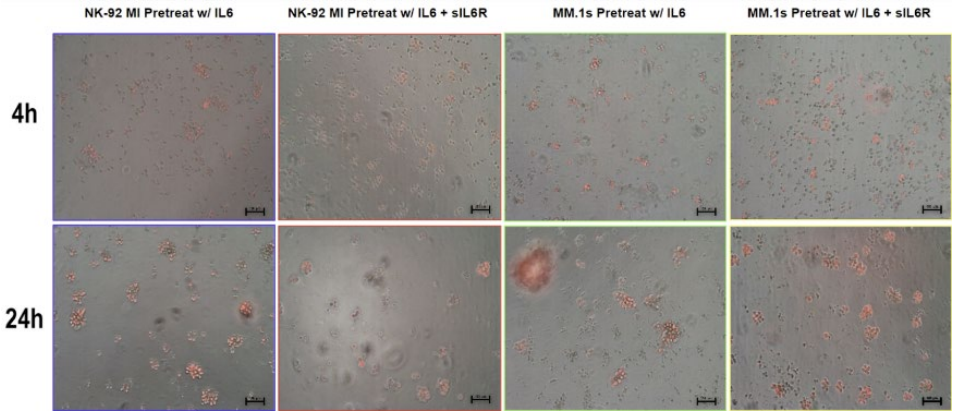

Figure 8. NK-92 MI and MM.1s co-cultures at 4h and 24h timepoints following pre-treatment periods of 24 hours with IL-6 or IL-6 and sIL6-R, merged images with brightfield and TRITC at 10x.

As mentioned previously, osteoblasts can efficiently produce IL-6. In a triple culture of hFOB, NK-92 MI, and MM.1s cells in comparison with a co-culture of NK-92 MI and MM.1s, *Figure 9* shows that the addition of osteoblasts was shown to have a significant effect on MM.1s viability at 24 hours, which can indicate conference of resistance to MM cells through production of IL-6.

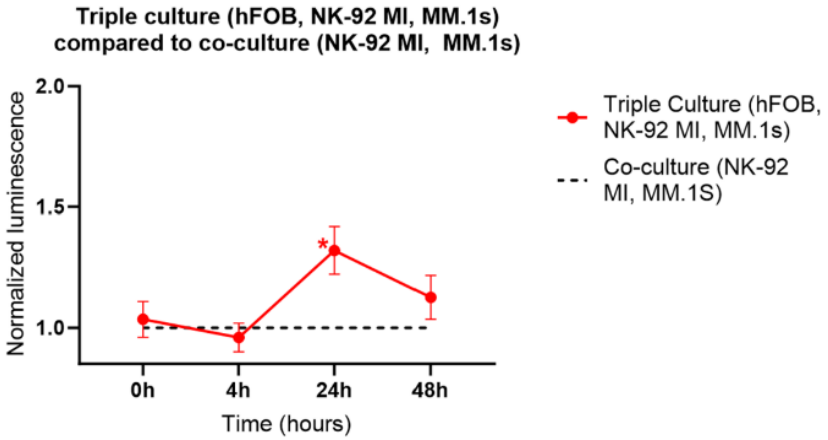

Figure 9. Triple culture of osteoblasts, multiple myeloma, and natural killer cells (red), compared to co-culture of multiple myeloma and natural killer cells (black) for 0 hours to 48 hours.

Discussion

The findings of this study shed light on the promising potential of natural killer (NK) cells in combating multiple myeloma (MM), a blood cancer characterized by the malignant transformation of plasma cells. The interplay between NK cells, MM cells, and the bone marrow microenvironment, specifically in the presence of interleukin-6 (IL-6), is crucial to understand for the development of innovative immune therapies. The inherent limitations of current MM treatments, which are predominantly reliant on proteasome inhibitors and steroids, show the urgent need for novel therapeutic approaches. NK cells, with their ability to recognize and attack malignant cells without prior activation, are a compelling option for research. Additionally, the impact of IL-6, a key cytokine in the inflammatory response, on NK cell cytotoxicity adds a layer of complexity to the study, given its potential influence on the bone marrow environment where multiple myeloma predominantly progresses.

The experimental design, as illustrated in *Figure 1*, implemented cultures of NK-92 MI, MM.1s, and hFOB cells, allowing for the assessment of NK cell cytotoxicity. Effector-to-target (E:T) ratios as 1:1:1 was chosen to provide valuable insights for potential clinical applications. The addition of exogenous IL-6 highlighted the impact of this cytokine on NK cell function and MM cell survival as it reflected elevated levels of pro-inflammatory IL-6 in multiple myeloma progression.

Microscopic imaging, as depicted in *Figure 2*, provides a clear visualization of the interactions between cells over time. Monocultures of MM.1s exhibited high proliferation and viability. Additionally, NK cells showed enlarged clusters. NK are known for forming clusters, and as seen in the literature, while the mechanism is not yet understood, clustering allows for cells to better coordinate immune responses and improve cytotoxic function (Kim et. al, 2017). Notably, the co-culture revealed a clear decrease in multiple myeloma cells, displaying the cytotoxic effects of NK cells on MM cells. Quantitative analysis through luminescence data in *Figure 3* reinforced the microscopic observations, indicating a significant decrease in MM viability over time in the presence of NK-92 MI cells. The statistical significance at the 48-hour time point highlights the sustained impact of NK cells on MM cells, suggesting a potential therapeutic window for intervention.

The RNA sequencing data revealed a high expression of IL-6 receptors in the MM.1s cell line, highlighting the potential significance of IL-6 signaling in MM progression. Subsequent experiments demonstrated that the addition of IL-6, particularly in combination with sIL-6R, promoted greater MM cell viability compared to IL-6 alone, suggesting a potential role for the IL-6/sIL-6R complex in enhancing MM cell survival. Notably, this effect was observed across various concentrations of IL-6, indicating a dose-dependent response.

Further investigation into the impact of pre-treatment with IL-6 and sIL-6R on MM cell viability revealed sustained increases in viability over time, particularly in comparison to untreated controls. Microscopic imaging corroborated these findings, revealing an increase in MM proliferation following treatment with IL-6 or IL-6/sIL-6R. These results suggest that IL-6 signaling may not only promote MM cell survival but also facilitate their proliferation, potentially contributing to disease progression. While pre-treatment with IL-6 or IL-6/sIL-6R conferred increased viability to both MM and NK cells in co-culture settings, MM cells exhibited higher viability compared to NK cells, indicating a preferential effect of IL-6 on MM cell survival. Additionally, the inclusion of osteoblasts in triple cultures further highlighted the impact of IL-6-producing cells on MM cell viability. The presence of osteoblasts significantly enhanced MM cell viability, suggesting a potential mechanism for the conferment of drug resistance through IL-6 production.

Conclusion

In conclusion, this research signifies a crucial step towards understanding the complex interactions between NK cells, MM cells, and the bone marrow microenvironment. The observed cytotoxic effects of NK-92 MI cells on MM.1s cells demonstrate the potential of NK cells as a novel immunotherapy for multiple myeloma. However, the observed increases in MM cell viability following treatment with IL-6 in conjunction with sIL-6R emphasize the combination therapeutic potential of targeting the IL-6 pathway in MM management. Moving forward, further understanding of the molecular mechanisms underlying IL-6-mediated effects on MM cells holds promise for the development of novel therapeutic approaches aimed at improving outcomes for clinical applications.

Limitations

The limitations of this study must be considered with interpretation of the findings. Firstly, this study uses single cell lines of each cell type, such as NK-92 MI, MM.1s, and hFOB, which may not capture the complexity of primary cell lines derived from multiple myeloma patients. Secondly, the controlled, in vitro use of interleukin-6 and soluble interleukin-6 receptor may not represent the entire array of cytokines and receptors present in the tumor microenvironment in vivo. Additionally, the concentrations of cytokines and receptors used in the study are limited to specific concentrations chosen to replicate elevated levels of IL-6 and sIL-6 receptor, and variations in these concentrations could influence the results. Furthermore, these results involve only short-term analyses of cell interactions, not greater than 48 hours. Finally, while this research points towards the potential use of NK cells as a multiple myeloma therapy, the safety concerns and effects on human or animal tissues in vivo are not assessed within this study. These limitations necessitate further research into cell-cell interactions and environments.

Acknowledgements

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