PROTECTIVE *RENALASE*-DEFICIENCY IN BETA CELLS SHAPES IMMUNE METABOLISM AND FUNCTION IN AUTOIMMUNE DIABETES

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1. Introduction

1.1 Overview about Diabetes

1.1.1 Definition and classification of diabetes

Diabetes is a chronic condition that arises when the pancreas does not create enough insulin or when the body does not use the insulin that is produced adequately. Insulin is a hormone that controls blood glucose levels. Hyperglycemia, also known as elevated blood glucose or raised blood sugar, is a common side effect of uncontrolled diabetes that can cause catastrophic damage to many of the body's systems, particularly the neurons and blood vessels, over time. (1)

There are two Types of Diabetes:

The pancreas does not produce insulin in type 1 diabetes because the body's immune system destroys the insulin-producing islet cells in the pancreas.
In type 2 diabetes, the pancreas produces less insulin than usual, and your body develops insulin resistance. This indicates that your body contains insulin but is unable to use it.

This paper is going to focus on Diabetes Type 1.

1.1.2 Epidemiology and prevalence statics

Although much attention has been placed on the global pandemic of type 2 diabetes, there has also been a similar increase in the incidence of type 1 diabetes — of roughly 3-4% every year. Diabetes affects almost three million people in the United Kingdom, with type 1 diabetes accounting for 10% of the total.
There are substantial differences in the disease's prevalence around the world. Finland and Scandinavia have the highest rates (48-49 per 100,000 persons each year), which is about 100 times higher than rural China. The United Kingdom is considered to have a high incidence, with children having the sixth highest prevalence (24.5 cases per 100,000 persons per year).
Type 1 diabetes is most commonly diagnosed in childhood or adolescence, but it can emerge at any age. The presentation age range is usually around 10-14 years. The most recent increase in the incidence of type 1 diabetes has been among children under the age of five, probably due to increased exposure to environmental factors. Increased bodyweight and subsequent insulin resistance in early children may possibly contribute to this change toward earlier onset.
The onset of the disease is more aggressive in children and adolescents than in adults – another peak in the incidence of type 1 diabetes has been recorded at roughly 40 years. The onset of the disease is more aggressive in children and adolescents than in adults – another peak in the incidence of type 1 diabetes has been recorded at roughly 40 years. This latter group of people is not uncommon to be diagnosed with type 2 diabetes. Testing positive for islet cell antibodies, on the other hand, suggests type 1 diabetes, and these patients require insulin therapy much sooner. Patients in this category may have latent autoimmune diabetes of the young, often known as type 1.5 diabetes. (2)

1.1.3 Pathophysiology of Type 1 Diabetes

Insulin is typically produced in the pancreas by the islets of Langerhans b-cells in response to a glucose stimulation. The autoimmune destruction of these insulin-producing cells is the root cause of type 1 diabetes. This causes an utter lack of the hormone, leaving patients dependent on exogenous supplies for the rest of their lives.

The current understanding of the pathogenesis of type 1 diabetes is based on a concept first proposed in the 1980s by American immunologist George Eisenbarth. In genetically predisposed individuals, autoimmune b-cell destruction is assumed to be triggered by an infective or environmental stimulation. Weight gain and insulin resistance have also been identified as major accelerators of b-cell death in type 1 and type 2 diabetes, according to a recent study.

Type 1 diabetes is frequently thought to be a disease with a quick onset; nevertheless, its emergence is a much slower process requiring progressive immunological damage. It is postulated that an infective or environmental trigger causes insulitis, which is defined as T-lymphocyte invasion of pancreatic islets leading to b-cell death. This process, which can last months or years, is characterized by the production of islet cell antibodies (which are found in 85% of patients at diagnosis). Clinical symptoms will appear after roughly 80% of pancreatic b-cells have been killed, generally at a period of high insulin demandd3 (e.g., during an illness). (3)

1.2 Autoimmune basis of Diabetes Type 1

1.2.1 Destruction of pancreatic beta cells and insulin deficiency

A beta cell-specific autoimmune mechanism causes type 1 diabetes by destroying the pancreatic beta cells that produce insulin. It has been established that beta cell autoantigens, macrophages, dendritic cells, B lymphocytes, and T lymphocytes have a role in the development of autoimmune diabetes. Antigen-presenting cells are considered to process and present beta cell autoantigens to T helper cells after beta cells release them as a result of cellular turnover or injury. The first cell types to colonize the pancreatic islets are macrophages and dendritic cells. Naive CD4+ T cells may be able to detect beta cell and major histocompatibility complex peptides provided by dendritic cells and macrophages in the islets as they circulate in the blood and lymphoid organs, such as the pancreatic lymph nodes. Interleukin (IL)-12 produced by dendritic cells and macrophages can activate these CD4+ T cells. Beta cell antigen-specific CD8+ T cells are attracted into the pancreatic islets during this phase when they are activated by the IL-2 generated by the activated TH1 CD4+ T cells. These CD8+ cytotoxic T cells and TH1-activated CD4+ T cells are responsible for killing beta cells. Granzymes and perforin generated from CD8+ cytotoxic T cells as well as soluble mediators like cytokines and reactive oxygen species released from activated macrophages in the islets can also harm beta cells. Thus, autoimmune type 1 diabetes is brought on by a coordinated attack on beta cells by activated macrophages, TH1 CD4+ T cells, and beta cell cytotoxic CD8+ T cells. (4)

1.2.2 Genetic predisposition and environmental triggers

Genetic predisposition is an increased likelihood of developing a particular disease based on the genetic make-up of an individual.

Family history: The child of a T1D parent may have a genetic predisposition, which means they are more likely to develop type 1 diabetes if one or both parents or their twin sibling have the condition. When there is no family history of T1D, the chances are less than 1%, but they rise to 3-5% if a parent has it and close to 50% if an identical twin has it.

Ethnicity: Caucasians are the most common ethnic group to be diagnosed with type 1 diabetes, according to U.S. statistics. People of Chinese and South American heritage are the least vulnerable.

Environmental factors are elements that have a potentially adjustable source and do not result from factors present at birth.

Viral infections can elicit an aberrant immune response in individuals with a predisposition, as exemplified by certain viruses such as rubella virus, coxsackie virus, and mumps virus. This phenomenon occurs when the immune system erroneously targets and eliminates the beta cells of the pancreas, rather than directing its attack towards the virus. The atypical immune response may be attributed to the structural resemblances between the viral envelope and the outer membrane of the beta cells in the pancreas.

The intestinal microbiota, also referred to as the intestinal flora, is comprised predominantly of bacteria that naturally inhabits the gastrointestinal tract. The makeup of an individual's food is intricately associated with their personal variation. The influence of the gut microbiota on the immune system and its potential role in initiating the start of Type 1 Diabetes (T1D) has been proposed by researchers. This implies that dietary factors may contribute to the development of this condition. The association between diet and Type 1 Diabetes (T1D) is a subject of debate. Regrettably, initial investigations examining the delayed introduction of specific foods in childhood have yielded equivocal findings regarding their potential for preventing T1D. (3)

2 Beta Cells

Beta cells are a specific cellular population located within the pancreas, which play a crucial role in the synthesis and secretion of insulin, a hormone that exerts regulatory control over glucose concentrations in the bloodstream. The cellular entities in question are situated within the islets of Langerhans, which are dispersed throughout the pancreatic tissue. The production of insulin by beta cells is crucial for the metabolic processes of the human body, facilitating the uptake of glucose by cells and its subsequent use as an energy source. (5)

2.1 The anatomical structure of beta cells

Beta cells have distinct morphological and functional attributes. They are around 10 to 15 micrometers. Beta cells are found near one another within the islets of Langerhans, which are structures that possess a rich blood supply, facilitating the effective transportation of insulin and various other hormones.

Beta cells have the capacity to synthesize substantial quantities of insulin in reaction to fluctuations in glucose concentrations. The ability of pancreatic beta cells to secrete insulin is attributed to their possession of specialized machinery, characterized by the presence of insulin granules, voltage-gated calcium channels, and a complex network of signaling molecules. (6)

2.2 Role in Glucose Regulation

Beta cells are of utmost importance in the regulation of glucose levels within the human body. Upon an increase in glucose levels, such as in the postprandial state, beta cells secrete insulin into the circulatory system. Insulin serves as a signaling molecule that facilitates glucose uptake by cells in various tissues, hence promoting its utilization as an energy source. During episodes of fasting, beta cells decrease the quantity of insulin they secrete in response to a decline in glucose levels.

The regulation of this process is intricately governed by a sophisticated interaction between hormones and signaling molecules. An illustration of this phenomenon can be observed in the case of glucose, which elicits the secretion of insulin from beta cells via a mechanism known as glucose-induced insulin secretion (GIIS). This process entails the closure of potassium channels located in the cell membrane, resulting in cellular depolarization and subsequent insulin release. (5)

2.3 Beta cell dysfunction in Diabetes

The impairment of beta cell function is a characteristic feature observed in both type 1 and type 2 diabetes. Type 1 diabetes is characterized by an autoimmune response wherein the immune system targets and eradicates beta cells, resulting in a total absence of insulin synthesis. The initiation of this process is believed to be influenced by a confluence of genetic and environmental elements, including viral infections.

In the context of type 2 diabetes, beta cells exhibit reduced sensitivity to insulin, perhaps resulting in insufficient insulin production by the pancreas to adequately counterbalance this diminished responsiveness. This phenomenon frequently arises as a r-esult of various circumstances, including obesity and a lack of physical activity, which contribute to the development of insulin resistance and the eventual exhaustion of beta cells. (6)

3 Beta Cell Replacement

Autoimmune diabetes, results from the autoimmune destruction of insulin-producing beta cells in the pancreas. This destruction leads to a chronic deficiency of insulin, necessitating lifelong insulin replacement therapy. However, insulin therapy cannot perfectly mimic the body's natural insulin production, often resulting in complications such as hypoglycemia and diabetic ketoacidosis. Beta cell replacement therapy offers a promising alternative, aiming to restore the body's ability to produce insulin endogenously.

3.1 Historical Perspective and Current Challenges

Beta cell replacement therapy began with the first human trials of islet transplantation in the 1970s. The Edmonton Protocol, introduced in 2000, demonstrated that T1DM patients can attain insulin independence using a glucocorticoid-free immunosuppressive regimen. Despite developments, the therapy still faces problems such as donor shortage, graft rejection, and the side effects of immunosuppressive drugs. (5) (6) (7)

The Edmonton Protocol was a major advancement in islet transplantation, greatly raising insulin independence rates. Prior to its launch, just 8% of patients remained off insulin for more than a year following transplantation. The Edmonton Protocol resulted in insulin independence for 50% of patients after a year and 25% after five years. The frequency of islet cell transplantation surgeries and centers has declined over time, raising questions about the approach's long-term viability (5)

The greatest significant advance in islet cell transplantation has been in safety. When compared to the early 2000s, the technique now resulted in considerably fewer adverse outcomes. General immunosuppression is still required, but has improved with more localised medication regimes. (5) Despites improvements, the Edmonton Protocol's long-term effectiveness is restricted by the steady loss in graft function over time. (7)

Cadaveric human islets have traditionally served as the principal source of beta cells for transplants. However, the shortage of donor pancreases restricts the general implementation of this method. (5) Graft rejection and the return of autoimmunity remain serious challenges. Immunosuppressive medication is necessary to prevent allograft rejection, but it has side effects such as increased susceptibility to infections and cancers. (6)
The reduction in the number of islet transplantation procedures and the heterogeneity in outcomes indicate the need for more sustainable and repeatable beta cell replacement techniques. (5) (7)

3.2 Immunological Considerations

3.2.1 Immunosuppression

To prevent allograft rejection and recurrent autoimmunity, immunosuppressive therapy is essential. However, the side effects of these drugs, including increased susceptibility to infections and malignancies, pose significant concerns. (8) Research is focused on immune-isolation techniques, such as encapsulation of islet cells, and genetic engineering to create hypoimmunogenic cells, to reduce the need for systemic immunosuppression. (9) Clinical trials have shown that beta cell replacement can achieve insulin independence or significantly reduce insulin requirements in T1DM patients. However, the variability in outcomes and the gradual decline in graft function over time highlight the need for further research (8).

4 Natural Killer Cells

Natural killer cells (NK cells) are a type of immune cell that plays a role in the body's defense against infections and cancer. Natural killer cells, also known as NK cells, are a type of leukocyte that eliminate contaminated and cancerous cells within the body. Natural killer (NK) cells play a crucial role as effector cells in the immune system. The immune system serves as a defense mechanism against detrimental intruders, such as pathogens (viruses, bacteria, and parasites) and cancerous cells.
NK cells are a subset of lymphocytes, a distinct category of white blood cells that also included B-cells and T-cells. NK cells are referred to as "innate" assassins due to their ability to eliminate potential dangers without prior encounter with a specific infection. Cytotoxic T-cells, among other lymphocytes, require prior exposure to a pathogen in order to effectively eliminate it. (10)

4.1 Function of NK Cells:

Natural killer cells eliminate previously healthy cells that now present a danger. This encompasses cells that have been infected by viruses and cells that have undergone malignancy (cancerous transformation). Natural Killer (NK) cells eradicate these deleterious cells during the initial phases, hence impeding their dissemination.

Natural killer cells are crucial components of the innate immune system, playing a vital role in defending against pathogens. The innate immune system serves as the initial barrier of protection in your body against any potential dangers. The human body possesses physical barriers, such as the integumentary system and mucous membranes, that effectively prevent the entry of pathogens. It comprises certain immune cells, such as NK cells, which eliminate pathogens that have breached the body's defenses.

Although the major function of an NK cell is cytotoxicity, these cells also engage in intercellular communication. Cytokines, which are proteins, are released by cells to instruct other cells in the immune system to target and eliminate dangerous cells and pathogens. (10)

4.2 The mechanism of action of natural killer cells in the immune system

Natural killer cells actively survey the body, scrutinizing cells for specific signals that signify the presence of either healthy or sick cells. Upon detecting indications of cellular injury, they promptly release lethal substances within the cell to terminate it. The cytotoxicity of an NK cell is contingent upon the presence or absence of signals it receives from the target cell it is surveying. An NK cell's surface is equipped with several receptors that collaborate to either activate or hinder the NK cell's ability to eliminate a target. (10) (11)

4.3 Inhibition of NK cells

Natural killer (NK) cells refrain from targeting cells that possess signs indicating their status as healthy cells that are part of the body. NK cells primarily identify MHC-1 as a cell signal indicating self-identity. The Major Histocompatibility Complex class I (MHC-1) on the target cell interacts to an inhibitory receptor on a Natural Killer cell. The binding inhibits the cytotoxic activity of the NK cell. Rather than engaging in an offensive action, the NK cell proceeds to the subsequent cell. (11)

4.4 Activation of NK cells

Natural killer cells are triggered to eliminate target cells that they do not identify as part of the body.
NK cells can be stimulated to attack by the release of activating signals from cancer cells and infected cells.
Cells with missing or degraded MHC-1: Natural Killer (NK) cells will eliminate cells that lack Major Histocompatibility Complex class 1 (MHC-1) expression. Occasionally, a cell may possess MHC-1 molecules, but they undergo a reduction in their expression level. This implies that there is a lower quantity of it compared to what is typically considered as standard. For instance, a viral infection might reduce the level of MHC-1 expression in a healthy cell.

Natural Killer (NK) cells employ the secretion of perforin and granzymes as a means to eliminate a specific target cell. Perforin facilitates the formation of a pore in the target cell, enabling the NK cell to introduce granzymes. The granzyme induces apoptosis in the cell.
Activated natural killer (NK) cells secrete cytokines that stimulate other leukocytes to assist in eliminating the danger from your body. (10)

4.5 Anatomy of NK Cells

Natural Killer (NK) cells undergo maturation within the hematopoietic tissue located in certain bones known as bone marrow. As they mature, NK cells may reside within the confines of the bone marrow. They have the potential to migrate to different tissues and organs inside the lymphatic system, including:

- Lymph nodes
- Spleen
- Tonsils
- The thymus gland

After reaching maturity, the body releases natural killer (NK) cells into the bloodstream. Lymphoid tissue and related organs also harbor fully developed NK cells. They are situated within organs such as the liver and lungs. Approximately 5% to 10% of the lymphocytes present in the bloodstream are natural killer (NK) cells. Their lifecycle is brief, lasting approximately two weeks. Adults possess over 2 billion natural killer (NK) cells at any given moment. (10)

5 Natural Killer cells and Autoimmune Diabetes

Natural Killer (NK) cells play an important role in the pathogenesis of autoimmune diseases, including Type 1 Diabetes. These innate immune cells have a multifaceted interaction with the adaptive immune system, which influences the onset and progression of autoimmune diseases. Investigating the role of NK cells in autoimmune diabetes is critical for understanding disease mechanisms and exploring potential therapeutic options. NK cells have been found to have complex roles in autoimmune diseases, such as autoimmune diabetes. On one side, they can provide a shield against the onset of autoimmune diabetes, while on the other side, they might contribute to the disease in specific circumstances. (12)

Role of Protection

NK cells have the ability to provide protection against autoimmune diabetes through various mechanisms. A noteworthy mechanism involves the activation of NK cells using polyinosinic:polycytidylic acid (poly I:C). This activation provides protection against diabetes in NOD mice, a model for T1D, by triggering the secretion of interferon-gamma (IFN-γ). These findings indicate that in specific circumstances, NK cells have the ability to influence the immune response in a way that reduces its harmful effects. (12)

Role of Promotion

On the other hand, NK cells have also been linked to the development of autoimmune diabetes. In the NOD mouse model, the absence of NK cells led to a heightened form of arthritis, indicating that NK cells may play a role in promoting autoimmune conditions. 1. The intricate role of NK cells is made more complex by their interactions with other immune cells and the presence of different receptors that can impact their function in the context of autoimmune diabetes. (13)

Understanding the mechanisms of action

The role of NK cells in autoimmune diabetes is shaped by an intricate interplay of activating and inhibitory receptors, cytokine production, and interactions with other immune cells. NK cells possess a diverse range of receptors, including NKG2D and NKp46, that have the ability to detect stress-induced ligands on β-cells. The activation of these receptors can result in the direct destruction of β-cells or the alteration of the immune response by producing cytokines. In addition, NK cells have the ability to impact the adaptive immune response through their interactions with dendritic cells (DCs) and T cells. As an example, NK cells have the ability to influence the maturation and function of DCs. This, in turn, has an impact on T cell activation and the potential development of autoimmune diabetes. (14)

6 RNLS Gene

Renalase is a FAD-dependent amine oxidase that is largely released by the kidneys and has a role in catecholamine metabolism. RNLS plays a role in regulating blood pressure, heart function, and glucose metabolism, in addition to its enzymatic action. The gene encoding renalase, RNLS, has been related with numerous cardiovascular and metabolic illnesses, highlighting its potential systemic influence. (18)

6.1 Genetic Association and Beta Cell Protection

Type 1 diabetes is an autoimmune disease in which the immune system incorrectly assaults and destroys beta cells. Research has identified RNLS as a potential moderator of beta cell sensitivity to autoimmune assaults. A genome-scale in vivo CRISPR screen identified RNLS as a target for protecting beta cells in T1D. (19)

6.2 Clinical Trials and Drug Development

The discovery of RNLS as a therapeutic target has prompted the development of medicines that imitate or augment its protective effects. The FDA-approved medication pargyline has been demonstrated to recapitulate the protective benefits of RNLS deletion, suggesting a potential pharmacological approach to prevent or delay the onset of T1D in high-risk people. Ongoing clinical trials are looking into the efficacy and safety of these approaches, which could lead to new T1D therapy techniques. (19) (20)

7 Materials and Methods

7.1 Isolation and Preparation of Natural Killer (NK) Cells

Natural Killer (NK) cells were isolated from murine spleens using aseptic techniques. Mice were euthanized in accordance with institutional guidelines, and spleens were excised and placed in phosphate-buffered saline (PBS) within a sterile Petri dish. Spleens were mechanically dissociated to create single-cell suspensions, which were then filtered through a 70 µm cell strainer to remove tissue debris. The cell suspensions were centrifuged at 300 rpm for 5 minutes, and the supernatant was discarded. The cell pellet was resuspended in ACK lysing buffer to lyse red blood cells and incubated at room temperature for 5 minutes. Following lysis, the suspension was diluted with NIT-Media, filtered, and centrifuged again to wash the cells. The final cell pellet was resuspended in PBS for subsequent counting and analysis.

7.2 Cell Counting and Viability Assessment Using CellDrop™ Automated Cell Counter

Cell counting and viability assessment were performed using the CellDrop™ Automated Cell Counter. The cells were resuspended in PBS, and an aliquot was mixed with trypan blue dye to distinguish viable from non-viable cells. The mixture was loaded directly into the CellDrop™ sample chamber without the need for slides. The CellDrop™ software was used to calculate the total cell count and viability percentage based on the number of viable (unstained) and non-viable (blue-stained) cells.

7.3 Magnetic Labeling and Separation

For magnetic labeling, cells were incubated with biotinylated NK cell-specific antibodies at 4°C. After washing, cells were incubated with anti-biotin microbeads and subjected to magnetic separation using a MACS column placed in a magnetic field. The column was rinsed with buffer, and the cell suspension was applied to the column to allow for the retention of labeled cells. Unlabeled cells were washed away, and the magnetically retained NK cells were eluted and collected for further use.

7.4 Fluorescent Labeling

For fluorescent labeling, cells were incubated with Tag-it Violet proliferation and cell tracking dye. The working solution of the dye was prepared by diluting the stock solution in PBS. Cells were incubated with the dye at 37°C, protected from light, to allow for the incorporation of the dye into the cell membranes. After incubation, the reaction was quenched with media, and cells were washed to remove excess dye.

7.5 NK activation assay Co Culture/ Transwell

NIT-1 cells were sourced from The American Type Culture Collection (ATCC) under the catalog number CRL-2055. These cells were cultured in DMEM containing high glucose and pyruvate (catalog number 11-995-073 from Gibco), enriched with 2 mM L-glutamine (catalog number 25-030-081 from Gibco), 10% fetal calf serum (FCS) (catalog number 10-082-147 from Gibco), 50 µM 2-Mercaptoethanol (catalog number 60-24-2 from Sigma-Aldrich), and a combination of penicillin and streptomycin (catalog number 15140122 from Thermo Fisher Scientific), all within a controlled environment maintained at 37 °C and 5% CO2. Additionally, mutant NIT-1 cells deficient in Renalase ($Rnls^{mut}$) were previously developed.

To conduct direct co-culture experiments, 10^4 NIT-1 cells (WT and $Rnls^{mut}$) were seeded in each well of a 96-well round-well plate. $5*10^4$ NK cells were activated with Recombinant mouse (m)InterLeukin-2 (IL-2) (catalog number 575406; Biolegend) at a concentration of 100 ng/ml and added to the NIT-1 cell-containing wells. Transwell assays were performed on the same 96-well plate with Transwell inserts (0.4 µm pore size; Corning). NIT-1 cells were seeded in the bottom wells, while IL-2 activated NK cells were placed in the Transwell inserts, allowing cell types to communicate without physical contact.

IL-2 supplementation

Recombinant mouse IL-2 was added daily to both co-culture and Transwell setups at a final concentration of 100 ng/ml. The rationale for daily IL-2 supplementation was to maintain NK cell activation and proliferation throughout the experiment. IL-2 stimulates NK cell proliferation, survival, and effector functions such as cytotoxic activity and cytokine production. The continuous presence of IL-2 was required to simulate an environment that supports sustained NK cell activity, mimicking conditions that may occur in vivo during immune responses.

Incubation and Analysis

Both the co-culture and Transwell systems were incubated for 72 hours at 37°C in 5% CO_2. Following incubation, NK cell proliferation, activation status, and cytotoxic activity against NIT-1 cells were determined. The Tag-it Violet Proliferation and Cell Tracking Dye (catalog number 425101; Biolegend) was used to assess NK cell proliferation, following the manufacturer's instructions. A BD LSR II flow cytometer was used to examine activation markers and cytotoxic degranulation, which included staining for CD45, CD107a, and other surface markers. To identify dead cells, a propidium iodide (PI) viability dye was used. The effect of the Rnls mutation on NIT-1 cells' susceptibility to NK cell-mediated cytotoxicity was also investigated, by comparing the responses of wild-type and $Rnls^{mut}$ NIT-1 cells to NK cell activity. The acquired data were processed and analyzed using FlowJo software version 10.6.1 (FlowJo LLC).

7.6 Conditioned Media Assay

Conditioned media experiments were carried out by introducing media from either wild-type (WT) or Renalase mutant ($Rnls^{mut}$) NIT-1 cells, which were mixed at a 1:1 ratio with the medium used to culture NK cells. The conditioned media was further enriched at a 3:1 ratio after 24 hours and again at a 4:1 ratio after 48 hours, both times with continuous IL-2 stimulation at a concentration of 100 ng/ml, for a total incubation period of 72 hours. Throughout the experiments, cells were co-cultured in an incubator set to 37°C and 5% CO_2 for 72 hours. Cells were harvested and stained with specific antibodies, as described in Table 1. To identify dead cells, a propidium iodide (PI) viability dye was used and flow cytometry was performed using a BD LSR II flow cytometer. The acquired data were processed and analyzed using FlowJo software version 10.6.1 (FlowJo LLC).

7.7 Surface Expression Assay

NIT-1 WT and $Rnls^{mut}$ cells were seeded in 24-well plates, with each well receiving $2x10^5$ cells of the respective genotypes. These cells were treated with varying concentrations of 2-Deoxy-D-glucose (2DG) to determine its effect on glucose metabolism in NIT-1 cells. The 2DG concentrations used in this experiment were 1 mM, 5 mM, and 10 mM. The experiment was set to last 48 hours, during which cells were either treated with the specified 2DG concentrations or given sterile deionized water (dH_2O) as a control.

Following the 48-hour treatment period, cell surface staining was used to identify specific molecules of interest, as previously described. This procedure involved the use of antibodies directed at molecules expressed on the cell surface, which allowed for the analysis of changes in expression levels in response to 2DG treatment. The goal of using 2DG, a glucose analog that inhibits glycolysis, was to disrupt glucose metabolism in NIT-1 cells, allowing the study of how such metabolic inhibition affects the expression of specific cell.

2-Deoxy-D-glucose (2-DG) is a glucose molecule analog in which the 2-hydroxyl group is replaced with hydrogen, preventing further glycolysis. This modification enables 2-DG to act as a competitive inhibitor of glucose metabolism, particularly inhibiting the production of glucose-6-phosphate at the phosphoglucoisomerase level, a critical step in the glycolytic pathway. (15)

7.8 Cell Staining and Flow Cytometry Analysis

Cell suspension is prepared by suspending cells in phosphate-buffered saline (PBS) containing 10% fetal calf serum. During the antibody incubation steps, FCS is used to block nonspecific binding sites and reduce background staining. To avoid non-specific binding of antibodies to Fc receptors (FcR) on the cell surface, which could result in false-positive results, the cells are pre-incubated for 5 minutes with an FcR-blocking antibody solution (Biolegend catalog number 156604). FcR-blocking is an important step, especially when staining cells with high levels of Fc receptors, such as B cells, NK cells, macrophages, and dendritic cells. After blocking, cells are stained with antibodies against the cell surface molecules of interest (refer to Table 1) for 20 minutes on ice. The antibodies are diluted in the FcR-blocking solution to ensure that Fc receptors are blocked during the staining process. This step enables the specific labeling of target molecules on the cell surface.

8 Results

8.1 NKp46 innate lymphoid cells are required for Rnls beta cells' protective immune regulation.

Previous studies on the effect of Rnls deletion on immune cell dynamics were carried out using the NIT-1 mouse beta cell line transplanted into non-obese diabetic (NOD) mice, a model of Type 1 Diabetes (T1D). Single-cell RNA sequencing of cells infiltrating the grafts revealed that Rnls mutant beta cells significantly influence immune cell activation and metabolism towards an anti-inflammatory state, characterized by increased oncostatin M and Tgfβ1 expression, as well as upregulation of glycolysis-related genes. This change in the immunological milieu was followed by a large increase in CD4 T cells and tolerogenic PD-L1+ antigen-presenting cells (APCs), whereas NK cells were significantly reduced. Given the established role of PD-L1 blockade in reducing the survival benefits of Rnls mutant beta cells against autoimmunity, as well as the known function of NK cells in influencing APC maturation and activation, a more in-depth investigation into the protective role of NK cells for Rnls mutant beta cells was conducted. The analysis revealed a significant difference in the gene expression profiles of graft-infiltrating NK cells and closely related type 1 innate lymphoid cells (ILC1), with wild-type beta cell graft-derived cells expressing more genes associated with cellular activation and inflammation than Rnls mutant cells. Notably, the frequency of NK cells in Rnls mutant grafts was reduced not only in later stages, but also at earlier time periods, prior to any discernible variations in graft weight between control and Rnls mutants. In contrast, the frequency of ILC1 remained constant between grafts, implying that ILC1 plays only a limited role in regulating Rnls mutant beta cells' protective immunological control. This led to the hypothesis that NK cells play an important role in controlling autoimmunity in Rnls grafts. To investigate this, autoreactive splenocytes were depleted of NKp46+ innate lymphoid cells before being adoptively transferred into mice with NIT-1 beta cell grafts. Surprisingly, depleting NKp46+ cells reduced the protective effect against autoimmunity in both immunodeficient NOD-Prkdc (NOD.scid) and NOD.scid gamma (NSG) mice, which lack functioning T, B, and NK cells. This finding emphasizes the critical function of NKp46+ innate lymphoid cells in promoting a tolerogenic APC phenotype necessary for reducing autoimmune assaults on Rnls mutant beta cells. (18)

As seen in *Figure 1*, Gene expression analysis of natural killer (NK) cells produced from NIT-1 beta cell grafts reveals up to 10 significantly altered pathways ($-\log10(FDR) \geq 2$) utilizing 213 (Rnls) or 506 (WT) elevated genes ($p \leq 0.05$) as input. This figure uses raw data from a previous 3' gene expression single cell RNA sequencing experiment. (B) A schematic diagram of the experimental design comparing paired WT and Rnls NIT-1 beta cell grafts. Immunodeficient mice were injected s.c. into opposite sides, followed by i.v. injection of autoreactive splenocytes 4 days later. NKp46 innate lymphoid cells were either depleted or not. Grafts were collected, scaled, and immune cells were analyzed using flow cytometry. (c) The weight of paired grafts from mice with and without NKp46 cell depletion from autoreactive splenocytes is displayed. The results are the average of nine (control) or five (NKp46+ depletion) paired biological replicates from two independent experiments using NOD.scid or NSG recipient mice, respectively. (D and E) Quantification of immune cell subpopulations based on five paired controls. Flow cytometry was used to identify NIT-1 beta cell grafts (D) and NKp46 deficient grafts (E). The results are the average of five paired biological replicates. (F-H) Flow cytometry data (F) and quantification (G and H) show the expression level of PD-L1 on immune cells from matched WT and Rnls-deficient grafts.

The results are the average of five paired biological replicates. * p<0.05, ** p<0.01, and ns p>0.05 (paired two-tailed t-test).
The data from scRNAseq (A) and NKp46 depletion experiments utilizing NSG (C-E) or NOD.scid (C, F-H) recipient mice are from independent experiments. The data for the Ctrl. condition in (C) are a combination of prior experiments and a fresh independent experiment.

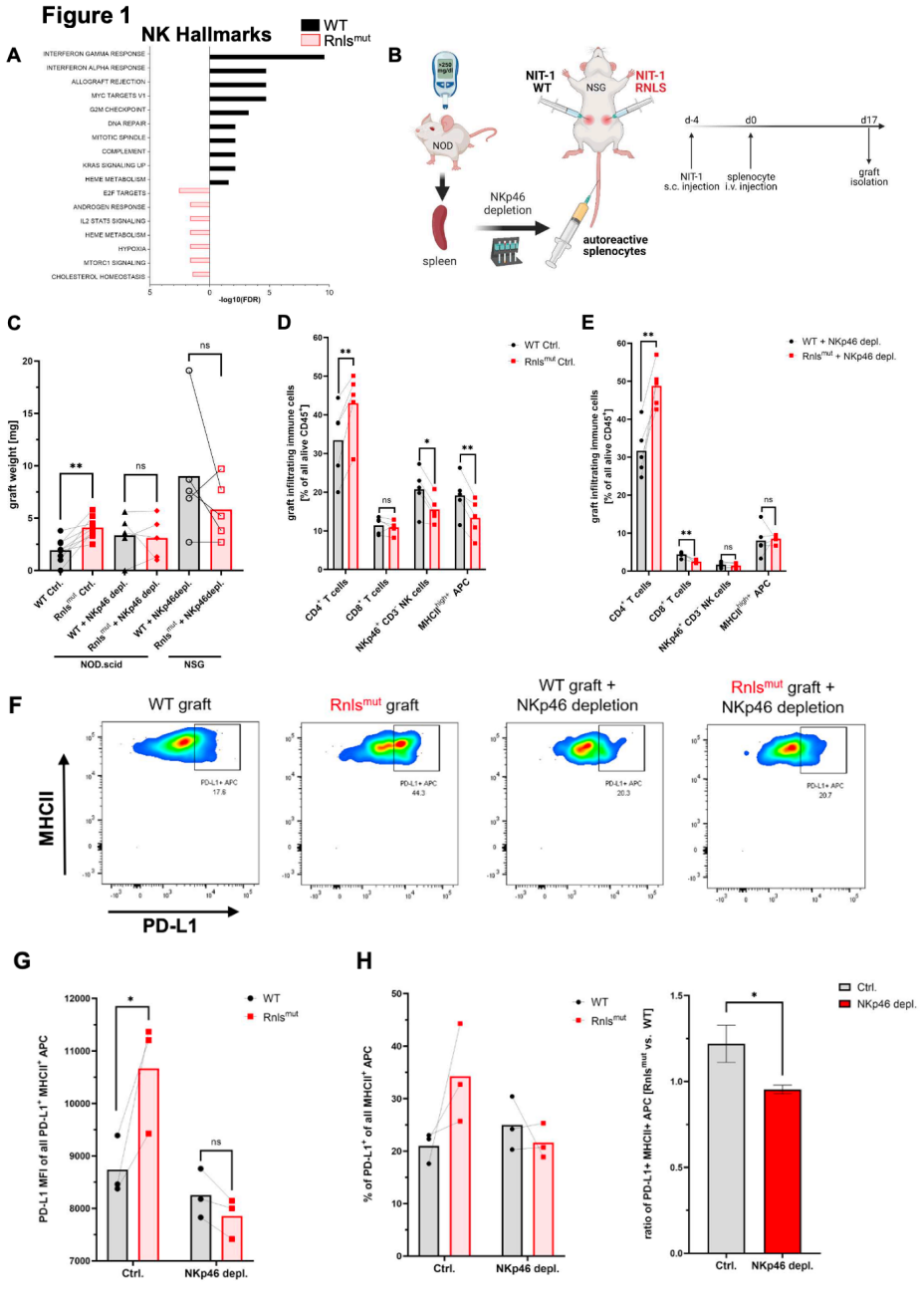

Figure 1: NKp46 innate lymphoid cells are crucial for protective immune-regulation by Renalase mutant (Rnls^{mut}) NIT-1 beta cells in vivo

8.2 Rnls^{mut} NIT-1 beta cells shape NK cell activation towards a regulatory phenotype

Following the discovery that NKp46 innate lymphoid cells play an important role in mediating protective immunological control and extending the survival of Rnls beta cell grafts, more study was done to establish whether Rnls beta cells directly impact the activation of innate lymphoid cells. Splenic NKp46 cells, which are mostly made up of NK cells because ILC1 accounts for just 5-10% of all NKp46 cells in the spleen, were extracted via negative selection to produce unmodified cells for in vitro co-culture investigations. These NK cells were activated using interleukin-2 (IL2), a cytokine that is known to increase NK cell proliferation and effector function. Co-culturing with Rnls NIT-1 beta cells resulted in a considerable reduction in NK cell activation, as demonstrated by lower expression of the activation markers CD44 and CD69, although it did not fully suppress NK cell activation. Notably, the expression of the degranulation marker CD107a on NK cells, which indicates cytotoxic potential, was significantly reduced in the presence of Rnls NIT-1 beta cells. Co-culture with Rnls NIT-1 beta cells inhibited NK cells' ability to proliferate after repeated IL2 stimulations, an effect that was dependent on direct cell-cell contact, as demonstrated by the restoration of NK cell proliferation when the two cell types were separated in a transwell system. This contact-dependent control of NK cells by Rnls NIT-1 beta cells was also seen with the activation marker CD69.

Tgfβ1 was shown to be more abundant in NK cells penetrating Rnls NIT-1 beta cell grafts, as revealed by differential gene expression analysis. To see if Tgfβ1 was also raised at the protein level, NK cells were stained for latency-associated peptide (LAP), which represents membrane-bound Tgfβ1. Co-culturing NK cells with Rnls NIT-1 beta cells resulted in higher Tgfβ1 expression on the cell surface compared to co-culture with wild-type cells. Unlike proliferative modulation, Rnls NIT-1 beta cells induced Tgfβ1 expression regardless of cell-cell interaction. This was validated using two methods: a trans-well experiment and the use of conditioned media from Rnls NIT-1 beta cells rather than supernatant from wild-type cells. In conclusion, Rnls NIT-1 beta cells influence NK cell activity via methods that are both dependent and independent of direct cell interaction. When NK cells interact with Rnls-deficient NIT-1 beta cells, they lose cytotoxic activity and proliferate less in response to IL2, while increasing the expression of the anti-inflammatory chemical TGFβ1.

Figure 2 shows that interleukin 2 (IL2)-stimulated primary mouse NK cells were co-cultured with the indicated NIT-1 beta cells for 72 hours. (A-D) Data from co-culture experiments treated with IL2 (20 ng/ml) show flow cytometry plots (A and C) and corresponding quantifications (B and D) of activation markers (A and B) and cytotoxic activity markers (C and D). (E-I) NK cells were activated with IL2 (100 ng/ml) every 24 hours in a co-culture or trans-well environment, alongside designated NIT-1 cells. (E & F) Representative flow cytometry data (E) or quantifications (F) showing NK cell growth under the given circumstances. (G) Quantifying the expression of activation marker CD69 on NK cells. (H & I) Flow cytometry results from trans-well conditions (H) and quantifications of indicated circumstances (I) demonstrate the expression of membrane-bound latency-associated peptide (LAP)/Tgfβ1 on NK cells. (J) Conditioned media experiments reveal LAP/Tgfβ1 surface expression in NK cells. Results are the mean ± SD of one or two independent experiments (A, B, E-I) or three (C, D, and J) (n = 3). *** $p<0.001$, ** $p<0.01$, * $p<0.05$, and ns $p>0.05$ (unpaired, two-tailed t-test). (18)

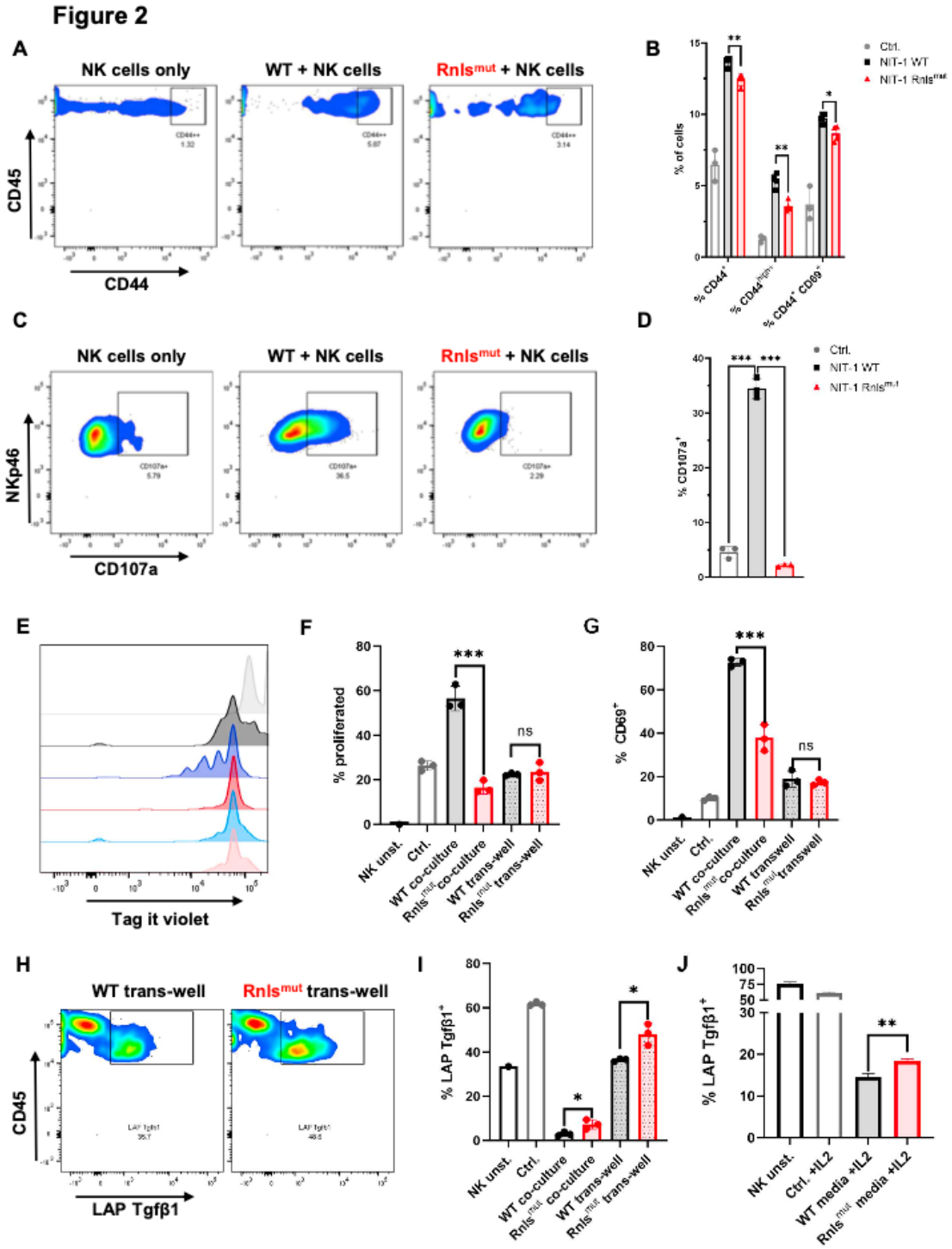

Figure 2: Rnls^{mut} NIT-1 beta cells broadly affect natural killer (NK) cell function in vitro.

8.3 NIT-1 beta cells lacking in Rnls exhibit overexpression of inhibitory immunological checkpoint molecules that limit NK cell activity.

$Rnls^{mut}$ NIT-1 beta cells have been demonstrated to be effective modulators of NK cell activation. An examination of bulk RNA sequencing data comparing differential gene expression between WT and $Rnls^{mut}$ NIT-1 cells indicated that $Rnls^{mut}$ NIT-1 beta cells are enriched in genes linked with immune-regulatory interactions between lymphoid and non-lymphoid cells. This shows that the increased production of inhibitory cell surface chemicals may influence NK cell stimulation in a cell contact-dependent manner. Using the GSEA-MSigDB database, we identified the top 1500 most substantially elevated genes in Rnls NIT-1 cells, which are known to modulate immune cell activity. CD44 and Itga4, which are critical for cell-cell adhesion, were among the genes that showed an increase in expression. Furthermore, $Rnls^{mut}$ NIT-1 beta cells increased the expression of important immunological checkpoint surface markers as Ceacam1, CD47, and CD200, all of which are known to prevent NK cell activation.

To determine if the increased mRNA expression levels equated to higher protein expression, cell surface labeling for these three important inhibitory NK cell ligands was conducted, followed by flow cytometry analysis. The findings revealed a considerable increase in CD47 surface expression, both in terms of cell percentage and mean fluorescence intensity. Similarly, Ceacam1 surface expression was significantly higher in terms of the amount of Ceacam1-expressing cells, with an increase in MFI on Rnls NIT-1 beta cells. While the number of cells positive for CD200 did not differ significantly between WT and Rnls mutants, CD200 MFI was much higher on Rnls NIT-1 beta cells. These findings imply that Rnls NIT-1 beta cells can regulate NK cell activation by upregulating several inhibitory NK cell ligands on their surface.

When investigating the basis for the overexpression of NK inhibitory molecules by Rnls cells, it was postulated that alterations in $Rnls^{mut}$ beta cell metabolism toward higher glycolysis could affect the cell surface expression of CD47. Given that CD47 expression is known to be regulated similarly to the immunological checkpoint PD-L1, and that glycolysis promotes PD-L1 expression, NIT-1 cells were treated with 2-deoxy-d-glucose (2DG) to limit glucose metabolism. Surprisingly, 2DG treatment significantly reduced CD47 surface expression on both live WT and $Rnls^{mut}$ NIT-1 cells. A lower concentration of 2DG lowered the percentage of Rnls cells that expressed CD47 to levels comparable to untreated WT cells, whereas tiny concentrations of 2DG had no effect on CD47 expression in WT cells. This suggests that increased glycolysis in Rnls beta cells contributes to the elevation of CD47 surface expression. Figure 3 (A) shows the top 1500 elevated immune cell function-related genes in $Rnls^{mut}$ NIT-1 beta cells compared to the WT control. A red box surrounds cell surface chemicals that have been shown to inhibit NK cell function. The red dotted line marks the threshold for substantial gene changes. Colored dots indicate the fold change of gene expression, from low (purple) to high (yellow). This figure's raw data came from a previous bulk RNA sequencing experiment. (B-J)
Flow cytometry was used to analyze inhibitory NK cell ligands expressed on the surface of Rnls and WT NIT-1 cells. The results are the mean ± SD of one of three separate studies (n = 3). *$p < 0.05$ (unpaired, two-tailed t-test). (18)

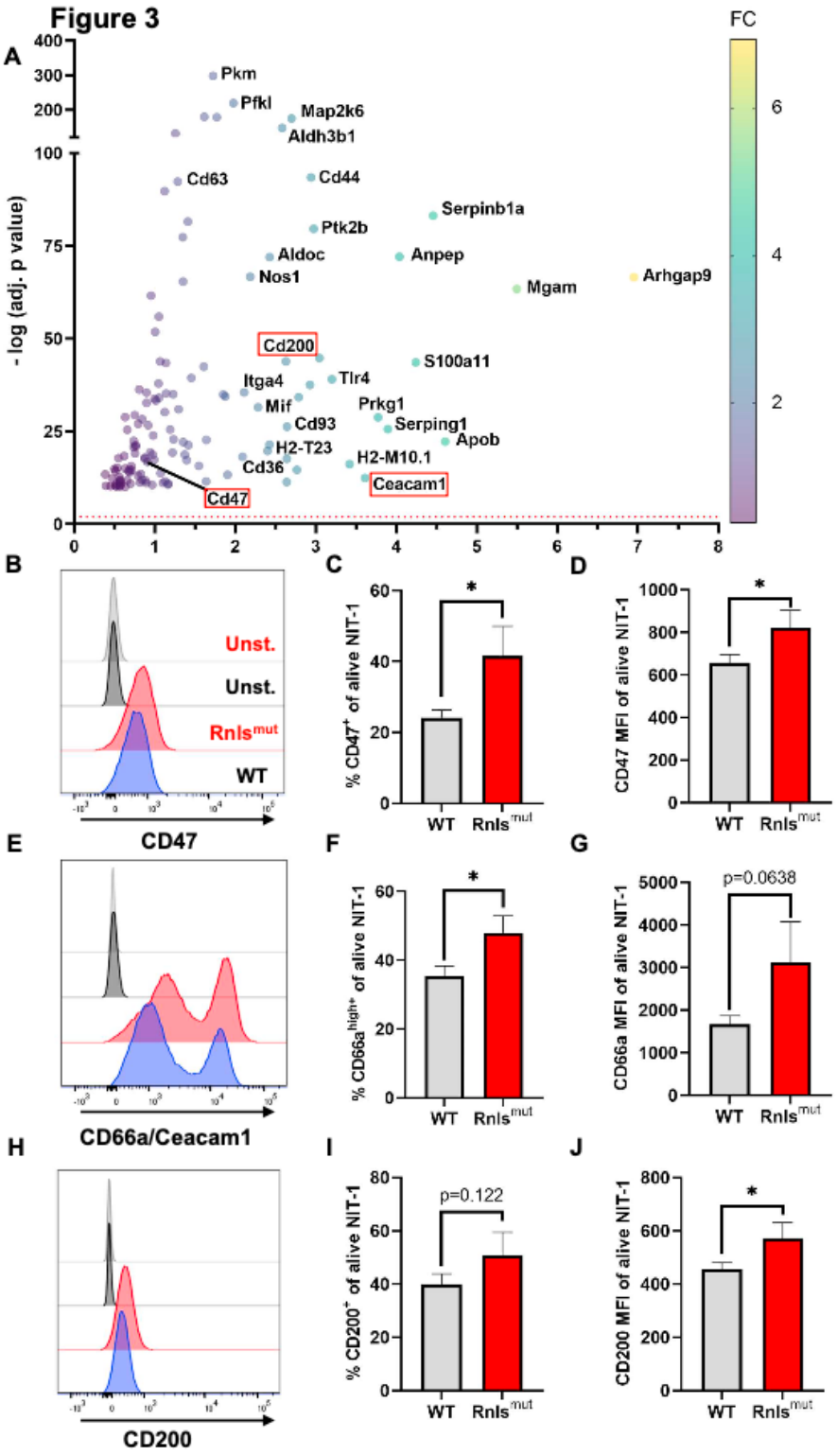

Figure 3: Rnls^{mut} NIT-1 beta cells show upregulated expression of key NK inhibitory ligands on their cell surface

9 Discussion

The findings of this study provide substantial evidence that highlights the pivotal role that natural killer (NK) cells play in shaping the immune-regulatory environment that surrounds renalase-deficient (Rnls) beta cells. In previous observations, it was found that the presence of natural killer (NK) cells in Rnls beta cell grafts was significantly lower than in wild-type (WT) grafts. This finding suggests that there may be a connection between NK cells and the protective properties of Rnls beta cells themselves. In this study, additional analysis of graft-infiltrating natural killer (NK) cells revealed distinct patterns of gene expression. Specifically, the NK cells derived from Rnls beta cell grafts exhibited elevated levels of the immune-regulatory cytokine TGF-β1, which plays a crucial role in reducing the severity of autoimmune reactions. There is abundant evidence that demonstrates the significance of TGF-β1 in the preservation of immune equilibrium and the prevention of autoimmunity. Furthermore, the dysregulation of this protein has been linked to a number of autoimmune diseases.

The finding that depleting NKp46+ cells rendered the protective effects of Rnls beta cells against autoimmunity null and void brought to light the indispensable role that innate lymphoid cells, and in particular NK cells, play in the process of mediating immune regulation within the milieu of the beta cell graft. This study further clarifies the influence of NKp46+ innate lymphoid cells on the phenotype of antigen-presenting cells (APCs) within beta cell grafts. On the basis of previous research that identified the PD-1/PD-L1 checkpoint as a critical mediator of immune regulation in beta cell graft survival, this study builds upon that research. In the presence of graft-infiltrating NKp46+ cells, it was observed that the frequency of mature MHCII+ APCs within Rnls beta cell grafts was significantly reduced. This reduction was contingent on the presence of NKp46+ cells. The complex interaction between NK cells and APCs is highlighted by the fact that TGF-β1, which is secreted by NK cells, is known to inhibit dendritic cell maturation and MHCII expression. This suggests that TGF-β1 may induce a tolerogenic APC phenotype within Rnls beta cell grafts.

Through the use of in vitro co-culture experiments, mechanistic insights were obtained regarding the manner in which Rnls beta cells directly modulate the activity of NK cells. It was discovered that beta cells that lacked Rnls were able to decrease the activation and cytotoxicity of natural killer (NK) cells, while simultaneously promoting the expression of the anti-inflammatory cytokine TGF-β1. In addition, Rnls beta cells were found to upregulate inhibitory immune checkpoint molecules like CD47, Ceacam1, and CD200, which provides additional evidence that these cells play a role in suppressing the activity of NK cells and immune responses. It has been reported in the past that the overexpression of CD47 on hypo-immunogenic MHCI/II-deficient islets inhibits natural killer cells and prevents the rejection of transplanted islets in humanized mice. However, the inhibition of NK cell activity may also be caused by other factors, such as changes in the metabolic processes of Rnls beta cells of the immune system.

The findings of the study indicate that increased glucose metabolism in Rnls beta cells leads to increased CD47 expression. This is because the inhibition of glycolysis by 2DG treatment significantly reduced the amount of CD47 surface expression. The expression of PD-L1 is known to be regulated by glucose metabolism; however, this study is the first to suggest that CD47 expression is also influenced in a similar manner. The expression of PD-L1 and CD47 is frequently regulated by mechanisms that are similar to one another, such as the stimulation of IFNγ. However, additional research is required to completely understand the molecular connection that exists between glucose metabolism and the upregulation of CD47 on beta cells.

To summarize, the findings of this research shed light on the intricate interactions that take place between Rnls beta cells and NKp46+ NK cells in the process of promoting protective immune regulation. This research also reveals potential therapeutic targets for autoimmune diabetes. The research opens up new avenues for the development of new strategies to preserve beta cell function and enhance graft survival in type 1 diabetes. This is accomplished by uncovering the mechanisms of immune modulation that occur within the environment of beta cells. (18)

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