12-2015

ROLE OF STAT3 IN HUMAN NK CELL FUNCTIONS

Prasad V. Phatarpekar

ROLE OF STAT3 IN HUMAN NK CELL FUNCTIONS

By

Prasad Vijaykumar Phatarpekar, M.Sc, M.S.

APPROVED:

Dean Anthony Lee, M.D, Ph.D.
Advisory Professor

Kimberly Schluns, Ph.D.

Michele Redell, M.D

Joseph L. Alcorn Jr, Ph.D.

Shulin Li, Ph.D.

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

ROLE OF STAT3 IN HUMAN NK CELL FUNCTIONS

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

And

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Prasad Vijaykumar Phatarpekar

M.Sc., M.S.

Houston, Texas

December, 2015

DEDICATION

To the love of my life

my wife

Chhaya

ACKNOWLEGMENTS

The journey which started quite some time back is finally coming to an end. Such a long course, I could not have traversed without the guiding hands, help, kindness, understanding, and steadfast support of my mentor, colleagues, friends, and most importantly my family.

First and foremost, my sincerest gratitude to my advisor, Dr. Dean Anthony Lee. After two false starts in graduate school, I finally found a lab and a mentor to suit my academic needs. Dr. Dean Lee, my advisor, provided me the space, literal and metaphorical, to think, work, and grow for the last five years. Ph.D. is such an undertaking, during the pursuit of which, it is easy to lose sight of the goal. The student needs firm, experienced hands to steer him back on course, a word of counseling when the going gets tough, empathy during distress, and encouragement during success. In short, one needs a shrink, a parental figure and an excellent teacher all rolled into one as your mentor. I found one in Dr. Lee. After two failed projects in two different labs, the trust he put in me gave me the hope and strength to reach my goal. When my project ran into rough weather, his scientific acumen, advice and reassurance brought me back on track. His ever accessibility and perseverance saw me through this arduous journey. I owe resurrection of my graduate career to him.

I would like to thank my present advisory committee members, Dr. Kimberly Schlun, Dr. Michelle Redell, Dr. Joseph Alcorn, and Dr. Shulin Li for their advice and suggestions at every stage of my research project. Thanks are also due to the past members of my advisory committee, Dr. Michael Blackburn, Dr. Roger O'Neil, Dr. Laurence Copper, and Dr. Dapeng Zhou for their guidance and encouragement.

When I started my research project five years back, other than studying immunology in a didactic course during my undergraduate years, I did not have much background in immunology in general and NK cell field in particular. Along with my mentor, Dr. Dean Lee, the other person who made my transition possible is my senior colleague in the lab and a close friend, Dr. Srinivas Somanchi. Right from training me in NK cell experimental techniques to introducing me to key NK cell concepts, designing the experiments and troubleshooting, his help and sound advice every step of the way were critical to the successful completion of my research project. Our daily coffee breaks aka brainstorming sessions were a vital part of my graduate education. I benefitted immensely from his expertise and astute intelligence.

I also owe thanks to Anitha Somanchi, a fellow graduate student in the lab and dear friend, for her technical expertise, ever readiness to help and encouragement. Thanks are also due to Neda Dastgheyb for her technical assistance and Dr. Vladimir Senyukov for his advice, whenever I asked for it.

I would also like to thank my senior colleagues in the Pediatrics Department, Dr. Shankar Kannan and Dr. Abhishek Mitra. I drew upon Dr. Kannan's scientific expertise and relied upon his kindness to borrow reagents and supplies innumerable times, which he provided willingly. A helping hand was always extended by Dr. Abhishek Mitra whenever I sought it.

My work would not have come to fruition without the invaluable contributions of my collaborators, Dr. Malini Mukherjee, Dr. Emily Mace, Dr. Pinaki Banerjee, and Dr. Jordan Orange, Section of Immunology, Allergy and Rheumatology at the Baylor College of Medicine, Houston, TX, and Dr. Gabrielle Romain and Dr. Navin Varadrajan, Department of Chemical and Biomolecular Engineering, University of Houston,

Houston, TX. Thanks are also due to Dr. Alexandra Freeman and Dr. Steven Holland, National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Health (NIH), Bethesda, MD for providing Job syndrome patients' blood samples, without which the work would not have been possible.

I would like to express my gratitude to Dr. John Weiner, past Assistant Dean, and Dr. Victoria Knutson, past Associate Dean, GSBS, UTHSC for their support during the difficult times of changing mentors. In these vulnerable situations, a student needs an assurance that somebody has his back. Without their help, my graduate education would have ended prematurely. My sincere thanks to Elisabet Lau, Senior Administrative Manager, Carol Helton, Senior Academic Coordinator, and Brenda Gaughan, Director of Academic Affairs, GSBS, UTHSC, for their "student first" policy and kindness.

I take this opportunity to thank Schissler Foundation, Houston, TX for providing me financial assistance for the academic year 2012-13 in the form Fellowship in the Genetics of Human Disease.

Big thanks to Renee Davis**,** Melinda Deel, Donna Boone, and Dr. Abby Geltemyer at the UT Student Health Clinic, for looking after my health with utmost kindness and warmth.

I would like to express my gratitude to my dear friend Dr. R.A. Sreepada, Goa, India, who always encouraged me to pursue Ph.D.

I am grateful to Dr. Dana Garcia, Texas State University-San Marcos, my past advisor during my Masters at Texas State, who has taken keen interest in the progress of my Ph.D and encouraged me throughout. I consider her as a living example of teacher's investment in a student.

My very good friends and well-wishers, Dr.Veena Ghai, Abhay and Dolly Ghai, Prasanna Kode, Eric and Dora Gonzalez, Jayaprakash and Anna Prathipati, Paritosh and Dr. Pratixa Joshi, Roland Tamez, Dr.Gena Jerkins, Divya Hanabe and Dr. Anil Gowda, Smita and Dr. Biplendu Jena, Mousoomi and Dr. Mukulesh Baruah, Mitalee Bhaid and Rohan Oak, Sucheta and Ravi Kini, Ayesha and Dr. Anish Ray, Dr. Pinki Chowdhury and Dr. Kallol Seth, Kelton and Janice Sams, Lee and Dr. Hardy Loe, Cherry and Siegfried Steinwender, and Jackie Newberry supported and encouraged me all along. Thank you so much!

Thanks to my family here in the United States, whom I know I can always count on, Sunil (Babu dada), Sunita and Neil Narvekar, Nisha and Niranjan Hiras and Dr. Kamalakar Baikerikar (Baikerikar kaka).

My family back in India: My grandmother, Late Geeta Y. Prabhu (Atu aji) for her unconditional love and, who along with my parents instilled in me the thirst for academic excellence. My aunt, Usha P. Prabhu (Usha maushi) for her unconditional love and support throughout my life. My elder brother, Rajendra V. Phatarpekar (Raju) for his absolute love. I know he has my back no matter what. My mother, Padma V. Phatarpekar (Aai), whose sole purpose in life is her children and who has been longing to see and address me as “Dr. Prasad Phatarpekar”, more than my own desire to see myself in the new avatar. My mother, who inspite of having a full time job as a teacher, worked very hard to make sure her children got all the attention they deserved while growing up. Her absolute love and drive to realize the dream kept me going. My father, Vijaykumar Narsinh Phatarpekar (Baba), who had to forgo his own attempt to do Ph.D. in physical chemistry for the sake of his family. A father, who, despite knowing the risk of delay, wanted to postpone his angioplasty until after the completion of his son’s

Ph.D., lest that news disturb his son. Their innumerable sacrifices, known and unknown, made my dream possible. They put their life on hold so that I could live mine.

Acknowledgements won't be complete without the mention of "Shimbu", who appeared one fine day at our doorstep to keep my spirits up. A huge stress burster!

That brings me to the most important person in my life, my dear wife Chhaya. The journey we started together is finally coming to an end. With me you toiled, in fact more so. I can't count the number of late nights you kept me company in the lab or waited for me at home and the number of weekends we spent in the lab. Because of me, we celebrated most of the Diwalis, Thanksgivings, birthdays, and marriage anniversaries in the lab. You endured through all for me. Through the ups and downs you stood unwavered. I could not have done what I did without you. So this is for you my dear.

ROLE OF STAT3 IN HUMAN NK CELL FUNCTIONS

Prasad Vijaykumar Phatarpekar, Ph.D.*

Advisory Professor: Dean Anthony Lee, M.D, Ph.D.

Natural Killer (NK) cells are cytotoxic lymphocytes, which play a critical role in the immune response against malignant cells and microbial infections, particularly viral. NK cells are equipped with germline encoded activating receptors, which upon detecting ligands expressed on stressed cells induce cytolytic activity of NK cells. Stimulation of NK cell proliferation and priming of NK cytolytic capability are accomplished by cytokines, which mediate their signals mainly through JAK-STAT signaling pathway. We found that K562 cells genetically modified to express membrane bound IL-21 (mbIL-21), which predominantly activates STAT3, induce robust expansion and activation of human NK cells. Further investigations in the lab, revealed role of STAT3 in the transcriptional regulation of NKG2D, a primary activating NK receptor. Based on these findings, I hypothesized that STAT3 signaling plays a critical role in human cytolytic function and proliferation.

I analyzed NK cells from Job syndrome patients to test my hypothesis. Job syndrome caused by dominant negative STAT3 mutations is a naturally occurring STAT3 deficient genetic model. Assessment of cytolytic activity revealed impaired cytolytic function in Job Syndrome patients' NK cells. Investigations into the probable underlying causes of impaired cytotoxicity showed deficient NKG2D receptor expression and impaired polarization of cytolytic granules to the immune synapse formed between Job syndrome patients' NK cell and target cell. I validated these finding in STAT3 knock-down primary human NK cells, which also displayed impaired cytolytic function and impaired cytolytic granule polarization phenotype.

Expansion of Job syndrome patients' NK cells with mbIL21 stimulation restored NKG2D expression to normal levels, corrected impaired cytolytic granule polarization and enhanced cytolytic activity. As constitutively active STAT3 is oncogenic, STAT3 is major drug target in cancer therapeutics. To assess a probable side effect of pharmacological inhibition of STAT3, I assessed its effect on human NK cell cytolytic function. Treatment of primary human NK cells with small molecule STAT3 inhibitor S3I-201 suppressed NK cytolytic function.

I employed pharmacological and genetic models of STAT3 deficiency to study the role of STAT3 in human NK cell proliferation. Treatment with STAT3 inhibitor S3I-201 reduced expansion of human NK cells stimulated not only with mbIL21 that predominantly activates STAT3, but also the expansion stimulated with membrane bound IL-15 (mbIL15), which predominantly activates STAT5 and activates STAT3 only marginally. mbIL21 and mbIL15 induced expansion was also found to be deficient in Job syndrome patients' NK cells. Thus, both pharmacological and genetic models complemented each other in underlying role of STAT3 signaling in human NK cell proliferation. Percentage of NK cells was also found to be lower in the peripheral blood of Job syndrome patients' compared to normal donors.

Employing pharmacological and genetic approaches, I showed that STAT3 deficiency in primary human NK cells causes impairment of cytolytic function and cytokine induced expansion. This is the first report to demonstrate the role of STAT3 in the transport of cytolytic granules in NK cell and NK cell functional deficiency in Job syndrome patients, which may provide an immunological basis for their proclivity to cancer. Restoration of NK cell function upon mbIL21 stimulation,

suggests adoptive NK cell therapy as a treatment option for Job syndrome patients. By assessing the effect of pharmacological STAT3 inhibition on NK cytotoxicity and proliferation, this study provides potential biomarkers for monitoring side effects of STAT3 inhibition, which is fast emerging as a therapeutic approach in cancer treatment.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

BACKGROUND

Background

Protection from the non-self and aberrant self is provided by the immune system. Non-self and aberrant self come in different forms and to counter them, the immune system has multiple tools in its arsenal. It is a complex organization of diverse types of cells, tissues and organs. Physical barriers such as skin and mucous membrane, mechanical processes including coughing and sneezing, biochemical defenses such as anti-microbial peptides, complement system, antibodies and cytokines, and cellular component comprising phagocytes and cytotoxic lymphocytes, all come under a big umbrella called the immune system. Some of these defense mechanisms such as physical barriers and anti-microbial compounds are common to all living organisms, while more evolved means of defense, such as phagocytic cells, are found in multi-cellular organisms, both vertebrates and invertebrates. Another component of cellular immunity, lymphocytes, which include cytolytic effector cells and antibody producing cells, are unique to vertebrates (1).

The human immune system has all the aforementioned modes of defense at its disposal. The generic mechanisms, which are the first line of defense against the invading pathogens, belong to the innate or natural branch of the immune system, while the pathogen specific mechanisms that are deployed during the later phase of the infection belong to the adaptive or acquired branch of the immune system. Antigen specificity and immune memory formation are the characteristics that distinguish adaptive immunity from innate immunity. Dendritic cells, mast cells, macrophages and neutrophils, the cellular mediators of innate immunity do not possess these immunological features, while lymphocytes, T and B cells, which execute the functions of adaptive immunity, do. However, the third type of lymphocyte, which was till recently

considered to be a part of the innate immune system, has been shown to have the attributes of the adaptive immune system as well. This unique cell, which belongs to both, innate and adaptive, immune systems, is Natural killer (NK) cell (2).

1.1 NK CELL

Observation of cell mediated cytotoxicity against tumor cells without prior sensitization to the tumor antigen in assays performed with human peripheral blood cells led to the discovery and coinage of the term natural killer cells (3). Since, the discovery in the early 70s the NK cell field has come a long way. In the beginning also monikered as null cells for having no T and B cell markers (3), NK cells now have an established identity as a subset of lymphocytes, defined by the expression of CD56, an isoform of neural cell adhesion molecule (4), and the absence T-cell co-receptor CD3 (5). NK cells are granular lymphocytes found in bone marrow, blood, peripheral organs such as liver, lung, peritoneal cavity and placenta, and secondary lymphoid organs including lymph nodes, tonsils, thymus, and spleen (4, 6, 7). In the peripheral blood of humans, NK cells comprise 2-18% of the lymphocyte population (8). Like CD8 T cells, they are cytolytic effector lymphocytes. The primary cytolytic mechanism is through exocytosis of lytic granules, which contain a number of cytotoxic proteins such as perforin and granzymes. Even though, both, NK and CD8 T cells are cytolytic lymphocytes that primarily kill their targets through exocytosis of lytic granules, there is a clear distinction as to the mode of activation between the two. Like T cell receptor (TCR) on T cells, NK cells do not exclusively express receptors specific for a single antigen, but express a multitude of activating receptors, which provide a broader ability to detect target cells expressing diverse types of ligands. Also, like TCR, antigen recognition by NK cell receptors is not dependent on major histocompatibility complex

(MHC) molecule dependent pathway of antigen presentation. NK cell receptors are germ-line encoded and like T cell receptor genes do not undergo somatic rearrangement and recombination (2, 9). As some of the activating ligands are expressed by normal cells, to differentiate normal self from abnormal cells, NK cells are also equipped with cell surface inhibitory receptors that recognize MHC class I classical and non-classical ligands on cells and mediate inhibitory signals to block cytolytic activity of NK cells. Thus, the cytolytic activity induced by NK cells is the net result of the balance between the strengths of activating and inhibitory signals (8). NK cells also kill target cells through ligands belonging to tumor necrosis factor (TNF) family. Three TNF-family ligands, viz. FAS ligand (FASL), TNF and TNF related apoptosis inducing ligand (TRAIL) either expressed on NK cells surface or secreted in the extracellular environment mediate apoptosis in target cells through the interaction with their respective receptors (6).

Along with cytolytic capability, NK cells can exert their effect through secretion of cytokines and chemokines in response to stimulation by target cells and cytokines secreted by accessory cells (10). NK cell-generated cytokines include interferon (IFN)-γ, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin (IL)-10 and IL-3, while chemokines such as CCL2, CCL3, CCL4, CCL5, XCL1 and CXCL8 are secreted by NK cells (2).

1.1.1 NK Cell Receptors

NK cells detect abnormal cells through the interactions of a large array of activating receptors expressed on NK cell surface with their corresponding ligands, whose expression is generally increased on the surface of stressed cells. Natural

cytotoxicity receptor (NCR) family, which includes NKp30, NKp44 and NKp46, is a major group of activating receptors. These receptors interact with pathogen-derived antigens such as viral hemagglutinins, cell wall core components of bacteria and malaria parasite, *Plasmodium falciparum* derived erythrocyte membrane protein-1. These receptors also interact with cellular ligands such as heparan sulphates and vimentin (11). Another primary activating receptor is natural killer group 2D (NKG2D). Its ligands include MHC class-I-related chain (MIC) A and MICB and the UL-16 binding protein (ULBP) 1, 2, 3, or 4. Expression of these ligands is induced by cellular stress such as heat shock, DNA damage, transformation and viral and bacterial infections (12). Other activating receptors that play an important role in NK cell activation include DNAX accessory molecule-1 (DNAM-1), 2B4 and CD160 (13, 14). DNAM-1 interacts with poliovirus receptor and nectin-2, whose expression is up-regulated on some tumor cells (13). 2B4 recognizes CD48 (13), while CD160 binds human leukocyte antigen (HLA) C (14). In NK cells, cell adhesion receptors such as lymphocyte function-associate antigen 1 (LFA-1) and CD2 mediate activating signals (13). In addition to antigen recognition receptors, NK cells express FcγRIIIA (CD16), a low affinity receptor for the Fc region of IgG, which enables NK cells to mediate antibody dependent cellular cytotoxicity (ADCC) (15). Most of these receptors (NCRs, NKG2D and CD16) associate with signaling adaptor proteins containing tyrosine-based activation motifs, which upon receptor engagement get phosphorylated by Src-family kinases. Phosphorylated tyrosines provide docking sites for more signaling proteins, which activate signaling pathways involving phosphatidyl- inositol-3-OH kinase (PI3K), phospholipase C and Vav. These signaling cascades affect exocytosis of lytic granules. Some of the activating receptor (2B4 and DNAM-1) do not couple with tyrosine-based activation motif containing adaptor protein, but possess such motifs in their own cytoplasmic

regions (13).

To recognize normal cells and thereby prevent their killing, NK cells are equipped with inhibitory receptors such as killer cell immunoglobulin-like receptors (KIRs) and CD94-NKG2A that recognize MHC class I classical and non-classical ligands on cells and mediate inhibitory signals to block cytolytic activity of NK cells. Inhibitory receptors do not associate with signaling adaptors, but contain tyrosine based motifs in their own cytoplasmic region. Instead of being activating, these motifs are inhibitory. These tyrosine residues are phosphorylated upon engagement of the inhibitory receptors and recruit tyrosine phosphatases SHP-1 and SHP-2, which in turn dephosphorylate tyrosine residues associated with activating receptor signaling complex thereby switching off the activation signals (13).

Activating, adhesion and inhibitory receptors are listed in Table 1.

TABLE 1. NK CELL RECEPTORS

Type	Receptors	Ligands
Activating	NKp30	Viral HA, pp65, BAT3, B7-H6, Heparin & heparan sulphates (11)*
	NKp44	Viral HA, Heparin & heparan sulphates (11)*
	NKp46	Viral HA, Heparin & heparan sulphates, Vimentin (11)*
	NKG2D	MICA, MICB, ULBP (8)*
	DNAM-1	Poliovirus receptor, Nectin-2 (8)*
	2B4	CD48 (8)*
	CD16	Immunoglobulin G (8)*
	CD27	CD70 (8)*
	CD160	HLA-C (8)*
	CD94-NKG2C	HLA-E (8)*
	CD94-NKG2E	HLA-E (8)*
Adhesion	CD11a-CD18	ICAM-1 (8)*
	CD11b-CD18	ICAM-1 (8)*
	CD2	CD58 (16)*
	CD62L	GlyCAM-1(17)*
Inhibitory	KIR	HLA-A, B & C (8)*
	CD94-NKG2A	HLA-E (18)*
	CD94-NKG2B	HLA-E (18)*
	LIR-1	HLA Class I (8)*

Abbreviations: CD- Cluster of differentiation; HA- Hemagglutinin; HLA- Human leukocyte antigen; BAT3- HLA-B associated transcript 3; MICA and MICB- MHC Class I polypeptide-related sequence A and B; ULBP- UL16 binding protein; KIR-Killer-cell immunoglobulin-like receptor; LIR- Leukocyte immunoglobulin-like receptor; ICAM-Intercellular adhesion molecule; DNAM-1- DNAX accessory molecule-1; GlyCAM-Glycosylation-dependent cell adhesion molecule

* The Number in the parenthesis indicates reference.

1.1.2 NK Cell Differentiation

NK cells are derived from hematopoietic stem cells (HSCs), primarily in the bone marrow, but evidence also points to their differentiation in secondary lymphoid tissues (SLTs) such as lymph nodes and tonsils (4). Based on the differentiation capability and commitment and gradual acquisition of NK specific phenotype, the human NK differentiation process is divided into 5 distinct stages. HSCs in response to cytokines such as Flt3 ligand, IL-6 and IL-7 differentiate into NK cell progenitors. Even though labeled NK progenitors for the ease of classification, these cells in reality are pluripotent as they are capable of differentiating into T cells and plasmacytoid dendritic cells. The stage 2 or precursor-NK (pre-NK) cell is marked by the acquisition of IL-15 responsiveness. At this stage, cells express CD122, the β receptor required for IL-2 and IL-15 signal mediation. The stage 2 cells are distinguished by their ability to differentiate into stage 3 in response to soluble IL-15 or IL-2 and in the absence of other cytokines and stroma. Even at this stage, the cells are not strictly committed to the NK cell lineage, but can also differentiate into T cells and dendritic cells. The other defining feature of this stage is the expression of CD56, which has come to define NK cells in the absence of T and B cell specific markers. The stage 3 is NK cell commitment stage. By differentiating into stage 3, the cells commit to NK cell lineage, as cells at this stage are incapable of differentiating into any other cell type. The stage 3 cells express some NK associated antigens like CD2, 2B4 and NKp44, but not others such as NKG2D, NKp30, NKp46, CD16, CD94-NKG2 complexes and KIRs. Due to their inability to secrete IFN-γ and effect perforin mediated cytotoxicity, the functional attributes of mature NK cells, these cells are identified as immature NK cells. Transitioning into the 4^{th} stage is marked by the expression of intracellular perforin,

IFN-γ, and surface expression of activating receptors NKG2D and NKp46. This stage is also characterized by the acquisition of the ability to mediate perforin and Fas ligand mediated cytotoxicity and IFN-γ secretion. Expression of CD94-NKG2A complex at this stage makes sure that attainment of functional capability is accompanied by the ability to be inhibited through MHC-I on the target cells. Due to the lack of CD16 expression, the NK cells at this stage are not capable of ADCC. The 5th and the terminal stage of differentiation is defined by the expression of CD16 and KIRs, equipping the cells with ADCC and MHC-I mediated licensing and inhibition (19).

1.1.3 NK Cell Functions

Diverse types of activating receptors enable NK cells to recognize pathogen infected, stressed and transformed cells. NK cells are also endowed with inhibitory receptors to identify self-signals. Calibration of activating and inhibitory signals determines whether NK cell sees a target cell as a self or non-self. Expression of MHC class I molecules, the predominant self-signals recognized by NK cells, is usually down-regulated in diseased cells, particularly virally infected cells, and transformed cells. During NK cell contact with such cells, lack of or diminished inhibitory signals tilt the balance in favor of NK cell activation, resulting in the target killing. This mode of target detection is called missing self recognition (2). NK cells employ this mechanism in the immunosurveillance of virally infected cells and tumor cells.

1.1.3.1 Anti-Cancer Activity of NK Cells

Equipped with cell surface receptors that detect danger signals on malignant cells and activate cytolytic activity, the inherent function of NK cells is immune-surveillance of transformed cells (6). Tumor recognition and rejection by NK cells have

been demonstrated both *in vitro* and *in vivo* in mice and rats (20-22). In animal models, anti-tumor activity of several cytokines such as IL-2, IL-10, IL-12, IL-18 and IL-21 has been shown to be mediated in a NK cell-dependent manner (8, 23-26). Expression of ligands recognized by activating receptors, NKG2D and DNAM1, is often up-regulated on malignant cells and as a result these receptors play a major role in anti-cancer activity of NK cells (8, 13). Mice deficient in NKG2D and DNAM1 receptors have impaired tumor immunosurveillance by NK cells (27-29). In humans, the critical role played by NK anti-cancer activity was brought to light by an 11 year follow-up study, which showed an association of higher incidence of cancer with low NK cytotoxicity in peripheral blood (30). Also, high levels of tumor-infiltrating NK cells are associated with positive prognosis (31-33). In acute myeloid leukemia (AML) patients, receiving HLA haplotype mismatched hematopoietic transplantation, donor derived NK cells, with mismatched KIR for recipient's HLA, have been shown to deliver graft-versus-leukemia effect (34). Anti-tumor effects of therapeutic monoclonal antibodies such as rituximab (anti-CD20) are also predominantly achieved through NK cell-mediated ADCC (35). Due to their anti-tumor activity, NK cells have emerged as candidate effector cells for immune therapy against cancer. Adoptive transfer of activated autologous or allogeneic NK cells has proven to be a safe and potentially efficacious immunotherapy for cancer (36, 37). NK cell therapy has been or being tested against multiple types of cancers (35).

1.1.3.2 Anti-Viral Activity of NK Cells

Along with lysis of malignant cells, NK cells also detect and lyse virally-infected cells. NK cells are part of the first line of defense against viral infections. A large body of work in mouse and human has firmly established role of NK cells in the immune

response against viral infections, particularly herepesviridae infections (38). Studies with mouse model of mouse cytomegalovirus (MCMV) have been instrumental in expanding our knowledge of the dynamics of NK cell response to viral infections. NK cells are activated during the early phase of viral infection and are recruited to the site of infection. NK cells contain viral replication, before the adaptive immune response kicks in, by killing virally-infected cells (39). To evade recognition and killing by $CD8^+$ cells, MCMV blocks MHC class I expression on the infected cell. But, reduction in MHC class I expression makes the cell susceptible to lysis by NK cell. To avoid NK cell mediated lysis, MCMV encodes MHC class I like proteins m157 and m144, which engage Ly49 receptors, the counterparts of human KIRs in mouse, to transmit inhibitory signals and thereby block NK cytolytic activity. Mouse NK cells counter this virus strategy by expressing an activating form of Ly49 receptor, Ly49H that couples with signaling adaptor protein DAP12 to transmit activating signals (39, 40). $Ly49H^+$ NK cells are selectively expanded in the later phase of MCMV infection. This strategy and counterstrategy by the virus and NK cells underscore the selective evolutionary pressure; both, MCMV and NK cells are subjected to, and also highlight the critical role played by NK cells in the defense against MCMV (40). The importance of NK cells in anti-viral defense is also apparent from the viral strategy of reducing expression of ligands for NK activating receptors on the infected cell. Expression of ligands for the mouse NKG2D receptor, retinoic acid early inducible-1 (Rae-1) family of proteins, H60, and murine ULBP-like transcript 1 (MULT1), is down-regulated by MCMV encoded proteins (40).

The essentiality of NK cells in anti-viral defense in humans was first brought to light by the susceptibility to herpesviruses observed in a patient, who had normal T and B cells, but was devoid of any NK cells. This patient suffered from varicella zoster virus

(VZV), human cytomegalovirus (HCMV) and herpes simplex virus (HSV) infections (41). Like MCMV, HCMV employs similar survival strategies such as down-regulation of expression of human NKG2D receptor ligands, whose expression tend to be up-regulated on stressed cells. Unlike in mouse NK cells, a receptor that recognizes HCMV encoded ligand has not been identified in human NK cells. However, CD94-NKG2C, a heterodimeric receptor, which usually recognizes endogenous ligand HLA-E and transmits activating signals, is the likely candidate as CD94-NKG2C^{+} NK cells are preferentially expanded in HCMV infected patients. Viral ligands are also recognized by other NK cell receptors such as NCRs. NCRs, NKp46 and NKp44, bind viral hemagglutinins, while NKp30 binds HCMV encoded pp65 (40). Other than containing HCMV, VZV and HSV infections, NK cells have also been suggested to play a role in controlling infections by Epstein-Barr virus, human papilloma virus and human immunodeficiency virus due to the low NK cell activity observed in individuals susceptible to infections by these viruses (38, 42).

1.1.3.3 NK Cells As Regulators of Innate And Adaptive Immunity

NK cell interactions with cells of innate and adaptive immune systems shape immune responses. NK cells secreted cytokines and direct cell-to-cell contact play a major role in the regulation of innate and adaptive immunity. Interactions of NK cells with dendritic cell (DC) are mutually beneficial. NK cells come in contact with DCs at the site of inflammation and in secondary lymphoid tissues. At the site of inflammation DCs are activated through their contact with microbes and microbial-infected cells. Activated DCs through cytokine secretion and direct contact with NK cells induce cytolytic capability, cytokine secretion, particularly IFN-γ and proliferation in NK cells. NK cells in return help DCs mature through IFN-γ and TNF-α secretion and also

through NK-DC contact in which NKp30 plays a critical role. Expression of antigen presenting molecules, MHC class I and class II, and co-stimulatory signals such as CD86 is up-regulated on mature DCs (43). NK cells also influence antigen presentation by DCs. Cell debris generated by NK mediated lysis of malignant and virally infected cells is internalized by DCs and then processed and presented on the cell surface bound to MHC molecules. Mature DCs migrate to secondary lymphoid organs such as lymph nodes where they present antigens to T cells leading to their activation. Thus, NK cell mediated maturation of DCs and NK cell influenced antigen presentation by DCs indirectly influence maturation and activation of T cells (8). In lymph nodes, mature DCs come in contact with NK cells in the parafollicular region of the T cell area. Here too, DC secreted cytokines induce proliferation, cytolytic capability and cytokine secretion in NK cells. Due to close proximity of DCs, NK and T cells in lymph nodes, the cells influence each other's activation. DC induced secretion of IFN-γ by NK cells help $CD4^+$ cells acquire Th1 phenotype, while DC activated secretion of IL-2 by $CD4^+$ cells stimulate proliferation and cytolytic capability of NK cells (44, 45). NK cells are also known to activate IgM and IgG synthesis in B cells and activated B cells have been shown to enhance IFN- γ secretion by NK cells (46).

Along with promoting maturation and activation of innate and adaptive immune cells, NK cells can regulate immune responses by secreting immune suppressive cytokine IL-10 and by killing immature DCs and MHC deficient $CD4^+$, $CD8^+$ and B cells (8, 47). In turn, T regulatory cells are known to inhibit NK cell cytolytic activity through TGF-β mediated mechanism (48). Thus, NK cells acts as a bridge between innate and adaptive immunity through their contact with various cell types and have both positive and negative influence on immune responses. In return, NK cell mediated immune response is also shaped by other immune cells.

1.1.3.4 Role of NK Cells In Pregnancy

NK cells are the predominant leukocytes found at the maternal-fetus interface. NK cells comprise >70% of the leukocytes in the decidua. The cytokines and chemokines secreted by these decidual NK cells (dNK) have been suggested to play a role in successful trophoblast invasion. Decidual NK cell secreted vascular endothelial growth factors promote angiogenesis of the placenta. Due to the association of low number of dNK cells with spontaneous abortion, immunologic tolerance of the fetus has also been attributed to dNK cells. Along with protecting the fetus from immune assault, NK cells are also deemed to protect the fetus from the transmission of pathogens from mother such as HCMV (49).

1.1.3.5 NK Cell Memory

Immune response mediated by clonally expanded cells armed with the same receptor that recognizes an identical antigen and the ability to mount a rapid and robust response upon exposure to the same antigen in the future due to the formation of long lasting memory cells during the initial exposure are the distinguishing characteristics of the adaptive immune system. The other defining feature of adaptive immune system is the mode of generating antigen receptor diversity. In T and B cells, recombination activating genes (RAGs) encoded enzymes mediated somatic non-homologous recombination and rearrangement of variable (V), diversity (D) and joining (J) segments of T and B cell receptor genetic locus generate the immense antigen receptor diversity. NK cell shares some features with T and B cells such as differentiation from common lymphoid progenitor and like T cell it is a cytotoxic effector lymphocyte. While NK cells express a large repertoire of receptors with distinct antigen specificity, this receptor diversity emanates from germ-line encoded receptors, which

do not undergo somatic recombination and rearrangement. Due to the lack of RAG mediated recombination and the ability of NK cells to kill malignant and virally infected cells without prior sensitization to the antigen, NK cells are categorized as innate lymphocytes (50). However, recently NK cells have been shown to possess the distinctive attribute of the adaptive immune system, the formation of immunological memory. The initial evidence supporting memory NK cell formation was acquired in T and B cell lacking mice showing hapten induced contact hypersensitivity (51). A hapten is a small protein modifying chemical. Haptenated proteins elicit immune response. Initial sensitization with a hapten leads to generation of long living memory T and B cells, which mount a more potent response upon future challenge with the same hapten. Microscopic analysis of leukocyte accumulation at the hapten challenged tissue and NK cell depletion experiments indicated that in T and B cell lacking hapten sensitized mice, contact hypersensitivity to the subsequent exposure to the same hapten was mediated by NK cells (51). A recall response observed in these mice four weeks after initial sensitization demonstrated existence of long lasting memory NK cells. Contact hypersensitivity observed in naive mice adoptively transferred with NK cells from sensitized mice further confirmed the identity of these memory cells as NK cells. The researchers further showed that memory NK cells reside in the liver (51). Following this initial study, existence of memory NK cells was reported in MCMV infected mice (52). Like clonal T cell expansion, NK cells expressing Ly49H receptor, which recognizes MCMV encoded protein m157, are selectively expanded upon MCMV infection (53), but it was not known whether these cells bear any antigenic memory. Sun et al. (52) showed that $Ly49H^{+}$ cells linger for a long time after infection and are far more effective than naïve cells in rendering protection upon adoptive transfer. Also, the

recall response was antigen specific. Thus, along with the attributes of an innate effector lymphocyte, NK cells also have the characteristics of adaptive immunity.

1.1.4 Cytokine Signaling in NK Cells

Orchestrating the immune response is a complex task as it involves regulation and coordination of multiple cellular processes such as differentiation, proliferation, migration, activation and suppression in multiple cell types belonging to both, innate and adaptive, arms of the immune system. This task is accomplished by cytokine-mediated signaling. Cytokines are small extracellular proteins secreted by immune and non-immune cells. It is a large group of proteins with diverse structures and functions. NK cell development, homeostasis, and NK cell functions are regulated by cytokines secreted by immune and non-immune cells.

1.1.4.1 Cytokines Involved in NK Development

Many different cytokines such as Flt3 ligand (Flt3L), stem cell factor (SCF), IL-12 and common cytokine receptor γ chain family of cytokines (IL-2, IL-4, IL-7, IL-15, and IL-21) are known to play a role at different stages of NK differentiation process (19, 54). Few of these cytokines, IL-2 and IL-15, have been shown to induce NK differentiation from HSCs *in vitro* in the absence of other cytokines, while the rest of the cytokines work in combination with IL-2 or IL-15 (19). NK cell generation, homeostasis and development is known to be IL-15 dependent as has been demonstrated by reduced NK cell numbers and impaired NK cell cytotoxicity in mice lacking IL-15 or IL-15 receptor α chain (54-56). IL-15 responsiveness acquired during the NK precursor stage is considered a major step towards NK lineage commitment (19). IL-2 has also been reported to play a role in NK cell differentiation and activation (57), but the number of

mature NK cells is not affected in IL-2 deficient mice and humans, thereby ruling out any major role of IL-2 in NK cell development *in vivo* (54, 58). Even though, IL-4 and IL-7 have been suggested to have a role in terminal differentiation and homeostasis of NK cells, studies in mice lacking IL-4 and IL-7, show that these cytokines are dispensable in NK cell generation and maturity (54). IL-21 has been demonstrated to play a synergizing role with IL-15 and Flt3L (59) and IL-15, Flt3L and SCF (60) in *in vitro* differentiation of NK cells from human HSCs. The evidence implicating IL-21 in human NK cell development *in vivo* is provided by NK cells from human subjects with loss of function mutations in IL-21 receptor gene (61). These patients had normal number of NK cells, but the IL-21 non-responsive NK cells showed diminished basal cytotoxicity, suggesting a flaw in functional maturation (61). In mouse too, IL-21, in combination with IL-2 or IL-15, has been shown to help in the functional maturation of NK cells *in vitro* and promote functional maturation *in vivo* (62). But this evidence in support of the role of IL-21 in NK differentiation is contrary to the finding of normal number of mature, functionally valid NK cells in IL-21 receptor knockout mice (63). This finding in mice is also contrary to the impaired NK cytotoxicity observed in IL-21 receptor deficient humans (61). The dissimilarity underscores the difference that may exist in the NK developmental process between mouse and human and serves as a caution while applying lessons learned from mouse to humans.

1.1.4.2 Cytokines Involved in NK Homeostasis

The homeostatic NK cell survival and proliferation are dependent on IL-15 signaling in mice (64-66), but are independent of IL-15 in humans (67), again highlighting the difference in the physiology of NK cells between the two. However, NK cell proliferation in response to viral infections in both, mice and humans has been

shown to be dependent on IL-15 signaling (68, 69). IL-12 (70, 71) and IL-18 (70) have also been demonstrated to play a role in NK proliferation in response to MCMV infection in mice. *In vitro* studies have demonstrated that cytokines, which have not yet been shown to play a physiological role in NK cell proliferation *in vivo*, could also induce proliferation of NK cells. One of the potent activators of NK cell proliferation is IL-2 (72, 73). It is used to expand NK cells *ex vivo* for immunotherapy in cancer (74, 75). Some cytokines do not induce proliferation acting alone, but in combination with other cytokines they do. For instance, IL-10, IL-12 and IL-18 stimulate proliferation of mice NK cells pre-activated with IL-2 (76). IL-18 also induces proliferation of mice NK cell in combination with other cytokines such as IL-12 and IL-15 (77-79). IL-21 too does not induce NK cell proliferation by itself, but does in combination with IL-2 in human NK cells (80). IL-21 also supports NK cell survival and enhances it further in combination with IL-2 (81).

1.1.4.3 Cytokines Involved in NK Functions

Unlike cytolytic T cells, NK cells do not need prior sensitization with target antigen before killing a target cell. Upon detecting stress signals through interactions of NK activating receptors with their cognate ligands on target cells, NK cells deliver their cytolytic function. This ready to kill approach makes it necessary for NK cells to be equipped with the detection tool, the activating receptors, and the effector tool, lytic granules containing perforin and granzymes, while on immunosurvelliance duty. Surface expression of activating receptors and pre-formation of lytic granules occur as a result of priming of NK cells by cytokines present in their microenvironment (8). Type 1 interferons (IFNα and IFNβ), common γ-chain cytokines (IL-2, IL-15 and IL-21), IL-6, IL-12 and IL-18 are known to activate cytolytic capability of NK cells (82). These

cytokines induce expression of lytic effector proteins such as perforin (IFNα, IL-2, IL-6, IL-12, IL-15 and IL-21) and granzymes (Type I IFNs, IL-2, IL-15, IL-21) (81, 83-86). Along with the induction of expression of lytic molecules, cytokines also stimulate expression of NK receptors, whose interactions with their cognate ligands on the target cells are required for the degranulation of the lytic proteins. NKG2D expression on NK cells is up-regulated in response to IFN-α, IL-2, IL-15, IL-12 and IL-21 (81, 87-90). NCRs' expression is induced by IL-2, IL-15, IL-12 and IL-21 (82), while DNAM-1 and 2B4 expression is modulated by IL-2 and IL-21, respectively (91, 92).

NK cells also effect their functions through cytokine secretion. NK cells are one of the primary producers of IFN-γ, which is a modulator of innate and adaptive immunity. IFN-γ shapes innate immunity through activation of macrophage killing ability. IFN-γ influences adaptive immunity by stimulating antigen presentation through induction of expression of MHC class I and II proteins in antigen presenting cells and by promoting acquisition of Th1 phenotype by $CD4^+$ T cells (4). It also exerts direct effect on virally-infected and malignant cells by inhibiting their proliferation and increasing their sensitivity to apoptosis (6). The major inducer of IFN- γ generation in NK cell is IL-12, secreted by activated DCs and macrophages (44, 93). Other cytokines such as IL-2, IL-15, IL-18 and IL-21 also stimulate expression of IFN- γ in NK cells (4).

The recently discovered feature of memory formation in NK cell has been shown to be regulated by cytokines. Adoptively transferred mouse NK cells, pre-stimulated with IL-12 in combination with IL-18 and IL-15, have been shown to retain priming memory. Stimulation with the same cytokines three weeks after initial stimulation elicits amplified IFN- γ secretion by these cells (94). A similar recall response has also been demonstrated in human NK cells (95). Viral antigen-specific memory in mouse NK cells is also dependent on IL-12 signaling (96).

Some of the cytokines, which play a critical role in the regulation of NK cell functions, are depicted in Figure 1.

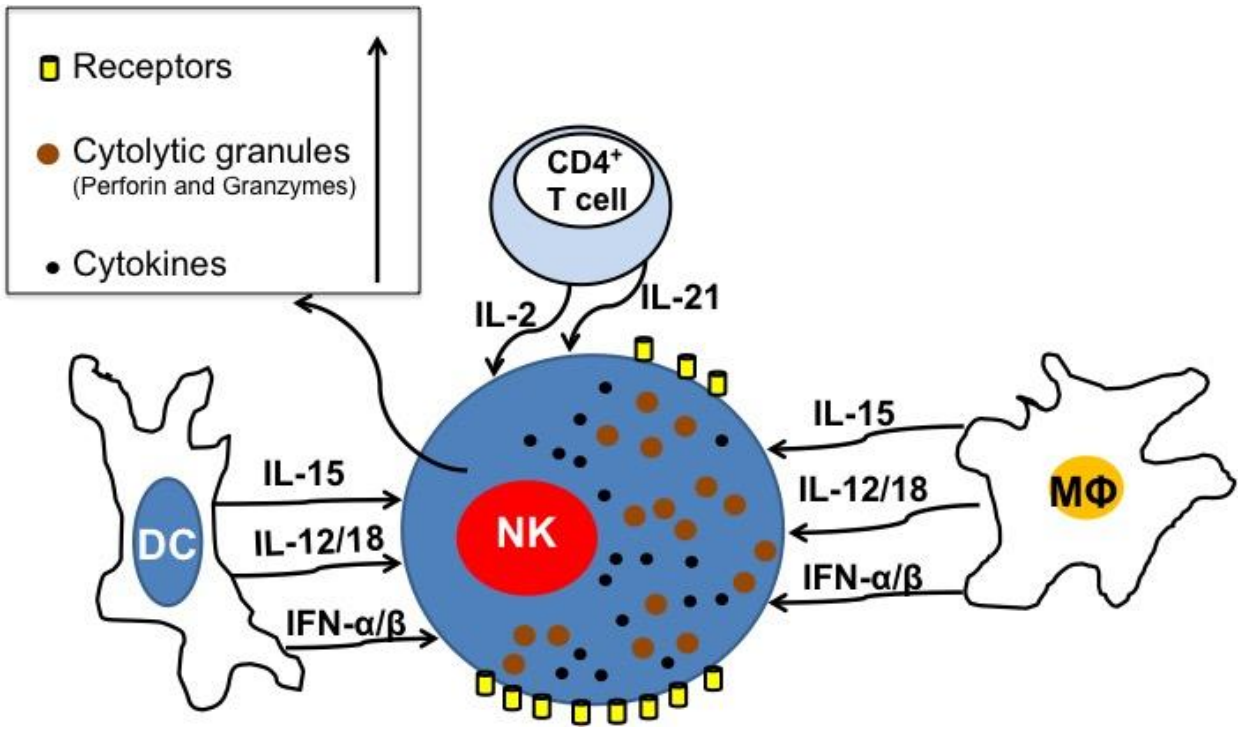


Figure 1. Regulation of NK cell functions by cytokines. IL-2 and IL-21 secreted by $CD4^+$ helper T cells and dendritic cell (DC) and macrophage (MΦ) derived IL-15, IL-12, IL-18 and IFN-α/β prime NK cells for their functions by inducing expression of NK receptors, perforin, granzymes and cytokines.

1.2 JAK-STAT SIGNALING

Cytokines mediate their signals by binding and activating their specific trans-membrane receptors on the cell surface and thereby triggering a cascade of molecular events inside the cell. Down-stream of an activated cell surface receptor, signals are relayed in a sequential pathway through a chain of effector molecules. One such pathway is Janus kinase (JAK)-signal transducer and activator of transcription (STAT), which is shared by many cytokines with different structures, receptors and functions to mediate signals in multiple types of immune and non-immune cells. Cytokines transmitting signals through receptors belonging to type I and II cytokine receptor superfamily primarily mediate their signals through JAK-STAT pathway. This is a large group of cytokines, which includes interferons, most of the interleukins and colony stimulating factors. Type 1 (IFNα and IFNβ) and 2 (IFN-γ) interferons, common γ-chain cytokines (IL-2, IL-4, IL-7, IL-15 and IL-21), IL-6, IL-10, IL-12 and IL-18, the cytokines with indispensible roles in NK cell development, homeostasis, activation and function, mediate their signals pre-dominantly through JAK-STAT signaling system (97).

The JAKs are a family of tyrosine kinases constitutively associated with cytoplasmic regions of the cytokine receptors. The family is comprised of four members, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). Cytokine-bound activated receptors activate JAKs' kinase activity and activated JAKs in turn phosphorylate specific tyrosine residues in the cytoplasmic domain of the cytokine receptors. These phosphorylated tyrosine residues provide docking sites for latent cytoplasmic transcription factors STATs through the interaction of their Src homology 2 (SH2) domains. Conserved tyrosine residues on receptor-bound STATs are phosphorylated by JAKs. STAT molecules dimerize by reciprocal interactions of their SH2 domains and

phosphotyrosines. The dimerized STATs translocate to the nucleus, where they bind to specific DNA elements to regulate transcription of target genes. There are seven members in the STAT family, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (98). All STATs share a domain architecture comprising of amino terminal (N-terminal), coiled-coil, DNA binding, linker, SH2 and transactivation domains from amino terminus to carboxy terminus (99).

1.2.1 Regulation of JAK-STAT Signaling

Regulation of JAK-STAT pathway occurs at multiple levels and by multiple mechanisms. JAKs are inhibited by suppressor of cytokine signaling (SOCS) family of proteins. Different SOCS use different mechanisms to block JAK activation. SOCS1 through its SH2 domain binds directly to the phosphorylated tyrosine on the JAKs and thus directly inhibits the kinase, while SOCS3 inhibits JAKs indirectly by binding to the activated receptor. Transcription of SOCS is up-regulated by JAK-STAT signaling thereby providing a negative feedback loop to regulate its own signaling. Dephosphorylation of tyrosines on JAKs by protein tyrosine phosphatases (PTPs) such as SH2 domain containing PTPs SHP-1 and SHP-2 also terminates JAK activation. JAK-STAT signaling is also regulated through degradation of JAKs via ubiquitin-proteasome pathway. Like JAKs, activated STATs are also dephosphorylated by PTPs such as SHP2 and T cell PTP (TCPTP). STATs are deactivated through dephosphorylation of tyrosine both in the cytoplasm and in the nucleus by SHP2, while TCPTP activity is restricted in the nucleus. Activated STATs are also disabled by protein inhibitors of activated STAT (PIAS) family of proteins, which bind to dimerized STATs in the nucleus (98). STAT family members can also regulate each other's activation. STAT1 and STAT3 have reciprocal inhibitory effects either due to

competition for the same docking sites on the cytokine receptor and/or sequestration of each other due to STAT1:STAT3 heterodimerization. Deficiency in one of these STATs enhances temporal activation and signaling response by the other (100). STATs are also known to activate their own transcription as is evident from the increased STAT1 protein expression in cells stimulated with STAT1 activating cytokines. Thus, there is a positive feedback mechanism that enhances signaling by a particular STAT (101).

1.2.2 Functions of JAK-STAT Signaling

Immune disorders and disease phenotypes reported in patients carrying mutations in the individual components of JAK-STAT signaling pathway brought to light the central role played by JAK-STAT signaling in the differentiation and development of immune cells, homeostasis, and co-ordination of immune response. Loss of function and hypomorphic JAK3 mutations found in severe combined immune deficiency (SCID) patients, characterized by extreme susceptibility to microbial infections due to lack of or diminished number and function of T and NK cells, established role of JAK3 signaling downstream of common γ-chain cytokines in lymphocyte development (97, 102). Susceptibility to mycobacterial, salmonella, Staphylococcal and viral infections and unresponsive cells to IFN-α, IFN-β and IL-12 stimulation observed in patients with TYK2 loss of function mutations were instrumental in elucidating the function of TYK2 signaling in anti-bacterial and anti-viral defense of above mentioned cytokines (102). A similar bacterial and viral infection and cell signaling phenotype detected in patients carrying loss of function and hypomorphic STAT1 mutations confirmed STAT1 as a signal relaying STAT downstream of TYK2 in response to IFN-α, IFN-β and IFN- γ signaling (102). Loss of function mutation in STAT2, another STAT activated by IFN-α and IFN-β signaling, results in susceptibility to vaccine-strain measles (103). Though,

most of the JAK-STAT signaling family members have roles restricted to the immune system, some components such as STAT5 have non-immune functions along with a role in the immune system. Along with activation, downstream of common γ chain cytokines (IL-2, IL-7, IL-9, IL-15) (104), STAT5 is also activated in response to growth hormone receptor stimulation (97). As a result, patients carrying loss of function STAT5b mutations have non-immunological phenotypes such as growth retardation. These patients also display complex immune deficiency symptoms. They are vulnerable to microbial infections, a sign of immune deficiency, and are also prone to autoimmune disorders such as autoimmune thyroiditis, idiopathic thrombocytopenic purpura, lymphocytic interstitial pneumonitis, and severe eczema. This seemingly contradictory immune phenotype is explained by the role played by STAT5b signaling in the generation of T regulatory (Treg) cells as well as in T cell homeostasis. The requirement of STAT5b signaling downstream of IL-2 activation for induction of Foxp3, a transcription factor necessary for Treg differentiation, underlies the low number of Treg cells, accounting for autoimmune disorders observed in STAT5b deficient patients. STAT5b signaling is also required for T cell ($CD4^+$ and $CD8^+$) homeostasis and as a result STAT5b deficient patients suffer from T cell lymphopenia, which accounts for their susceptibility to microbial infections (102).

Another constituent of JAK-STAT pathway with immune and non-immune functions is STAT3. Perhaps, STAT3 is the member of the JAK-STAT family with the most diverse functions that best exemplifies the reach and indispensability of JAK-STAT pathway in systemic cell signaling.

1.2.3 STAT3

STAT3 was discovered as an activator of acute phase gene expression in hepatocytes in response to IL-6 stimulation and hence was initially named as acute phase response factor. Since its discovery, numerous *in vitro* and *in vivo* studies have revealed STAT3 as a transcription factor relaying signals downstream of diverse types of cellular signals in multitude of tissues (105). The functional diversity is a result of STAT3's ability to be activated by different signaling mechanisms. STAT3 is activated not only by JAKs activated by cytokines, but also by receptor tyrosine kinases activated by growth factors (Epidermal and platelet-derived growth factor) and src tyrosine kinases (Src, Lck, Hck, Lyn, Fyn, and Fgr) either independently or downstream of activated receptor tyrosine kinases and G-protein coupled receptors (106). Despite its response to varied stimuli and signaling pathways, mode of STAT3 activation remains the same. The canonical STAT3 activation mechanism involves phosphorylation of tyrosine (Y) residue located at the interface of SH-2 and transactivation domains (Y705 in human STAT3, which has 770 amino acids) by a tyrosine kinase (107, 108). In addition to the canonical pathway, STAT3 is activated by other mechanisms. Phosphorylation of a serine (S) residue located in the transactivation domain (S727 in human STAT3) unleashes maximal transcription activation potential of STAT3 (106). The serine can be phosphorylated by kinases such as ERK, CDK1, ATM and ATR independent of signal promoting tyrosine phosphorylation (109). Unphosphorylated STAT3 has been shown to form homodimers and translocate to the nucleus and regulates gene transcription either by itself or by interacting with other transcription factors such as NFκB (101). Along with homodimerized form, STAT3 regulates gene expression by forming heterodimers with STAT1 and STAT5 (110). Extranuclear

function of STAT3 has been reported recently, wherein STAT3 located in mitochondria was shown to be required for activation of electron transport chain and this function was dependent upon phosphorylation of S727 (111). Even though STAT3 can be activated in various ways and has a function outside of its role as a transcription activator, STAT3 pre-dominantly delivers its function as a transcription factor upon phosphorylation of its tyrosine residue (107).

1.2.3.1 Functions of STAT3

STAT3 regulates expression of genes involved in fundamental cellular processes such as cell cycle, apoptosis, cell signaling, transcription, angiogenesis, lipid metabolism, remodeling of extracellular matrix and cell migration (112). STAT3 also plays a role in self-renewal of embryonic stem cells by blocking differentiation (105). Given STAT3's pleiotropic role, it is not surprising that systemic deletion of STAT3 results in embryonic lethality (113). Due to this limitation, researchers generated tissue specific deletion of STAT3 in mouse, which has been instrumental in our understanding of physiological role of STAT3. STAT3 has non-redundant functions in multiple organs ranging from skin to nervous system. In skin, STAT3 is required for wound healing and keratinocyte migration, while in thymic and memory epithelium and in sensory and motor neurons STAT3 is required for pro-survival signals. In liver, STAT3-activated gene expression promotes acute phase response (107).

1.2.3.2 Role of STAT3 in Cancer

STAT3 is activated by mitogenic signals such as cytokines and growth factors and activates expression of genes promoting proliferation, survival, migration and angiogenesis; the processes when out of control give rise to cancers. Constitutive or

elevated activity and/or expression of STAT3 are associated with various types of cancers including leukemia, lymphomas, multiple myeloma, melanoma and cancers in breast, lung, liver, pancreas, prostate, ovary, and head and neck. In many of these cancers high STAT3 activity and expression are markers of poor prognosis (106). Oncogenic STAT3 activity could be a result of one or a combination of multiple processes. It could be secondary to deregulated upstream signaling pathway triggered by cytokines, growth factors and oncogenic Srcs. Loss of regulation in STAT3 signaling through repression or silencing of inhibitors of STAT3 signaling such as SOCS3, PTPs and PIAS3 have been shown to be responsible for over active STAT3. STAT3 by up-regulating expression of itself and by activating expression of STAT3 activating cytokines such as IL-6 and IL-10 promotes its overexpression and sustained activation through positive feedback. Somatic mutations leading to constitutively active STAT3 have also been reported in some cancers (108). STAT3 also drives oncogenesis by promoting inflammation in the tumor microenvironment. Persistent inflammation has been shown to be a cause of certain cancers such as gastric and liver and aids oncogenesis in others (114). STAT3 regulated IL-6 is one of the main drivers of inflammation in tumor microenvironment. IL-6, a pro-inflammatory cytokine not only mediates inflammatory conditions in the malignant cells, but also in the stromal and immune cells in the tumor microenvironment. IL-6 activates STAT3 in these cells, which activates expression of genes responsible for inflammation in a positive feedback loop (114). STAT3 also activates expression of IL-10, an immune suppressive cytokine. IL-10 secreted by cancer cells and immune cells suppress anti-cancer response of immune cells in the tumor microenvironment. IL-10 also activates STAT3 thereby propagating immune suppression in a positive feedback loop (115).

1.2.3.3 Role of STAT3 in Immune System

STAT3 mediates signals down-stream of cytokines belonging to different families such as common γ-chain cytokines (IL-2, IL-7, IL-9, IL-15 and IL-21), gp130 receptor utilizing cytokines (IL-6, IL-11, IL-27, IL-31, oncostatin M, leukemia inhibitory factor), Type I IFNs (IFNα and β), Type II IFN (IFNγ), IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26), IL-12, IL-23, Flt3 ligand and colony stimulating factors (G-CSF and M-CSF). These cytokines play an important role in the differentiation, proliferation, survival and modulation of activation of myeloid and lymphoid cells (116). Like tissue specific deletion in non-immune tissues, STAT3 deletion in hematopoietic cells and specific types of immune cells in mouse have brought to light the critical role played by STAT3 signaling in immune function.

T cell specific deletion of STAT3 revealed its requirement in IL-6 and IL-2 induced proliferation of T cells. In response to IL-2, STAT3 induces expression of CD25, the private IL-2 receptor, which is required for high affinity binding of IL-2 (107). STAT3 deficiency in $CD4^+$ T cells impairs generation of Th17 cells. Th17 cells mediate anti-microbial immunity, mostly at the epithelial barriers, by secreting pro-inflammatory cytokines IL-17A, IL-17F, IL-21 and IL-22. These cytokines help recruit and activate neutrophils at the site of infection and also induce secretion of anti-microbial peptides from epithelial cells (116). Cytokines involved in Th17 differentiation, IL-6, IL-21 and IL-23 mediate their signal through STAT3, which activates transcription of retinoic acid-related orphan nuclear receptors (RORs) RORγt and RORα, the transcription factors that drive the genetic program for Th17 differentiation. The cytokines secreted by Th17 cells, IL-17A, IL-17F and IL-21 are also STAT3 targets (117, 118). STAT3 deficiency in $CD4^+$ helper cells also impairs their ability to secrete IL-10 in response to IL-6 and IL-

27. Pro-inflammatory cytokines such IL-6 and IL-27 along with the induction of inflammation mediators also stimulate secretion of immune-suppressive cytokine IL-10 from helper T cells, which prevents a protective inflammatory response from becoming a pathological one (119). Thus, in helper T cells, STAT3 is required for initiating an inflammatory response as well as for its regulation. $CD8^+$ T cell specific STAT3 deletion helped elucidate role of STAT3 in memory T cell formation. Cytokines IL-10 and IL-21 through STAT3 activation trigger the genetic program required for memory T cell differentiation (120). B cell specific STAT3 deletion impairs T cell dependent differentiation of B cells into plasma cells (121).

Along with cells of lymphoid origin, STAT3 signaling is also critical for development and immune response of myeloid cells. STAT3 deficient macrophages and neutrophils lose their ability to respond to IL-10, which signals through STAT3 to rein in inflammation. Loss of STAT3 in these cells results in excessive secretion of pro-inflammatory cytokines such TNF-α, lL-6 and IL-1, leading to inflammatory disorders (122). Employing bone marrow specific deletion, STAT3 has been shown to be required for neutrophil mobilization in response to G-CSF and chemokines (123). Hematopoietic cell specific STAT3 deletion results in decreased number of dendritic cells (DCs). Flt3 ligand, which mediates its signals through STAT3 activation, is a major regulator of DC development and as a result STAT3 deficiency leads to impaired development of DCs (124).

Most of the knowledge about the role of STAT3 in immune function has been obtained from mouse experiments. Even though mouse is a preferred animal model due to the ease of handling, genetic maneuverability, and mainly because of the many similarities between mouse and human physiology, not everything learned from a mouse model can be extrapolated to humans due to the differences that exist between

these two species. Monogenic mutations, nature's own experiment, found in humans offer an opportunity to study role of the protein coded by that gene in human physiology. STAT3 gain of function germline mutations were recently discovered in humans, which caused multiorgan autoimmunity and lymphoproliferation and in one patient, susceptibility to mycobacterial infections (125, 126). Even though all pathogenic mechanisms are not yet known, few insights into immune dysregulation caused by hypermorphic STAT3 have been obtained. The autoimmunity is due to the low number of Treg cells in these human subjects (125, 126) and Treg deficiency is likely caused by suppressed STAT5 activation in these patients (125). Suppression of STAT1 activation was also reported in these patients, but its implications, if any, are not known (125). Even though any mutation that affects normal function of the protein provides important information about the physiological role of that protein, more telling are the loss of function or hypomorphic mutations, which clearly highlight the necessity of the protein.

There are naturally occurring, albeit rare, heterozygous dominant negative STAT3 mutations in humans, which cause an immunodeficiency called Job syndrome.

1.3 JOB SYNDROME

Job syndrome is a rare immune disorder, initially characterized by the triad of eczema and recurrent skin and lung infections (127). As boils due to recurrent skin infections is a cardinal feature of this immunodeficiency, the immune disorder was named after the biblical character Job, whose body was covered with boils. Later investigation also identified increased serum levels of IgE as a clinical symptom, which lent hyper IgE syndrome as an alternative name to the disorder (128). The skin and lung infections are primarily caused by *Staphylococcus aureus.* Lungs are also infected

with *Streptococcus pneumoniae* and *Haemophilus* species, albeit less frequently. Hard to treat secondary *Pseudomonas aeruginosa* and mycobacterial infections further exacerbate poor pulmonary conditions and are the main cause of mortality. Fungal infections such as mucocutaneous candidiasis and Aspergillus are also common (129). Job syndrome is also characterized by various non-immunological features such as facial asymmetry, scoliosis, osteopenia, minor trauma fractures, hyperextensibility, retention of primary teeth and aneurysm (129-131).

In 2007, two groups independently discovered dominant negative STAT3 mutations as the causative factor for the Job syndrome (130, 131). The mutations are found to be sporadic as well as familial and are mostly restricted to the DNA binding and SH2 domains (132). Investigations into the pathogenesis of immunodeficiency of Job syndrome patients have delineated the role of STAT3 signaling in the generation and function of immune cells of lymphoid and myeloid origin (133). Cells from Job syndrome patients serve as a naturally occurring STAT3 deficient genetic model, which has helped decipher role of STAT3 in human physiology.

1.4 GENESIS OF HYPOTHESIS AND SPECIFIC AIMS

NK cells play a crucial role in immune response to virally infected and malignant cells. Cytolytic activity of NK cells is a result of the balance between the activation of inhibitory and activating receptors expressed on NK cell surface. The innate ability of NK cells to use these receptors to recognize and destroy malignant cells has made them excellent candidate immune cells to be used in adoptive immunotherapy against cancer. Due to the limited number of NK cells in the peripheral blood, *ex vivo* expansion and activation of autologous or allogeneic NK cells before their transfer into the patient forms the basis of NK cell adoptive immunotherapy.

Research efforts in our lab are focused on developing NK cell adoptive immunotherapies against cancers. Activation and expansion of patient- or donor-derived NK cells under laboratory conditions to a sufficiently large number is a pre-requisite for the success of such a therapeutic approach. Understanding the signaling mechanisms involved in NK cell proliferation, survival and activation is a key to optimizing the *ex vivo* culture conditions of NK cells, which will translate to improved clinical applications of NK cell immunotherapy.

We previously described robust *ex vivo* expansion and activation of human NK cells by co-cultivation with K562 (erythroleukemic cell line) based artificial antigen presenting cells (aAPC) genetically modified to express membrane-bound IL21 (mbIL21). We found that mbIL21 results in greater proliferation, longer telomere length, and less senescence than NK cells expanded with aAPC expressing membrane-bound IL15 (mbIL15) (134). Within the STAT family, IL21 activates STAT3 most efficiently (135).

Investigations in our lab also revealed that STAT3 signaling activates expression of NKG2D (136), a primary activating receptor involved in the cytolytic immune response mediated by NK cells to infections and malignant cells (12). We showed that STAT3 regulates NKG2D expression at the transcriptional level (136).

Based on these findings, **I hypothesized that STAT3 signaling is critical for human NK cell cytolytic function and proliferation.**

I proposed the following specific aims to investigate this hypothesis:

Aim #1: To evaluate the role of STAT3 signaling in cytolytic function of NK cells

Aim #2: To evaluate the role of STAT3 signaling in NK cell proliferation

NK cells from Job syndrome patients carrying dominant negative STAT3 mutations provided an opportunity to test my hypothesis in a naturally occurring genetic

model. Along with Job syndrome patients' NK cells, I also validated my hypothesis in STAT3 knock-down primary human NK cells. Along with genetic models, I also employed pharmacological approach wherein STAT3 activation was blocked using small molecule STAT3 inhibitor.

CHAPTER 2

ROLE OF STAT3 SIGNALING IN HUMAN NK CELL CYTOLYTIC FUNCTION

"This chapter is partly based on the already published results in the Journal *Blood*. Zhu S, **Phatarpekar PV***, Denman CJ, Senyukov VV, Somanchi SS, Nguyen-Jackson HT, Mace EM, Freeman AF, Watowich SS, Orange JS, Holland SM, Lee DA. Transcription of the activating receptor NKG2D in natural killer cells is regulated by STAT3 tyrosine phosphorylation. *Blood*. 2014;124(3):403-411. © the American Society of Hematology."

***Co-first Author**

Role of STAT3 Signaling In Human NK Cell Cytolytic Function

2.1 INTRODUCTION

Cytokines, the central regulators of immunity, predominantly employ Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway to mediate signals in immune cells. In canonical JAK-STAT signaling, signals are relayed from the extracellular environment to the nucleus to regulate gene expression in target cells. Upon binding of cytokines to their specific multimeric receptors, JAKs, tyrosine kinases, constitutively associated with cytoplasmic regions of cytokine receptors, are activated. Activated JAKs phosphorylate specific tyrosine residues in the cytoplasmic domain of the cytokine receptors to provide docking sites for latent cytoplasmic transcription factors STATs through the interaction of their Src homology 2 (SH2) domains. Conserved tyrosine residues on receptor bound STATs are phosphorylated by JAKs. Phosphorylated STATs dissociate from receptors and dimerize by reciprocal interactions of their SH2 domains and phosphotyrosines. dimerized STATs translocate to the nucleus and bind to specific DNA elements to regulate transcription of target genes. Four JAKS, JAK1, JAK2, JAK3 and TYK2 through seven STATS, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 mediate signals from diverse types of cytokines in multiple types of immune cells (137).

STATs are composed of multiple domains. The N-terminal domain facilitates binding of two adjacent DNA bound dimers, which stabilizes DNA-STAT interaction. The coiled-coil domain which succeeds the N-terminal domain is involved in

interactions with different proteins essential for forming transcription activation complex. C-terminus to the coiled-coil domain is the DNA binding domain and as the name indicates is at the core of the function of the protein (99). The linker domain, which bridges the DNA binding domain with the SH2 domain, is essential for the transcriptional activation (138). The SH2 domain serves two critical functions; initial binding of the latent STAT to the phosphorylated tyrosine on the cytoplasmic portion of the cytokine receptor that leads to the phosphorylation of its own tyrosine by JAKs and upon activation it enables dimer formation with other activated STAT through reciprocal interactions of SH2 domain and phosphotyrosine. The tyrosine residue, whose phosphorylation by JAKs activates the STATs, is located at the interface of SH2 domain and the transcriptional activation domain (TAD). The TAD lies at the C-terminus of the protein and as the name suggests is critical for the transcriptional activation of target genes (139).

Among STATs, STAT3 is the most pleotropic transcription factor regulating genes involved in fundamental cellular processes such as proliferation, apoptosis, differentiation and migration in multiple cell lineages downstream of a plethora of extracellular signals (107). Given its critical role in cellular processes, it is not surprising that germline STAT3 deletion is embryonically lethal in mouse (113). Targeted tissue specific deletion has revealed STAT3's role in the development and function of multiple organs as diverse as skin and brain (107). STAT3 is activated by several families of cytokines that employ JAKs to mediate their signals (116). In addition to JAKs, receptor tyrosine kinases are known to activate STAT3 downstream of some extracellular ligands (EGF, M-CSF, PDGF and Flt3 ligand) and tyrosine kinases belonging to src family also activate STAT3 either directly or downstream of receptor tyrosine kinases or G-protein coupled receptors (105, 106). As STAT3 relays proliferative and anti-

apoptotic signals initiated by different cytokines and growth factors in various cell types, dysregulation of these signaling pathways often leads to constitutive hyper-activation of STAT3 resulting in oncogenic transformation of various tissues. Increased expression and activation of STAT3 is associated with multiple types of cancers and besides mediating pro-proliferative and anti-apoptotic signals, STAT3 also promotes growth and spread of these cancers by activating expression of genes involved in angiogenesis and metastasis (106). Development of STAT3 inhibitors is a very active area of cancer therapeutics and many such drugs are at different stages of pre-clinical and clinical studies (140).

Natural defense against cancer, the Immune system, whose failure to detect and destroy transformed cells manifests as the disease, is also under the influence of STAT3 signaling. Many cytokines responsible for initiating and modulating innate and adaptive immune responses by inducing differentiation and/or regulating activation of different types of immune cells mediate their signals through STAT3. Cytokine families critical for normal immune function such as common γ-chain cytokines (IL-2, IL-7, IL-15 and IL-21), IL-6/gp130 (IL-6, IL-11, IL-27, IL-31, oncostatin M and leukemia inhibitory factor), IFNs (IFN-α, IFN-β and IFN-Y), IL-10 (IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26), IL-12, IL- 23, colony stimulating factors (G-CSF and M-CSF) and Flt3 ligand employ STAT3 down-stream of their receptors (116). These signaling molecules play an important role in the differentiation, proliferation, survival and modulation of activation of myeloid and lymphoid cells (116). Cell lineage specific STAT3 deletion in mice has revealed role of STAT3 signaling in differentiation and function of myeloid and lymphoid cells. STAT3 signaling plays a critical role in dendritic cell differentiation (124), neutrophil mobilization (123), and IL-10 mediated negative modulation of macrophage and neutrophil activity (122). In lymphoid cells, STAT3 signaling is

required for the generation and maintenance of Th17 cells, a subset of T helper cells responsible for immune response against bacteria and fungi at epithelial surfaces (117, 118) and T cell dependent differentiation of B cells into plasma cells (121).

Dominant negative mutations in STAT3 cause a sporadic or autosomal dominant primary immunodeficiency called Job syndrome or Hyper IgE syndrome (HIES) characterized by immunological features such as eczema, recurrent bacterial and fungal skin and lung infections and elevated serum IgE as well as non-immunological manifestations such as facial asymmetry, scoliosis, osteopenia, minor trauma fractures, hyperextensibility, retention of primary teeth and aneurysms (129-131). Majority of the STAT3 mutations found in Job syndrome are one amino acid change causing missense mutations, while few have been found to cause in frame deletion of single amino acid (129, 132, 141). The mutations are mostly restricted to the DNA binding and SH2 domains, with few in the transcriptional activation domain (142-146). A single mutation each has been reported in the coiled-coil (145) and linker domain (144). Though most of the mutations are in the exons, few intronic mutations causing exon-skipping leading to short in frame deletions have also been reported (142, 145, 146). Despite the large heterogeneity and their locations in different domains, the mutations have been shown to act in a dominant negative manner and give rise to the same immunological phenotype (142, 144, 146). The dominant negative mutations do not totally abolish STAT3 DNA binding activity in Job syndrome patients. As DNA binding and transcriptional activation is mediated by STAT3 dimers, the normal STAT3 allele enables dimer formation between wild type STAT3 and retains residual STAT3 activity, which is about 25% of the activity found in normal individuals (131).

Since the discovery of deficient STAT3 signaling as a causative factor, investigations into the pathogenesis of immunological dysfunctions in Job syndrome

patients have revealed role of STAT3 in the development and function of multiple types of immune cells. Recurrent bacterial and fungal infections in Job syndrome patients prompted analysis of Th17 cells, which were found to be absent in these patients. Cellular and molecular evaluation delineated a direct role of STAT3 in the differentiation and function of these cells (147, 148). Similarly, inquiries into the molecular mechanisms underlying defective T cell dependent B cell activation and differentiation into plasma cells in Job syndrome patients established a fundamental role of STAT3 in the generation and function of T follicular helper cells (149), while investigations into the etiology of atopic dermatitis revealed requirement of STAT3 in IL-10 directed dendritic cell induced differentiation of T regulatory cells (150). Decreased number of central memory $CD4^+$ and $CD8^+$ cells in Job syndrome patients were pivotal in deciphering the requirement of STAT3 for the differentiation and proliferation of human central memory T cells and found to be the causative factor for vulnerability of Job syndrome patients to the varicella zoster virus and Epstein-Barr virus reactivation (151). Likewise, efforts to unravel the molecular mechanisms causing deficiency of memory B cells and antigen dependent antibody production in Job syndrome patients led to the establishment of role of IL-21 induced STAT3 signaling in the differentiation of human memory B cells and antigen specific plasma cells from naïve B cells (152, 153). Along with the cells of lymphoid origin, immunological defects in Job syndrome patients also provided insights into the role of STAT3 in cells of myeloid origin. Investigations of cellular and molecular mechanisms causing atopic conditions in Job syndrome patients revealed requirement of STAT3 in IL-10 induced generation of tolerogenic dendritic cells (150) and in IgE mediated signaling in mast cells (154). Despite having reactivation of herpes virus infections and increased risk of malignancies as clinical symptoms (144, 151, 155, 156), surprisingly, the immune cell

that plays critical role in immune responses against these risks, namely the natural killer (NK) cell, hasn't received any research attention in Job syndrome patients.

NK cells, cytotoxic lymphocytes of the innate immune system, play a crucial role in immune response to viruses and tumors, destroying virally infected cells and neoplasms. NK cells detect abnormal cells through the interactions of activating receptors expressed on NK cell surface with their corresponding ligands, whose expression is generally increased on the surface of stressed cells. A large array of activating receptors such as natural cytotoxicity receptor (NCR) family (NKp30, NKp44, NKp46), NKG2D, DNAM1, 2B4, CD160, CD16, CD2, and CD94-NKG2C are expressed on NK cell surface providing broader ability to detect target cells expressing diverse types of ligands. Upon binding of activating receptors to their cognate ligands, signals are transmitted inside NK cells to trigger cytolytic activity. As some of these activating ligands are expressed by normal cells, to differentiate normal self from abnormal cells, NK cells are also equipped with cell surface inhibitory receptors such as killer cell immunoglobulin-like receptors (KIRs) and CD94-NKG2A that recognize major histo-compatibility (MHC) class I classical and non-classical ligands on cells and mediate inhibitory signals to block cytolytic activity of NK cells. Thus, the cytolytic activity of NK cells is the net result of the balance between the strengths of activating and inhibitory signals (8). NK cells use multiple ways to kill the target cells. NK cells possess specialized lysosomal organelles called lytic granules containing cytotoxic molecules. The process of releasing the lytic content in the extracellular space through lytic granule exocytosis is called degranulation. The major cytolytic proteins in the lytic granules are perforin and granzymes. NK cells also express apoptosis inducing ligands belonging to the tumor necrosis factor (TNF) family viz. FAS ligand (FASL) and TNF related apoptosis inducing ligand (TRAIL) either in a membrane bound form and/or as a

part of lytic granules. Of the multiple mechanisms at NK cell's disposal for target cell lysis, release of death inducing proteins, perforin and granzymes, into the cleft of the synapse formed between NK and target cell through the exocytosis of lytic granules, is the predominant one (6, 157). Upon release, perforin forms pores in the plasma membrane or in the endosomal membrane and facilitate entry of granzymes into the cytosol of target cells. Granzymes are serine proteases that induce apoptosis in caspase dependent and independent manner (157).

The process of directed secretion of cytotoxic molecules at the interface of NK and target cell through degranulation is a well-regulated multistep process. The process is set in motion by the initial contact between NK cell and a potential target cell, which is likely mediated through tethering receptors like CD2 and receptors belonging to selectin family such as CD62L. Lymphocyte function associated antigen 1 (LFA1; CD11:CD18) and macrophage receptor 1 (MAC1; CD11b:CD18), receptors expressed on NK cells belonging to integrin family, establish firm adhesion between NK and target cells through their interactions with intercellular adhesion molecule 1 (ICAM1) expressed on target cells (158). Activating receptors such as NCRs, NKG2D, DNAM1 also contribute to NK adhesion through their interactions with their corresponding ligands on target cells and these receptors along with LFA1 transmit activating signals inside the NK cells to initiate immune synapse formation. The activating signals trigger F-actin reorganization, which is the first critical step required for the commitment to synapse formation (159). During the contact between NK and target cell, along with activating receptors, inhibitory receptors also engage their cognate ligands on target cells and mediate inhibitory signals. Integration of activating and inhibitory signals at this stage decides the fate of the immune synapse. Strong inhibitory signal blocks actin reorganization, clustering of activating receptors and

thereby prevents further activation of NK cells and synapse maturation, which results in NK detachment from the target (158). In the absence of overriding inhibitory signals, activating signals induce formation of polymerized filamentous actin (F-actin) from monomeric globular actin and accumulation of polymerized actin at the NK cell synapse. Accumulation of F-actin at the synapse is a pre-requisite for the clustering of activating receptors. Synergistic signals from the cluster of activating receptors stimulate polarization of cytolytic granules to the synapse. For directed secretion of perforin and granzymes into the synaptic cleft, cytolytic granules dispersed throughout the cell have to be congregated near the synapse. This is achieved first by movement of granules along microtubules to converge at microtubule organizing center (MTOC), a cellular structure where microtubule formation begins and from which microtubules radiate in all directions, and then by polarization of MTOC along with lytic granules to the synapse. Once near the synapse, the granules traverse the clearings created by F-actin remodeling to reach the plasma membrane and secrete their lytic content into the synaptic cleft through exocytosis (158, 159).

Unlike cytolytic T cells, NK cells do not need prior sensitization with target antigen before killing a target cell. Upon detecting stress signals through interactions of NK activating receptors with their cognate ligands on target cells, NK cells deliver their cytolytic function. This ready to kill approach makes it necessary for NK cells to be equipped with the detection tool, the activating receptors, and the effector tool, lytic granules containing perforin and granzymes, while on immunosurvelliance duty. Surface expression of activating receptors and pre-formation of lytic granules occur as a result of priming of NK cells by cytokines present in their microenvironment (8). Type 1 interferons (IFNα and IFNβ), common γ-chain cytokines (IL-2, IL-15 and IL-21), IL-6, IL-12 and IL-18 are known to activate cytolytic capability of NK cells and that is mainly

achieved through STAT mediated signaling (82, 160). Cytokines induce perforin expression through STAT mediated signaling. IFNα and IL-6 stimulate perforin gene expression through STAT1 (83, 161), IL-2 and IL-15 through STAT5 (83, 84, 162), while IL-12 activates via STAT4 (84). Perforin gene has a STAT binding site in its enhancer region and cytokines have been shown to induce perforin expression through STAT mediated transcriptional activation (83, 84). Like perforin, granzyme B expression has also been demonstrated to be modulated through STAT signaling. During viral infection, type I IFNs stimulate granzyme B expression in NK cells via STAT1 signaling (86) and granzyme B production is significantly impaired in STAT1 deficient NK cells in response to viral infection (163). Along with perforin and granzyme, cytokines also induce expression of activating receptors in NK cells. NKG2D expression on NK cells is up-regulated in response to IFN-α, IL-2, IL-15 and IL-12 (87-90). IL-2 stimulation also increases expression of other activating receptors such as DNAM1, NKp44 and NKp30 (91). IL-21, which also modulates NK cell cytotoxicity, has been shown to up-regulate expression of activating receptors NKp30, NKp46, NKG2D and 2B4 (81, 92). Even though role of STATs in cytokine regulated expression of NK activating receptors has not been experimentally established yet, given that these cytokines predominantly signal through STAT family members (104, 116), their role in the regulation of expression of these receptors is very likely. The critical role played by STATs in cytokine mediated arming of NK cell for cytotoxicity is reflected in the impaired cytolytic activity of STAT deficient NK cells. NK cells from STAT1 deficient mouse and human had lower basal (164-166) as well as induced cytotoxicity, particularly during viral infections (68, 86, 163). Diminished basal and IL-2 and IL-15 induced cytolytic activity was also observed in NK cells from STAT5b deficient mouse (162), while IL-12 failed to augment cytotoxicity of NK cells from STAT4 deficient

mouse (167, 168). Cytokines display signaling promiscuity by activating multiple STATs. Type 1 interferons (IFNα and IFNβ), common γ-chain cytokines (IL-2, IL-15 and IL-21), IL-12, IL-18 and IL-10, cytokines known to stimulate cytolytic capability of NK cells, in addition to other STATs also activate STAT3 in NK cells (80, 136, 161, 169-173). Despite being activated by several cytokines critical for NK cytolytic function, very little research effort has gone into understanding the role of STAT3 signaling in NK cytotoxicity, particularly in human NK cells.

Beyond serving just the academic interests, investigating the role of STAT3 in NK cytolytic function has far reaching clinical applications. Constitutively active STAT3 is oncogenic (174). Constitutively active STAT3 drives development, maintenance and spread of multiple cancers by activating a transcriptional profile that promotes proliferation, survival, angiogenesis and metastasis (106). Besides malignant cell intrinsic effects, STAT3 signaling also influences tumor microenvironment. STAT3 plays a critical role in tumor microenvironment to promote tumor growth and is generally considered to be a negative regulator of immunity against tumor. Tumor secreted factors such as VEGF, IL-6 and IL-10 activate STAT3 signaling in tumor cells, which helps them escape immune surveillance and activation of STAT3 by tumor associated factors in immune cells suppresses their activation and thereby the immune response (115). Thus, STAT3 plays a crucial role at the interface of tumor biology and immunity. STAT3 is a major drug target in cancer therapeutics and multiple approaches to block STAT3 signaling are at different stages of clinical development (106, 140, 175). Equally promising therapeutic prospects against cancer are offered by NK cell immunotherapy. Equipped with cell surface receptors that detect danger signals on malignant cells and activate cytolytic activity, the inherent function of NK cells is immune- surveillance of transformed cells. This innate role of NK cells is evident due to

the fact that low NK cytotoxicity in peripheral blood is associated with higher incidence of cancer as was observed in an 11 year follow-up study (30). High levels of tumor infiltrating NK cells are associated with positive prognosis (31-33). Due to their anti-tumor activity, NK cells have emerged as candidate effector cells for immune therapy against cancer. Tumor recognition and rejection by NK cells have been demonstrated both *in vitro* and *in vivo* in mice and rats (20-22). The anti-tumor activity of several cytokines such as IL-2, IL-12, IL-18 and IL-21 is mediated by NK cells (8, 26). Adoptive transfer of activated autologous or allogeneic NK cells has proven to be a safe and potentially efficacious immunotherapy for cancer (36, 37). NK cell therapy has been or being tested against multiple types of cancers (35). In the light of the role NK cells play in controlling malignancy, it becomes critical to understand the role of STAT3, which drives malignant growth and also regulates body's immune response to it, in NK cell cytolytic function. It is all the more important when STAT3 inhibition and NK cell immunotherapy are emerging as treatment modalities for cancer.

Due to the limited number of NK cells in the peripheral blood, expanding NK cells to a sufficiently large number is imperative to the success of NK cell immunotherapy. Expanding and activating NK cells *ex vivo*, before their transfer into the patients, forms the basis of NK immunotherapy. Research efforts in our lab are focused on developing an *ex vivo* NK cell expansion platform. We previously described robust *ex vivo* expansion and activation of NK cells by co-cultivation with K562 (erythroleukemic cell line) based artificial antigen presenting cells (aAPC) genetically modified to express membrane-bound IL-21 (mbIL21) (134). Within the STAT family, IL-21 activates STAT3 most efficiently (104, 135). Intrigued by this initial finding, our further investigations revealed that STAT3 signaling activates expression of NKG2D (136), a primary activating receptor involved in the immune response mediated by NK

cells to infections and tumors (12). In this study the inquiries have been taken further to probe the role of STAT3 in NK cytolytic function. Job syndrome patients carrying dominant negative STAT3 mutations offer a naturally occurring genetic model of STAT3 deficiency. Employing NK cells from Job syndrome patients and siRNA mediated STAT3 knock-down in primary human NK cells, I studied role of STAT3 in NK cytolytic activity and the cellular processes involved in delivering the cytolytic function. The evidence presented in this study establishes a fundamental role of STAT3 in the cytotoxicity of human NK cells and the cellular processes underlying it.

2.2 MATERIAL AND METHODS

2.2.1 Cell and Cell Lines

Anonymized normal donor buffy coats were obtained from the Gulf Coast Regional Blood Center (Houston, TX) under a protocol approved by the Institutional Review Board (IRB) of UT MD Anderson Cancer Center. Peripheral blood was obtained from STAT3 genotyped Job's syndrome patients at National Institute of Allergy and Infectious Diseases and Children's Hospital of Philadelphia under protocols approved by the IRB of each respective institution. IRB approval was obtained from UT MD Anderson Cancer Center to acquire Job's syndrome samples from collaborators and to perform this research. The parental K562 cell line was obtained from the American Type Culture Collection (ATCC). This study was conducted in accordance with the Declaration of Helsinki.

2.2.2 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) from buffy coats and blood samples were obtained by buoyant density centrifugation on Ficoll-Paque following

previously described protocol (176). Briefly, buffy coats or blood samples mixed with phosphate buffer saline (PBS) were loaded on top of Ficoll-Paque in a 50 ml tube and centrifuged at 400g for 20 minutes without brake. The PBMCs settled at the interface of Ficoll-Paque and plasma were recovered and washed three times with PBS. The RBCs settled below Ficoll-Paque were used to isolate NK cells from PBMCs.

2.2.3 NK Cell Purification

NK cells were purified from PBMCs by enriching them to ≥ 90% purity ($CD3^{-}CD16/56^{+}$) with RosetteSep Human NK Cell Enrichment Cocktail (STEMCELL Technologies, Vancouver, BC, Canada) following manufacturer's instructions. In brief, PBMCs were mixed at 1:100 ratio with red blood cells (RBCs) obtained from the same sample. To the PBMCs:RBCs mixture, Human NK Cell Enrichment Cocktail was added at 1 µl/10^6 PBMCs. The solution was mixed well and incubated at room temperature for 20 minutes with gentle mixing every five minutes. After adding PBS with 2% fetal bovine serum (FBS) to the PBMCs:RBCs mix, the solution was layered on top of Ficoll-Paque and centrifuged at 400g for 20 minutes. The NK cells were recovered from top of the Ficoll-Paque layer.

2.2.4 Cell Culture Media

The NK and K562 cells were cultured in RPMI 1640 (Cellgro/Mediatech, Manassas, VA) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM l-glutamine (Gibco/Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin (Cellgro/Mediatech). This media is hereinafter referred to as NK cell media.

2.2.5 Antibodies

Fluorochrome conjugated mouse monoclonal antibodies (mAb) against human CD3, CD11b, CD16, CD25, CD27, CD56, CD57, CD62L, CD69, 2B4, DNAM1, NKG2D, NKp30, NKp44 and NKp46 were obtained from BD Biosciences (San Jose, CA). Fluorochrome conjugated mouse monoclonal antibodies against human KIR2DL1, KIR2DL2/3, KIR3DL1 were obtained from Miltenyi (San Diego, CA). Fluorochrome conjugated mouse monoclonal antibodies against human CD160 was obtained from BioLegend (San Diego, CA), while fluorochrome-conjugated mouse monoclonal antibodies against human NKG2A were obtained from R&D (Minneapolis, MN). Rabbit monoclonal antibodies against human β-actin and STAT3 were obtained from Cell Signaling Technology (Danvers, MA).

2.2.6 CD3 Depletion

NK cells expanded from PBMCs were depleted of T cells using magnetic separation system from Miltenyi Biotec (San Diego, CA) following manufacture's protocol. Briefly, cells were stained with CliniMACS CD3 microbeads before loading them onto the MACS LD separation column. The CD3 microbead stained cells were separated from rest of the cells using QuadroMACS separator.

2.2.7 *Ex vivo* Expansion of Human NK Cells

NK cells were expanded as previously described with few modifications (176). Briefly, PBMCs were co-cultured with irradiated (100 cGy) K562 artificial antigen presenting cells (aAPCs), genetically modified to express membrane bound IL-21 (mbIL-21), at 1:1 (PBMCs:aAPCs) ratio and 50 U/ml recombinant human IL-2 in a T-75 flasks (Corning, Corning, NY) at 10^6 cells/ml. The K562s expressing mbIL-21 were

generated as previously described (134). Culture was refreshed with new media containing 50 U/ml IL-2 every three to four days. At the end of one week of expansion, PBMCs were re-stimulated with K562s expressing mbIL-21 and 50 U/ml IL-2.

2.2.8 Cell Count and Cell Viability

Cells were counted using hemocytometer. Cell viability was determined by Trypan blue exclusion method. Briefly, cells were stained with trypan blue and number of cells stained blue and total number of cells were counted using hemocytometer. Percentage viability was calculated using the formula [((Total number of cells-number of blue cells)/Total number of cells) *100].

2.2.9 Cellular Cytotoxicity Assay

NK cell cytolytic activity was determined using the Calcein-release assay as previously described (176). Briefly, NK cells treated with STAT3 inhibitor S3I-201 and NK cells electroporated STAT3 siRNA were co-incubated with the target cells (K562). Before co-incubation with effector cells, the target cells (K562) were labeled with 2 µg/mL of calcein-AM (Sigma-Aldrich, St. Louis, MO) for 1 hour at 37 °C with occasional shaking. The target cells were dispensed in a 96 well round bottom microplate in such a way that each well contained 10000 cells. Effector and target cells were co-incubated at the indicated effector to target (E:T) ratios at 37 °C for 4 hours in a 96 well round bottom microplate. After incubation, 100 µL of the supernatant was collected and transferred to a new plate. Absorbance at 570 nm was determined using a SpectraMAX plus384 (Molecular Devices, Sunnyvale, CA). Percent specific lysis was calculated according to the formula [(experimental release-spontaneous release) / (maximum release – spontaneous release)] x 100.

2.2.10 Lytic Unit Calculation

To obtain a uniform measure to compare NK cytotoxicity assessed in assays performed at different times, lytic units were calculated. The number of NK cells required to kill a specified percentage of target cells was delineated as one lytic unit (177). To determine the lytic unit, a standard curve was plotted using percent specific lysis versus the log values of their corresponding E:T ratio for each sample. A specific percentage of target cell lysis was selected based on the criterion that the standard curves of all the samples in the relevant data set crossed the reference percentage exactly once (177). The E:T ratio corresponding to the reference percentage was interpolated from the standard curve. In the assays, where purified NK cells were used, the number of NK cells required to kill the specified percentage of target cells was calculated by multiplying the interpolated E:T ratio by 10000 (the number of target cells in each well). In the assays, where PBMCs were used, the number of NK cells were obtained using the formula [(E:T ratio x 10000 x % of NK cells in PBMCs)/100]. Lytic units per one million cells were obtained according to the formula (1,000,000/number of NK cells in one lytic unit) (177).

2.2.11 siRNA Mediated Gene Silencing

Primary human NK cells isolated from PBMCs and cultured overnight in complete NK cell media with 50 U/ml IL-2 were nucleofected with siRNA using Amaxa human NK cell nucleofector kit from Lonza (Allendale, NJ, USA). Before electroporation, the cells were spun at 100 g for 10 minutes and washed once with NK media without serum and antibiotics. The cells were resuspended in human NK cell nucleofector solution at a density of 3-4 x 10^6 cells/100 µl. The cell suspension was mixed with the indicated concentration of On-Targetplus SMART pool siRNA for STAT3

or On-Targetplus non-targeting SMART pool siRNA for control from Dharmacon (Pittsburg, PA, USA) in a cuvette supplied with the nucleofector kit. The nucleofection was performed using program X-01 in an Amaxa Nucleofector from Lonza. Upon nucleofection, the cells were cultured for 2 hours in NK media without serum and antibiotics in a 12-well plate. After 2 hours, cells were resuspended in RPMI with 10% serum and 50 U/ml recombinant human IL-2 (Proleukin, Novartis Vaccines and Diagnostics, Inc.) at a density of 1x 10^6 cells/ml. The cells were cultured for 48 hours before use.

2.2.12 Flow-Cytometry Analysis

Flow cytometry buffer, PBS with 2mM EDTA and 0.1% sodium azide, was used for blocking, staining, washing and resuspending the cells. Before staining with antibodies, the cells were blocked with 50% FBS for 30 minutes at 4°C. Blocked cells were stained with appropriate fluorochrome conjugated antibodies for 30 minutes at 4°C, washed twice and resuspended in flow cytometry buffer. Data was acquired using a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ), and was analyzed using FlowJo Version 9.2 software (FlowJo, Ashland, OR, USA).

2.2.13 RT-PCR

RNA was isolated using RNeasy plus mini kit from Qiagen (Valencia, CA, USA) and its concentration was measured using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Primers were designed using Primer-BLAST software (178) available at NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Semi-quantitative reverse transcription PCR (RT-PCR) was performed using OneStep RT-PCR kit from Qiagen (Valencia, CA, USA) with primers listed in Table 2.

The reactions were run in PTC-200 peltier thermal cycler (GMI, Ramsey, MN). The RT-PCR products were analyzed by electrophoresis through 1.2% agarose gel. Gel was imaged in Remi molecular imager using ChemiDoc XRS+ system (Bio-Rad, Hercules, CA). The RT-PCR bands were quantified using ImageJ software (179).

For quantitative real-time PCR, cDNA was synthesized using SuperScript III first strand synthesis supermix (Invitrogen, Life technologies, Grand Island, NY). The real-time PCR reaction was set up with cDNA and primers listed in Table 2 using IQ SYBR green supermix in iCycler real time PCR detection system (Bio-Rad, Hercules, CA). Relative gene expression was quantified using ΔC_T method (180).

2.2.14 Western-Blot Analysis

NK cells were lysed with RIPA buffer (Thermo Fisher Scientific, Grand Island, NY) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail. The protein was quantified using BCA assay kit (Thermo Fisher Scientific, Grand Island, NY). Proteins were resolved using sodium dodecyl sulfate- Polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed first with unconjugated primary and then with horseradish peroxidase conjugated secondary antibody. The protein on the membrane was detected using enhanced chemiluminescence (ECL) reagents following manufacture's protocol (Amersham, GE Healthcare Life Sciences, Pittsburgh, PA). The protein bands were quantified using ImageJ software (179).

2.2.15 Pharmacological Inhibition of STAT3 in NK Cells

S3I-201 (Calbiochem, Gibbstown, NJ), a small molecule STAT3 inhibitor, reconstituted in DMSO was used to block STAT3 signaling. Purified NK cells were incubated overnight with or without S3I-201. The control cells were treated with DMSO.

2.2.16 Single Cell Analysis

The single-cell cytotoxicity assay was performed using fabricated nanowell arrays as described previously (181, 182). Briefly, K562 tumor cells (targets) and STAT3 siRNA – or control siRNA- transfected NK cells (effectors) were labeled with 1 μM of red PKH26 and green PKH67 respectively for 2 minutes (Sigma-Aldrich, St. Louis, MO). NK and K562 cells were loaded sequentially onto the nanowell array at a concentration of 10^6 cells/mL, let to settle for 3 minutes and the entire chip was immersed in phenol red free complete medium containing 1/60 (V/V) AnnexinV-AlexaFluor-647 (Life Technologies, Carlsbad, CA). Images were acquired on an AxioObserver mounted with a 5% CO_2, 37 ^{0}C and 100% humidity incubator, piloted with Zen software (Carl Zeiss, Jena, Germany) and fitted with a Hamamatsu Orca Flash 4.0 sCMOS camera and a 20x 0.8 NA objective. Timelapse imaging microscopy in nanowell grids (TIMING) images were taken for 8 hours at intervals of 6 minutes and data were processed and analyzed as described previously (183).

Data were post-processed using Excel and GraphPad Prism. Statistical testing was run using Fisher's exact test when contingency numbers were compared.

2.2.17 Analysis of Subcellular Cytolytic Processes

Analysis of synaptic strength, granule convergence to MTOC and MTOC polarization to the synapse were performed as previously described (184-186). Briefly,

NK and K562 cells were incubated together in suspension at 2:1 (PBMC:K562) ratio for 15 minutes to form conjugates between the two. The cell suspension was then placed on poly-L-lysine coated glass slide (Sigma-Aldrich, St. Louis, MO) for 15 minutes to let the cells adhere to the slide. All the steps were carried out at 37^{o}C. Cells attached to the slides were fixed and permeabilized with 4% formaldehyde, 0.1% saponin and 0.1% triton X-100 in PBS for 15 minutes. Before staining with the antibodies, cells were blocked with 10% heat-inactivated mouse serum (Sigma-Aldrich, St. Louis, MO) for 30 minutes. Blocked cells were first stained with biotinylated anti-tubulin mAb (Invitrogen, Waltham, MA) for 1 hour followed by rinsing and incubation with streptavidin-conjugated Pacific blue 405 (Invitrogen, Waltham, MA). Slides were rinsed and incubated with Alexa Fluor 568-conjugated phalloidin (Invitrogen, Waltham, MA) and FITC-conjugated anti-perforin mAb (BD Biosciences, San Jose, CA) for 1 hr. After rinsing the slides, cells were covered with 0.15 mm glass coverslips (VWR Scientific, Radnor, PA) using Prolong Antifade reagent (Invitrogen, Waltham, MA) (185).

Cell conjugates were imaged with a laser scanning confocal microscope (DMIRE2; Leica, Buffalo Grove, IL). The acquired images were analyzed using Volocity software (PerkinElmer, Waltham, MA). In an image, NK and K562 cells were distinguished on the basis of size difference (NK cell smaller compared to K562) and the presence of perforin (present in NK and absent in K562).

To determine actin deposition at the interface of NK and K562, the entire interface was divided into squares, each measuring 1 μm x 1 μm. In each square, mean fluorescence intensity (MFI) of phalloidin, which stained F-actin, and the area occupied by phalloidin were quantified. Actin deposition in the square was estimated by the formula MFI of phalloidin x area occupied by phalloidin. The actin deposited at the NK-K562 interface, the immune synapse, was quantified by summing up actin

deposition in all the squares the interface was divided into. The quantified actin deposition at the interface included actin deposited by NK and K562. To estimate contribution of NK cell in actin deposition at the synapse, contribution of K562 to actin deposition and background actin deposition in unconjugated NK cells were subtracted from the quantified actin deposition at the interface. K562 contributed actin and background actin deposition in NK were estimated by quantifying actin deposited in equal number of squares in the cortex of unconjugated K562 cells and unconjugated NK cells, respectively (184).

Microtubule organizing center (MTOC) was visualized by staining it with biotinylated anti-tubulin mAb and streptavidin-conjugated Pacific blue 405. During image analysis, MTOC was delineated as tubulin labeled region with a pixel size threshold that eliminated measurement of individual microtubules (185). MTOC polarization was defined as the distance between MTOC and immune synapse and was measured by drawing a line between MTOC and the center of immune synapse using Volocity software (185, 186).

Lytic granules were identified by staining with FITC-conjugated anti-perforin mAb. Granule convergence to MTOC was defined as the shortest distance between each individual granule and MTOC in the NK cell in conjugation with K562. To measure the distance, x and y coordinates of the MTOC and all the lytic granules in the cell were obtained using Volocity software and the mean MTOC to granule distance (MGD) for the cell was measured using the following formula (186)

$$MGD = \frac{\left(\sum_{i=1}^{n} \sqrt{(x - x_i)^2 + (y - y_i)^2}\right)}{n}$$

x and y denote coordinates for the MTOC; x_i and y_i denote coordinates of each individual granule; n denote number of granules in the cell (186).

2.2.18 Statistical Analysis

Statistical analysis was performed for the indicated statistical tests using GraphPad Prism for Macintosh, version 5.0a. P values <.05 were considered significant.

TABLE 2. PRIMERS USED FOR RT-PCR AND REAL TIME PCR ANALYSIS

Primer	Sequence 5' to 3'	Product length (base pairs)
RT-PCR		
STAT1 Forward primer	TCAGGCTCAGTCGGGGAATA	801
STAT1 Reverse primer	ATCACTTTTGTGTGCGTGCC	
STAT3 Forward primer	GGAGCTGGCTGACTGGAAGAG	856
STAT3 Reverse primer	CTCGATGCTCAGTCCTCGCTTG	
STAT5a Forward primer	GAGGATCAAGCGTGCTGACCGGC	670
STAT5a Reverse primer	TGGTTTCAGGTTCCACAGGTTGC	
STAT5b Forward primer	CCGCAATGATTACAGTGGCG	828
STAT5b Reverse primer	TTCAAGTCTCCCAAGCGGTC	
Real Time PCR		
Arl8B Forward primer	GACTGGTTCCGTTCGCTCTTCTGG	241
Arl8B Reverse primer	TGACTCCTCTGCAATACCGCTCCC	
CIP4 Forward primer	TGGTGAAAAAATATCTGCCCAAGAGAC	293
CIP4 Reverse primer	CCGGCAGTCCCGCTCAAATT	
IQGAP1 Forward primer	GGAGGCACTGGCTAAGACGGAAG	220
IQGAP1 Reverse primer	TCTCTGCATGGCTCTCTGATGTTCTG	
KIF5B Forward primer	AAATAGGAATTGCTGTGGGAAATAATGATG	217
KIF5B Reverse primer	ATACGAAGCTGACATGCTGCTAACTCC	
SKIP Forward primer	AGCTATTTTGCTGCATGTGAGGATGAG	276
SKIP Reverse primer	CTGGAACAACCGCAGGTAGCTCTC	
GAPDH Forward primer	GGAGAAGGCTGGGGCTCATTTG	244
GAPDH Reverse primer	CTTCTGGGTGGCAGTGATGGC	

2.3 RESULTS

In our previous work, we showed that membrane bound IL-21, a STAT3 activating cytokine, stimulates expression of activating receptors and cytolytic activity of human NK cells (134) and STAT3 activation plays a critical role in the transcriptional regulation of basal as well as induced expression of the primary activating receptor NKG2D in human NK cell (136). As cytolytic activity of NK cells is triggered by the signals transmitted by the activating receptors upon binding to their cognate ligands on target cells, our previous findings led me to hypothesize that STAT3 activation may play a role in the regulation of cytolytic function of NK cells. Availability of NK cells from Job syndrome patients carrying dominant negative STAT3 mutations (Table 3) offered me an opportunity to test my hypothesis in a physiologically relevant genetic model.

Table 3. STAT3 mutations in Job syndrome patients

		Mutation		
Sample ID	**Source**	**Nucleotide, cDNA location**	**Amino acid**	**Domain**
Job C001	NIAID/NIH	1027 G-T	V343P	DNA binding
Job C002	NIAID/NIH	1144 C-T	R382W	DNA binding
Job C003	NIAID/NIH	1144 C-T	R382W	DNA binding
Job C004	NIAID/NIH	1970 A-G	Y657C	SH2
Job C005	NIAID/NIH	1909 G-A	V637M	SH2
Job C006	NIAID/NIH	2003 C-T	S668F	SH2
Job C007	NIAID/NIH	1865 C-T	T622I	SH2
Job C008	NIAID/NIH	1387-1389 delGTG	V463del	DNA binding
Job C009	NIAID/NIH	1144 C-T	R382W	DNA binding
Job C010	NIAID/NIH	2137 G-A	V713M	Transactivation
Job C011	NIAID/NIH	1294 G-A	V432M	DNA binding
Job C012	NIAID/NIH	1144 C-T	R382W	DNA binding
Job C013	NIAID/NIH	1145 G-A	R382Q	DNA binding
Job C014	NIAID/NIH	1145 R-A	R382Q	DNA binding

NIAID: National Institute of Allergy and Infectious Diseases; NIH: National Institute of Health

Nucleotide Key: A- Adenine, G- Guanine, C- Cytosine, T- Thymine

Amino acid key: C-Cysteine, F- Phenylalanine, I- Isoleucine, M- Methionine, P- Proline, Q- Glutamine, R- Arginine, S- Serine, T- Threonine, V- Valine, W- Tryptophan, Y- Tyrosine

del: deletion

2.3.1 NK Cells from Job Syndrome Patients

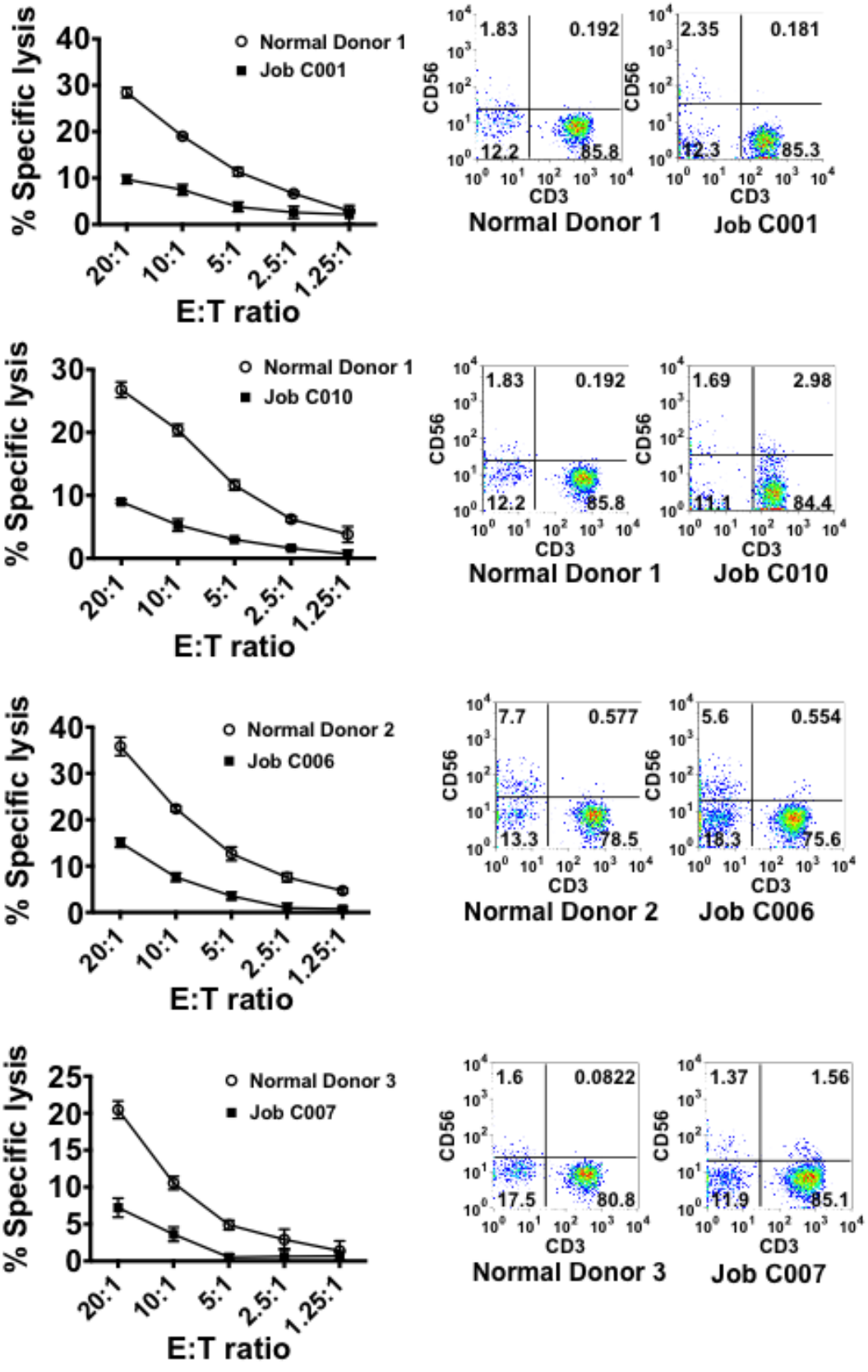
2.3.1.1 Impaired Cytotoxicity of NK Cells in Job Syndrome Patients

I assessed basal cytotoxicity of NK cells from Job syndrome patients against K562, an NK sensitive human erythroleukemic cell line (187), which is a standard target routinely used to measure NK cytolytic function using purified NK cells (188, 189) as well as PBMCs (190, 191). Due to limited availability of Job syndrome blood samples, instead of purified NK cells, PBMCs were used in the cytotoxicity assays. Due to varying percentage of NK cells in the PBMCs, assays were performed in pairs of PBMCs from one Job syndrome patient and PBMCs from a normal, healthy donor containing similar percentage of NK cells (Figure 2a). Number of PBMCs used in the paired assay was adjusted in order to get identical number of NK cells for both, Job syndrome and normal donor. Comparison between Job syndrome patient and its correspondingly matched normal donors showed diminished NK cytotoxicity in Job syndrome patients over a wide range of effector: target ratios (Figure 2a). As the assays were performed with PBMCs with varying number of NK cells, for bulk comparison between all the Job syndrome donors and normal donors, lytic unit, defined as the number of NK cells required to kill a specified percentage of target cells, was determined from the cytotoxicity curve of each individual sample. Job syndrome patients were found to have significantly reduced number of lytic units compared to normal donors (Figure 2b). These results showed that NK cells with dominant negative STAT3 mutations have impaired cytolytic function.

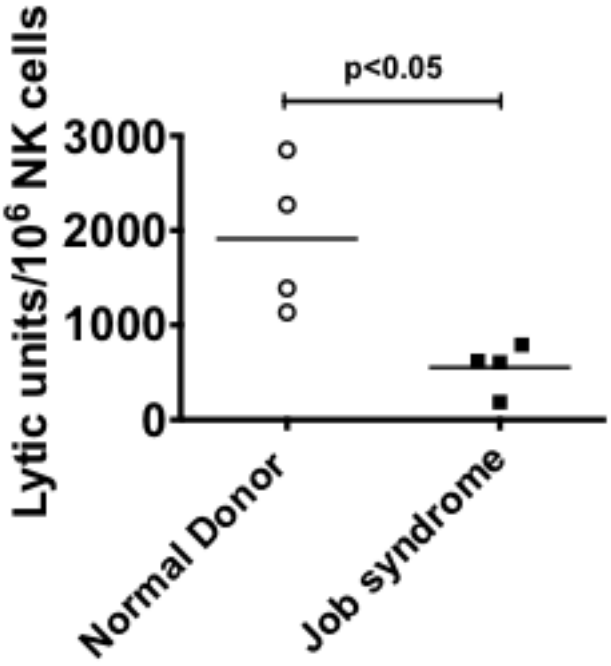
Figure 2. Deficient cytolytic function in NK cells from Job syndrome patients.

(a) Cytotoxicity of NK cells was assessed against K562 using PBMCs employing calcein release assay at different effector to target ratios (E:T). Assay for each donor was performed in triplicates for each E:T ratio. Data is presented as mean ± standard deviation (SD) for each E:T ratio for each donor. Due to varying percentage of NK cells in the PBMCs, assays were performed in pairs of PBMCs from one Job syndrome patient and PBMCs from a normal, healthy donor containing similar percentage of NK cells. Number of PBMCs used in the paired assay was adjusted in order to get identical number of NK cells for both, Job syndrome and normal donor. Data is presented in a graphical form by plotting percentage specific lysis against its corresponding E:T ratio. The flow-cytometry graphs beside the cytotoxicity plots show the percentage of NK cells ($CD3^-$ $CD56^+$) in PBMCs. **(b)** Significantly reduced number of lytic units in Job syndrome patients. Lytic unit, defined as the number of NK cells required to kill a specified percentage of target cells, was determined from the cytotoxicity curve of each individual sample obtained in the calcein assay. A specific percentage of target cell lysis was selected based on the criterion that the standard curves of all the samples in the relevant data set crossed the reference percentage exactly once. Based on this criterion, number of NK cells required to kill 5% of target cells were defined as one lytic unit for the comparison between normal donors and Job syndrome patients. The E:T ratio corresponding to the reference percentage was interpolated from the standard curve. The number of NK cells were obtained using the formula [(E:T ratio x 10000 x % of NK cells in PBMCs)/100]. Lytic units per one million NK cells were obtained according to the formula (1000,000/number of NK cells in one lytic unit). Lytic unit of each donor is shown along with the mean for the group. P value indicated is for two-tailed Student's t test.

a


% Specific lysis
Normal Donor 1
Job C001
E:T ratio
20:1
10:1
5:1
2.5:1
1.25:1
CD56
CD3
1.83
0.192
12.2
85.8
2.35
0.181
12.3
85.3
Normal Donor 1
Job C001
Normal Donor 1
Job C010
1.69
2.98
11.1
84.4
Normal Donor 1
Job C010
Normal Donor 2
Job C006
7.7
0.577
13.3
78.5
5.6
0.554
18.3
75.6
Normal Donor 2
Job C006
Normal Donor 3
Job C007
1.6
0.0822
17.5
80.8
1.37
1.56
11.9
85.1
Normal Donor 3
Job C007

b


p<0.05
Lytic units/10^6 NK cells
3000
2000
1000
0
Normal Donor
Job syndrome

2.3.1.2 Receptor Expression in NK Cells from Job Syndrome Patients

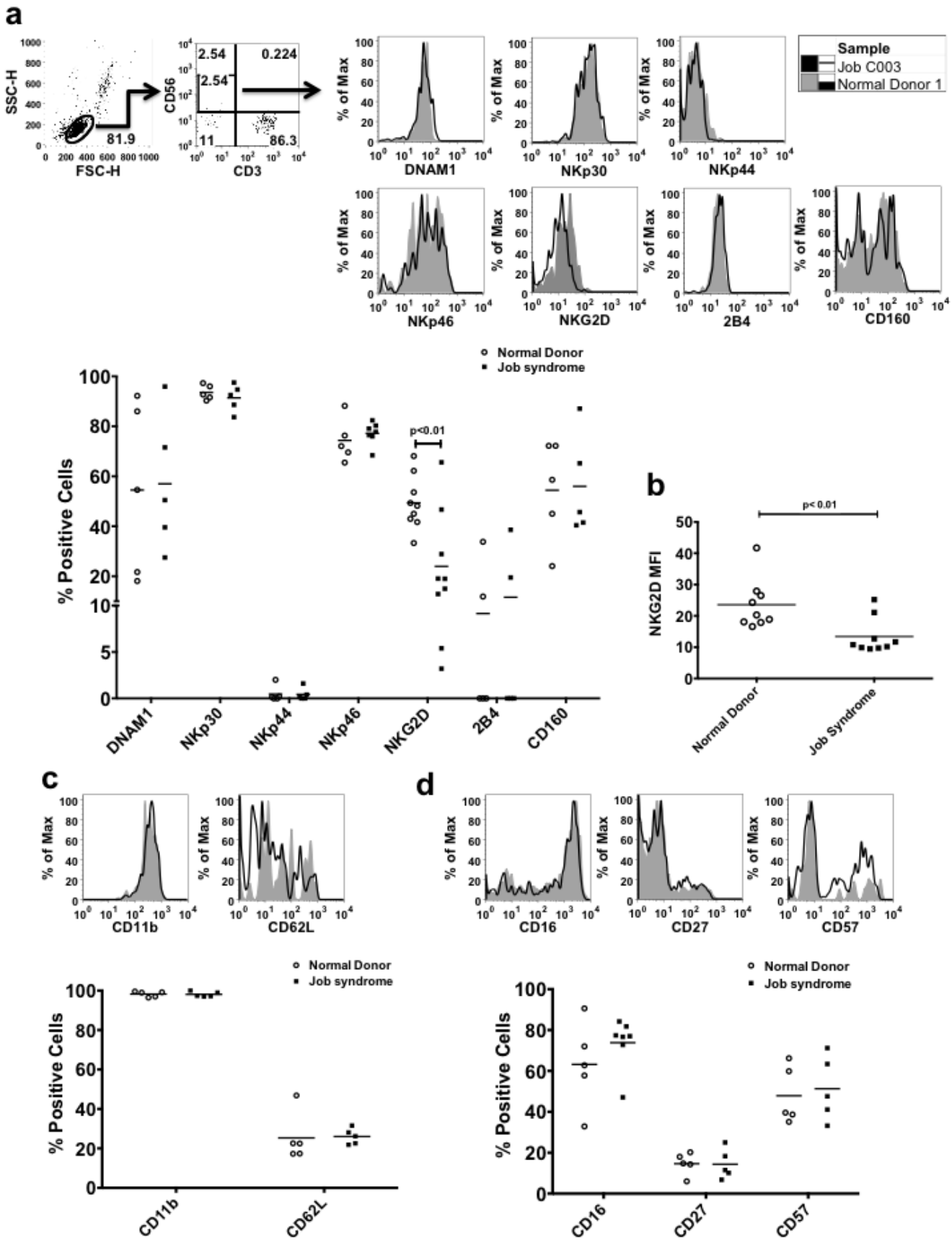
Target cell lysis by NK cell is the culmination of a complex series of events mediated by various types of receptors expressed on NK cell surface. Initial tethering and later firm adhesion with target cell are mediated through adhesion receptors such as L-selectin (CD62L) and macrophage receptor 1 (CD11b) (158). The activating receptors upon binding to their ligands transmit signals to activate exocytosis of lytic granules. Signals relayed by a large array of activating receptors such as natural cytotoxicity receptor (NCR) family (NKp30, NKp44, NKp46), NKG2D, DNAM1, 2B4 and CD160, expressed on NK cells, have been shown to induce NK cytotoxicity (14, 15). Signals transmitted by inhibitory receptors belonging to killer cell immunoglobulin-like receptor (KIR) family (KIR2DL1, KIR2DL2/3, KIR3DL1) and CD94-NKG2A inhibit the cytolytic process (15, 192). The cytolytic activity of NK cells is the net result of the balance between the strengths of activating and inhibitory signals (8). Cytotoxic potential of mature NK cells is indicated by the expression of low affinity Fc receptor CD16 (193) and epitope CD57 (194) and the absence of TNF receptor family member CD27 (195). Priming of NK cells for cytotoxic action is denoted by the expression of CD69 and absence of CD25 (196).

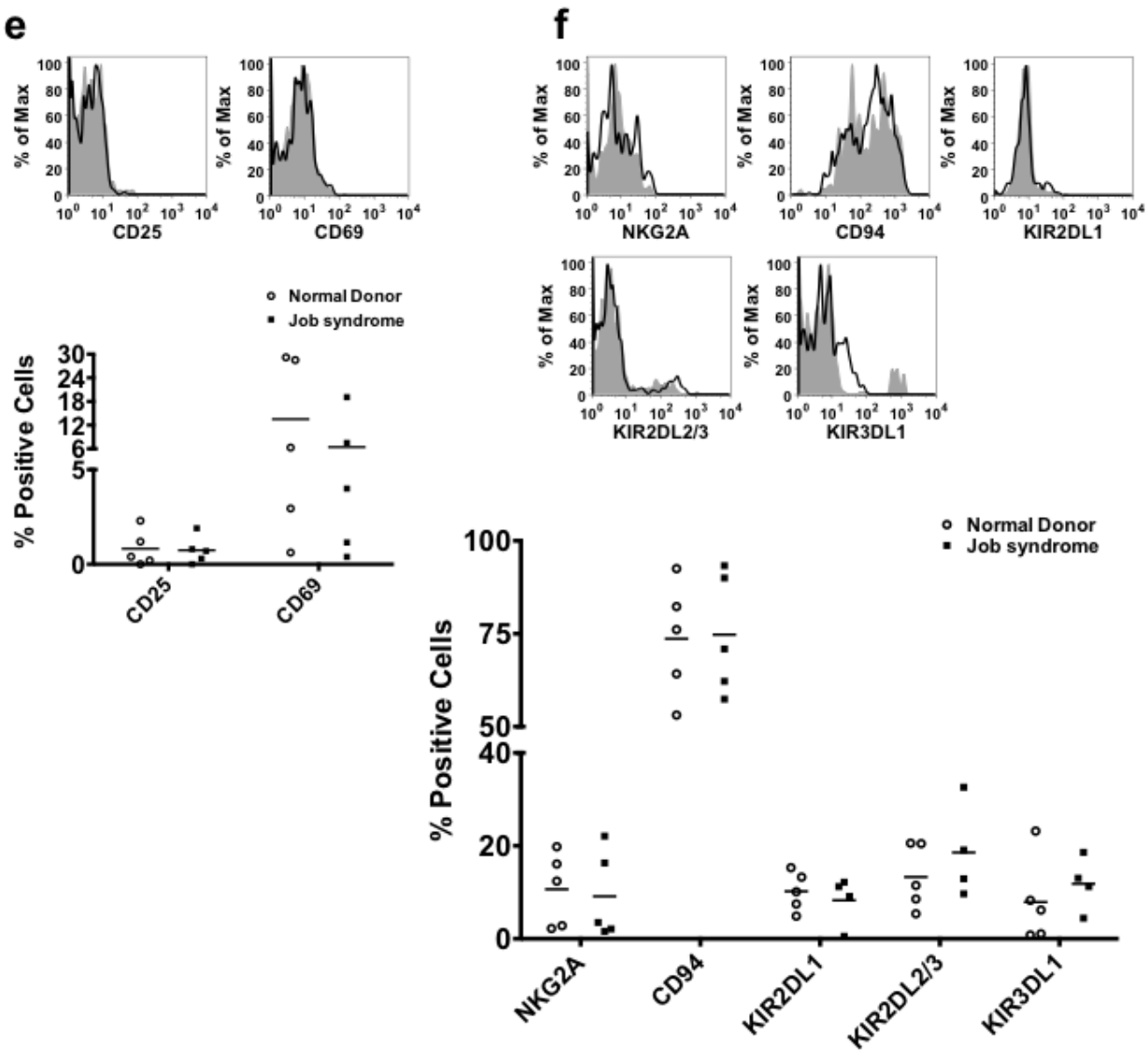
I assessed expression of the different receptors and maturation and activation markers in NK cells from Job syndrome patients by flow-cytometry to investigate whether the deficient cytotoxicity is due to the impaired receptor expression. The cells were found to have normal expression of all the surface molecules except the activating receptor NKG2D (Figure 3). Both, number of NK cells expressing NKG2D (Figure 3a) as well as receptor density per cell (Figure 3b) were found to be significantly reduced compared to NK cells from normal donors.

Figure 3. Significantly reduced expression of NKG2D receptor in Job syndrome patients' NK cells. Basal expression of receptors showed no difference between NK cells from normal donors and Job syndrome patients, except NKG2D. Expression of NKG2D was significantly reduced in Job syndrome patients' NK cells both in terms of number of NK cells expressing NKG2D, shown as percentage of NK cells expressing the receptor, as well as receptor density per cell, depicted as mean fluorescence intensity (MFI). PBMCs isolated from normal donors and Job syndrome patients were analyzed by flow-cytometry to assess baseline expression of different types of receptors on NK cells. In the flow-cytometric analysis of PBMCs, lymphocyte population was distinguished by their forward scatter and side scatter characteristics and from this gated population, NK cells were identified by gating $CD56^{+}$ $CD3^{-}$ cells **(a)**. Expression of different types of receptors; **(a)** activating, **(c)** adhesion **(d)** maturation markers **(e)** priming markers and **(f)** inhibitory was assessed and depicted as percentage of NK cells expressing the receptor. Each panel also shows representative flow charts comparing expression of the receptors in that particular category between a normal donor and Job syndrome patient. Panel **b** depicts receptor density per cell, shown as mean fluorescence intensity (MFI), of NKG2D.

In each graph values for individual donor are shown along with the mean for the group. P value indicated is for two-tailed Student's t test.

"The percentage of NK cells expressing NKG2D receptor (Panel a) and NKG2D MFI (Panel b) shown in this figure were originally published in *Blood*. Zhu S, **Phatarpekar PV***, Denman CJ, Senyukov VV, Somanchi SS, Nguyen-Jackson HT, Mace EM, Freeman AF, Watowich SS, Orange JS, Holland SM, Lee DA. Transcription of the activating receptor NKG2D in natural killer cells is regulated by STAT3 tyrosine phosphorylation. *Blood.* 2014;124(3):403-411. © the American Society of Hematology." *__Co-first Author__


a
SSC-H
FSC-H
81.9
CD56
CD3
2.54
0.224
2.54
11
86.3
Sample
Job C003
Normal Donor 1
% of Max
DNAM1
NKp30
NKp44
NKp46
NKG2D
2B4
CD160
Normal Donor
Job syndrome
% Positive Cells
p<0.01
b
NKG2D MFI
p< 0.01
Normal Donor
Job Syndrome
c
CD11b
CD62L
d
CD16
CD27
CD57


e
% of Max
CD25
CD69
Normal Donor
Job syndrome
% Positive Cells
f
NKG2A
CD94
KIR2DL1
KIR2DL2/3
KIR3DL1

2.3.1.3 Cellular Processes Involved in Cytolytic Function of Job Syndrome Patients' NK Cells

Degranulation at the interface of NK and target cell leading to target cell lysis is the end result of a series of cellular processes. The process is set in motion by immune synapse formation between NK and target cells. Accumulation of F-actin at the synapse leads to its maturation. Signals transmitted by activating receptors clustered at the mature synapse cause convergence of lytic granules dispersed throughout the cell to MTOC and subsequent polarization of MTOC to synapse for directed secretion of lytic granule contents into the synaptic cleft. Defect in all or any of these processes affects NK cytotoxicity (158). To investigate whether these cellular processes are intact in Job syndrome patients' NK cells, I studied synaptic strength, granule convergence to MTOC and MTOC polarization to synapse in fixed conjugates of NK cells with K562 using confocal microscopy

2.3.1.3.1 Synaptic Strength

F-actin accumulation at the interface of NK and target cell is the pivotal step in synapse formation, which enables clustering of activating receptors and subsequent integration of activating signals that mediate granule convergence to MTOC and later MTOC polarization to the synapse (159). As a result, amount of F-actin deposited at the NK: target interface reflects strength of the synapse and is used as a measure of it (184). To measure amount of F-actin, in fixed, permeabilized NK: K562 conjugates fluorescent phalloidin, an F-actin binding peptide (197), was used. The signal intensity of phalloidin fluorophore at the interface of NK and target and the area occupied by fluorescent phalloidin at the interface were measured in confocal micrographs. The F-actin density at the synapse was estimated by multiplying signal intensity of fluorescent

phalloidin with the area occupied by F-actin (184). Synapse strength estimated by this method in >60 conjugates, pooled from four donors each, of Job syndrome patients' and normal donors' NK cells showed no difference between the two (Figure 4e). Thus, upon binding to the target formation of synapse appeared to be normal in Job syndrome patients' NK cells.

2.3.1.3.1 Granule Convergence

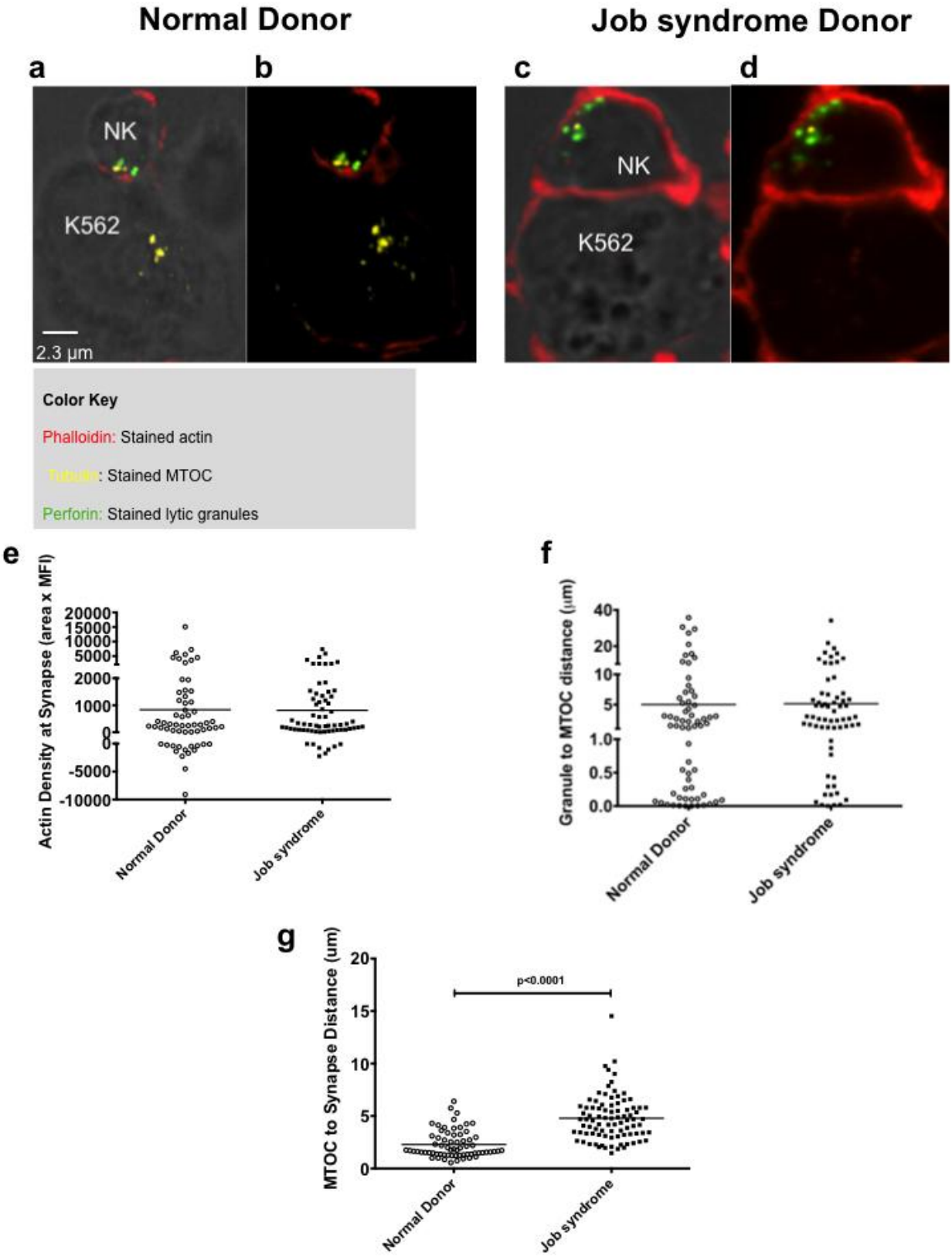
Directed secretion of perforin and granzyme into the synaptic cleft is initiated by recruitment of lytic granules dispersed throughout the cell to the MTOC, called as granule convergence (158). Granule convergence to MTOC was measured as the distance between a lytic granule, visualized by staining with fluorochrome conjugated perforin antibody, and the MTOC, visualized by staining with biotinylated conjugated anti-tubulin antibody and fluorochrome conjugated streptavidin, in a confocal micrograph of a fixed conjugate between NK and K562. Average of granule-MTOC distance for all the granules in a cell was denoted as the mean granule to MTOC distance for the cell. Granule convergence measured in >60 conjugates, pooled from four donors each, of Job syndrome patients and normal donors NK cells showed no difference between the two (Figure 4f).

Figure 4. Normal actin deposition at the synapse and granule convergence to microtubule organizing center (MTOC), but impaired MTOC polarization in Job syndrome patients' NK cells. PBMCs and K562 cells were incubated together to facilitate conjugate formation between the two. The cells were placed on poly-L-lysine coated glass slides. The cells attached to the slide were permeabilized and stained with fluorochrome conjugated phalloidin, fluorochrome conjugated anti-perforin antibody and biotinylated anti-tubulin antibody and fluorochrome-conjugated streptavidin. Phalloidin, anti-tubulin and anti-perforin were used to stain F-actin, MTOC and lytic granules, respectively. The conjugates were imaged using scanning confocal microscope. The images were analyzed using Volocity software. Representative brightfield image and its corresponding confocal image show conjugate formed between an NK cell from normal donor and K562 **(a and b)** and an NK cell from Job syndrome patient and K562 (**c and d**). In the image, NK cell was distinguished from K562 on the basis of cell size (NK smaller compared to K562) and the presence of perforin (present in NK and absent in K562). F-actin deposited at the interface of the conjugate, immune synapse, is stained with phalloidin (red). MTOC is stained with anti-tubulin (yellow) and lytic granules are stained with anti-perforin (green). **(e)** Synaptic strength was measured in terms of amount F-actin deposited at the interface of NK and K562. To quantify F-actin, signal intensity of phalloidin fluorophore at the interface of NK and target and the area occupied by fluorescent phalloidin at the interface were measured in confocal micrographs using Volocity software. The F-actin density at the synapse was estimated by multiplying signal intensity of fluorescent phalloidin with the area occupied by F-actin. Synapse strength estimated by this method in conjugates, pooled from four donors each, of normal donors and Job syndrome patients is represented in graphical form. **(f)** Granule convergence to MTOC was defined as the shortest distance between

each individual granule and MTOC in the NK cell in conjugation with K562. Lytic granules were identified by staining with anti-perforin antibody and MTOC was recognized by staining with anti-tubulin antibody. The distance between MTOC and each lytic granule in a conjugated NK cell was measured using Volocity software. Average of granule-MTOC distance for all the granules in a cell was denoted as the mean granule to MTOC distance for the cell. Granule convergence measured in conjugates, pooled from four donors each, of normal donors and Job syndrome patients is represented in graphical form. Each data point represents mean granule to MTOC distance for an individual NK cell in conjugation with K562. **(g)** MTOC polarization was defined as the distance between the MTOC of an NK cell, visualized by staining with anti-tubulin antibody, and the immune synapse, demarcated as the contact area between the NK and K562 in a conjugate and stained with fluorochrome conjugated phalloidin. The distance was measured by drawing a line between the MTOC and the center of immune synapse in a confocal image using Volocity software. MTOC polarization measured in conjugates, pooled from four donors each, of normal donors and Job syndrome patients is represented in graphical form.

In each graph mean for the group is denoted. P value indicated is for two-tailed Student's t test.

Confocal microscopic analysis of subcellular cytolytic processes and data processing were performed by my collaborator, Dr. Malini Mukherjee in Dr. Jordan Orange laboratory at the section of Immunology, Allergy, and Rheumatology, Baylor College of Medicine, Houston, Texas.


Normal Donor
Job syndrome Donor
a
b
c
d
NK
K562
2.3 µm
Color Key
Phalloidin: Stained actin
Tubulin: Stained MTOC
Perforin: Stained lytic granules
e
Actin Density at Synapse (area x MFI)
20000
15000
10000
5000
2000
1000
0
-5000
-10000
Normal Donor
Job syndrome
f
Granule to MTOC distance (µm)
40
20
10
5
1.0
0.5
0.0
Normal Donor
Job syndrome
g
MTOC to Synapse Distance (um)
p<0.0001
20
15
10
5
0
Normal Donor
Job syndrome

2.3.1.3.2 MTOC Polarization to the Synapse

Signals transmitted by activating receptors, clustered at the mature synapse, induce migration of MTOC in NK cell towards the synapse. MTOC along with it also brings the converged granules to the synapse. It is a critical step in the directed secretion of lytic content (185). MTOC polarization to the synapse was assessed by measuring the distance between the MTOC and the center of the synapse in confocal micrographs of NK cells conjugated with K562. MTOC polarization measured in >60 conjugates, pooled from four donors each, of Job syndrome patients and normal donors NK cells showed MTOC to be at a significantly greater distance from the synapse in Job syndrome patients NK cells compared to normal donors (Figure 4g). The larger distance between the MTOC and synapse in Job syndrome patients' NK cells indicated impaired polarization of MTOC and lytic granules.

Thus, even though the initial events of synapse formation and granule convergence occurred normally, transport of lytic cargo to the synapse was found to be defective in NK cells from Job syndrome patients.

2.3.2 STAT3 Knock-Down in NK Cells

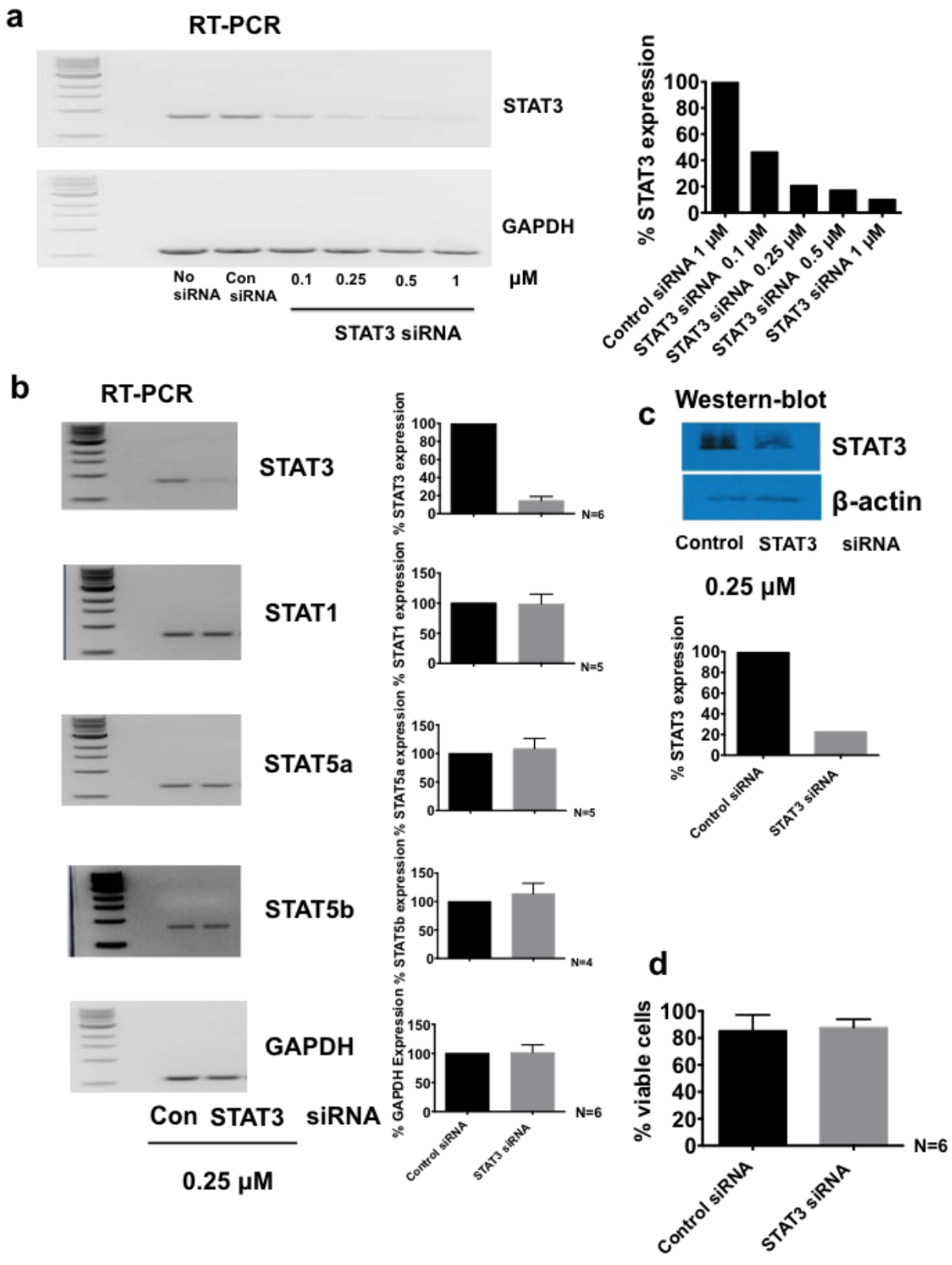
To corroborate my finding of impaired cytotoxicity and cellular processes involved in cytolytic function in NK cells with dominant negative STAT3 mutations and to obtain unequivocal evidence for the role of STAT3 in the regulation of NK cytolytic function, I also evaluated cytolytic function in STAT3 knock-down (STAT3 KD) primary human NK cells.

2.3.2.1 siRNA Mediated Knock-Down of STAT3

Purified human NK cells from normal donors maintained overnight with 50 U/ml IL-2 were electroporated with control and STAT3 siRNA and cultured thereafter with 50 U/ml IL-2 for 48 hours before analysis. Calibration of STAT3 siRNA concentration showed incremental decrease in STAT3 mRNA expression in RT-PCR analysis. However, at concentration above 0.25 μm along with STAT3, GAPDH mRNA also started decreasing indicating non-specific effect (Figure 5a). As a result, 0.25 μm siRNA was used for rest of the studies. At 0.25 μm siRNA, STAT3 mRNA expression, quantified by ImageJ software and normalized to GAPDH, showed about 80% decrease compared to control siRNA (Figure 5a and 5b). STAT3 protein expression analyzed by western-blot also showed a corresponding decrease (Figure 5c). RT-PCR analysis showed no effect of STAT3 siRNA on the expression of STAT1, STAT5a and STAT5b mRNA (Figure 5b), STAT family members closely related to STAT3 (198, 199). Cell viability, analyzed by trypan blue exclusion method, was not affected by STAT3 knock-down 48 hr post electroporation (Figure 5d).

Figure 5. STAT3 siRNA mediated about 80% decrease in STAT3 mRNA and protein expression in primary human NK cells without any effect on the expression of other STATs and cell viability. Purified human NK cells from normal donors maintained overnight with 50 U/ml IL-2 were electroporated with control or STAT3 siRNA and cultured thereafter with 50 U/ml IL-2 for 48 hours before analysis. Knock-down of mRNA was assessed by semi-quantitative RT-PCR using gene specific primers. The RT-PCR products were analyzed by electrophoresis through 1.2% agarose gel. Gel was imaged and the RT-PCR bands were quantified using ImageJ software. Relative band intensities of target genes were normalized to GAPDH and converted to percentage expression by considering expression in control siRNA treated cells as 100%. **(a)** Calibration of STAT3 siRNA concentration showed incremental decrease in STAT3 mRNA as visualized in the gel image and its quantification plotted as percentage STAT3 expression. However, at concentration above 0.25 μm along with STAT3, GAPDH mRNA also started decreasing indicating non-specific effect. As a result, 0.25 μm siRNA concentration was used for rest of the studies **(b)** mRNA levels of STAT3, STAT1, STAT5a, STAT5b, and GAPDH in NK cells electroporated with 0.25 μm siRNA were analyzed by semi-quantitative RT-PCR. The gels were imaged and the bands were quantified as described above. The gel image shown is a representative of multiple experiments. The graph beside the gel depicts quantification of RT-PCR bands normalized to GAPDH and represented as percentage expression. N denotes number of times the experiment was repeated **(c)** STAT3 knock down was also analyzed at the protein level by western-blot. Protein lysate, 15 μg/lane, resolved by SDS-PAGE and transferred to PVDF membrane, was probed with anti-STAT3 and anti-β-actin (loading control) antibodies. The bands were quantified by ImageJ software. Relative band intensities were normalized to β-actin and converted to percentage expression by

considering expression in control siRNA treated cells as 100%. The upper panel shows western gel image and lower panel depicts quantification in graphical form **(d)** Cell viability of siRNA treated cells was assessed by trypan blue exclusion method. N denotes number of times the experiment was repeated.


a
RT-PCR
STAT3
GAPDH
No siRNA
Con siRNA
0.1
0.25
0.5
1
μM
STAT3 siRNA
% STAT3 expression
100
80
60
40
20
0
Control siRNA 1 μM
STAT3 siRNA 0.1 μM
STAT3 siRNA 0.25 μM
STAT3 siRNA 0.5 μM
STAT3 siRNA 1 μM
b
RT-PCR
STAT3
STAT1
STAT5a
STAT5b
GAPDH
Con
STAT3
siRNA
0.25 μM
% STAT3 expression
% STAT1 expression
% STAT5a expression
% STAT5b expression
% GAPDH Expression
N=6
N=5
N=5
N=4
N=6
Control siRNA
STAT3 siRNA
c
Western-blot
STAT3
β-actin
Control
STAT3
siRNA
0.25 μM
% STAT3 expression
Control siRNA
STAT3 siRNA
d
% viable cells
N=6
Control siRNA
STAT3 siRNA

2.3.2.2 Impaired Cytotoxicity of STAT3 Knock-Down NK Cells

Assessment of cytolytic activity by calcein release assay against K562 revealed small but consistent decrease in cytotoxicity of STAT3 knock-down NK cells compared to control cells over a wide range of E:T ratios (Figure 6a). Comparison between control cells and STAT3 knock-down cells also showed significantly diminished number of lytic units in the latter (Figure 6b).

2.3.2.3 Single Cell Analysis of Cytotoxicity of STAT3 Knock-Down NK Cells

In bulk electroporation, as was performed in the present study to knock-down STAT3 in primary human NK cells, all the cells may not be electroporated, leaving some cells without knock-down of the target mRNA. These cells, electroporated in bulk, if used in a bulk assay such as the calcein assay employed in the present study to assess the cytotoxicity of NK cells, then the cells with intact STAT3, even if few, could skew the output. The possibility of existence of cells with intact STAT3 in STAT3 knock-down bulk population may explain the difference between the cytotoxicity of control cells and STAT3 knock-down cells being smaller as these cells with intact killing capacity through encounters with multiple target cells may compensate for the cytolytic deficiency of rest of the cells. Therefore, cytotoxicity of STAT3 knock-down NK cell at a single cell level was also analyzed. NK cell and K562 cell interactions were studied using the single cell assay, TIMING (Timelapse Imaging Microscopy in Nanowell Grids), as described previously (182) (Figure 7a**)**. Among NK cells that established at least one contact with the target cell (K562) in nanowells containing exactly 1 NK cell and 1 K562 cell (number of nanowells=791 and 1010, respectively for conditions with STAT3 knock-down and control NK cells), STAT3 knock-down NK cells were able to perform lysis (detected using Annexin V staining) at a lower frequency (35%) compared

to control NK cells (43%) (Figure 7b). Serial killing ability of NK cells was also assessed by measuring killing frequency in nanowells containing exactly 1 NK cell and 2 K562 cells and where NK cell made at least 2 contacts with the target cells (number of nanowells=463 and 388, respectively for conditions with STAT3 knock-down and control NK cells). STAT3 knock-down NK cells were able to perform lysis of both the target cells at a lower frequency (28.7%) compared to control NK cells (37.4%) (Figure 7c).

Figure 6. Impaired cytolytic function in STAT3 knock-down NK cells.

(a) Purified human NK cells from normal donors maintained overnight with 50 U/ml IL-2 were electroporated with control or STAT3 siRNA and cultured thereafter with 50 U/ml IL-2 for 48 hours before assessment of their cytotoxicity against K562 using calcein assay. Assay for each donor was performed in triplicates for each E:T ratio. Data is presented as mean ± standard deviation (SD) for each E:T ratio for each donor. The experiment was repeated three times with three different donors.

(b) Lytic unit, defined as the number of NK cells required to kill a specified percentage of target cells, was determined from the cytotoxicity curve of each individual sample obtained in the calcein assay. A specific percentage of target cell lysis was selected based on the criterion that the standard curves of all the samples in the relevant data set crossed the reference percentage exactly once. Based on this criterion, number of NK cells required to kill 75% of target cells were defined as one lytic unit for the comparison between control and STAT3 knock-down (STAT3 KD). The E:T ratio corresponding to the reference percentage was interpolated from the standard curve. The number of NK cells in the lytic unit were obtained by multiplying the interpolated E:T ratio by 10000 (the number of target cells in each well). Lytic units per one million NK cells were obtained according to the formula (1,000,000/number of NK cells in one lytic unit). Lytic unit of each donor is shown along with the mean for the group. P value indicated is for two-tailed paired Student’s t test.

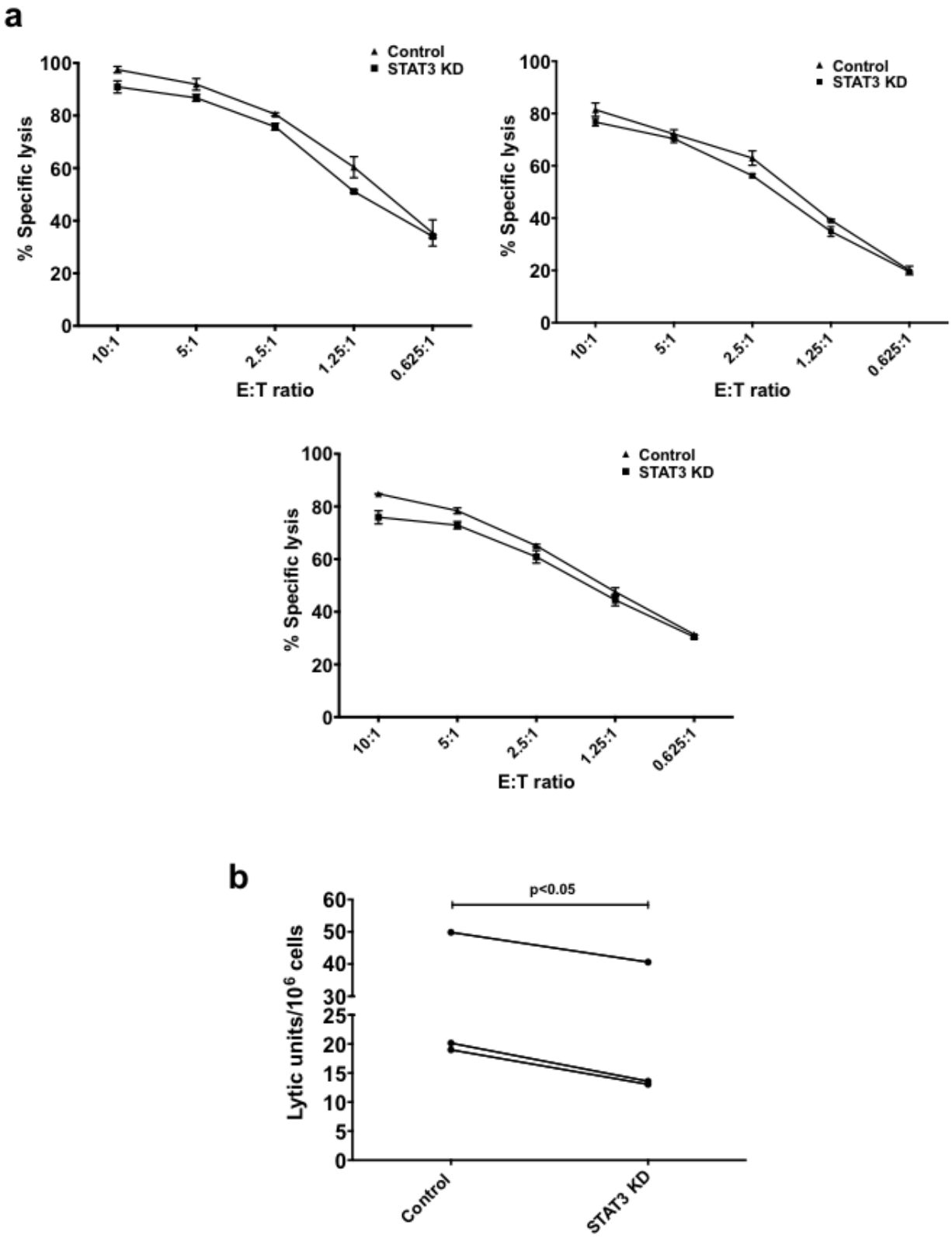
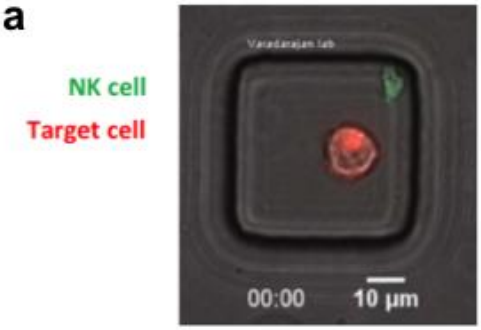

a
Control
STAT3 KD
% Specific lysis
100
80
60
40
20
0
10:1
5:1
2.5:1
1.25:1
0.625:1
E:T ratio
b
p<0.05
Lytic units/10^6 cells
60
50
40
30
25
20
15
10
5
0
Control
STAT3 KD

Figure 7. Analysis of cytotoxicity at the single cell level demonstrated cytolytic function and serial killing ability of STAT3 knock-down NK cells to be impaired.

The single-cell cytotoxicity assay was performed using fabricated nanowell arrays. STAT3 siRNA – or control siRNA- electroporated NK cells (effectors) and K562 cells (targets), labeled with green PKH67 and red PKH26, respectively, were loaded sequentially onto the nanowell array. The entire array chip was immersed in phenol red free complete medium containing AnnexinV-AlexaFluor-647. Timelapse imaging microscopy in nanowell grids (TIMING) images were taken for 8 hours at intervals of 6 minutes **(a)** Representative example of a nanowell containing a K562 cell (red, stained with PKH red) and a control NK cell (green, stained with PKHgreen). Scale bar 10 µm **(b)** Among NK cells that established at least one contact with the target cell in nanowells containing exactly 1 NK cell and 1 K562 cell (E:T -1:1), cytotoxicity of NK cell was assessed by detecting lysis of K562, identified using Annexin V staining, and by counting the number of wells in which the lysis occurred. The tabular presentation of the data shows the total number of wells, the number of wells in which the target was killed and not killed and the percentage of target cells killed for STAT3 knock-down (STAT3 KD) and control conditions. The charts beside the tables represent the same data in a graphical form **(c)** The serial killing ability of NK cells was assessed by measuring killing frequency in nanowells containing exactly 1 NK cell and 2 K562 cells (E:T-1:2) and where NK cell made at least 2 contacts with the target cells resulting in the death of both. The tabular presentation of the data shows the total number of wells, the number of wells in which the target was killed and not killed and the percentage of target cells killed for STAT3 knock-down (STAT3 KD) and control conditions. The charts beside the tables represent the same data in a graphical form. P values indicated are for Fisher's exact test used for the analysis of contingency tables.

The single cell cytotoxicity assay was performed by collaborators Drs. Gabrielle Romain and Navin Varadrajan at the Department of Chemical and Biomolecular Engineering, University of Houston, Houston, TX.

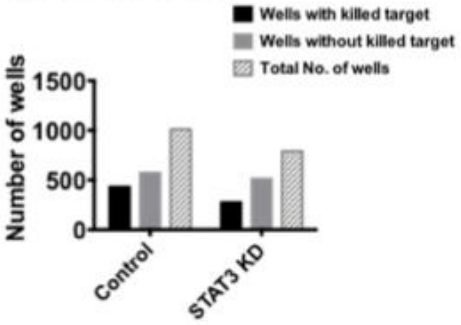
a

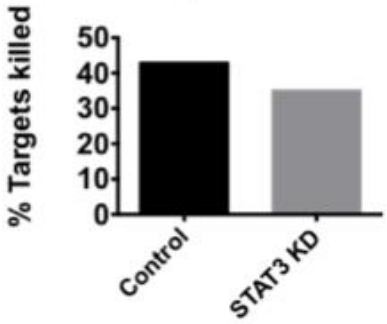


b

E:T- 1:1 one contact made between NK and target

Experimental condition	Number of wells		Total number of wells	% Targets killed
	Target killed	Target not killed		
Control	434	576	1010	42.9703
STAT3 KD	278	513	791	35.14539
p= 0.0008				

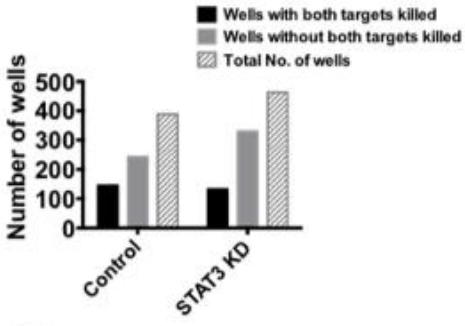


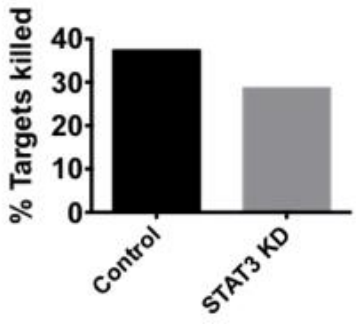


c

E:T- 1:2 Two contacts made between NK and target

Experimental condition	Number of wells		Total number of wells	% Targets killed
	Both Targets killed	Both Target not killed		
Control	145	243	388	37.37114
STAT3 KD	133	330	463	28.7257
p=0.0082				





2.3.2.1 Receptor Expression in STAT3 Knock-Down NK Cells

No difference in the expression of adhesion, activating and inhibitory receptors and maturation and activation markers was observed between STAT3 knock-down NK cells and control cells (Figure 8a-e). Unlike in Job syndrome patients' NK cells, NKG2D was normally expressed in STAT3 knock-down NK cells (Figure 8a).

2.3.2.2 Cellular Processes Involved in Cytolytic Function of STAT3 Knock-Down NK Cells

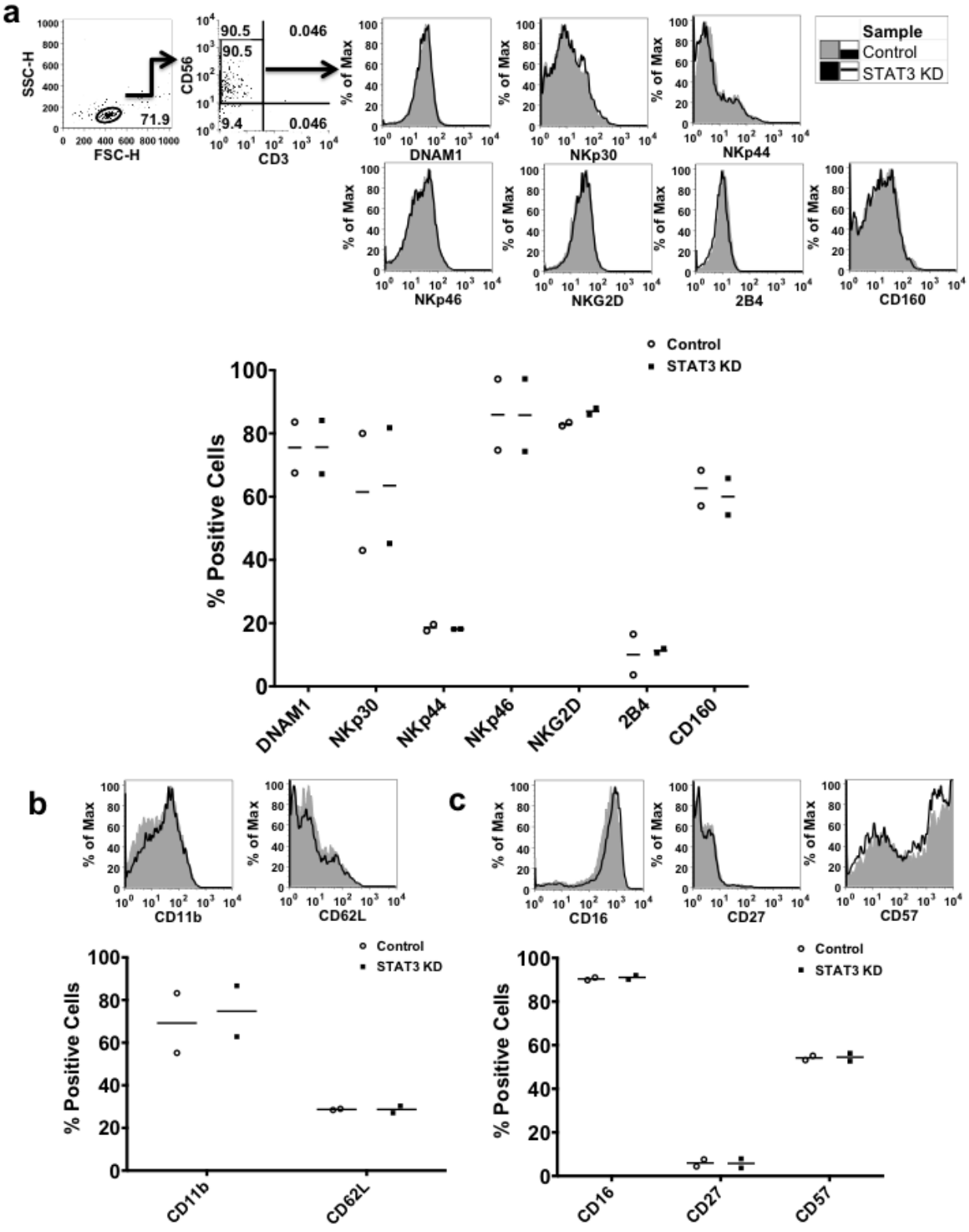
Analysis of synapse strength, granule convergence and MTOC polarization in STAT3 knock-down NK cells showed recapitulation of cellular process phenotype that was observed in Job syndrome patients' NK cells.

Synapse strength measured in terms of actin density at the synapse was found to be no different between STAT3 knock-down NK cells and control cells conjugated with K562, indicating no effect of STAT3 deficiency on synapse strength (Figure 9e and h).

Comparison of granule convergence estimated as the average of lytic granule to MTOC distance for all the granules in the cell was found to be greater in STAT3 knock-down NK cells compared to control cells. However, the difference was statistically not significant (Figure 9f and I).

MTOC polarization to the synapse assessed by measuring the distance between the MTOC and the center of the synapse was found to be significantly greater in STAT3 knock-down NK cells compared to normal donors (Figure 9g and J). This finding corroborated impaired MTOC polarization observed in NK cells from Job syndrome patients and revealed requirement of STAT3 for this cellular process.

Figure 8. No difference in the expression of receptors between control and STAT3 knock-down (STAT3 KD) NK cells. Purified human NK cells from normal donors maintained overnight with 50 U/ml IL-2 were electroporated with control or STAT3 siRNA and cultured thereafter with 50 U/ml IL-2 for 48 hours before assessment of expression of different types of receptors by flow-cytometry. In the flow-cytometric analysis, lymphocyte population was distinguished by their forward scatter and side scatter characteristics and from this gated population, NK cells were identified by gating $CD56^{+}$ $CD3^{-}$ cells **(a)**. Expression of different types of receptors; **(a)** activating, **(b)** adhesion **(c)** maturation markers **(d)** priming markers and **(e)** inhibitory was assessed and depicted as percentage of NK cells expressing the receptor. Each panel also shows representative flow charts comparing expression of the receptors in that particular category between control and STAT3 knock-down (STAT3 KD) NK cells. In each graph values for individual donor are shown along with the mean for the group.


a
SSC-H
FSC-H
71.9
CD56
CD3
90.5
0.046
90.5
9.4
0.046
% of Max
DNAM1
NKp30
NKp44
NKp46
NKG2D
2B4
CD160
Sample
Control
STAT3 KD
% Positive Cells
b
CD11b
CD62L
c
CD16
CD27
CD57

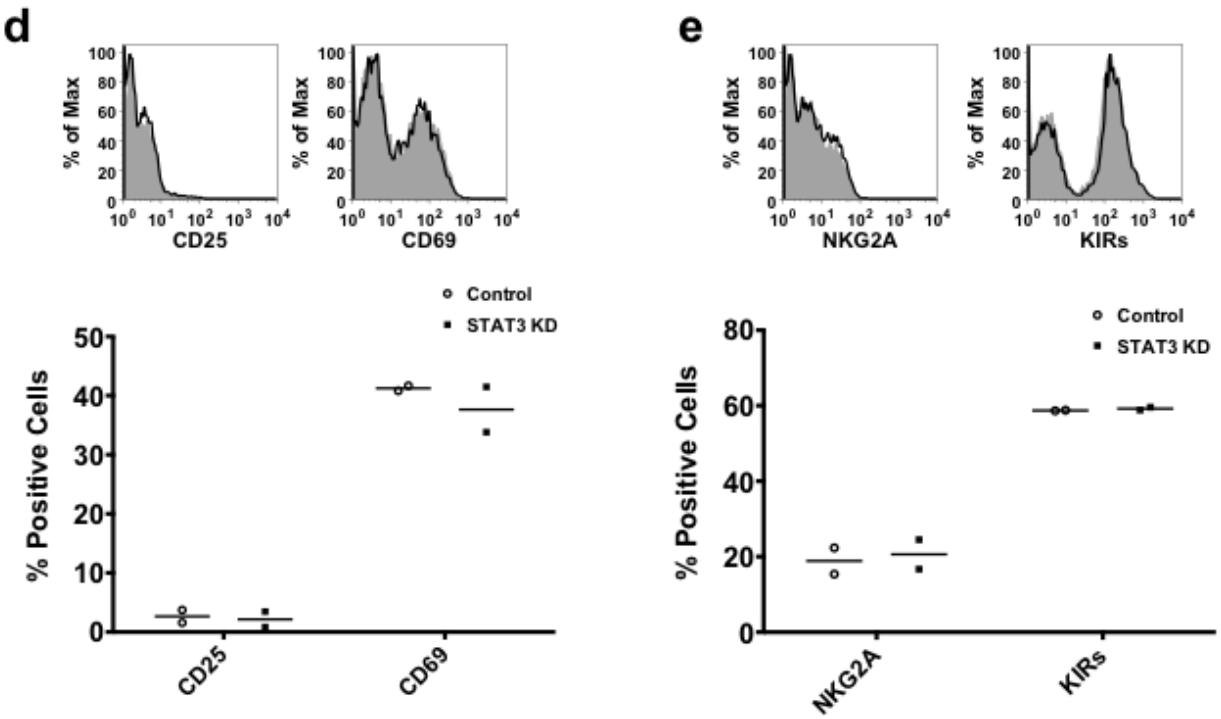
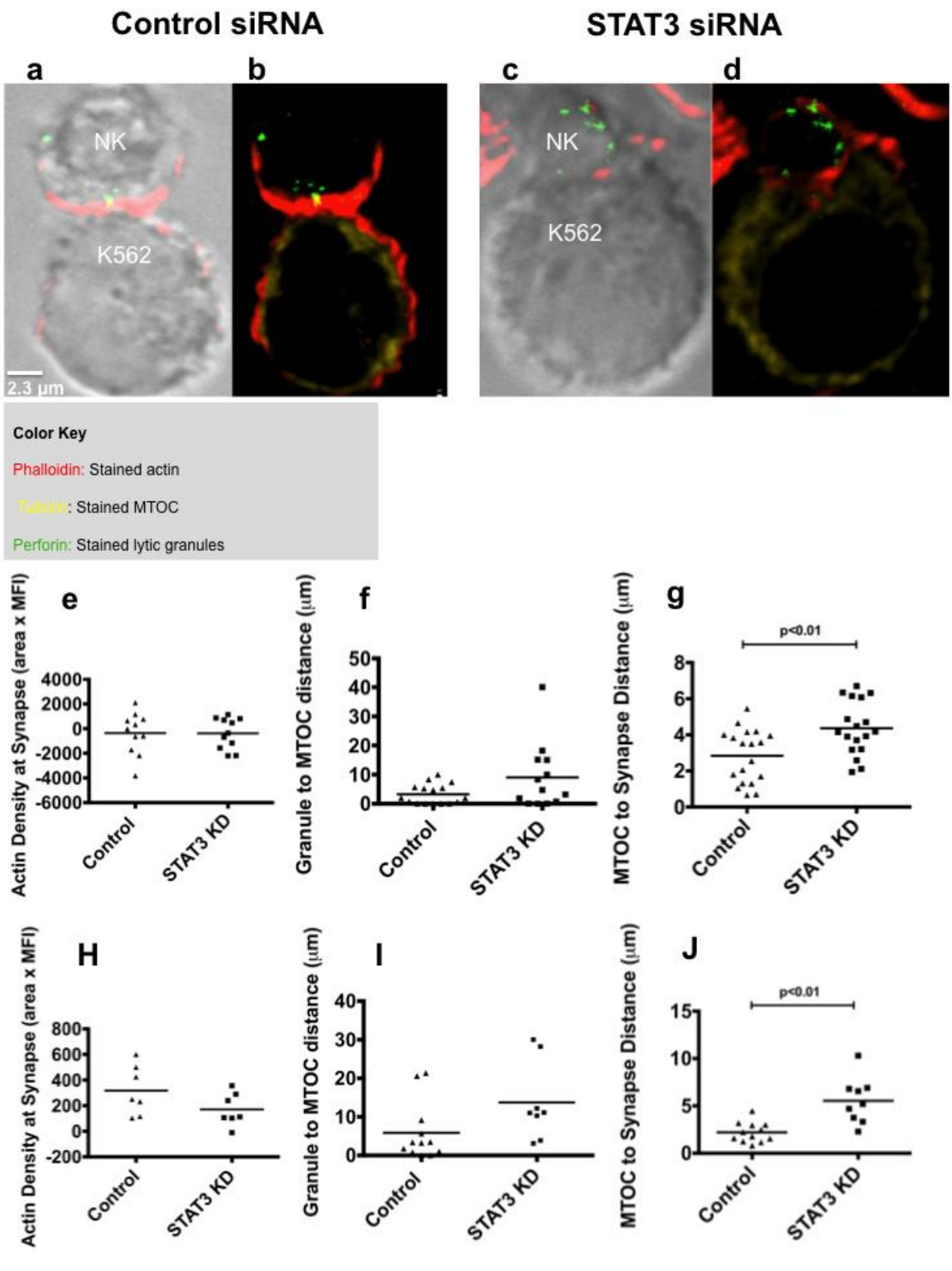

d
% of Max
CD25
CD69
Control
STAT3 KD
% Positive Cells
e
NKG2A
KIRs

Figure 9. Normal actin deposition at the synapse and granule convergence, but impaired microtubule organizing center (MTOC) polarization in STAT3 knock-down NK cells. Purified human NK cells from normal donors maintained overnight with 50 U/ml IL-2 were electroporated with control or STAT3 siRNA and cultured thereafter with 50 U/ml IL-2 for 48 hours before assessment of cellular processes involved in cytolytic function. NK and K562 cells were incubated together to facilitate conjugate formation between the two. The cells were placed on poly-L-lysine coated glass slides. The cells attached to the slide were permeabilized and stained with fluorochrome conjugated phalloidin, fluorochrome conjugated anti-perforin antibody and biotinylated anti-tubulin antibody and fluorochrome conjugated streptavidin. Phalloidin, anti-tubulin and anti-perforin were used to stain F-actin, MTOC and lytic granules, respectively. The conjugates were imaged using scanning confocal microscope. The images were analyzed using Volocity software. The experiment was repeated two times with two different donors. Representative brightfield image and its corresponding confocal image show conjugate formed between a control NK cell and K562 **(a and b)** and a STAT3 knock-down NK cell and K562 (**c and d**). In the image, NK cell was distinguished from K562 on the basis of cell size (NK smaller compared to K562) and the presence of perforin (present in NK and absent in K562). F-actin deposited at the interface of the conjugate, immune synapse, is stained with phalloidin (red). MTOC is stained with anti-tubulin (yellow) and lytic granules are stained with anti-perforin (green). **(e,h)** Synaptic strength was measured in terms of amount F-actin deposited at the interface of NK and K562. To quantify F-actin, signal intensity of phalloidin fluorophore at the interface of NK and target and the area occupied by fluorescent phalloidin at the interface were measured in confocal micrographs using Volocity software. The F-actin density at the synapse was estimated by multiplying signal

intensity of fluorescent phalloidin with the area occupied by F-actin. Synapse strength estimated by this method in conjugates is represented in graphical form. **(f,i)** Granule convergence to MTOC was defined as the shortest distance between each individual granule and MTOC in the NK cell in conjugation with K562. Lytic granules were identified by staining with anti-perforin antibody and MTOC was recognized by staining with anti-tubulin antibody. The distance between MTOC and each lytic granule in a conjugated NK cell was measured using Volocity software. Average of granule-MTOC distance for all the granules in a cell was denoted as the mean granule to MTOC distance for the cell. Granule convergence measured in conjugates is represented in graphical form. Each data point represents mean granule to MTOC distance for an individual NK cell in conjugation with K562. **(g,j)** MTOC polarization was defined as the distance between the MTOC of an NK cell, visualized by staining with anti-tubulin antibody, and the immune synapse, demarcated as the contact area between the NK and K562 in a conjugate and stained with fluorochrome conjugated phalloidin. The distance was measured by drawing a line between the MTOC and the center of immune synapse in a confocal image using Volocity software. MTOC polarization measured in conjugates is represented in graphical form.

In each graph mean for the group is denoted. P value indicated is for two-tailed Student's t test.

Confocal microscopic analysis of subcellular cytolytic processes and data processing were performed by my collaborator, Dr. Malini Mukherjee in Dr. Jordan Orange laboratory at the section of Immunology, Allergy, and Rheumatology, Baylor College of Medicine, Houston, Texas.


Control siRNA
STAT3 siRNA
a
b
c
d
NK
K562
2.3 µm
Color Key
Phalloidin: Stained actin
Tubulin: Stained MTOC
Perforin: Stained lytic granules
e
f
g
Actin Density at Synapse (area x MFI)
Granule to MTOC distance (µm)
MTOC to Synapse Distance (µm)
p<0.01
Control
STAT3 KD
H
I
J

2.3.3 Molecular Mechanism Underlying Impaired MTOC Polarization

Upon converging to the MTOC, lytic granules along with MTOC start polarizing toward the immune synapse. The molecular players involved in this mobilization have been identified. CDC42 interacting protein 4 (CIP4), is one of the essential components of the molecular machinery driving MTOC polarization. Silencing of CIP4 expression in NK cells inhibits MTOC polarization (185). Lytic granules and MTOC polarize to immune synapse by moving along microtubules with the help of the motor protein kinesin family member 5B (KIF5B). A lytic granule associated small G-protein, ADP-ribosylation factor like 8b (Arl8b), links the lytic granule to KIF5B. Silencing of Arl8b and KIF5B in NK cells impairs lytic granule and MTOC polarization to the immune synapse signifying their key role in the process (200). Another member of the MTOC polarizing machinery is IQGAP1, a multi-domain scaffolding protein that interacts with both F-actin and microtubules, whose silencing also inhibits MTOC polarization (201).

To investigate the cause for impaired MTOC polarization in STAT3 knock-down NK cells, I analyzed gene expression of Arl8b, KIF5B, CIP4 and IQGAP1 by real-time PCR. Expression of Arl8b, KIF5B and IQGAP1 was similar in STAT3 knock-down and control cells (Figure 10a-c). But the expression of CIP4 was significantly lower in STAT3 knock-down cells (Figure 10d), suggesting it as a likely cause for impaired MTOC polarization in STAT3 knock-down NK cells.

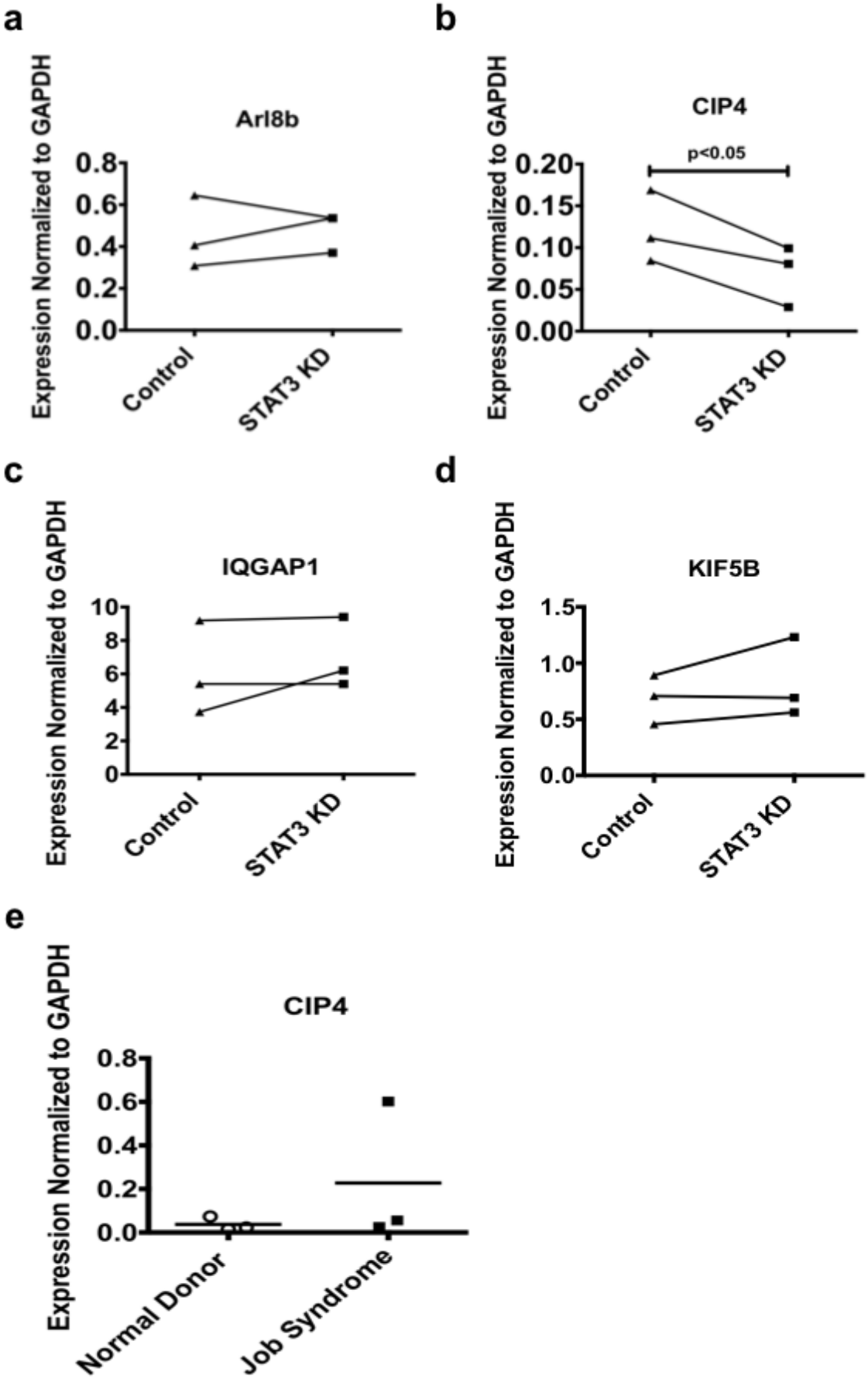
I also assessed expression of CIP4 in Job syndrome patients' NK cells. However, unlike STAT3 knock-down NK cells, CIP4 gene expression was found to be normal in Job syndrome patients' NK cells (Figure 10e). This result indicated that a

different mechanism is responsible for impaired MTOC polarization in Job syndrome patients' NK cells.

Figure 10. No difference in the gene expression of Arl8b, IQGAP1 and KIF5B, molecules involved in MTOC polarization, between control and STAT3 knock-down (STAT3 KD) NK cells, except significantly reduced gene expression of CIP4, another molecule required for MTOC polarization, in STAT3 KD NK cells, but not in NK cells from Job syndrome patients. Purified human NK cells from normal donors maintained overnight with 50 U/ml IL-2 were electroporated with control or STAT3 siRNA and cultured thereafter with 50 U/ml IL-2 for 48 hours before assessment of gene expression by real-time PCR. For real-time PCR, cDNA was synthesized using SuperScript III first strand synthesis supermix. The real-time PCR reaction was set up with cDNA and primers listed in Table 2 using IQ SYBR green supermix in iCycler real time PCR detection system. Relative gene expression was quantified using ΔC_T method with GAPDH as the housekeeping gene. Gene expression of **(a)** Arl8b **(b)** CIP4 **(c)** IQGAP1 and **(d)** KI5FB was measured. **(e)** CIP4 gene expression was also evaluated in NK cells from normal donors and Job syndrome patients.

In each graph values for individual donor are shown along with the mean for the group. P value indicated is for two-tailed paired Student's t test.

Real-time PCR



2.3.3.1 mbIL21 Expanded Job Syndrome Patients' NK Cells

Previous work in our lab demonstrated robust expansion of human NK cells stimulated with K562 genetically modified to present membrane bound IL-21 (mbIL21). These expanded cells had high expression of activating receptors and enhanced cytolytic activity (134). IL-21 predominantly activates STAT3 in NK cells (80, 136, 171) and as expected mbIL21 induced expansion and activation of NK cells is mediated through STAT3 signaling (202). Job syndrome patients have residual STAT3 activity, which is 25% of the normal (131). To explore whether stimulation of the residual STAT3 activity by mbIL21 improves deficient NKG2D receptor expression, MTOC polarization and cytotoxicity of Job syndrome patients' NK cells, I expanded NK cells from PBMCs of normal donors and Job syndrome patients, by stimulating them with mbIL21 over three weeks. After three weeks of expansion, T cells were depleted to obtain pure NK cell population.

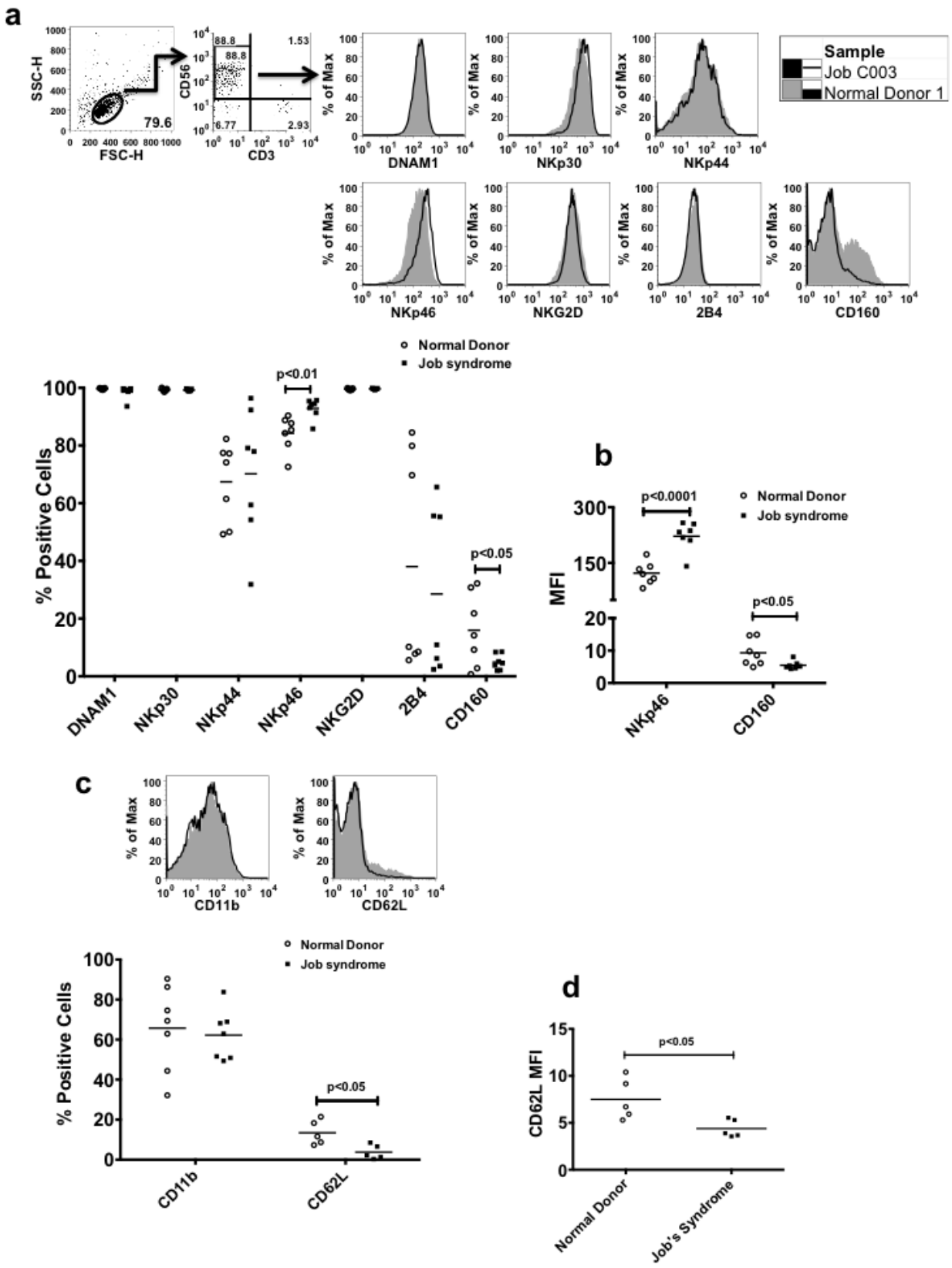
2.3.3.2 Receptor Expression in mbIL21 Expanded NK Cells

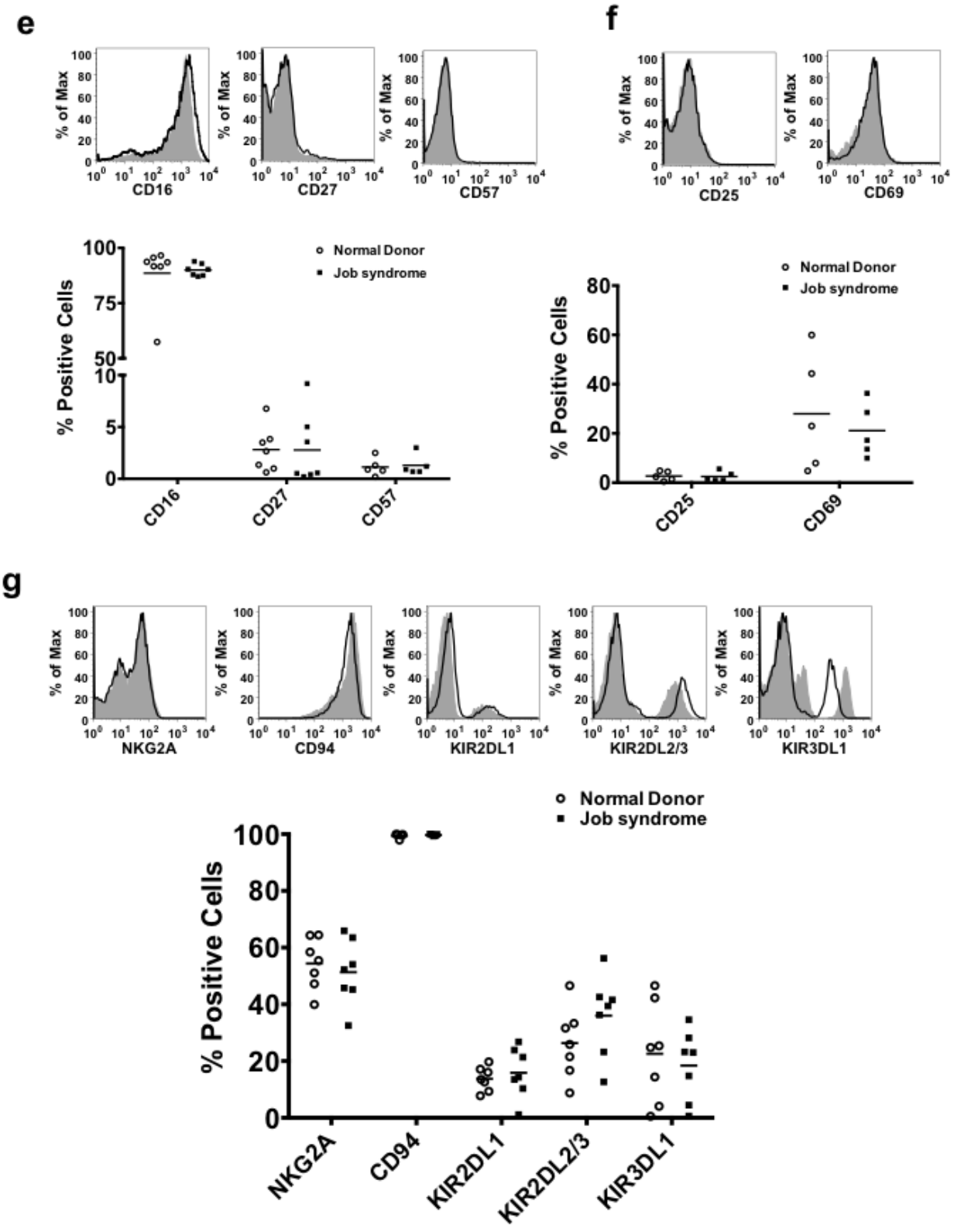
mbIL21 expanded NK cells showed increased expression of all the activating receptors analyzed except CD160 over basal expression. CD160 expression decreased after expansion. Deficient basal expression of NKG2D in Job syndrome patients' NK cells was restored in mbIL21 expanded NK cells. Almost entire NK cell population was NKG2D positive and no difference in expression was observed between NK cells from Job syndrome patients and normal donors. Expression levels of other activating receptors were also found to be similar between NK cells from Job syndrome patients and normal donors, except NKp46 and CD160 (Figure 11a). NKp46

expression was significantly higher in Job syndrome patients' NK cells, while CD160 expression was significantly higher in NK cells from normal donors (Figure 11a-b). Compared to basal expression levels, expression of adhesion receptors, CD11b and CD62L, decreased in mbIL21 expanded NK cells. No difference in the expression of CD11b was found between NK cells from Job syndrome patients and normal donors, but CD62L expression was significantly higher in NK cells from normal donors (Figure 11c-d). Among maturation markers, compared to basal expression levels, CD16 expression showed increased, while CD27 and CD57 expression decreased in mbIL21 expanded NK cells. No difference in expression was observed between NK cells from Job syndrome patients and normal donors (Figure 11e). Expression of activation marker CD25 did not change upon expansion, while CD69 expression increased over the basal levels. No difference in expression was observed between NK cells from Job syndrome patients and normal donors (Figure 11f). Expression of all the inhibitory receptors assessed increased in mbIL21 expanded NK cells and similar expression levels were observed in NK cells from Job syndrome patients and normal donors (Figure 11g).

Figure 11. Job syndrome NK cells expanded with mbIL21 stimulation had enhanced expression of NKG2D receptor, which was similar to the expression of NKG2D in mbIL21 expanded NK cells from normal donors. The level of expression of other receptors in mbIL21 expanded NK cells was also similar between Job syndrome and normal donors, except NKp46, whose expression was higher in the former and CD160 and CD62L, whose expression was higher in the latter. NK cells were expanded from PBMCs stimulated with K562s, genetically modified to present membrane bound IL-21 (mbIL21), over a three week period. Receptor expression was measured by flow-cytometry. In the flow-cytometric analysis, lymphocyte population was distinguished by their forward scatter and side scatter characteristics and from this gated population, NK cells were identified by gating $CD56^{+}$ $CD3^{-}$ cells **(a)**. Expression of different types of receptors; **(a)** activating, **(c)** adhesion **(e)** maturation markers **(f)** priming markers and **(g)** inhibitory was assessed and depicted as percentage of NK cells expressing the receptor. Each panel also shows representative flow charts comparing expression of the receptors in that particular category between a normal donor and Job syndrome patient. Panel **b** depicts receptor density per cell, shown as mean fluorescence intensity (MFI), of NKp46 and CD160. Panel **d** portrays MFI of CD62L.

In each graph values for individual donor are shown along with the mean for the group. P values indicated are for two-tailed Student's t test.


a
SSC-H
FSC-H
79.6
CD56
CD3
88.8
88.8
1.53
6.77
2.93
% of Max
DNAM1
NKp30
NKp44
NKp46
NKG2D
2B4
CD160
Sample
Job C003
Normal Donor 1
Normal Donor
Job syndrome
% Positive Cells
p<0.01
p<0.05
b
MFI
p<0.0001
p<0.05
c
CD11b
CD62L
d
CD62L MFI
Normal Donor
Job's Syndrome


e
f
% of Max
CD16
CD27
CD57
CD25
CD69
Normal Donor
Job syndrome
% Positive Cells
g
NKG2A
CD94
KIR2DL1
KIR2DL2/3
KIR3DL1

2.3.3.3 Cellular Processes Involved In Cytolytic Function of mbIL21 Expanded NK Cells

Comparison between mbIL21 expanded NK cells from Job syndrome patients and normal donors, pooled from three donors each, showed no difference in the synaptic strength and granule convergence (Figure 12e-f). Impaired MTOC polarization observed in un-stimulated Job syndrome patients' NK cells were corrected in the mbIL21-expanded cells. The MTOC to synapse distance was similar in NK cells from Job syndrome patients and normal donors after mbIL21 expansion (Figure 12g).

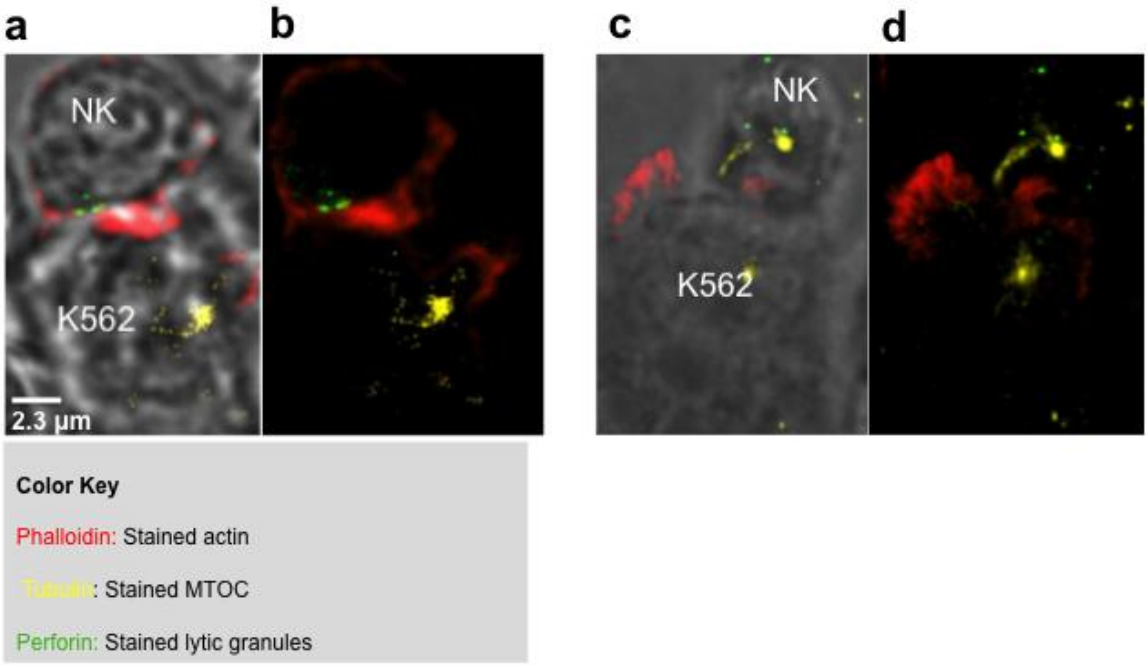
2.3.3.4 Cytotoxicity of mbIL21 Expanded NK Cells

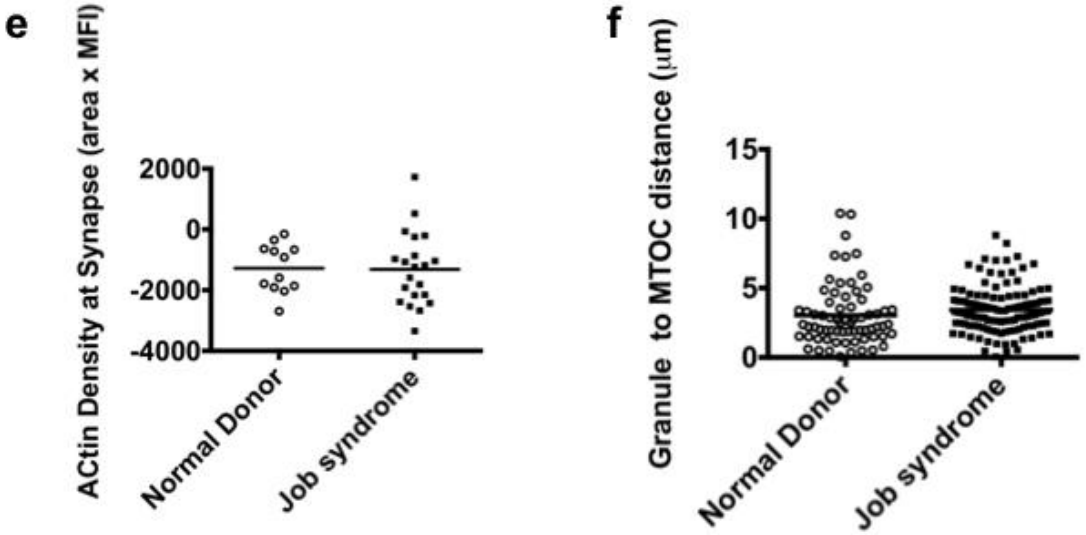
mbIL21 expanded NK cells from Job syndrome patients and normal donors showed enhanced cytotoxicity. The cytotoxicity of NK cells from normal donors was marginally higher than the NK cells from Job syndrome patients (Figure 13a). NK cells from normal donors had marginally higher number of lytic units than NK cell from Job syndrome patients, but the difference was statistically not significant (Figure 13b).

Figure 12. Impaired MTOC polarization observed in un-stimulated Job syndrome patients' NK cells was corrected in the mbIL21 expanded cells. The MTOC to synapse distance was similar in NK cells from Job syndrome patients and normal donors after mbIL21 expansion. NK cells were expanded from PBMCs stimulated with K562s, genetically modified to present membrane bound IL-21 (mbIL21), over a three week period. NK and K562 cells were incubated together to facilitate conjugate formation between the two. The cells were placed on poly-L-lysine coated glass slides. The cells attached to the slide were permeabilized and stained with fluorochrome conjugated phalloidin, fluorochrome conjugated anti-perforin antibody and biotinylated anti-tubulin antibody and fluorochrome conjugated streptavidin. Phalloidin, anti-tubulin and anti-perforin were used to stain F-actin, MTOC and lytic granules, respectively. The conjugates were imaged using scanning confocal microscope. The images were analyzed using Volocity software. Representative brightfield image and its corresponding confocal image show conjugate formed between an NK cell from normal donor and K562 (a and b) and an NK cell from Job syndrome patient and K562 (c and d). In the image, NK cell was distinguished from K562 on the basis of cell size (NK smaller compared to K562) and the presence of perforin (present in NK and absent in K562). F-actin deposited at the interface of the conjugate, immune synapse, is stained with phalloidin (red). MTOC is stained with anti-tubulin (yellow) and lytic granules are stained with anti-perforin (green). (e) Synaptic strength was measured in terms of amount F-actin deposited at the interface of NK and K562. To quantify F-actin, signal intensity of phalloidin fluorophore at the interface of NK and target and the area occupied by fluorescent phalloidin at the interface were measured in confocal micrographs using Volocity software. The F-actin density at the synapse was estimated by multiplying signal intensity of fluorescent phalloidin with the area occupied by F-

actin. Synapse strength estimated by this method in conjugates, pooled from three donors each, of normal donors and Job syndrome patients is represented in graphical form. (f) Granule convergence to MTOC was defined as the shortest distance between each individual granule and MTOC in the NK cell in conjugation with K562. Lytic granules were identified by staining with anti-perforin antibody and MTOC was recognized by staining with anti-tubulin antibody. The distance between MTOC and each lytic granule in a conjugated NK cell was measured using Volocity software. Average of granule-MTOC distance for all the granules in a cell was denoted as the mean granule to MTOC distance for the cell. Granule convergence measured in conjugates, pooled from three donors each, of normal donors and Job syndrome patients is represented in graphical form. Each data point represents mean granule to MTOC distance for an individual NK cell in conjugation with K562. (g) MTOC polarization was defined as the distance between the MTOC of an NK cell, visualized by staining with anti-tubulin antibody, and the immune synapse, demarcated as the contact area between the NK and K562 in a conjugate and stained with fluorochrome conjugated phalloidin. The distance was measured by drawing a line between the MTOC and the center of immune synapse in a confocal image using Volocity software. MTOC polarization measured in conjugates, pooled from three donors each, of normal donors and Job syndrome patients is represented in graphical form.

Confocal microscopic analysis of subcellular cytolytic processes and data processing were performed by my collaborator, Dr. Malini Mukherjee in Dr. Jordan Orange laboratory at the section of Immunology, Allergy, and Rheumatology, Baylor College of Medicine, Houston, Texas.


a
b
c
d
NK
K562
2.3 µm
NK
K562
Color Key
Phalloidin: Stained actin
Tubulin: Stained MTOC
Perforin: Stained lytic granules


e
ACtin Density at Synapse (area x MFI)
2000
0
-2000
-4000
Normal Donor
Job syndrome
f
Granule to MTOC distance (µm)
15
10
5
0
Normal Donor
Job syndrome

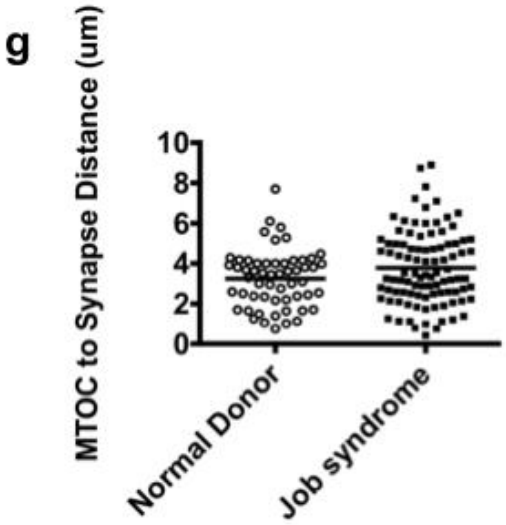
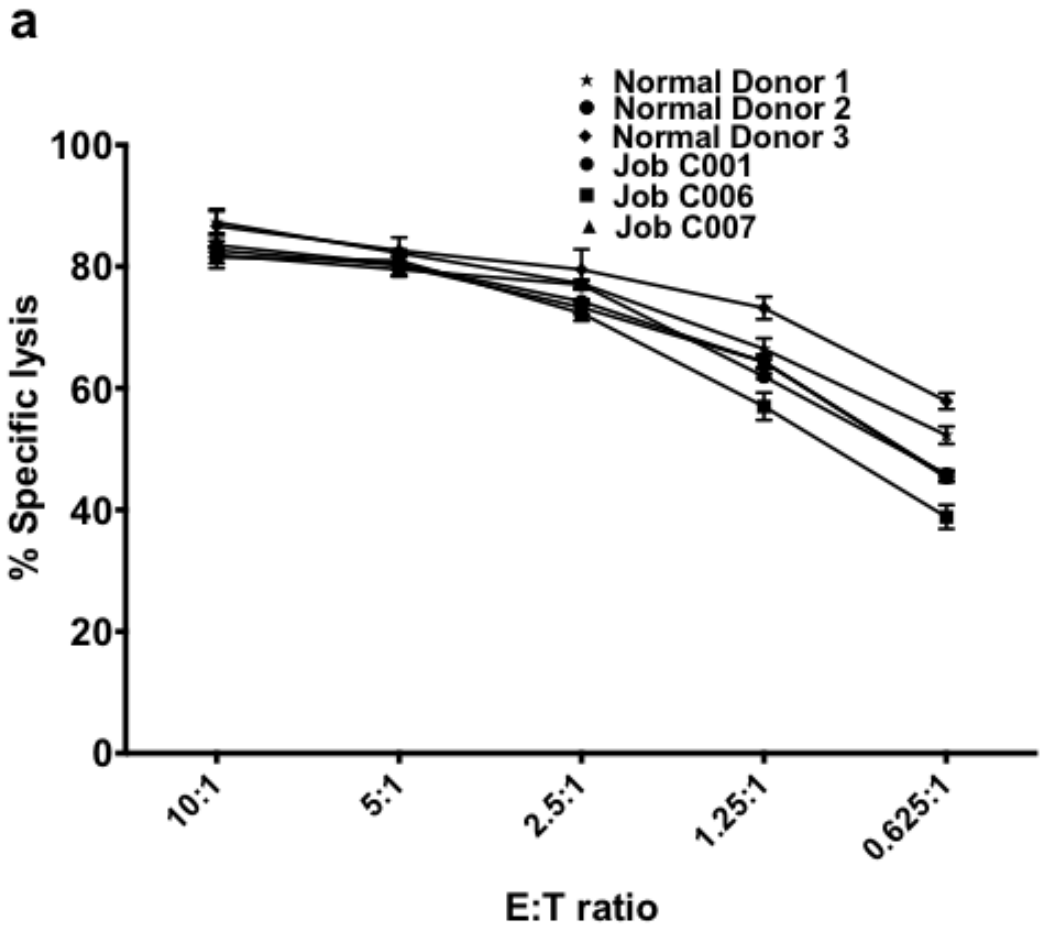
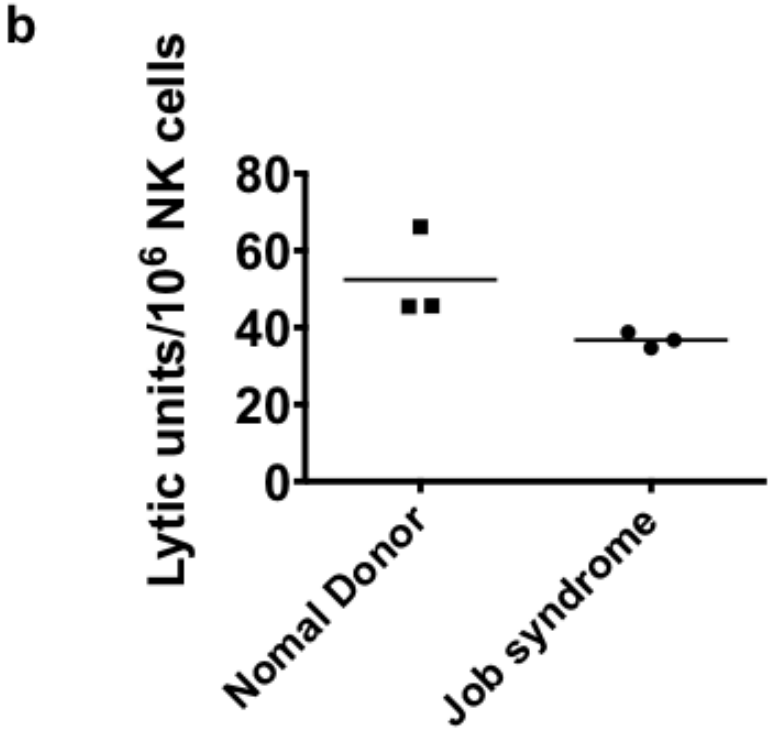

g
MTOC to Synapse Distance (um)
10
8
6
4
2
0
Normal Donor
Job syndrome

Figure 13. Job syndrome NK cells expanded with mbIL21 stimulation had enhanced cytotoxicity, which was marginally lower than the cytotoxicity of mbIL21 expanded NK cells from normal donors (a) NK cells were expanded from PBMCs stimulated with K562s, genetically modified to present membrane bound IL-21 (mbIL21), over a three week period. The expanded cells were CD3 depleted. Cytotoxicity was assessed using calcein assay. Assay for each donor was performed in triplicates for each E:T ratio. Data is presented as mean ± standard deviation (SD) for each E:T ratio for each donor. **(b)** Lytic unit, defined as the number of NK cells required to kill a specified percentage of target cells, was determined from the cytotoxicity curve of each individual sample obtained in the calcein assay. A specific percentage of target cell lysis was selected based on the criterion that the standard curves of all the samples in the relevant data set crossed the reference percentage exactly once. Based on this criterion, number of NK cells required to kill 75% of target cells were defined as one lytic unit for the comparison between control and STAT3 knock-down (STAT3 KD). The E:T ratio corresponding to the reference percentage was interpolated from the standard curve. The number of NK cells in the lytic unit were obtained by multiplying the interpolated E:T ratio by 10000 (the number of target cells in each well). Lytic units per one million NK cells were obtained according to the formula (1000,000/number of NK cells in one lytic unit). In the graph lytic unit of each donor is shown along with the mean for the group.


a
Normal Donor 1
Normal Donor 2
Normal Donor 3
Job C001
Job C006
Job C007
% Specific lysis
100
80
60
40
20
0
10:1
5:1
2.5:1
1.25:1
0.625:1
E:T ratio

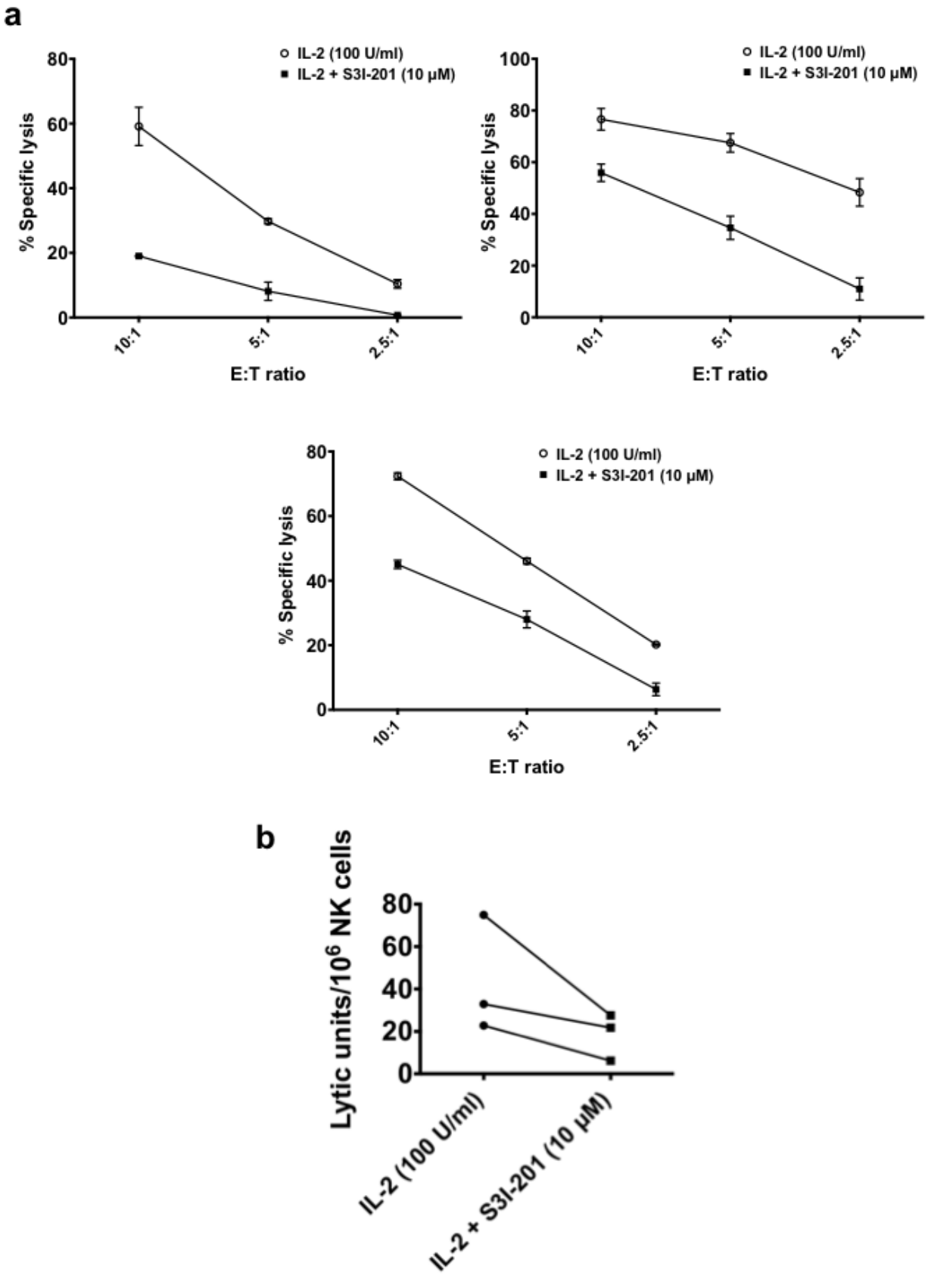

b
Lytic units/10^6 NK cells
80
60
40
20
0
Nomal Donor
Job syndrome

2.3.4 Pharmacological Inhibition of STAT3 Inhibits NK Cytotoxicity

Constitutively active STAT3 has been shown to be oncogenic in multiple cancers and as a result, STAT3 inhibitors have been tested in pre-clinical and clinical studies against various types of cancers (140). NK cells due to their inherent anti-cancer activity are also fast emerging as a candidate cell for immunotherapy against multiple types of cancer (35). The anti-tumor activity of several cytokines is also mediated by NK cells (8). As cancer treatment many a times involves combination of different treatment modalities, it is imperative to determine the compatibility of different modes of treatments with each other. To determine the effect of pharmacological STAT3 inhibition on NK cytolytic function, I assessed cytotoxicity of NK cells treated with STAT3 inhibitor S3I-201. It binds SH2 domain of STAT3 to block dimerization and thereby inhibits DNA binding and transcriptional activity of STAT3 (203). It has been shown to inhibit tumor growth in pre-clinical studies (198, 203).

I evaluated cytolytic activity of primary human NK cells from normal donors treated overnight with and without 10 μM S3I-201, a concentration shown not to affect viability of NK cells in a previous study from our lab (136). Along with the drug treatment, the cells were also activated overnight with 100 U/ml IL-2, a concentration commonly used to activate cytolytic function of NK cells (204-206). Comparison between S3I-201 treated and non- treated NK cells showed a substantial decrease in cytotoxicity in S3I-201 treated NK cells against K562 at all the E:T ratios tested (Figure 14a). S3I-201 treated NK cells had reduced number of lytic units (Figure 14b). The results show that pharmacological inhibition STAT3 adversely affects NK cytolytic function.

Figure 14. **Pharmacological inhibition of STAT3 reduces cytolytic function of human NK cells (a)** Purified human NK cells from normal donors were treated overnight with IL-2 in the presence and absence of small molecule STAT3 inhibitor S3I-201. Control cells were treated with the solvent for the drug DMSO. Cytotoxicity against K562 was assessed using calcein assay. Assay for each donor was performed in triplicates for each E:T ratio. Data is presented as mean ± standard deviation (SD) for each E:T ratio for each donor. The experiment was repeated three times with three different donors. **(b)** Lytic unit, defined as the number of NK cells required to kill a specified percentage of target cells, was determined from the cytotoxicity curve of each individual sample obtained in the calcein assay. A specific percentage of target cell lysis was selected based on the criterion that the standard curves of all the samples in the relevant data set crossed the reference percentage exactly once. Based on this criterion, number of NK cells required to kill 25% of target cells were defined as one lytic unit for the comparison between control and STAT3 knock-down (STAT3 KD). The E:T ratio corresponding to the reference percentage was interpolated from the standard curve. The number of NK cells in the lytic unit were obtained by multiplying the interpolated E:T ratio by 10000 (the number of target cells in each well). Lytic units per one million NK cells were obtained according to the formula (1000,000/number of NK cells in one lytic unit). In the graph lytic unit of each donor is shown along with the mean for the group.


a
IL-2 (100 U/ml)
IL-2 + S3I-201 (10 μM)
% Specific lysis
E:T ratio
10:1
5:1
2.5:1
b
Lytic units/10^6 NK cells
IL-2 (100 U/ml)
IL-2 + S3I-201 (10 μM)

2.4 *DISCUSSION*

In this study, I showed that Job syndrome patients' NK cells with dominant negative STAT3 mutations have impaired cytolytic function, NKG2D receptor expression, and MTOC polarization. I confirmed these findings in STAT3 knock-down primary human NK cell, which also showed deficient cytolytic function and MTOC polarization. I further showed that membrane bound IL-21 stimulation of Job syndrome patients' NK cells with dominant negative STAT3 mutations enhances NK cytotoxicity, restores NKG2D receptor expression to normal levels and corrects defective MTOC polarization. Finally, I showed that pharmacological inhibition of STAT3 reduces NK cytolytic function.

Cytokine signaling drives differentiation, homeostasis, and functional maturation of NK cells (207). As cytokine signals are pre-dominantly mediated through activation of members of STAT family, multiple STATs play a critical role in the regulation of basal as well as induced cytotoxicity of NK cells. IL-2 and IL-15, cytokines belonging to common γ chain family, are implicated in the differentiation, proliferation, survival and functional activation of NK cells (58). Both cytokines mainly activate STAT5 (104) and this signaling preference is reflected in the diminished basal as well as IL-2 and IL-15 induced cytolytic activity of NK cells from STAT5b deficient mice (162) and loss of anti-tumor activity in STAT5 deficient mice NK cells (208). The other STAT that has been shown to be important for NK cytolytic function is STAT1. Anti-viral response of NK cells is activated by IFN α/β (38, 86), which predominantly mediate their signals through STAT1 activation (173, 209). IFN α activates cytolytic activity of NK cells through STAT1 signaling (161, 165). The dependence on STAT1 signaling is manifested in the reduced NK cytotoxicity during viral infection in STAT1 deficient mice

(68, 163). STAT1 signaling also plays a role in NK cell development by enabling functional maturation (210). As a result, not only induced, but also basal NK cytotoxicity decreases in STAT1 deficient mice (164, 165). In humans too, STAT1 deficiency results in diminished basal as well as induced NK cytotoxicity (166). IL-12, a potent activator of NK cytolytic activity (211-214), predominantly stimulates STAT4 (173). Even though STAT4 is not known to influence basal NK cytolytic function, in STAT4 deficient mouse, NK cells fail to augment cytotoxicity in response to IL-12 (167, 168). Compared to these STATs, very little is known about the function of STAT3 signaling in NK cytolytic activity, especially in human NK cells, and as a result its role is not well defined.

Monogenic immune deficiencies have helped delineate the role of number of biomolecules in the immune system. Immune disorders caused by genetic deficiencies of different components of JAK-STAT signaling pathway are instrumental in expanding our understanding of the function of the individual members in the biology of multiple types of immune cells (97, 102, 137). Investigations into the pathogenesis of immunological dysfunctions in Job syndrome patients have revealed the role of STAT3 in the development and function of immune cells of lymphoid origin, Th17, T follicular helper cells, T regulatory cells, central memory T cells, memory B cells, and antigen specific plasma cells (147-153), as well as of myeloid origin, tolerogenic dendritic cells and mast cells (150, 154). I took advantage of nature's own genetic experiment to study the role of STAT3 in NK effector function. The cytolytic functional defect reported in this study is the first NK cell deficiency reported in this immune disorder carrying dominant negative STAT3 mutations. Due to the paucity of PBMCs from Job syndrome patients, instead of using purified NK cells, the cytotoxicity assays in this study were performed with PBMCs. As other types of immune cells in the PBMCs could influence

cytotoxicity of NK cells, as has been demonstrated by the inhibition of NK cytotoxicity by T regulatory cells (215, 216), it was difficult to infer whether the diminished cytotoxicity is due to NK cell intrinsic STAT3 deficiency or indirect effect of STAT3 deficiency in other immune cells. Thus, to confirm whether the effect is due to NK cell intrinsic STAT3 deficiency and to further validate the results obtained with Job syndrome patients' NK cells, I evaluated cytotoxicity of STAT3 knock-down primary NK cells from normal human donors. The diminished cytolytic function observed in these cells unequivocally showed that NK cell intrinsic STAT3 deficiency impairs cytotoxicity. The degree of inhibition of cytotoxicity observed in STAT3 knock-down NK cells was not to the extent as was observed in Job syndrome patients' NK cells. There could be two plausible reasons for the dissimilar decrease observed; the different types of STAT3 deficiencies in the two genetic models and different culture conditions. The presence of dominant negative STAT3 allele in Job syndrome patients' NK cells renders these cells chronically deficient in STAT3 signaling, while siRNA mediated STAT3 knock-down creates an acute STAT3 deficiency. The ability to affect the cytolytic ability in a fundamental way such as impairment of functional maturity during development is possible in the former, but not in the latter case. The other reason could be the presence of 50 U/ml IL-2 in the culture medium of STAT3 knock-down NK cells. As nucleofection process puts cells under stress and decreases cell viability (Weaver, 1993), to increase the chances of cell survival cytokine support was provided to the siRNA nucleofected cells 24 hrs before and 48 hrs after nucleofection i.e. throughout the culture period. The Job syndrome patients' PBMCs on the other hand were not stimulated with any cytokine before the assessment of basal cytotoxicity. IL-2 is a potent activator of NK cells. Along with promoting cell survival and proliferation, it stimulates NK cytotoxicity (217-219). Though, IL-2 activates STAT3 in NK cells (80,

169, 173, 206, 220), the pre-dominant STAT activated by IL-2 is STAT5 (80, 173) and it activates NK cytotoxicity mainly through STAT5 signaling (162). As STAT5 expression is intact in STAT3 knock-down NK cells, IL-2 activated STAT5 may have alleviated the adverse effect of STAT3 deficiency on NK cytotoxicity.

Yet another reason that could explain the smaller difference in cytotoxicity between STAT3 knock-down and control NK cells is the possibility of cells with intact STAT3 in STAT3 knock-down NK population. In bulk electroporation, as was performed in the present study to knock-down STAT3 in primary human NK cells, all the cells may not be electroporated, leaving some cells without knock-down of the target mRNA. These cells, electroporated in bulk, if used in a bulk assay such as the calcein assay, employed in the present study to assess the cytotoxicity of NK cells, then the cells with intact STAT3, even if few, could skew the output. The possibility of existence of cells with intact STAT3 in STAT3 knock-down bulk population may explain the difference between the cytotoxicity of control cells and STAT3 knock-down cells being smaller as these cells with intact killing capacity through encounters with multiple target cells may compensate for the cytolytic deficiency of rest of the cells. To overcome this limitation of calcein assay, cytolytic activity of STAT3 knock-down NK cells was assessed at the single cell level, which also revealed lower frequency of killing in STAT3 knock-down NK cells compared to control cells.

This is the first study to report that STAT3 deficiency causes impaired cytotoxicity in primary human NK cells. Very few studies have investigated the role of STAT3 in the cytolytic function of human NK cells. Similar to my results, silencing of STAT3 expression in NK92 cells, a human NK cell line, using siRNA has been shown to reduce cytotoxicity, while silencing of suppressor of cytokine signaling 3 (SOCS3), a negative regulator of STAT3 signaling (221), increased NK92 cytotoxicity against K562

(222). Pharmacological inhibition of STAT3 in primary human NK cells has also been shown to decrease basal cytotoxicity (223). However, the evidence presented in this particular study is not very conclusive due to the type of STAT3 inhibitor used. Iannello et al. (223) used cucurbitacin or JSI-124 to block STAT3. Even though JSI-124 is used as a STAT3 specific inhibitor (224, 225), along with STAT3, it also inhibits activation of JAK2 and JAK3 and thus actually is a JAK-STAT inhibitor (226, 227). In fact, JSI-124 blocks activation of STAT5 (228) at a concentration well below the one used by Iannello et al. (10 μM) (223). As STAT5 deficiency impairs basal NK cytotoxicity (162), the effect of JSI-124 could have been mediated through STAT5 inhibition. The only other study to implicate STAT3 in human NK cell cytotoxicity reported a decrease in IL-2 induced STAT3 activation and a concurrent reduction in cytotoxicity of primary human NK cells treated with immunosuppressant FK506 (170). Though the evidence implicating STAT3 in NK cytotoxicity was not direct, the fact that only STAT3 activation was inhibited, while IL-2 induced STAT1 and STAT5 activation remained unaffected in FK506 treated NK cells suggested requirement of STAT3 activation in IL-2 induced human NK cell cytotoxicity (170).

Contrary to human NK cells, STAT3 deficiency in mouse NK cells either enhances cytolytic function or does not have any effect. Ablation of STAT3 in hematopoietic cells of mice did not affect NK cell cytolytic activity in tumor free mice, but in tumor bearing mice, STAT3 ablation increased cytolytic activity compared to wild type (229). This observation suggests that STAT3 deficiency by itself may not impart any beneficiary effect on NK cell cytotoxicity, but in the context of tumor microenvironment, it does in mouse NK cells. STAT3 deficiency in mouse NK cells does not seem to have an uniform effect on the cytotoxicity, but is rather influenced by the type of target cells as it is apparent from a study in which NK cell specific deletion

of STAT3 did not show any effect on cytotoxicity assessed *in vitro* against different targets such RMA, RMA-Rae1, YAC-1 and v-abl cell lines, but increased cytotoxicity against a melanoma cell line B16F10 (230). This increase was due to the high expression level of ligands for DNAM-1 receptor on the target cell and enhanced expression of the receptor on STAT3 deficient NK cells (230). Interestingly, no difference was observed in the cytotoxicity of STAT3 deficient and wild type NK cells against v-abl cells in assays performed *in vitro*, but *in vivo*, STAT3 deficient cells displayed enhanced anti-tumor activity against the same cells (230). The different outcomes in *in vitro* and *in vivo* settings indicate that cell extrinsic factors play a critical role in determining the effect of STAT3 deficiency on mouse NK cytotoxicity. Even though STAT3 deficiency does not always equate to an increase in mouse NK cytotoxicity, it does so depending upon the target and the microenvironment in which NK interacts with the target, whereas in human NK cells, reported results so far associate STAT3 deficiency with impaired cytotoxicity. As there are similarities between mouse and human immune systems so are the differences (231). With respect to NK cells, the differences are highlighted by the structurally divergent families of inhibitory receptors, KIRs in human and Ly49s in mouse, different types of ligands for human and mouse NKG2D receptor (231), and difference in the function of 2B4 receptor, mainly activating in human and inhibitory in mouse, due to the coupling with divergent types of intracellular signaling molecules (232). Cytolytic activity of NK cells is a result of the balance between the activation of inhibitory and activating receptors expressed on NK cell surface (8) and as a result the tumor model used to assess cytolytic activity may play a major role in the outcome of NK cell activity, particularly with respect to the modulation of specific activating or inhibitory ligands. The difference in the structure, function, and ligands for some of the receptors between mouse and human NK cells

underscores the caution that should be exercised while applying lessons learned from mouse to humans. It may also be possible that STAT3 deficiency may affect expression of receptors and intracellular signaling molecules differently in mouse and human NK cells.

Cytolytic activity of NK cells is the net result of the balance between the strengths of signals mediated by activating and inhibitory receptors (8). To investigate the cause of impaired cytotoxicity in STAT3 deficient human NK cells, I assessed receptor expression. Out of all the activating, inhibitory, adhesion receptors and activation and maturation markers measured, expression of NKG2D receptor was found to be different between Job syndrome patients' NK cells and normal donors' NK cells. Previously, we reported transcriptional activation of NKG2D by STAT3 (136) and consistent with earlier finding NKG2D receptor expression was found to be impaired in NK cells carrying dominant negative STAT3 mutations. The decrease in Job syndrome patients' NK cell cytolytic activity against K562 could be attributed to the impaired NKG2D expression as NK cells lyse K562 pre-dominantly in a NKG2D dependent manner (233, 234). Despite of STAT3 deficiency NKG2D receptor expression was found to be normal in STAT3 knock-down NK cells. The normal expression may be attributed to IL-2 present in the culture medium. IL-2 modulates NKG2D expression at post-transcriptional level by increasing transcription and translation of DAP10, the adaptor protein NKG2D associates with to mediate signal. Pairing with DAP10 is required for NKG2D receptor expression and IL-2 induces NKG2D expression by enhancing availability of DAP10 (89). The normal expression of NKG2D may also explain the smaller decrease in cytotoxicity observed in STAT3 knock-down NK cells compared to the drastic reduction observed in Job syndrome patients' NK cells. The only other receptor that has been shown to be modulated by STAT3 is DNAM-1, which

showed up-regulated expression in STAT3 deficient mouse NK cells (230). No such change in the expression of DNAM-1 was observed in Job syndrome patients' NK cells as well as in STAT3 knock-down human NK cells, again emphasizing the difference between mouse and human NK cells.

Signaling cascades initiated by activating receptors enable synthesis and deposition of F-actin at the NK-target interface, convergence of lytic granules dispersed throughout the NK cell to MTOC and polarization of granules and MTOC towards the immune synapse. These are the critical cellular processes involved in NK lytic activity (158). Assessment of these cellular processes demonstrated normal deposition of F-actin at the synapse in STAT3 deficient NK cells. Normal deposition of F-actin suggests that the signaling axis of activating receptor signals- Src family kinase- Vav phosphorylation- Rac1 activation- actin nucleators- actin polymerization is intact and functional in STAT3 deficient NK cells (13, 235-239). Normal convergence of lytic granules to MTOC indicates that STAT3 deficiency does not affect Src family kinase mediated dynein motor dependent transport of lytic granules along microtubules (186, 240). Convergence of lytic granules to MTOC is the first step in the directed traffic of these lysosomal organelles towards immune synapse. Upon convergence, movement of MTOC along with the lytic granules in the direction of the synapse is called MTOC polarization. I observed impaired polarization of MTOC to synapse in both Job syndrome patients' and STAT3 knock-down NK cells. During polarization to the immune synapse, the transport of MTOC and lytic granules along the microtubules is powered by plus-end motor protein kinesin-1 (KIF5B), which associates with MTOC and lytic granules through a small G-protein, Arl8b (200). The transport of MTOC and lytic granules along the microtubules towards the actin rich immune synapse is facilitated by proteins, which could associate with both microtubules and actin. CDC42

interacting protein 4 (CIP4) and IQGAP1 are two such proteins. CIP4 can interact with actin through Wiskott-Aldrich syndrome protein (WASp), an actin associated actin polymerization activating protein (236), and with MTOC through its binding with tubulin (185). Activation of NK cells by a target cell triggers localization of CIP4 at MTOC and its interaction with WASp (185). IQGAP1 is a multi-domain scaffolding protein that can also interact with F-actin and microtubules (201). Individual silencing of these proteins have been shown to impair MTOC and lytic granule polarization in NK cells (185, 200, 201). To investigate whether the expression of these molecules is affected by STAT3 deficiency, I quantified mRNA expression by Real-time PCR. Expression of Arl8b, KIF5B and IQGAP1 was found to be similar in STAT3 knock-down and control NK cells, but CIP4 expression was found to be significantly reduced in STAT3 knock-down NK cells, suggesting it as a likely cause for impaired MTOC polarization. However, CIP4 gene expression was found to be normal in Job syndrome patients' NK cells indicating that a different mechanism is responsible for the impaired MTOC polarization observed in these cells. As discussed earlier, the two genetic models used in this study do not exactly mimic each other, which may result in differential regulation of expression of genes such CIP4. Signals mediated by NKG2D receptors trigger MTOC polarization (241, 242). However, the reduced expression of NKG2D observed in Job syndrome patients' NK cells as a possible cause of MTOC impairment is unlikely as STAT3 knock-down NK cells with normal expression of NKG2D receptor also had impaired MTOC polarization. Thus, the causative factor responsible for impaired MTOC polarization in Job syndrome patients' NK cells remains elusive.

Despite of impaired MTOC and lytic granule polarization in STAT3 knock-down NK cells, the decrease in cytotoxicity was marginal. This could be attributed to the lytic granules which are known to exist near the immune synapse in NK cell and do not

require MTOC polarization for their delivery to the synapse (243-245). These granules are responsible for initial rapid release of lytic molecules in the synaptic cleft (245). As suggested in an earlier study (185), pre-activation of NK cells during the culture period such as IL-2 activation as was done in this study may have increased the number of pre-docked lytic granules in STAT3 knock-down NK cells. Even though it is not known how many lytic granules are required to kill K562, the target cell used in the present study, considering its sensitivity to NK cell mediated lysis, exocytosis of few lytic granules may be sufficient to do the job.

One of the primary functions of NK cells is tumor immunosurveillance (6, 8, 246, 247). This innate role of NK cells is reflected in the association of low NK cytotoxicity in the peripheral blood with higher incidence of cancer as was observed in an 11-year follow-up study (30). NK immunosurveillance of tumor cells is pre-dominantly mediated through NKG2D receptor (27). NKG2D is a primary activating receptor on NK cells. In humans, its ligands include MHC class-I-related chain (MIC) A and MICB and the UL-16 binding proteins (ULBP). Expression of these ligands is induced by cellular stress such as heat shock, DNA damage, transformation, and viral and bacterial infection. Not surprisingly, NKG2D plays a critical role in the immune response mediated by NK to infections and tumors (12). As observed in the present study, Job syndrome patients with dominant negative STAT3 mutations have both the factors, impaired NKG2D receptor expression and NK cytotoxicity, that could cause a predisposition to cancer. In fact, autosomal dominant Job syndrome patients do carry higher risk of malignancy, particularly lymphoma (144, 155, 156, 248-251). Very few instances of other types of malignancies such as vulvar cancer (252) and pulmonary adenocarcinoma (253) have been reported. The possibility of deficient NK cytolytic function due to impaired NKG2D receptor expression as a putative cause of proclivity to lymphoma development in Job

syndrome patients is supported by the earlier reports of impaired NK cytotoxicity observed in lymphoma patients (254, 255) and a recent study linking the cytotoxic impairment to reduced NKG2D receptor expression on NK cells (256). The risk of lymphoma development is 259 fold higher in autosomal dominant Job syndrome patients compared to general population (155). Many cases of lymphoma in autosomal dominant Job syndrome patients were reported before the discovery of dominant negative STAT3 mutations as the causative factor of the syndrome, but confirmation of STAT3 mutations in subsequent cases has established a firm link between STAT3 deficiency and occurrence of lymphoma (144, 156, 250, 251). STAT3 deficiency not only inhibits NK cytotoxicity through NK intrinsic effects, but tumor specific STAT3 deletion also adversely affects NK infiltration and NK cytotoxicity as has been shown in a STAT3 deleted BCR/ABL induced lymphoma model (257). Thus, impaired cytotoxicity of STAT3 deficient NK cells and reduced NK immunosurveillance influenced by STAT3 deficient lymphoma could be promoting lymphogenesis in Job syndrome patients.

Even though Job syndrome patients carry dominant negative STAT3 mutations, they are not totally devoid of STAT3 activity. As STATs mediate their transcriptional activation function as dimers, the 25% probability of functional dimer formation, due to the one normal STAT3 allele present, retains residual STAT3 activity in Job syndrome patients' cells, which is 25% of the wild type (131). I wanted to investigate whether stimulation of the residual functional STAT3 by IL-21 could improve deficient NKG2D receptor expression, MTOC polarization, and cytotoxicity of Job syndrome patients' NK cells. IL-21, a cytokine belonging to common γ chain family, pre-dominantly activates STAT3 (80, 136, 171) and marginally activates STAT1, STAT4, and STAT5 in NK cells (80, 92, 136, 171, 258). IL-21 is a potent activator of NK cells. It induces expression of activating receptors, effector cytolytic proteins, granzymes and perforin, and cytolytic

activity of NK cells (80, 81, 92, 136, 223, 259). *In vivo* anti-tumor effects of IL-21 are mediated by NK cells (26) and these anti-tumor effects are NKG2D dependent (260). IL-21 induced expression of activating receptors and perforin, and activation of cytolytic function in NK cells is mediated through STAT3 activation (136, 223). Previous work in our lab demonstrated robust expansion of human NK cells stimulated with K562, genetically modified to present membrane bound IL-21 (mbIL21). These expanded cells had high expression of activating receptors and enhanced cytolytic activity (134). In the present study, NK cells, expanded from PBMCs stimulated with mbIL21 over a three-week period, were found to have similar expression of NKG2D receptor in Job syndrome patients and normal donors. MTOC polarization was also found to be normal in mbIL21 expanded Job syndrome patients' NK cells. Thus, the deficient NKG2D receptor expression and MTOC polarization in resting Job syndrome patients' NK can be corrected by mbIL21 stimulation. Consistent with these results, mbIL21 activation also enhanced cytolytic function of Job syndrome patients' NK cells. Though the cytotoxicity of mbILl21 expanded Job syndrome patients' NK cells was marginally lower than mbIL21 expanded NK cells from normal donors, there was no significant difference as was observed at basal level.

As discussed earlier, deficient NK cytotoxicity caused by impaired NKG2D receptor expression is the likely reason for Job syndrome patients' predisposition towards lymphoma development. The finding that mbIL21 activation restores NKG2D receptor expression to normal levels and enhances cytotoxicity of Job syndrome patients' NK cells offers an adoptive NK cell immunotherapy approach to treat lymphoma in these patients.

Due to oncogenic properties of STAT3 signaling in multiple cancers, inhibition of STAT3 is emerging as a therapeutic approach in cancer treatment. Multiple

approaches to block STAT3 signaling are at different stages of clinical development (106, 140, 175). In the light of the finding of the present study that STAT3 deficiency inhibits NK cytolytic activity, it is critical to determine the effect of this therapeutic approach on the function of the immune cell whose primary function is tumor immunosurveillance. It is all the more important when equally promising treatment options against cancer are offered by NK cells. High levels of tumor infiltrating NK cells are associated with positive prognosis (31-33). The anti-tumor activity of several cytokines such as IL-2, IL-12, IL-18, and IL-21 is mediated by NK cells (8, 26). Adoptive transfer of activated autologous or allogeneic NK cells has proven to be a safe and potentially efficacious immunotherapy for cancer (36, 37) and has been or being tested against multiple types of cancers (35). As cancer treatment many a times involves combination of different treatment modalities, it is imperative to determine the compatibility of different modes of treatments with each other. Development of Small molecule inhibitors of STAT3 is one of the most actively researched areas in cancer therapeutics (140). S3I-201 is one such inhibitor, which binds SH2 domain of STAT3 to block its dimerization and thereby inhibits DNA binding and transcriptional activity (203). It has been shown to inhibit tumor growth in pre-clinical studies (198, 203). In a previous study, we reported suppression of NKG2D receptor expression in S3I-201 treated primary human NK cells (136). Assessment of the effect of this drug on the cytotoxicity of human NK cells in the present study showed that S3I-201 reduces cytolytic activity of NK cells. Thus, pharmacological inhibition of STAT3 impairs NKG2D receptor expression and cytolytic function; the former could be the cause of the latter, thereby disarming NK cells. These results indicate that blocking STAT3 as a therapeutic approach in cancer treatment may have adverse effects on NK cell cytolytic function and caution needs to be exercised, while employing this mode of treatment.

2.5 CONCLUSION

The evidence presented in this report shows that STAT3 deficiency in human NK cells causes impairment of cytolytic function. I used two genetic models to study the role of STAT3 in NK cytolytic function; NK cells from Job syndrome patients carrying dominant negative STAT3 mutations, a naturally occurring genetic model of STAT3 deficiency, and siRNA mediated knock-down of STAT3 in primary human NK cells. NK cells from Job syndrome patients displayed significantly reduced cytotoxicity compared to NK cells from normal donors. Investigations into the probable underlying causes of impaired cytolytic function of Job syndrome patients' NK cells revealed reduced expression of NKG2D receptor, a primary activating receptor involved in activating cytotoxicity of NK cells. Among the subcellular cytolytic processes probed, synapse formation and granule convergence were found to be normal, while MTOC polarization to the synapse was defective. The cytolytic phenotypes of STAT3 deficient Job syndrome patients' NK cells were recapitulated in STAT3 knock-down primary human NK cells. Assessment of cytolytic activity by conventional bulk assay as well as single cell analysis showed suppressed cytolytic function in STAT3 knock-down NK cells. Impaired MTOC polarization was also observed in these cells. Thus, two genetic models complemented each other in highlighting role of STAT3 in MTOC polarization and cytolytic function of human NK cells. Job syndrome patients are not totally devoid of STAT3 activity. One normal allele of STAT3 enables them to retain about 25% activity of the wild type. In this study, I showed that stimulation of Job syndrome NK cells with membrane bound form of STAT3 activating cytokine IL-21 restores NKG2D expression and MTOC polarization to normal levels and improves cytolytic function. Lastly, I studied the effect of pharmacological inhibition of STAT3 on NK cell cytolytic

function. Deregulated STAT3 activity is associated with different types of cancers and as a result, STAT3 is considered a promising therapeutic target. Various ways to block STAT3 are under clinical consideration and small molecule inhibitors are one of the important ones. One of the major functions of NK cells is immunosurveillance of malignant cells. In the light of the finding of the present study, I found it imperative to evaluate the effect of pharmacological inhibition of STAT3 on NK cytolytic function. Treatment of primary human NK cells with small molecule STAT3 inhibitor S3I-201 suppressed NK cytolytic function, thereby showing a possible side effect of this therapeutic approach.

This is the first report to show that STAT3 deficiency in primary human NK cells causes impairment of MTOC polarization and cytolytic activity. This is also the first report of NK cell functional deficiency in Job syndrome patients. Reduced NKG2D expression and impaired NK cytolytic function, observed in this study, may provide an immunological basis for the proclivity of Job syndrome patients to lymphoma and restoration of NK cell function upon mbIL21 stimulation, as demonstrated in this study, suggests an adoptive NK cell therapy as a therapeutic approach to treat Job syndrome patients. By assessing the effect of small molecule STAT3 inhibitor on NK cytolytic function, this study provides a potential biomarker for monitoring side effects of STAT3 inhibition.

Chapter 3

ROLE OF STAT3 SIGNALING IN HUMAN NK CELL EXPANSION

Role of STAT3 Signaling In Human NK Cell Expansion

3.1 INTRODUCTION

NK cells, effector lymphocytes of the innate immune system, control microbial infections, particularly viral and are the first line of defense against cancer cells. Like T and B cells, NK cells do not require prior sensitization with antigen to execute their effector functions. NK cells are equipped with an array of germline encoded activating receptors to detect ligands expressed on stressed cells and inhibitory receptors to detect self-signals such as MHC class I molecules. The balance between the strength of signals mediated by the two types of receptors determines whether NK cells discharge their effector function upon contact with the target cell (8).

Immune response entails availability of a pool of effector cells in normal conditions and further expansion of these cells during infections. Steady-state number of NK cells is maintained through homeostatic proliferation as has been shown in both mice (65, 261, 262) and humans (263, 264). The homeostatic NK cell survival and proliferation are dependent on IL-15 signaling in mice (64-66), but are independent of IL-15 in humans (67).

In addition to proliferation during steady state, NK cells also proliferate in response to viral infections. NK proliferative responses to various types of viral infections in mice such as mouse cytomegalovirus (MCMV) (38, 53, 68, 265), lymphocytic choriomeningitis virus *(*LCMV*)* (266), and vaccinia virus (163) and a wide range of viral infection in humans such as human cytomegalovirus (HCMV) (267, 268), Epstein-Barr virus (EBV) (269), human immunodeficiency virus (HIV) (270), hepatitis C virus (271) and hantavirus (69) have been reported. In the early phase of viral infection,

NK cells proliferate in response to cytokine signaling. In MCMV and LCMV infections in mice this effect has been shown to be Type I interferon (IFNα/β) dependent (38, 272). Type I IFNs do not directly mediate pro-proliferative signals in NK cells, but promote proliferation by inducing generation of IL-15 (68). The role of IL-15 in NK proliferative response to viral infection has also been established in humans (69). IL-12 (70, 71) and IL-18 (70) have also been demonstrated to play a role in NK proliferation in response to MCMV infection in mice, but whether these cytokines directly act on NK cells or promote proliferation through an indirect mechanism remains to be determined. Cytokine induced NK cell proliferation in the early phase of viral infection is nonselective, while in the later phase NK cells expressing activating receptors that recognize virus induced ligands are selectively expanded (272). This is best exemplified by the well-studied preferential proliferation of $Ly49H^+$ NK cells in response to MCMV infection in mice (52, 53, 265, 273, 274). Ly49H is an activating receptor expressed in mouse NK cells that recognizes MCMV encoded ligand m157 (272). Ly49H transmits signals in NK cells by coupling with signaling adaptor protein DAP12 and these signals directly promote proliferation of NK cells (273). In humans too, selective expansion of NK cells such as KIR^+ NK cells in response to HIV (275) and $CD94/NKG2C^+$ in response to HCMV (267) infection have been reported, but evidence supporting direct involvement of these receptors' signaling in NK proliferation is lacking.

In vitro studies have demonstrated that cytokines and activating receptors, which have not yet been shown to play a physiological role in NK cell proliferation *in vivo,* could also induce proliferation of NK cells. One of the potent activators of NK cell proliferation is IL-2 (72, 73). It is used to expand NK cells *ex vivo* for immunotherapy in cancer (74, 75). Some cytokines do not induce proliferation acting alone but in combination with other cytokines they do. For instance, IL-10, IL-12, and IL-18

stimulate proliferation of mice NK cells pre-activated with IL-2 (76). IL-18 also induces proliferation of mice NK cell in combination with other cytokines such as IL-12 and IL-15 (77-79). IL-21 too does not induce NK cell proliferation by itself, but does in combination with IL-2 in human NK cells (80). Stimulation of activating receptors also promotes NK cell proliferation. Individual stimulation of NKG2D, NKp30, and NKp46 induces proliferation in human NK cells (276).

Cytokines known to induce proliferation of NK cells mediate their signals employing JAK-STAT pathway. IL-15, established to be the major endogenous cytokine responsible for survival and proliferation of NK cells *in vivo* in mice and IL-2 which is routinely used to expand NK cells from mouse and human *in vitro* activate STAT 1, 3, 4, and 5 in NK cells (171, 173, 277). Out of all the STATs, STAT5 is predominantly activated by IL-2 and IL-15 (171, 173). The importance of STAT5 signaling in NK cell proliferation is reflected in the reduced proliferative response of NK cells from STAT5 knock-out mice to IL-2 and IL-15 (162). Out of the two members of the STAT5 group, STAT5a and STAT5b, these effects are more pronounced in STAT5b knock-out mice, thereby signifying its role in mouse NK cell proliferation (162). IL-2 and IL-15 also support NK cell survival (58) and signals promoting survival are also pre-dominantly mediated through STAT5 (208, 278). Apart from STAT5, the only other STAT that has been implicated in NK cell proliferation is STAT1. NK proliferative response to viral infection is dependent on type I IFN mediated STAT1 signaling. The pro-proliferative effects are not due to NK cell intrinsic STAT1 signaling, but due to the availability of IL-15 that is generated as a result of Type I IFN mediated STAT1 signaling (68).

The cytokines, IL-2, IL-15, IL-10, IL-12 and IL-18, which have been shown to induce proliferation of NK cells either *in vivo* or *in vitro*, activate STAT3 in NK cells (80, 136, 169-173). STAT3 is considered to be a pro-proliferative and pro-survival

transcription factor. STAT3 target genes such as cyclin D1, D2, D3, cdc25A, and c-Myc (174, 279, 280) promote cell cycle progression, while Bcl-XL (281) and survivin (282) prevent apoptosis. However it is not known whether STAT3 signaling plays any role in NK cell proliferation and survival.

Anti-cancer activity of NK cells has generated a great deal of interest in NK cell immunotherapy against cancer. Due to the limited number of NK cells in the peripheral blood, expanding NK cells to a sufficiently large number is imperative to the success of NK cell immunotherapy. Expanding and activating NK cells *ex-vivo*, before their transfer into the patients, forms the basis of NK immunotherapy. Research efforts in our lab are focused on developing an *ex vivo* NK cell expansion platform. We previously described robust *ex vivo* expansion of NK cells by co-cultivation with K562 (erythroleukemic cell line) based artificial antigen presenting cells (aAPC) genetically modified to express membrane-bound cytokines (134). We observed significantly higher fold expansion of NK cells activated with membrane bound IL-21 (mbIL21) than those activated with membrane bound IL-15 (mbIL15) (134). As out of all the STATs, IL-21 activates STAT3 most efficiently (104, 135), I hypothesized that STAT3 may play a role in NK cell proliferation.

In this investigation, I studied role of STAT3 in membrane bound cytokine activated primary human NK cell proliferation by blocking STAT3 employing pharmacological and genetic tools. In the pharmacological model, I blocked STAT3 using a small molecule STAT3 inhibitor, while in the genetic model NK cells from Job syndrome patients carrying dominant negative STAT3 mutations were used. Both the approaches showed significant reduction in the proliferation of NK cells thereby underlining requirement of STAT3 in NK cell proliferation.

3.2 MATERIAL AND METHODS

3.2.1 Cell and cell lines

Anonymized normal donor buffy coats were obtained from the Gulf Coast Regional Blood Center (Houston, TX) under a protocol approved by the Institutional Review Board (IRB) of UT MD Anderson Cancer Center. Peripheral blood was obtained from STAT3 genotyped Job's syndrome patients at National Institute of Allergy and Infectious Diseases and Children's Hospital of Philadelphia under protocols approved by the IRB of each respective institution. IRB approval was obtained from UT MD Anderson Cancer Center to acquire Job's syndrome samples from collaborators and to perform this research. This study was conducted in accordance with the Declaration of Helsinki.

3.2.2 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) from buffy coats and blood samples were obtained by buoyant density centrifugation on Ficoll-Paque following previously described protocol (176). Briefly, buffy coats or blood samples mixed with phosphate buffer saline (PBS) were loaded on top of Ficoll-Paque in a 50 ml tube and centrifuged at 400g for 20 minutes without brake. The PBMCs settled at the interface of Ficoll-Paque and plasma were recovered and washed three times with PBS. The red blood cells (RBCs) settled below Ficoll-Paque were used to isolate NK cells from PBMCs.

3.2.3 NK Cell Purification

NK cells were purified from PBMCs by enriching them to ≥ 95% purity ($CD3^{-}CD16/56^{+}$) with RosetteSep Human NK Cell Enrichment Cocktail (STEMCELL Technologies, Vancouver, BC, Canada) following manufacturer's instructions. In brief, PBMCs were mixed at 1:100 ratio with red blood cells (RBCs) obtained from the same sample. To the PBMCs:RBCs mixture, Human NK Cell Enrichment Cocktail was added at 1 µl/10^6 PBMCs. The solution was mixed well and incubated at room temperature for 20 minutes with gentle mixing every five minutes. After adding PBS with 2% fetal bovine serum (FBS) to the PBMCs:RBCs mix, the solution was layered on top of Ficoll-Paque and centrifuged at 400g for 20 minutes. The NK cells were recovered from top of the Ficoll-Paque layer.

The NK cells were cultured in RPMI 1640 (Cellgro/Mediatech, Manassas, VA) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM l-glutamine (Gibco/Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin (Cellgro/Mediatech). This media is hereinafter referred to as NK cell media.

3.2.4 Antibodies

Murine anti-human CD3, CD56, and isotype control mAbs were obtained from BD Biosciences (Bedford, MA).

3.2.5 *Ex vivo* expansion of human NK cells

NK cells were expanded as previously described with few modifications (176). Briefly, PBMCs or purified NK cells were co-cultured with irradiated (100 cGy) K562 artificial antigen presenting cells (aAPCs), genetically modified to express membrane bound cytokines, at 1:2 (PBMCs/NK:aAPCs) ratio and 50 U/ml recombinant human IL-2

in a T-75 flask (Corning, Corning, NY) at 10^6 cells/ml. The K562s expressing membrane bound cytokines were generated as previously described (134). Culture was refreshed with new media containing 50 U/ml IL-2 every three to four days. In expansions extended beyond one week, a portion of the expanded cells was carried forward by recursive stimulation with K562s expressing membrane bound cytokine and 50 U/ml IL-2. In the expansions started with purified NK cells, fold expansion of NK cells was calculated by dividing the number of viable NK cells at the end of a weeklong expansion with the number of viable NK cells at the start of expansion. In the expansions started with PBMCs, fold expansion of NK cells was calculated by dividing the number of NK cells in the PBMCs at the end of weeklong expansion by the number of NK cells in the PBMCs at the start of expansion. Number of NK cells in the PBMCs were calculated by estimating the percentage of $CD3^-$ $CD56^+$ cells by flow-cytometric analysis. Inferred fold NK cell expansion was calculated from the resulting cultures as if all the cells were carried forward in the expansion (134).

3.2.6 Cell Count and Cell Viability

Cells were counted using hemocytometer. Cell viability was determined by Trypan blue exclusion method. Briefly, cells were stained with trypan blue and number of cells stained blue and total number of cells were counted using hemocytometer. Percentage viability was calculated using the formula [((Total number of cells-number of blue cells)/Total number of cells) *100].

3.2.7 Flow-Cytometry Analysis

Flow cytometry buffer, PBS with 2mM EDTA and 0.1% sodium azide, was used for blocking, staining, washing and resuspending the cells. Before staining with

antibodies, the cells were blocked with 50% FBS for 30 minutes at 4°C. Blocked cells were stained with appropriate fluorochrome conjugated antibodies for 30 minutes at 4°C, washed twice and resuspended in flow cytometry buffer. Data was acquired using a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ), and was analyzed using FlowJo Version 9.2 software (FlowJo, Ashland, OR, USA).

3.2.8 Pharmacological inhibition of STAT3 in NK cells

S3I-201 (Calbiochem, Gibbstown, NJ), a small molecule STAT3 inhibitor, reconstituted in DMSO was used to block STAT3 signaling. Purified NK cells were incubated for an hour with or without S3I-201 before stimulating them with aAPCs and IL-2. Cells were pre-treated with the drug every time media was refreshed. Control cells were treated with the solvent, DMSO.

3.2.9 Proliferation Assessment By CFSE Dilution

Proliferation of NK cells was assessed by the 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution method (283). Briefly, freshly purified NK cells, resuspended in pre-warmed PBS containing 0.1% BSA at 10^6 cells/ml, were stained with 2 μM CFSE (Molecular Probes, Eugene, OR) by incubating them at 37^{o}C for 10 minutes. At the end of incubation period, staining was quenched by adding cold NK cell media 5 times the volume of the staining solution and by incubating them on ice for 5 minutes. Cells were pelleted and washed three times with NK cell media. CFSE stained cells were expanded as described earlier. At the end of one week of expansion CFSE staining was measured by flow-cytometry.

3.2.10 Apoptosis Assessment

Apoptotic NK cells were identified using apoptosis detection kit from BD Pharmingen (Franklin Lake, NJ) following manufacturer's protocol with few modifications. Briefly, NK cells were washed twice with cold PBS and then resuspended in 1X binding buffer at a density of 10^6 cells/ml. 100 µl of cell suspension (10^5 cells) was transferred to a 5 ml flow cytometry tube. 3 µl of Phycoerythrin (PE) conjugated Annexin V was added to the tube and mixed by gentle vortexing. The cells were incubated in dark for 15 minutes at room temperature. After incubation, cells were washed twice with 1X binding buffer and then resuspended in 400 µl 1X binding buffer containing 5 µl 7-Amino-Actinomycin (7-AAD). The cells were incubated in dark for 10 minutes at room temperature. PE-Annexin V and 7-AAD staining were analyzed by flow-cytometry.

3.2.11 Statistical Analysis

Statistical analysis was performed for the indicated statistical tests using GraphPad Prism for Macintosh, version 5.0a. P values <.05 were considered significant.

3.3 RESULTS

Ex vivo NK cell expansion platform, developed in our lab, demonstrated robust expansion of human NK cells activated with K562 cells genetically modified to present membrane bound IL-21 (mbIL21) (134). As IL-21 pre-dominantly signals through STAT3 activation (104, 135), I hypothesized that STAT3 activation may play a role in the induction of proliferation of human NK cells. I used pharmacological and genetic models to test my hypothesis.

3.3.1 Pharmacological Inhibition of STAT3

3.3.1.1 STAT3 inhibitor S3I-201 reduces mbIL21 stimulated expansion of human NK cells

I employed pharmacological approach to block STAT3 with a small molecule inhibitor S3I-201. It binds SH2 domain of STAT3 to block dimerization and thereby inhibits DNA binding and transcriptional activity of STAT3 (203). Human NK cells purified from peripheral blood mononuclear cells (PBMCs) were expanded by stimulation with mbIL21 for one week in the presence and absence S3I-201. At the end of the week, viable cells were counted by differentiating viable cells from non-viable cells by staining the cells with trypan blue. The number of NK cells was significantly higher in the cultures without STAT3 inhibitor (Figure 15).

3.3.1.2 STAT3 inhibition decreases proliferation and survival of human NK cells stimulated with mbIL21

Expansion of cell number is the net result of two processes; increase in cell number due to proliferation and decrease in cell number due to cell death. I evaluated the effect of pharmacological inhibition of STAT3 on proliferation of NK cells using CFSE dilution assay. CFSE, a cell permeable fluorescent dye, reacts covalently with amine containing intracellular molecules thereby fluorescently labeling the cell. CFSE

fluorescence per cell is progressively halved after each cell division (284). Thus, more the number of cell divisions a cell undergoes, lesser the fluorescence intensity the daughter cells end up with. Flow-cytometric analysis revealed significantly higher CFSE fluorescence in S3I-201 treated NK cells than in control cells at the end of one week of expansion with mbIL21 indicating fewer cell divisions in S3I-201 treated NK cells compared to control cells (Figure 16b). This observation suggested that pharmacological blocking of STAT3 inhibits mbIL21 stimulated proliferation of human NK cells. I also evaluated the effect of STAT3 inhibition on the survival of NK cells by assessing viability and apoptosis. Viability of cells was evaluated by staining the cells with 7-AAD, a cell membrane impermeable fluorescent compound with strong affinity for DNA that stains non-viable cells with compromised cell membranes. Apoptotic cells were detected by staining the cells with fluorescent tagged Annexin V, a protein that binds phosphotidylserine that is exposed on the surface of apoptotic cells (285). Both, 7-AAD and Annexin V, stainings were measured by flow-cytometry. Percentage of viable cells was found to decrease (Figure 16d) and percentage of apoptotic cells was found to increase (Figure 16e) in S3I-201 treated NK cells indicating that pharmacological inhibition of STAT3 negatively affects survival of mbIL21 activated human NK cells.

Figure 15. Pharmacological inhibition of STAT3 decreases mbIL21 stimulated expansion of human NK cells. Purified NK cells were incubated for an hour with or without small molecule STAT3 inhibitor S3I-201 before stimulating them with irradiated K562 artificial antigen presenting cells (aAPCs), genetically modified to express membrane bound IL-21 (mbIL21), at 1:2 (NK:K562) ratio and 50 U/ml recombinant human IL-2. Control cells were treated with the solvent, DMSO. Culture was refreshed with new media containing 50 U/ml IL-2 every three to four days. Cells were pre-treated with the drug every time media was refreshed. At the end of one week of expansion, viable cells were counted by trypan blue exclusion method. Fold expansion of NK cells was calculated by dividing the number of viable NK cells at the end of a weeklong expansion with the number of viable NK cells at the start of expansion. P value indicated is for two-tailed paired Student's t test.

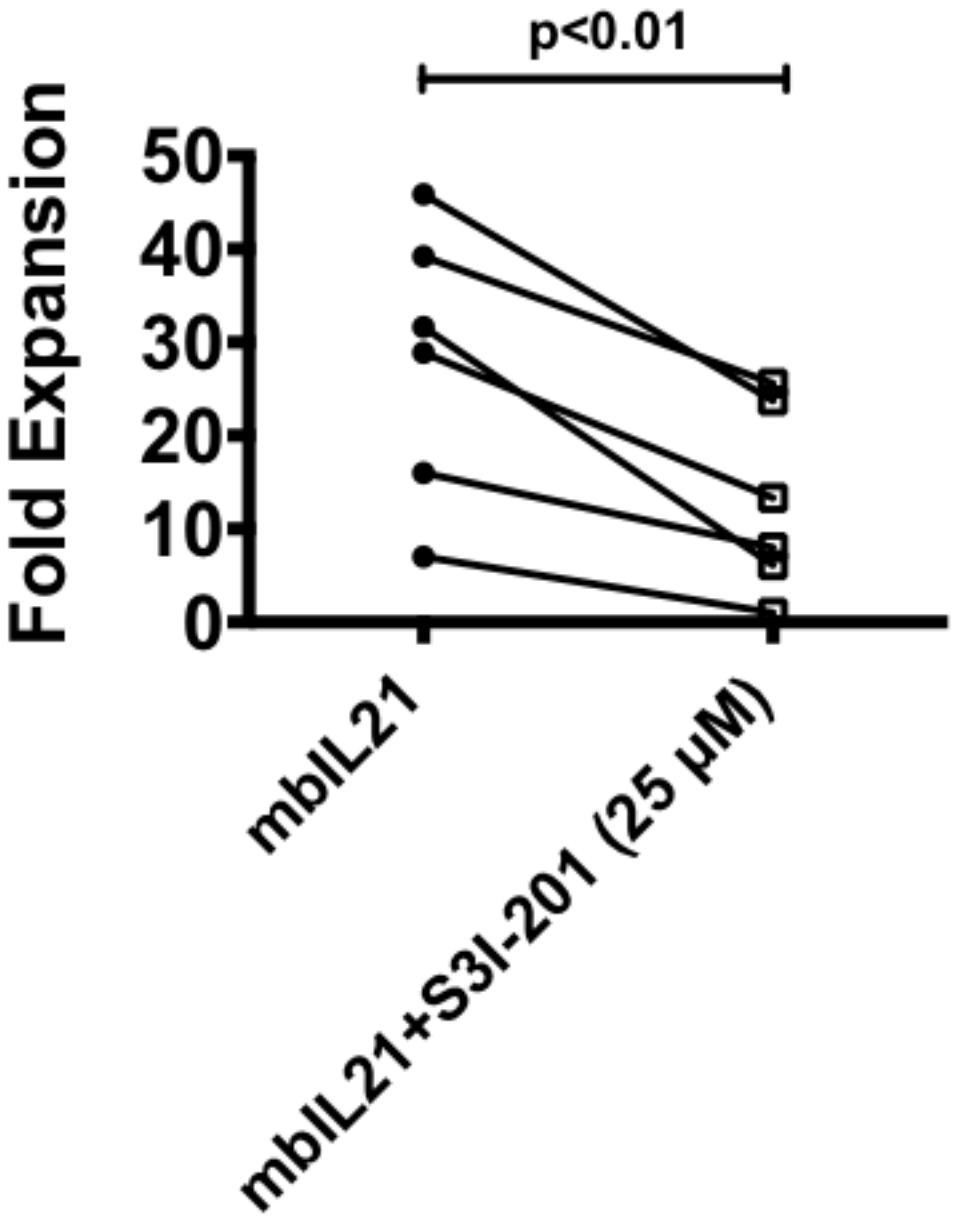
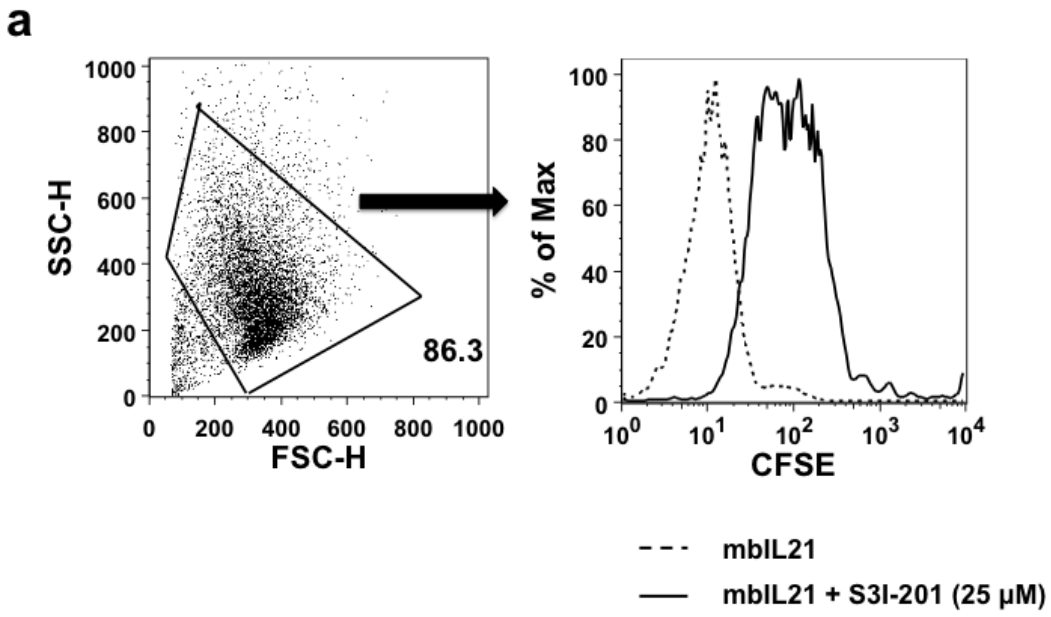
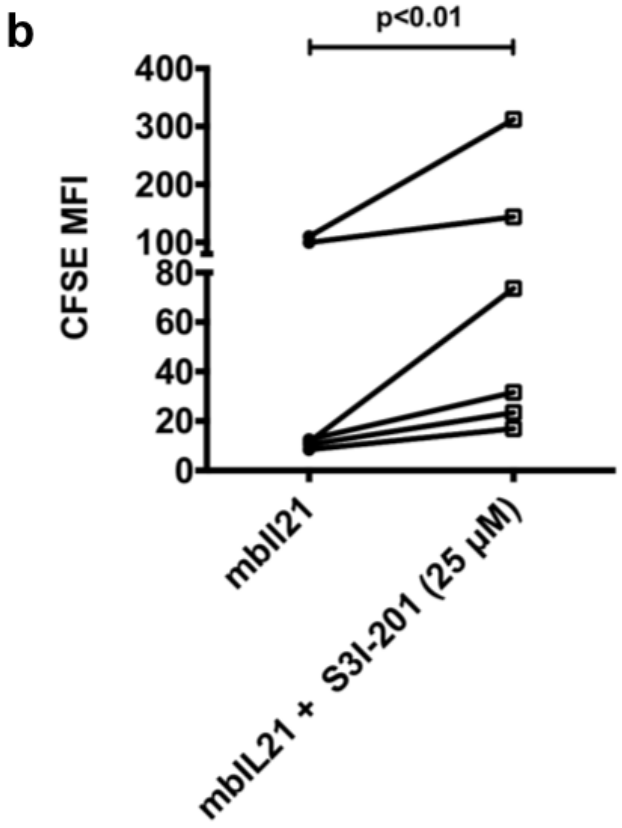
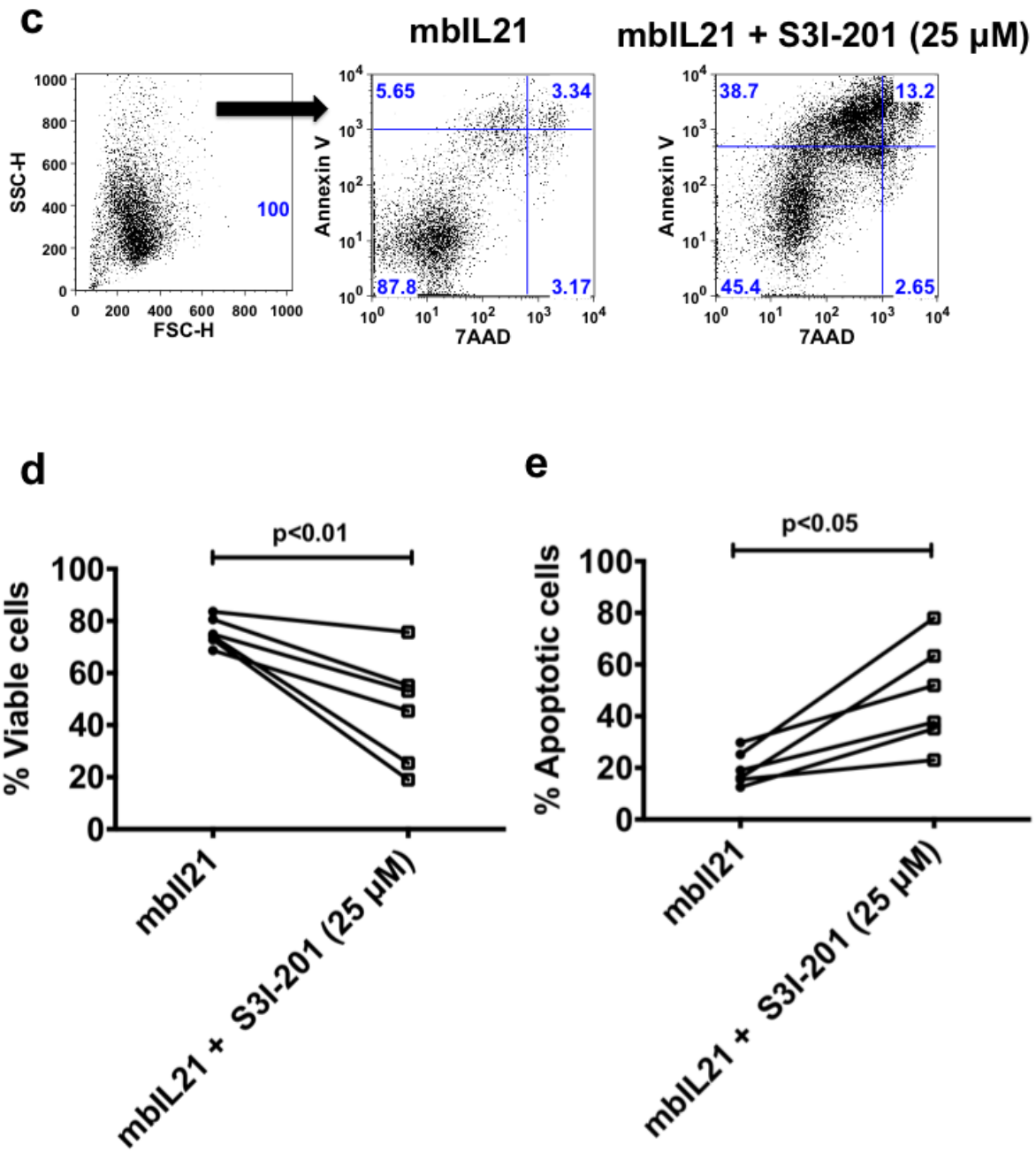

p<0.01
Fold Expansion
50
40
30
20
10
0
mbIL21
mbIL21+S3I-201 (25 μM)

Figure 16. Pharmacological inhibition of STAT3 decreases proliferation and survival of mbIL21 stimulated human NK cells. Significantly higher CFSE fluorescence in S3I-201 treated NK cells than in control cells at the end of one week of expansion with mbIL21, indicating fewer cell divisions in S3I-201 treated NK cells compared to control cells. Lower percentage of viable cells and higher percentage of apoptotic cells in S3I-201 treated NK cells at the end of one week of expansion with mbIL21, indicating reduced survival. Purified NK cells, stained with CFSE, were incubated for an hour with or without small molecule STAT3 inhibitor S3I-201, before stimulating them with irradiated K562 artificial antigen presenting cells (aAPCs), genetically modified to express membrane bound IL-21 (mbIL21), at 1:2 (NK:K562) ratio and 50 U/ml recombinant human IL-2. Control cells were treated with the solvent, DMSO. Culture was refreshed with new media containing 50 U/ml IL-2 every three to four days. Cells were pre-treated with the drug every time media was refreshed. At the end of one week of expansion mean fluorescence intensity (MFI) of CFSE was measured by flow cytometry **(a)** Representative flow-charts showing live NK cells gated on the basis of forward and side scatter and CFSE staining of this gated population for control and S3I-201 treated cells **(b)** CFSE MFI in control and S3I-201 treated cells **(c)** At the end of one week of expansion viability and apoptosis of the entire population (100%) were assessed by flow-cytometric analysis of 7AAD and Annexin V staining **(d)** Cells stained negative for both, 7AAD and Annexin V were considered viable. Percentage of viable cells at the end of one week expansion is shown **(e)** Cells stained with just Annexin V were considered apoptotic. Percentage of apoptotic cells at the end of one week expansion is shown. P values indicated are for two-tailed paired Student's t test.


a
SSC-H
FSC-H
86.3
% of Max
CFSE
mbIL21
mbIL21 + S3I-201 (25 μM)

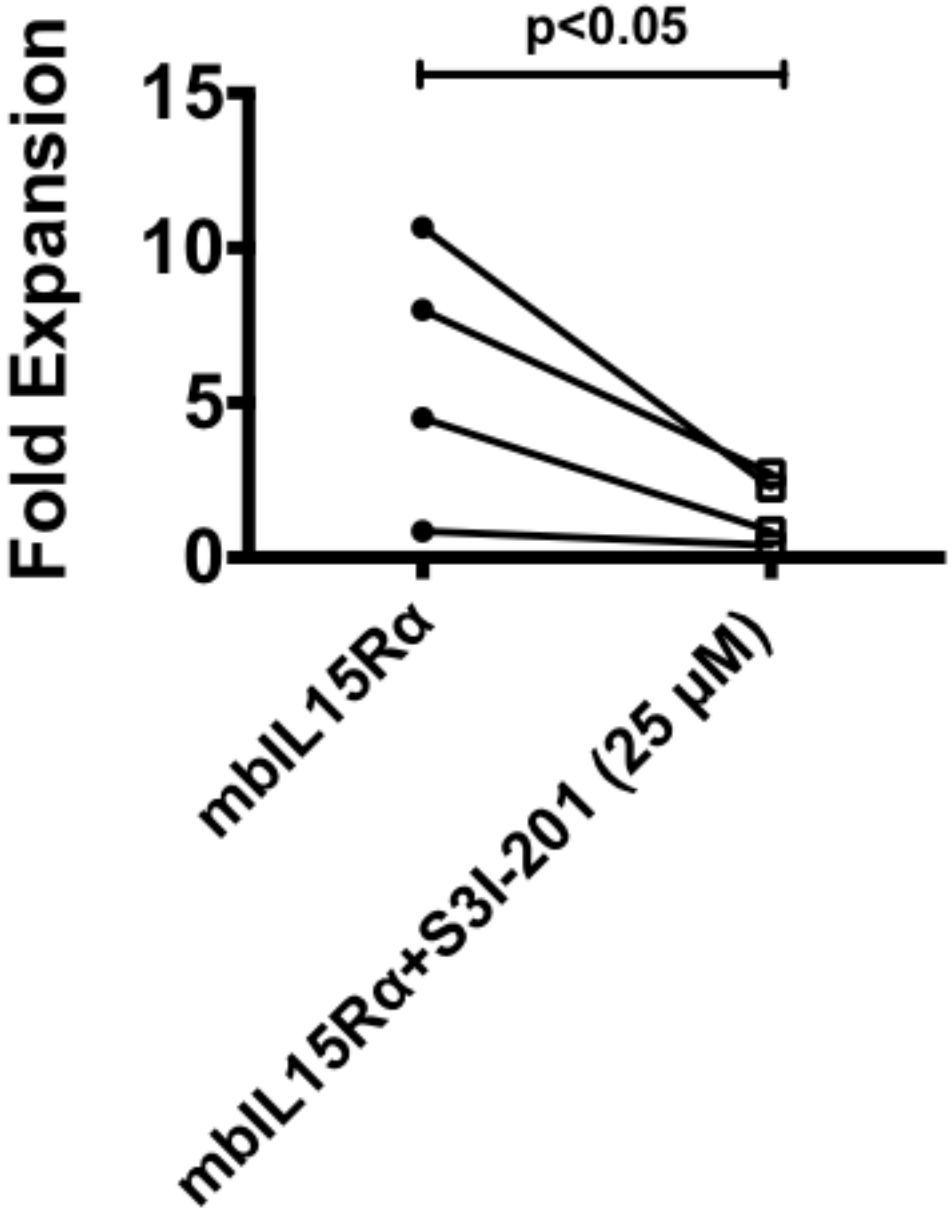

b
p<0.01
CFSE MFI
mbIl21
mbIL21 + S3I-201 (25 μM)


c
mbIL21
mbIL21 + S3I-201 (25 μM)
SSC-H
FSC-H
100
Annexin V
7AAD
5.65
3.34
87.8
3.17
38.7
13.2
45.4
2.65
d
p<0.01
% Viable cells
mbIl21
mbIL21 + S3I-201 (25 μM)
e
p<0.05
% Apoptotic cells
mbIl21
mbIL21 + S3I-201 (25 μM)

3.3.1.3 STAT3 inhibitor S3I-201 reduces mbIL15-15Rα stimulated expansion of human NK cells

To evaluate whether inhibition of STAT3 has any bearing on the expansion of NK cells stimulated with a cytokine, which does not pre-dominantly activate STAT3, I assessed the effect of S3I-201 on NK cells expanded with K562s genetically modified to present membrane bound IL-15 in complex with its receptor α (mbIL15-15Rα), the way IL15 is trans-presented to NK cells physiologically (286). IL-15 is a potent inducer of proliferation of mouse and human NK cells (58, 287, 288) and though it activates multiple STATs including STAT3 in NK cells (220), its pro-proliferative effect is mainly attributed to STAT5 signaling at least in mouse (162). NK cell expansion, measured by counting viable NK cells at the end of one week of activation with mbIL15-15Rα, showed significant reduction in the expansion of S3I-201 treated NK cells compared to control (Figure 17). Thus, pharmacological blocking of STAT3 not only inhibited expansion of NK cells stimulated with cytokine such as IL21, which pre- dominantly activates STAT3, but also of NK cells stimulated with cytokines such as IL15, which pre- dominantly activates STAT other than STAT3.

Figure 17. Pharmacological inhibition of STAT3 decreases mbIL15-15Rα stimulated expansion of human NK cells. Purified NK cells were incubated for an hour with or without small molecule STAT3 inhibitor S3I-201 before stimulating them with irradiated K562 artificial antigen presenting cells (aAPCs), genetically modified to express membrane bound IL-15 in complex with its receptor α (mbIL15-15Rα), at 1:2 (NK:K562) ratio and 50 U/ml recombinant human IL-2. Control cells were treated with the solvent, DMSO. Culture was refreshed with new media containing 50 U/ml IL-2 every three to four days. Cells were pre-treated with the drug every time media was refreshed. At the end of one week of expansion, viable cells were counted by trypan blue exclusion method. Fold expansion of NK cells was calculated by dividing the number of viable NK cells at the end of a weeklong expansion with the number of viable NK cells at the start of expansion. P value indicated is for two-tailed paired Student's t test.


p<0.05
Fold Expansion
15
10
5
0
mbIL15Rα
mbIL15Rα+S3I-201 (25 μM)

3.3.2 Impaired Expansion of Job Syndrome Patients' NK Cells

Availability of NK cells from Job syndrome patients carrying dominant negative STAT3 mutations (Table 2) offered me an opportunity to test my hypothesis in a physiologically relevant genetic model. I expanded NK cells from Job syndrome patients' PBMCs by stimulating them with membrane bound cytokine presenting K562 cells. The cells were expanded over a three-week period by carrying forward a portion of expanded cells from the previous week. Fold expansion of NK cells from Job syndrome patients was found to be lower than NK cells from normal donors. Impaired expansion of Job syndrome patients' NK cells was observed in mbIL21 (Figure 18) as well as in mbIL15-15Rα based expansion platforms (Figure 19). The fold expansion at the end of first and second week was lower in Job syndrome patients' NK cells than in NK cells from normal donors, but the difference was not statistically significant (Figure 18a and b for mbIL21 expanded cells and Figure 19a and b for mbIL15-15Rα expanded cells). A statistically significant difference in fold expansion was observed at the end of third week (Figure 18c for mbIL21 and Figure 19c for mbIL15-15Rα). Inferred fold expansion showed a widening difference between Job syndrome patient's NK cells and normal donor's NK cells over the three-week expansion period (Figure 18d for mbIL21 and Figure 19d for mbIL15-15Rα). Thus, consistent with the results obtained with pharmacological inhibition of STAT3, genetic deficiency of STAT3 also impaired expansion of NK cells activated with mbIL21 as well as mbIL15-15Rα.

Figure 18. Impaired expansion of Job syndrome patients' NK cells stimulated with mbIL21. The absolute fold expansion at the end of first and second week was lower in Job syndrome patients' NK cells than in NK cells from normal donors, but the difference was not statistically significant. A statistically significant difference in fold expansion was observed at the end of third week. Inferred fold expansion showed a widening difference between Job syndrome patient's NK cells and normal donor's NK cells over the three week expansion period. PBMCs were stimulated with irradiated K562 artificial antigen presenting cells (aAPCs), genetically modified to express membrane bound IL-21 (mbIL21), at 1:2 (PBMC:K562) ratio and 50 U/ml recombinant human IL-2. Culture was refreshed with new media containing 50 U/ml IL-2 every three to four days. Beyond one week, a portion of the expanded cells was carried forward by recursive stimulation with mbIL21 and 50 U/ml IL-2. At the end of one week of expansion, viable cells were counted by trypan blue exclusion method. Fold expansion of NK cells was calculated by dividing the number of NK cells in the PBMCs at the end of weeklong expansion by the number of NK cells in the PBMCs at the start of expansion. Number of NK cells in the PBMCs were calculated by estimating the percentage of $CD3^-$ $CD56^+$ cells by flow-cytometric analysis. Inferred fold NK cell expansion was calculated from the resulting cultures as if all the cells were carried forward in the expansion. **(a)** Fold expansion at the end of first week **(b)** Fold expansion at the end of second week **(c)** Fold expansion at the end of third week **(d)** Inferred fold expansion over the three week expansion period.

In each graph values for individual donor are shown along with the mean for the group. P value indicated is for two-tailed Student's t test.

Expansion with mbIL21 stimulation

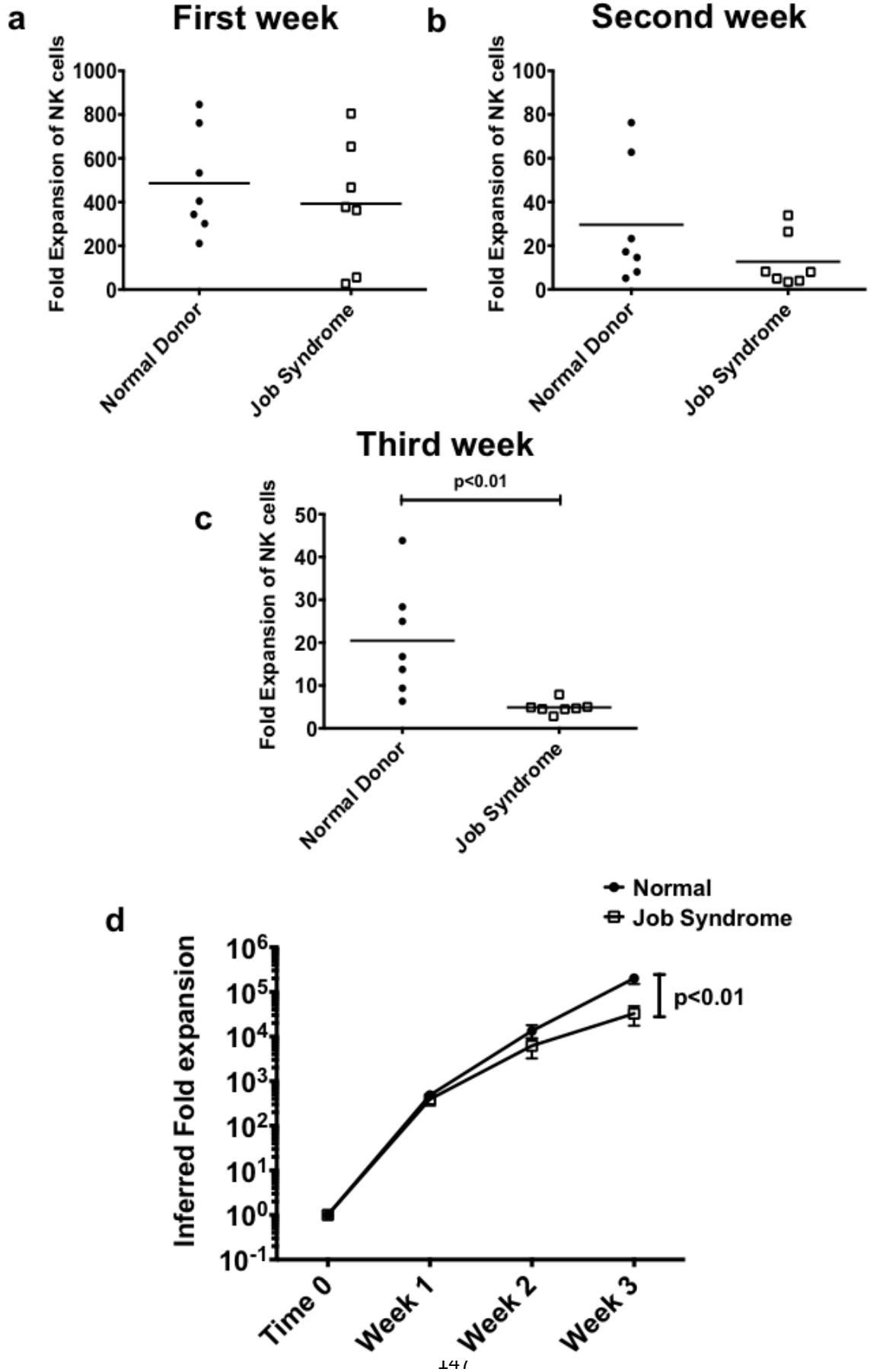
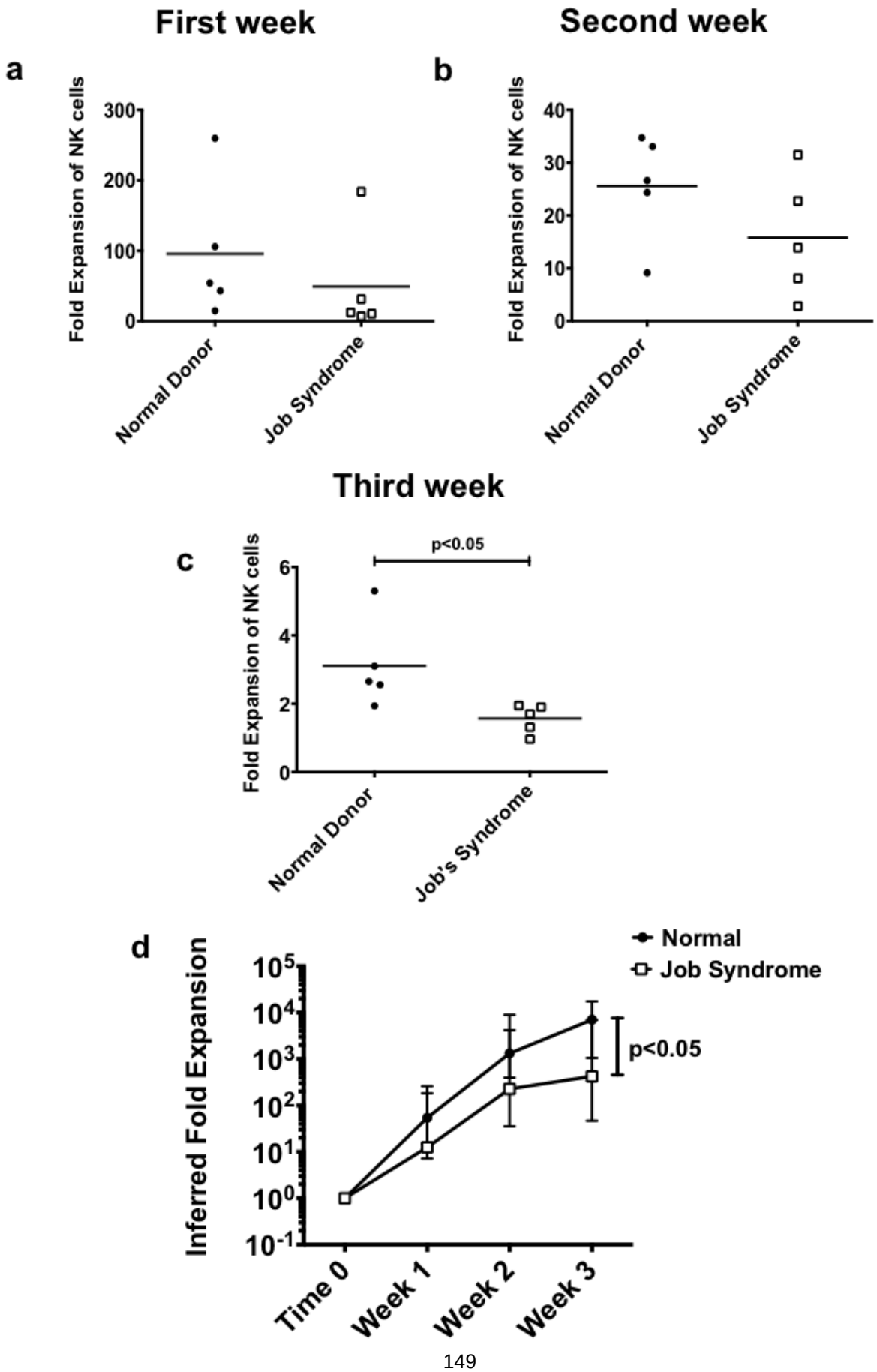


Figure 19. Impaired expansion of Job syndrome patients' NK cells stimulated with mbIL15-15Rα. The absolute fold expansion at the end of first and second week was lower in Job syndrome patients' NK cells than in NK cells from normal donors, but the difference was not statistically significant. A statistically significant difference in fold expansion was observed at the end of third week. Inferred fold expansion showed a widening difference between Job syndrome patient's NK cells and normal donor's NK cells over the three week expansion period. PBMCs were stimulated with irradiated K562 artificial antigen presenting cells (aAPCs), genetically modified to express membrane bound IL-15 in complex with its receptor α (mbIL15-15Rα), at 1:2 (PBMC:K562) ratio and 50 U/ml recombinant human IL-2. Culture was refreshed with new media containing 50 U/ml IL-2 every three to four days. Beyond one week, a portion of the expanded cells was carried forward by recursive stimulation with mbIL21 and 50 U/ml IL-2. At the end of one week of expansion, viable cells were counted by trypan blue exclusion method. Fold expansion of NK cells was calculated by dividing the number of NK cells in the PBMCs at the end of weeklong expansion by the number of NK cells in the PBMCs at the start of expansion. Number of NK cells in the PBMCs were calculated by estimating the percentage of $CD3^-$ $CD56^+$ cells by flow-cytometric analysis. Inferred fold NK cell expansion was calculated from the resulting cultures as if all the cells were carried forward in the expansion. **(a)** Fold expansion at the end of first week **(b)** Fold expansion at the end of second week **(c)** Fold expansion at the end of third week **(d)** Inferred fold expansion over the three week expansion period. In each graph values for individual donor are shown along with the mean for the group. P value indicated is for two-tailed Student's t test.

Expansion with mbIL15R stimulation



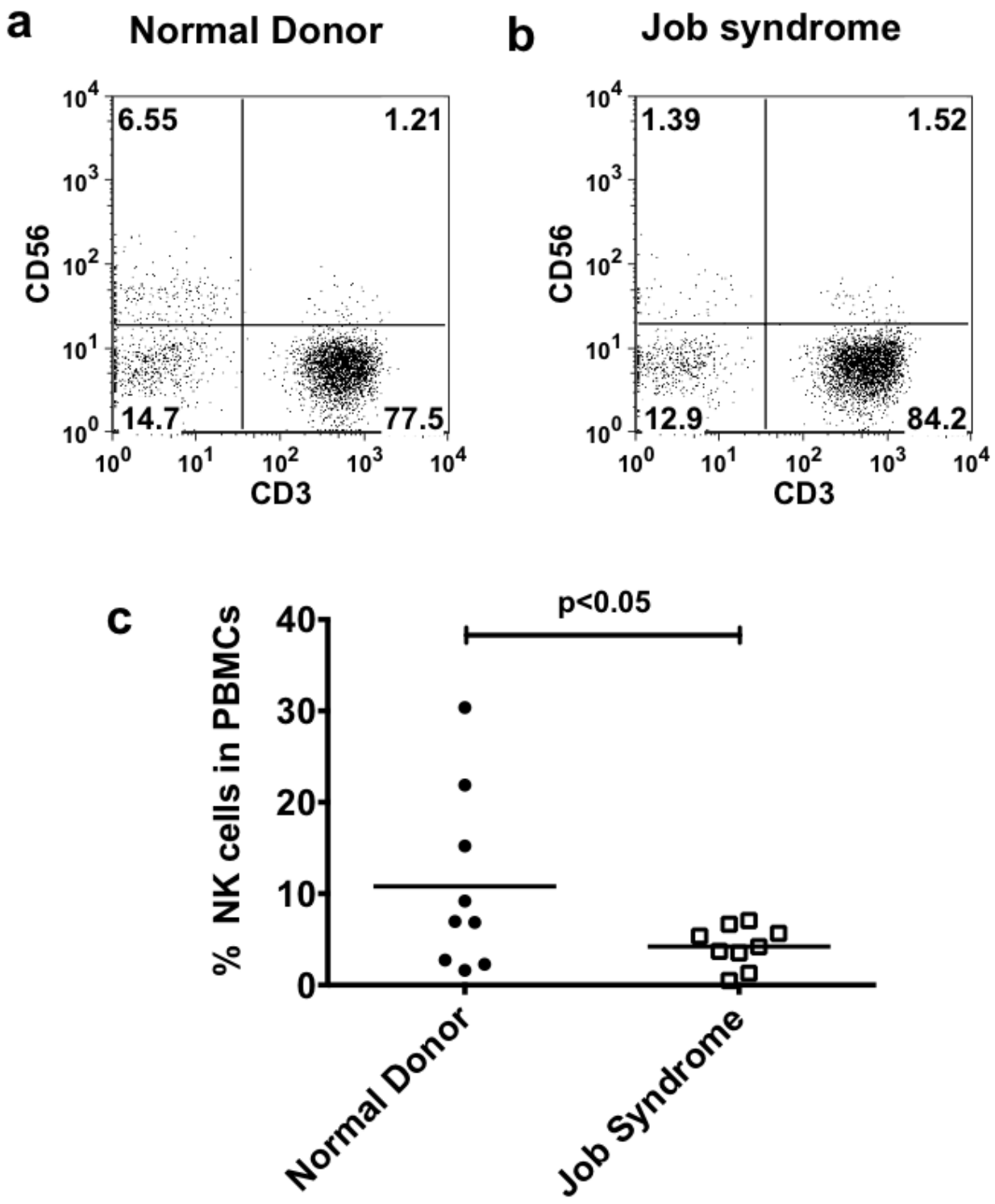
3.3.3 Lower percentage of NK cells in the peripheral Blood of Job Syndrome Patients

To investigate whether genetic defect in STAT3 has any bearing on the number of NK cells in the peripheral blood of Job syndrome patients, I evaluated the percentage of NK cells, defined as $CD3^-$ $CD56^+$, in the lymphocyte population. NK cells comprise around 2-18% of peripheral blood lymphocytes in human (8), which was reflected in the range of percentages of NK cells observed in normal donors (Figure 20c). Even though majority of Job syndrome patients had NK cell percentages within the normal range, they were mostly at the lower end of the scale. The difference in the percentage of NK cells between normal and Job syndrome donors was statistically significant (Figure 20c).

Figure 20. Lower percentage of NK cells in the PBMCs of Job syndrome patients. PBMCs isolated from normal and Job syndrome donors were analyzed by flow-cytometry. NK cells were identified as $CD3^{-}CD56^{+}$. Representative flow-chart showing percentage of $CD3^{-}CD56^{+}$ cells is shown for **(a)** normal and **(b)** Job syndrome donor. **(C)** Percentage of NK cells in the PBMCs is shown in graphical form.

In the graph values for individual donor are shown along with the mean for the group.

P value indicated is for two-tailed Student's t test.


a
Normal Donor
6.55
1.21
14.7
77.5
CD56
CD3
b
Job syndrome
1.39
1.52
12.9
84.2
CD56
CD3
c
p<0.05
% NK cells in PBMCs
Normal Donor
Job Syndrome

3.4 DISCUSSION

In this investigation, employing pharmacological blocking of STAT3, I showed that inhibition of STAT3 impairs human NK cells expansion stimulated with membrane bound cytokines, IL-21 and IL-15. I validated this finding in NK cells from Job syndrome patients carrying dominant negative STAT3 mutations. Both, pharmacological and genetic evidence underscored the importance of STAT3 in human NK cell expansion.

IL-21, a cytokine belonging to common γ chain family, has mitogenic effect on lymphocytes (59, 135, 289). IL-21 by itself cannot induce proliferation, but does so as a co-stimulator with other cytokines and mitotic factors (59, 135). Activation of JAK-STAT signaling pathway is central to the IL-21 mediated signals in immune cells. It activates multiple forms of STATs including STAT1, STAT3, STAT5a, and STAT5b. Out of these STATs, STAT3 is most strongly activated (135). In mouse T cells and human B cells, the mitogenic signals of IL-21 are mediated through activation of STAT1 and STAT3 (135, 153).

Like in B and T cells, IL-21 acts as a mitogen in NK cells. In mouse and human NK cells, IL-21 promotes proliferation in combination with either IL-2 or IL-15 (80, 290). We utilized the mitogenic property of IL-21 signaling to develop a human NK cell expansion platform in which cells are stimulated with K562s genetically modified to present membrane bound IL-21 (mbIL21) and soluble IL-2 (134). This expansion system supported log-phase growth in NK cell numbers over a six week period and resulted in significantly greater fold expansion than expansion of NK cells stimulated with K562s genetically modified to present membrane bound IL-15 (mbIL15) and soluble IL-2 (134). In NK cells, IL-21 activates STAT1, STAT3, STAT4, and STAT5, and out of all STATs, activates STAT3 predominantly (80, 92, 171). However, not much

is known about the role of individual STATs in IL-21 mediated pro-proliferative signals in NK cells. Very few studies have investigated the signaling mechanisms involved in IL-21 induced proliferation in NK cells.

In this study, using small molecule inhibitor S3I-201, I showed that pharmacological blocking of STAT3 inhibits proliferation of human NK cells stimulated with mbIL21. These results validated an earlier report, which suggested that mbIL21 induced expansion of human NK cells is mediated through STAT3 activation (202). However, the evidence presented in this particular study is not very conclusive due to the type of STAT3 inhibitor used. Wang et al. (202) used a small molecule inhibitor JSI-124 to block STAT3. Even though, JSI-124 is used as a STAT3 specific inhibitor (224, 225), along with STAT3, it also inhibits activation of JAK2 and JAK3 and thus actually it is a JAK-STAT inhibitor (226, 227). JSI-124 also blocks activation of STAT5 (228). As IL-21 activates STAT5 in NK cells (80, 92) and STAT5 deficiency is known to impair NK cell proliferation and survival (162, 208), the effect of JSI-124 could have been mediated through STAT5 inhibition. Compared to JSI-124, S3I-201 is a STAT3 specific inhibitor as it preferentially inhibits STAT3 DNA binding activity over that of other STATs with a two-fold higher potency (203). Along with proliferation, IL-21 has also been shown to support survival of human NK cells (81, 223). However, the signaling mechanism involved in the transmission of pro-survival signals was not known. In this investigation, along with the inhibition of proliferation, blockage of STAT3 also increased apoptosis of NK cells activated with mbIL21 thereby highlighting the critical role of activated STAT3 in the pro-proliferative as well as pro-survival signals mediated by IL-21. STAT3 is a pro-survival transcription factor as it activates expression of anti-apoptotic genes such as Bcl-2, Bcl-X_L (291), and survivin (282) and down-regulates expression of pro-apoptotic genes such as BCL2L11 (Bim) (225). IL-21 has also been

shown to promote survival of NK cells through the up-regulation of expression of anti-apoptotic proteins Bcl-2 and Bcl-X_L (223). The evidence presented in the current study suggests involvement of STAT3 in IL-21 mediated activation of expression of Bcl-2 and Bcl-X_L.

Pharmacological blocking of STAT3 not only inhibited expansion of NK cells stimulated with mbIL21, which pre- dominantly activates STAT3, but also of NK cells stimulated with mbIL15-15Rα, which pre- dominantly activates STAT5 and activates STAT3 only marginally (292). *In vivo* IL-15 is trans presented to NK cells by accessory cells such as dendritic cells, which express IL-15 in complex with the α subunit of its receptor on their membranes. The IL15-IL15Rα complex mediates signals in NK cells by interacting with β and γ subunits of IL-15 receptor expressed on the NK cell membrane (293). IL-15 induced proliferation and survival of NK cells are considered to be mediated mainly through the activation of STAT5 (162, 208). Along with STAT5, IL-15 also activates STAT3, albeit marginally (171). However, evidence presented in the current study suggests that STAT3 plays a major role, disproportionate to its activation, in IL-15 induced proliferation of NK cells. A recent study also implied involvement of STAT3 activation in the proliferation of mouse NK cells stimulated with IL-2 (294), a cytokine, which also activates STAT5 pre-dominantly, and STAT3 marginally in NK cells (80) and whose proliferative effects were considered to be mediated through STAT5 activation (162).

Even though, the concentration (25 μM) of the pharmacological inhibitor, S3I-201, used in the present study was well below its IC_{50} for STATs other than STAT3, a non-specific effect cannot be entirely ruled out due to the sequence similarity between SH2 domain of STAT3, the binding target of S3I-201, and SH2 domains of other STATs, particularly STAT1 (295). To validate the results obtained using

pharmacological inhibitor and to obtain unequivocal evidence in support of the role of STAT3 in NK cell expansion, I expanded NK cells from Job syndrome patients' carrying dominant negative STAT3 mutations. Both, mbIL21 and mbIL15-15Rα stimulation induced significant expansion of Job syndrome patients' NK cells, but the expansion was significantly impaired compared to NK cell from normal donors. These results were consistent with the results obtained with pharmacological inhibition. Thus, both pharmacological and genetic evidence signify requirement of STAT3 in NK cell expansion.

Steady-state number of NK cells *in vivo* is maintained through homeostatic proliferation (263, 264). To evaluate whether impaired expansion of Job syndrome patients' NK cell observed *ex vivo* has any bearing on NK cells *in vivo,* I assessed percentage of NK cells in the lymphocyte population of the peripheral blood. NK cells comprise around 2-18% of peripheral blood lymphocytes in human (8). Even though the percentages of NK cells in Job syndrome patients were within the normal range, they were at the lower end of the spectrum. The low NK percentage may result from a deficiency in either differentiation, proliferation or survival or a combination of these processes. Therefore, the low NK percentage cannot be attributed to just impaired proliferation unless all the aforementioned processes are studied.

3.5 CONCLUSION

In this investigation, using pharmacological and genetic models, I showed that inhibition of STAT3 significantly reduces expansion of human NK cells stimulated not only with IL-21 that predominantly activates STAT3, but also the expansion stimulated with IL-15, which predominantly activates STAT5 and activates STAT3 only marginally. Thus, STAT3 signaling is critical for human NK cell expansion *ex vivo*. In mbIL-21

stimulated NK cell expansion, STAT3 signaling is critical for proliferation and survival. Even though fold expansion of Job syndrome patients' NK cells was significantly reduced compared to NK cells from normal donors, Job syndrome patients' NK cells expanded considerably in response to cytokine stimulation, suggesting adoptive NK immunotherapy potential in Job syndrome patients. This treatment option is promising in the light of the finding of low percentage of NK cells in the peripheral blood of Job syndrome patients.

FUTURE DIRECTIONS

4.1 FUTURE DIRECTIONS

In this investigation, I evaluated the role of STAT3 in the functions of human NK cells. Findings of this study established role of STAT3 signaling in human NK cell cytolytic activity and expansion. However, much work remains to be done to understand the role of STAT3 in NK cell biology in its entirety.

In this study, to investigate the underlying causes of impaired cytotoxicity of STAT3 deficient NK cells, I explored function of STAT3 in NK receptor expression and subcellular cytolytic processes, both of which were found to be affected in STAT3 deficient NK cells. However, other factors that also have bearing on cytolytic function were not probed. Conjugate formation between NK and target cells, the very first step that sets the stage for the cytolytic encounter and expression of perforin and granzyme, the cytolytic molecules that effect the lysis (158), need to be assessed in STAT3 deficient cells. NK cells also mediate cytolysis through CD16, an Fc receptor, which upon binding to the antibody attached to a target cell activates degranulation (8). I did not assess antibody dependent cellular cytotoxicity in STAT3 deficient NK cells. Even though CD16 expression level, measured in the present study, was unchanged in STAT3 deficient cells, effect of STAT3 deficiency on signaling pathways triggered by CD16 cannot be ruled out.

In addition to cytolysis of infected and malignant cells, NK cells also mediate their functions through other means. In order to understand role of STAT3 in NK cell functions, it is critical to assess all NK cell effector functions in STAT3 deficient cells. NK cells are equipped with apoptosis inducing ligand FASL and TRAIL, which play important role in immunosurveillance of malignant cells, particularly in liver (296). Along with cytolytic capability, NK cells also exert their effect through secretion of cytokines

and chemokines in response to stimulation by target cells and cytokines secreted by accessory cells (10). NK cell generated cytokines include IFN-γ, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin (IL)-10 and IL-3, while chemokines such as CCL2, CCL3, CCL4, CCL5, XCL1, and CXCL8 are also secreted by NK cells (2). Compared to leukocytes from normal donors, leukocytes from Job syndrome donors have been reported to secrete higher levels of IFN-γ and TNF-α in response to lipopolysaccharide and IL-12 stimulation, indicating deregulation (130). In NK92 cells, an NK cell line, IL-2 stimulated IL-10 secretion requires STAT3 activation (222). As NK cells are the major source of cytokines, particularly IFN-γ, it would be of interest to assess cytokine secretion in STAT3 deficient NK cells in response to stimulation by cytokines and activating receptors. Assessment of expression of FASL, TRAIL, cytokines and chemokines in Job syndrome patients' NK cells will provide important insights into the regulation of NK function by STAT3.

NK cells from Job syndrome patients are an excellent genetic tool to study the role of STAT3 in NK cell function. However, Job syndrome is a rare immunodeficiency and hence availability of cells from Job syndrome patients is a major limiting factor. This constraint warrants procurement of alternative genetic model that is easily accessible. In the present investigation, I used siRNA mediated STAT3 knock-down in primary human NK cells to validate the results I obtained with Job syndrome patients' NK cells. But, the former genetic model does not exactly mimic the latter. siRNA mediated STAT3 knock-down generates acute STAT3 deficiency, while Job syndrome patients' NK cells have chronic STAT3 deficiency right from their inception. This difference may account for the differences observed between the two in the present investigation. A genetic model closely mimicking Job syndrome patients' NK cells

would require presence of the dominant negative STAT3 allele in NK cells throughout the development process, which entails transfection of dominant negative STAT3 in hematopoietic stem cells. Even then, the model will be far from perfect, as the cells would still have, unlike Job syndrome patients' cells, two normal copies of STAT3 gene and one copy of dominant negative STAT3. A mouse carrying dominant negative Job syndrome STAT3 gene has been generated (297), however, due to the differences between mouse and human NK cells, as discussed earlier, not everything learned in mouse can be extrapolated to humans. This is especially true in case of role of STAT3 in NK cells, as STAT3 deficiency in these two species has not generated uniform phenotypes.

In this investigation, I also studied proliferation of Job syndrome patients' NK cells and human NK cells treated with STAT3 inhibitor, both of which showed impaired proliferation. However, the molecular mechanisms involved were not investigated. STAT3 is a pro-proliferative and pro-survival transcription factor. Whole genome transcriptome analysis and gene transcription analysis by microarray of STAT3 deficient NK cells will help identify direct and indirect target genes of STAT3 involved in human NK cell proliferation and survival. A protein expression analysis by reverse phase protein array (RPPA) will further validate these targets at the functional level.

Constitutively active STAT3 and over activation of STAT3 are oncogenic. STAT3 is a therapeutic target in many cancers and various strategies to block STAT3 activation are under clinical consideration. In the light of the finding of my investigation that STAT3 deficiency causes impairment of NK cell cytolytic function, I was interested in determining the effect of pharmacological inhibition of STAT3 on NK cell cytolytic activity. *In vitro* cytotoxicity assays showed that treatment with small molecule STAT3 inhibitor S3I-201 suppresses cytolytic activity of human NK cells. This finding bears

importance because NK cells are the first line of defense against malignant cells. To determine whether impairment of NK cytolytic function is a likely adverse effect of STAT3 blocking in cancer treatment, cytotoxicity of NK cells from patients in clinical trials receiving STAT3 inhibitors should be assessed. Such an assessment will also help determine the compatibility of adoptive NK cell therapy, an emerging immunotherapy approach for cancer treatment, with the pharmacological blocking of STAT3, should these two treatment options be used in combination.

BIBLIOGRAPHY

1. Beck, G., and Habicht, G. S. (1996) Immunity and the invertebrates. *Scientific American* **275**, 60-63, 66
2. Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., Yokoyama, W. M., and Ugolini, S. (2011) Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44-49
3. Oldham, R. K. (1983) Natural killer cells: artifact to reality: an odyssey in biology. *Cancer metastasis reviews* **2**, 323-336
4. Caligiuri, M. A. (2008) Human natural killer cells. *Blood* **112**, 461-469
5. Farag, S. S., Fehniger, T. A., Ruggeri, L., Velardi, A., and Caligiuri, M. A. (2002) Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* **100**, 1935-1947
6. Smyth, M. J., Hayakawa, Y., Takeda, K., and Yagita, H. (2002) New aspects of natural-killer-cell surveillance and therapy of cancer. *Nature reviews. Cancer* **2**, 850-861
7. Gregoire, C., Chasson, L., Luci, C., Tomasello, E., Geissmann, F., Vivier, E., and Walzer, T. (2007) The trafficking of natural killer cells. *Immunological reviews* **220**, 169-182
8. Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. (2008) Functions of natural killer cells. *Nature immunology* **9**, 503-510
9. Long, E. O., Kim, H. S., Liu, D., Peterson, M. E., and Rajagopalan, S. (2013) Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annual review of immunology* **31**, 227-258
10. Fauriat, C., Long, E. O., Ljunggren, H. G., and Bryceson, Y. T. (2010) Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* **115**, 2167-2176
11. Kruse, P. H., Matta, J., Ugolini, S., and Vivier, E. (2014) Natural cytotoxicity receptors and their ligands. *Immunology and cell biology* **92**, 221-229
12. Ogasawara, K., and Lanier, L. L. (2005) NKG2D in NK and T cell-mediated immunity. *Journal of clinical immunology* **25**, 534-540
13. Watzl, C., and Long, E. O. (2010) Signal transduction during activation and inhibition of natural killer cells. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **Chapter 11**, Unit 11 19B
14. Le Bouteiller, P., Barakonyi, A., Giustiniani, J., Lenfant, F., Marie-Cardine, A., Aguerre-Girr, M., Rabot, M., Hilgert, I., Mami-Chouaib, F., Tabiasco, J., Boumsell, L., and Bensussan, A. (2002) Engagement of CD160 receptor by HLA-C is a triggering mechanism used by circulating natural killer (NK) cells to mediate cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16963-16968
15. Bryceson, Y. T., March, M. E., Ljunggren, H. G., and Long, E. O. (2006) Activation, coactivation, and costimulation of resting human natural killer cells. *Immunological reviews* **214**, 73-91
16. Kim, E. O., Kim, T. J., Kim, N., Kim, S. T., Kumar, V., and Lee, K. M. (2010) Homotypic cell to cell cross-talk among human natural killer cells reveals differential and overlapping roles of 2B4 and CD2. *The Journal of biological chemistry* **285**, 41755-41764

17. Nicholson, M. W., Barclay, A. N., Singer, M. S., Rosen, S. D., and van der Merwe, P. A. (1998) Affinity and kinetic analysis of L-selectin (CD62L) binding to glycosylation-dependent cell-adhesion molecule-1. *The Journal of biological chemistry* **273**, 763-770
18. Lion, E., Willemen, Y., Berneman, Z. N., Van Tendeloo, V. F., and Smits, E. L. (2012) Natural killer cell immune escape in acute myeloid leukemia. *Leukemia* **26**, 2019-2026
19. Freud, A. G., and Caligiuri, M. A. (2006) Human natural killer cell development. *Immunological reviews* **214**, 56-72
20. Trinchieri, G. (1989) Biology of natural killer cells. *Advances in immunology* **47**, 187-376
21. Barlozzari, T., Reynolds, C. W., and Herberman, R. B. (1983) In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM1-treated rats. *Journal of immunology* **131**, 1024-1027
22. Gorelik, E., Rosen, B., Copeland, D., Weatherly, B., and Herberman, R. B. (1984) Evaluation of role of natural killer cells in radiation-induced leukemogenesis in mice. *Journal of the National Cancer Institute* **72**, 1397-1403
23. Kundu, N., Beaty, T. L., Jackson, M. J., and Fulton, A. M. (1996) Antimetastatic and antitumor activities of interleukin 10 in a murine model of breast cancer. *Journal of the National Cancer Institute* **88**, 536-541
24. Zheng, L. M., Ojcius, D. M., Garaud, F., Roth, C., Maxwell, E., Li, Z., Rong, H., Chen, J., Wang, X. Y., Catino, J. J., and King, I. (1996) Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism. *The Journal of experimental medicine* **184**, 579-584
25. Heinzerling, L., Dummer, R., Pavlovic, J., Schultz, J., Burg, G., and Moelling, K. (2002) Tumor regression of human and murine melanoma after intratumoral injection of IL-12-encoding plasmid DNA in mice. *Experimental dermatology* **11**, 232-240
26. Wang, G., Tschoi, M., Spolski, R., Lou, Y., Ozaki, K., Feng, C., Kim, G., Leonard, W. J., and Hwu, P. (2003) In vivo antitumor activity of interleukin 21 mediated by natural killer cells. *Cancer research* **63**, 9016-9022
27. Guerra, N., Tan, Y. X., Joncker, N. T., Choy, A., Gallardo, F., Xiong, N., Knoblaugh, S., Cado, D., Greenberg, N. M., and Raulet, D. H. (2008) NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* **28**, 571-580
28. Gilfillan, S., Chan, C. J., Cella, M., Haynes, N. M., Rapaport, A. S., Boles, K. S., Andrews, D. M., Smyth, M. J., and Colonna, M. (2008) DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *The Journal of experimental medicine* **205**, 2965-2973
29. Iguchi-Manaka, A., Kai, H., Yamashita, Y., Shibata, K., Tahara-Hanaoka, S., Honda, S., Yasui, T., Kikutani, H., Shibuya, K., and Shibuya, A. (2008) Accelerated tumor growth in mice deficient in DNAM-1 receptor. *The Journal of experimental medicine* **205**, 2959-2964
30. Imai, K., Matsuyama, S., Miyake, S., Suga, K., and Nakachi, K. (2000) Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* **356**, 1795-1799
31. Coca, S., Perez-Piqueras, J., Martinez, D., Colmenarejo, A., Saez, M. A., Vallejo, C., Martos, J. A., and Moreno, M. (1997) The prognostic significance of

intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* **79**, 2320-2328
32. Ishigami, S., Natsugoe, S., Tokuda, K., Nakajo, A., Che, X., Iwashige, H., Aridome, K., Hokita, S., and Aikou, T. (2000) Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer* **88**, 577-583
33. Villegas, F. R., Coca, S., Villarrubia, V. G., Jimenez, R., Chillon, M. J., Jareno, J., Zuil, M., and Callol, L. (2002) Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung cancer* **35**, 23-28
34. Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W. D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., Martelli, M. F., and Velardi, A. (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **295**, 2097-2100
35. Cheng, M., Chen, Y., Xiao, W., Sun, R., and Tian, Z. (2013) NK cell-based immunotherapy for malignant diseases. *Cellular & molecular immunology* **10**, 230-252
36. Miller, J. S., Soignier, Y., Panoskaltsis-Mortari, A., McNearney, S. A., Yun, G. H., Fautsch, S. K., McKenna, D., Le, C., Defor, T. E., Burns, L. J., Orchard, P. J., Blazar, B. R., Wagner, J. E., Slungaard, A., Weisdorf, D. J., Okazaki, I. J., and McGlave, P. B. (2005) Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* **105**, 3051-3057
37. Rubnitz, J. E., Inaba, H., Ribeiro, R. C., Pounds, S., Rooney, B., Bell, T., Pui, C. H., and Leung, W. (2010) NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 955-959
38. Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P., and Salazar-Mather, T. P. (1999) Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual review of immunology* **17**, 189-220
39. Lanier, L. L. (2008) Evolutionary struggles between NK cells and viruses. *Nature reviews. Immunology* **8**, 259-268
40. Jost, S., and Altfeld, M. (2013) Control of human viral infections by natural killer cells. *Annual review of immunology* **31**, 163-194
41. Biron, C. A., Byron, K. S., and Sullivan, J. L. (1989) Severe herpesvirus infections in an adolescent without natural killer cells. *The New England journal of medicine* **320**, 1731-1735
42. Orange, J. S. (2006) Human natural killer cell deficiencies. *Current opinion in allergy and clinical immunology* **6**, 399-409
43. Brilot, F., Strowig, T., and Munz, C. (2008) NK cells interactions with dendritic cells shape innate and adaptive immunity. *Frontiers in bioscience : a journal and virtual library* **13**, 6443-6454
44. Walzer, T., Dalod, M., Robbins, S. H., Zitvogel, L., and Vivier, E. (2005) Natural-killer cells and dendritic cells: "l'union fait la force". *Blood* **106**, 2252-2258
45. Moretta, L., Ferlazzo, G., Bottino, C., Vitale, M., Pende, D., Mingari, M. C., and Moretta, A. (2006) Effector and regulatory events during natural killer-dendritic cell interactions. *Immunological reviews* **214**, 219-228
46. Yuan, D. (2004) Interactions between NK cells and B lymphocytes. *Advances in immunology* **84**, 1-42

47. Crouse, J., Xu, H. C., Lang, P. A., and Oxenius, A. (2015) NK cells regulating T cell responses: mechanisms and outcome. *Trends in immunology* **36**, 49-58
48. Pedroza-Pacheco, I., Madrigal, A., and Saudemont, A. (2013) Interaction between natural killer cells and regulatory T cells: perspectives for immunotherapy. *Cellular & molecular immunology* **10**, 222-229
49. Jabrane-Ferrat, N., and Siewiera, J. (2014) The up side of decidual natural killer cells: new developments in immunology of pregnancy. *Immunology* **141**, 490-497
50. Sun, J. C., Lopez-Verges, S., Kim, C. C., DeRisi, J. L., and Lanier, L. L. (2011) NK cells and immune "memory". *Journal of immunology* **186**, 1891-1897
51. O'Leary, J. G., Goodarzi, M., Drayton, D. L., and von Andrian, U. H. (2006) T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nature immunology* **7**, 507-516
52. Sun, J. C., Beilke, J. N., and Lanier, L. L. (2009) Adaptive immune features of natural killer cells. *Nature* **457**, 557-561
53. Dokun, A. O., Kim, S., Smith, H. R., Kang, H. S., Chu, D. T., and Yokoyama, W. M. (2001) Specific and nonspecific NK cell activation during virus infection. *Nature immunology* **2**, 951-956
54. Vosshenrich, C. A., Ranson, T., Samson, S. I., Corcuff, E., Colucci, F., Rosmaraki, E. E., and Di Santo, J. P. (2005) Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *Journal of immunology* **174**, 1213-1221
55. Kennedy, M. K., Glaccum, M., Brown, S. N., Butz, E. A., Viney, J. L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C. R., Brasel, K., Morrissey, P. J., Stocking, K., Schuh, J. C., Joyce, S., and Peschon, J. J. (2000) Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *The Journal of experimental medicine* **191**, 771-780
56. Lodolce, J. P., Boone, D. L., Chai, S., Swain, R. E., Dassopoulos, T., Trettin, S., and Ma, A. (1998) IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9**, 669-676
57. Granucci, F., Zanoni, I., Pavelka, N., Van Dommelen, S. L., Andoniou, C. E., Belardelli, F., Degli Esposti, M. A., and Ricciardi-Castagnoli, P. (2004) A contribution of mouse dendritic cell-derived IL-2 for NK cell activation. *The Journal of experimental medicine* **200**, 287-295
58. Becknell, B., and Caligiuri, M. A. (2005) Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Advances in immunology* **86**, 209-239
59. Parrish-Novak, J., Dillon, S. R., Nelson, A., Hammond, A., Sprecher, C., Gross, J. A., Johnston, J., Madden, K., Xu, W., West, J., Schrader, S., Burkhead, S., Heipel, M., Brandt, C., Kuijper, J. L., Kramer, J., Conklin, D., Presnell, S. R., Berry, J., Shiota, F., Bort, S., Hambly, K., Mudri, S., Clegg, C., Moore, M., Grant, F. J., Lofton-Day, C., Gilbert, T., Rayond, F., Ching, A., Yao, L., Smith, D., Webster, P., Whitmore, T., Maurer, M., Kaushansky, K., Holly, R. D., and Foster, D. (2000) Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* **408**, 57-63
60. Sivori, S., Cantoni, C., Parolini, S., Marcenaro, E., Conte, R., Moretta, L., and Moretta, A. (2003) IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. *European journal of immunology* **33**, 3439-3447

61. Kotlarz, D., Zietara, N., Uzel, G., Weidemann, T., Braun, C. J., Diestelhorst, J., Krawitz, P. M., Robinson, P. N., Hecht, J., Puchalka, J., Gertz, E. M., Schaffer, A. A., Lawrence, M. G., Kardava, L., Pfeifer, D., Baumann, U., Pfister, E. D., Hanson, E. P., Schambach, A., Jacobs, R., Kreipe, H., Moir, S., Milner, J. D., Schwille, P., Mundlos, S., and Klein, C. (2013) Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. *The Journal of experimental medicine* **210**, 433-443
62. Brady, J., Hayakawa, Y., Smyth, M. J., and Nutt, S. L. (2004) IL-21 induces the functional maturation of murine NK cells. *Journal of immunology* **172**, 2048-2058
63. Kasaian, M. T., Whitters, M. J., Carter, L. L., Lowe, L. D., Jussif, J. M., Deng, B., Johnson, K. A., Witek, J. S., Senices, M., Konz, R. F., Wurster, A. L., Donaldson, D. D., Collins, M., Young, D. A., and Grusby, M. J. (2002) IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity* **16**, 559-569
64. Cooper, M. A., Bush, J. E., Fehniger, T. A., VanDeusen, J. B., Waite, R. E., Liu, Y., Aguila, H. L., and Caligiuri, M. A. (2002) In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood* **100**, 3633-3638
65. Prlic, M., Blazar, B. R., Farrar, M. A., and Jameson, S. C. (2003) In vivo survival and homeostatic proliferation of natural killer cells. *The Journal of experimental medicine* **197**, 967-976
66. Ranson, T., Vosshenrich, C. A., Corcuff, E., Richard, O., Muller, W., and Di Santo, J. P. (2003) IL-15 is an essential mediator of peripheral NK-cell homeostasis. *Blood* **101**, 4887-4893
67. Lebrec, H., Horner, M. J., Gorski, K. S., Tsuji, W., Xia, D., Pan, W. J., Means, G., Pietz, G., Li, N., Retter, M., Shaffer, K., Patel, N., Narayanan, P. K., and Butz, E. A. (2013) Homeostasis of human NK cells is not IL-15 dependent. *Journal of immunology* **191**, 5551-5558
68. Nguyen, K. B., Salazar-Mather, T. P., Dalod, M. Y., Van Deusen, J. B., Wei, X. Q., Liew, F. Y., Caligiuri, M. A., Durbin, J. E., and Biron, C. A. (2002) Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *Journal of immunology* **169**, 4279-4287
69. Braun, M., Bjorkstrom, N. K., Gupta, S., Sundstrom, K., Ahlm, C., Klingstrom, J., and Ljunggren, H. G. (2014) NK cell activation in human hantavirus infection explained by virus-induced IL-15/IL15Ralpha expression. *PLoS pathogens* **10**, e1004521
70. Andrews, D. M., Scalzo, A. A., Yokoyama, W. M., Smyth, M. J., and Degli-Esposti, M. A. (2003) Functional interactions between dendritic cells and NK cells during viral infection. *Nature immunology* **4**, 175-181
71. Sun, J. C., Ma, A., and Lanier, L. L. (2009) Cutting edge: IL-15-independent NK cell response to mouse cytomegalovirus infection. *Journal of immunology* **183**, 2911-2914
72. London, L., Perussia, B., and Trinchieri, G. (1986) Induction of proliferation in vitro of resting human natural killer cells: IL 2 induces into cell cycle most peripheral blood NK cells, but only a minor subset of low density T cells. *Journal of immunology* **137**, 3845-3854
73. Talmadge, J. E., Wiltrout, R. H., Counts, D. F., Herberman, R. B., McDonald, T., and Ortaldo, J. R. (1986) Proliferation of human peripheral blood lymphocytes

induced by recombinant human interleukin 2: contribution of large granular lymphocytes and T lymphocytes. *Cellular immunology* **102**, 261-272
74. Klingemann, H. G., and Martinson, J. (2004) Ex vivo expansion of natural killer cells for clinical applications. *Cytotherapy* **6**, 15-22
75. Koehl, U., Esser, R., Zimmermann, S., Tonn, T., Kotchetkov, R., Bartling, T., Sorensen, J., Gruttner, H. P., Bader, P., Seifried, E., Martin, H., Lang, P., Passweg, J. R., Klingebiel, T., and Schwabe, D. (2005) Ex vivo expansion of highly purified NK cells for immunotherapy after haploidentical stem cell transplantation in children. *Klinische Padiatrie* **217**, 345-350
76. Cai, G., Kastelein, R. A., and Hunter, C. A. (1999) IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *European journal of immunology* **29**, 2658-2665
77. Tomura, M., Zhou, X. Y., Maruo, S., Ahn, H. J., Hamaoka, T., Okamura, H., Nakanishi, K., Tanimoto, T., Kurimoto, M., and Fujiwara, H. (1998) A critical role for IL-18 in the proliferation and activation of NK1.1+ CD3- cells. *Journal of immunology* **160**, 4738-4746
78. Lauwerys, B. R., Renauld, J. C., and Houssiau, F. A. (1999) Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. *Cytokine* **11**, 822-830
79. French, A. R., Holroyd, E. B., Yang, L., Kim, S., and Yokoyama, W. M. (2006) IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. *Cytokine* **35**, 229-234
80. Wendt, K., Wilk, E., Buyny, S., Schmidt, R. E., and Jacobs, R. (2007) Interleukin-21 differentially affects human natural killer cell subsets. *Immunology* **122**, 486-495
81. Skak, K., Frederiksen, K. S., and Lundsgaard, D. (2008) Interleukin-21 activates human natural killer cells and modulates their surface receptor expression. *Immunology* **123**, 575-583
82. Zwirner, N. W., and Domaica, C. I. (2010) Cytokine regulation of natural killer cell effector functions. *BioFactors* **36**, 274-288
83. Yu, C. R., Ortaldo, J. R., Curiel, R. E., Young, H. A., Anderson, S. K., and Gosselin, P. (1999) Role of a STAT binding site in the regulation of the human perforin promoter. *Journal of immunology* **162**, 2785-2790
84. Yamamoto, K., Shibata, F., Miyasaka, N., and Miura, O. (2002) The human perforin gene is a direct target of STAT4 activated by IL-12 in NK cells. *Biochemical and biophysical research communications* **297**, 1245-1252
85. Leong, J. W., and Fehniger, T. A. (2011) Human NK cells: SET to kill. *Blood* **117**, 2297-2298
86. Hwang, I., Scott, J. M., Kakarla, T., Duriancik, D. M., Choi, S., Cho, C., Lee, T., Park, H., French, A. R., Beli, E., Gardner, E., and Kim, S. (2012) Activation mechanisms of natural killer cells during influenza virus infection. *PloS one* **7**, e51858
87. Konjevic, G., Mirjacic Martinovic, K., Vuletic, A., and Babovic, N. (2010) In-vitro IL-2 or IFN-alpha-induced NKG2D and CD161 NK cell receptor expression indicates novel aspects of NK cell activation in metastatic melanoma patients. *Melanoma research* **20**, 459-467
88. Sutherland, C. L., Chalupny, N. J., Schooley, K., VandenBos, T., Kubin, M., and Cosman, D. (2002) UL16-binding proteins, novel MHC class I-related proteins,

bind to NKG2D and activate multiple signaling pathways in primary NK cells. *Journal of immunology* **168**, 671-679
89. Park, Y. P., Choi, S. C., Kiesler, P., Gil-Krzewska, A., Borrego, F., Weck, J., Krzewski, K., and Coligan, J. E. (2011) Complex regulation of human NKG2D-DAP10 cell surface expression: opposing roles of the gammac cytokines and TGF-beta1. *Blood* **118**, 3019-3027
90. Smyth, M. J., Swann, J., Kelly, J. M., Cretney, E., Yokoyama, W. M., Diefenbach, A., Sayers, T. J., and Hayakawa, Y. (2004) NKG2D recognition and perforin effector function mediate effective cytokine immunotherapy of cancer. *The Journal of experimental medicine* **200**, 1325-1335
91. Dybkaer, K., Iqbal, J., Zhou, G., Geng, H., Xiao, L., Schmitz, A., d'Amore, F., and Chan, W. C. (2007) Genome wide transcriptional analysis of resting and IL2 activated human natural killer cells: gene expression signatures indicative of novel molecular signaling pathways. *BMC genomics* **8**, 230
92. Burgess, S. J., Marusina, A. I., Pathmanathan, I., Borrego, F., and Coligan, J. E. (2006) IL-21 down-regulates NKG2D/DAP10 expression on human NK and CD8+ T cells. *Journal of immunology* **176**, 1490-1497
93. Michel, T., Hentges, F., and Zimmer, J. (2012) Consequences of the crosstalk between monocytes/macrophages and natural killer cells. *Frontiers in immunology* **3**, 403
94. Cooper, M. A., Elliott, J. M., Keyel, P. A., Yang, L., Carrero, J. A., and Yokoyama, W. M. (2009) Cytokine-induced memory-like natural killer cells. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 1915-1919
95. Romee, R., Schneider, S. E., Leong, J. W., Chase, J. M., Keppel, C. R., Sullivan, R. P., Cooper, M. A., and Fehniger, T. A. (2012) Cytokine activation induces human memory-like NK cells. *Blood* **120**, 4751-4760
96. Sun, J. C., Madera, S., Bezman, N. A., Beilke, J. N., Kaplan, M. H., and Lanier, L. L. (2012) Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *The Journal of experimental medicine* **209**, 947-954
97. O'Shea, J. J., Holland, S. M., and Staudt, L. M. (2013) JAKs and STATs in immunity, immunodeficiency, and cancer. *The New England journal of medicine* **368**, 161-170
98. Shuai, K., and Liu, B. (2003) Regulation of JAK-STAT signalling in the immune system. *Nature reviews. Immunology* **3**, 900-911
99. Levy, D. E., and Darnell, J. E., Jr. (2002) Stats: transcriptional control and biological impact. *Nature reviews. Molecular cell biology* **3**, 651-662
100. Regis, G., Pensa, S., Boselli, D., Novelli, F., and Poli, V. (2008) Ups and downs: the STAT1:STAT3 seesaw of Interferon and gp130 receptor signalling. *Seminars in cell & developmental biology* **19**, 351-359
101. Yang, J., and Stark, G. R. (2008) Roles of unphosphorylated STATs in signaling. *Cell research* **18**, 443-451
102. Casanova, J. L., Holland, S. M., and Notarangelo, L. D. (2012) Inborn errors of human JAKs and STATs. *Immunity* **36**, 515-528
103. Hambleton, S., Goodbourn, S., Young, D. F., Dickinson, P., Mohamad, S. M., Valappil, M., McGovern, N., Cant, A. J., Hackett, S. J., Ghazal, P., Morgan, N. V., and Randall, R. E. (2013) STAT2 deficiency and susceptibility to viral illness in humans. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 3053-3058

104. Rochman, Y., Spolski, R., and Leonard, W. J. (2009) New insights into the regulation of T cells by gamma(c) family cytokines. *Nature reviews. Immunology* **9**, 480-490
105. Akira, S. (2000) Roles of STAT3 defined by tissue-specific gene targeting. *Oncogene* **19**, 2607-2611
106. Johnston, P. A., and Grandis, J. R. (2011) STAT3 signaling: anticancer strategies and challenges. *Molecular interventions* **11**, 18-26
107. Levy, D. E., and Lee, C. K. (2002) What does Stat3 do? *The Journal of clinical investigation* **109**, 1143-1148
108. Zhang, H. F., and Lai, R. (2014) STAT3 in Cancer-Friend or Foe? *Cancers* **6**, 1408-1440
109. Carpenter, R. L., and Lo, H. W. (2014) STAT3 Target Genes Relevant to Human Cancers. *Cancers* **6**, 897-925
110. Delgoffe, G. M., and Vignali, D. A. (2013) STAT heterodimers in immunity: A mixed message or a unique signal? *Jak-Stat* **2**, e23060
111. Wegrzyn, J., Potla, R., Chwae, Y. J., Sepuri, N. B., Zhang, Q., Koeck, T., Derecka, M., Szczepanek, K., Szelag, M., Gornicka, A., Moh, A., Moghaddas, S., Chen, Q., Bobbili, S., Cichy, J., Dulak, J., Baker, D. P., Wolfman, A., Stuehr, D., Hassan, M. O., Fu, X. Y., Avadhani, N., Drake, J. I., Fawcett, P., Lesnefsky, E. J., and Larner, A. C. (2009) Function of mitochondrial Stat3 in cellular respiration. *Science* **323**, 793-797
112. Dauer, D. J., Ferraro, B., Song, L., Yu, B., Mora, L., Buettner, R., Enkemann, S., Jove, R., and Haura, E. B. (2005) Stat3 regulates genes common to both wound healing and cancer. *Oncogene* **24**, 3397-3408
113. Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3801-3804
114. Yu, H., Pardoll, D., and Jove, R. (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. *Nature reviews. Cancer* **9**, 798-809
115. Yu, H., Kortylewski, M., and Pardoll, D. (2007) Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nature reviews. Immunology* **7**, 41-51
116. Tangye, S. G., Cook, M. C., and Fulcher, D. A. (2009) Insights into the role of STAT3 in human lymphocyte differentiation as revealed by the hyper-IgE syndrome. *Journal of immunology* **182**, 21-28
117. Harris, T. J., Grosso, J. F., Yen, H. R., Xin, H., Kortylewski, M., Albesiano, E., Hipkiss, E. L., Getnet, D., Goldberg, M. V., Maris, C. H., Housseau, F., Yu, H., Pardoll, D. M., and Drake, C. G. (2007) Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. *Journal of immunology* **179**, 4313-4317
118. Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S., and Dong, C. (2007) STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *The Journal of biological chemistry* **282**, 9358-9363
119. Stumhofer, J. S., Silver, J. S., Laurence, A., Porrett, P. M., Harris, T. H., Turka, L. A., Ernst, M., Saris, C. J., O'Shea, J. J., and Hunter, C. A. (2007) Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nature immunology* **8**, 1363-1371

120. Cui, W., Liu, Y., Weinstein, J. S., Craft, J., and Kaech, S. M. (2011) An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity* **35**, 792-805
121. Fornek, J. L., Tygrett, L. T., Waldschmidt, T. J., Poli, V., Rickert, R. C., and Kansas, G. S. (2006) Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. *Blood* **107**, 1085-1091
122. Takeda, K., Clausen, B. E., Kaisho, T., Tsujimura, T., Terada, N., Forster, I., and Akira, S. (1999) Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* **10**, 39-49
123. Nguyen-Jackson, H., Panopoulos, A. D., Zhang, H., Li, H. S., and Watowich, S. S. (2010) STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. *Blood* **115**, 3354-3363
124. Laouar, Y., Welte, T., Fu, X. Y., and Flavell, R. A. (2003) STAT3 is required for Flt3L-dependent dendritic cell differentiation. *Immunity* **19**, 903-912
125. Milner, J. D., Vogel, T. P., Forbes, L., Ma, C. A., Stray-Pedersen, A., Niemela, J. E., Lyons, J. J., Engelhardt, K. R., Zhang, Y., Topcagic, N., Roberson, E. D., Matthews, H., Verbsky, J. W., Dasu, T., Vargas-Hernandez, A., Varghese, N., McClain, K. L., Karam, L. B., Nahmod, K., Makedonas, G., Mace, E. M., Sorte, H. S., Perminow, G., Rao, V. K., O'Connell, M. P., Price, S., Su, H. C., Butrick, M., McElwee, J., Hughes, J. D., Willet, J., Swan, D., Xu, Y., Santibanez-Koref, M., Slowik, V., Dinwiddie, D. L., Ciaccio, C. E., Saunders, C. J., Septer, S., Kingsmore, S. F., White, A. J., Cant, A. J., Hambleton, S., and Cooper, M. A. (2015) Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. *Blood* **125**, 591-599
126. Haapaniemi, E. M., Kaustio, M., Rajala, H. L., van Adrichem, A. J., Kainulainen, L., Glumoff, V., Doffinger, R., Kuusanmaki, H., Heiskanen-Kosma, T., Trotta, L., Chiang, S., Kulmala, P., Eldfors, S., Katainen, R., Siitonen, S., Karjalainen-Lindsberg, M. L., Kovanen, P. E., Otonkoski, T., Porkka, K., Heiskanen, K., Hanninen, A., Bryceson, Y. T., Uusitalo-Seppala, R., Saarela, J., Seppanen, M., Mustjoki, S., and Kere, J. (2015) Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. *Blood* **125**, 639-648
127. Davis, S. D., Schaller, J., and Wedgwood, R. J. (1966) Job's Syndrome. Recurrent, "cold", staphylococcal abscesses. *Lancet* **1**, 1013-1015
128. Buckley, R. H., Wray, B. B., and Belmaker, E. Z. (1972) Extreme hyperimmunoglobulinemia E and undue susceptibility to infection. *Pediatrics* **49**, 59-70
129. Sowerwine, K. J., Holland, S. M., and Freeman, A. F. (2012) Hyper-IgE syndrome update. *Annals of the New York Academy of Sciences* **1250**, 25-32
130. Holland, S. M., DeLeo, F. R., Elloumi, H. Z., Hsu, A. P., Uzel, G., Brodsky, N., Freeman, A. F., Demidowich, A., Davis, J., Turner, M. L., Anderson, V. L., Darnell, D. N., Welch, P. A., Kuhns, D. B., Frucht, D. M., Malech, H. L., Gallin, J. I., Kobayashi, S. D., Whitney, A. R., Voyich, J. M., Musser, J. M., Woellner, C., Schaffer, A. A., Puck, J. M., and Grimbacher, B. (2007) STAT3 mutations in the hyper-IgE syndrome. *The New England journal of medicine* **357**, 1608-1619
131. Minegishi, Y., Saito, M., Tsuchiya, S., Tsuge, I., Takada, H., Hara, T., Kawamura, N., Ariga, T., Pasic, S., Stojkovic, O., Metin, A., and Karasuyama, H.

(2007) Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature* **448**, 1058-1062
132. Freeman, A. F., and Holland, S. M. (2009) Clinical manifestations, etiology, and pathogenesis of the hyper-IgE syndromes. *Pediatric research* **65**, 32R-37R
133. Kane, A., Deenick, E. K., Ma, C. S., Cook, M. C., Uzel, G., and Tangye, S. G. (2014) STAT3 is a central regulator of lymphocyte differentiation and function. *Current opinion in immunology* **28**, 49-57
134. Denman, C. J., Senyukov, V. V., Somanchi, S. S., Phatarpekar, P. V., Kopp, L. M., Johnson, J. L., Singh, H., Hurton, L., Maiti, S. N., Huls, M. H., Champlin, R. E., Cooper, L. J., and Lee, D. A. (2012) Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PloS one* **7**, e30264
135. Zeng, R., Spolski, R., Casas, E., Zhu, W., Levy, D. E., and Leonard, W. J. (2007) The molecular basis of IL-21-mediated proliferation. *Blood* **109**, 4135-4142
136. Zhu, S., Phatarpekar, P. V., Denman, C. J., Senyukov, V. V., Somanchi, S. S., Nguyen-Jackson, H. T., Mace, E. M., Freeman, A. F., Watowich, S. S., Orange, J. S., Holland, S. M., and Lee, D. A. (2014) Transcription of the activating receptor NKG2D in natural killer cells is regulated by STAT3 tyrosine phosphorylation. *Blood* **124**, 403-411
137. Villarino, A. V., Kanno, Y., Ferdinand, J. R., and O'Shea, J. J. (2015) Mechanisms of Jak/STAT signaling in immunity and disease. *Journal of immunology* **194**, 21-27
138. Yang, E., Wen, Z., Haspel, R. L., Zhang, J. J., and Darnell, J. E., Jr. (1999) The linker domain of Stat1 is required for gamma interferon-driven transcription. *Molecular and cellular biology* **19**, 5106-5112
139. Horvath, C. M. (2000) STAT proteins and transcriptional responses to extracellular signals. *Trends in biochemical sciences* **25**, 496-502
140. Wang, X., Crowe, P. J., Goldstein, D., and Yang, J. L. (2012) STAT3 inhibition, a novel approach to enhancing targeted therapy in human cancers (review). *International journal of oncology* **41**, 1181-1191
141. Yong, P. F., Freeman, A. F., Engelhardt, K. R., Holland, S., Puck, J. M., and Grimbacher, B. (2012) An update on the hyper-IgE syndromes. *Arthritis research & therapy* **14**, 228
142. Renner, E. D., Rylaarsdam, S., Anover-Sombke, S., Rack, A. L., Reichenbach, J., Carey, J. C., Zhu, Q., Jansson, A. F., Barboza, J., Schimke, L. F., Leppert, M. F., Getz, M. M., Seger, R. A., Hill, H. R., Belohradsky, B. H., Torgerson, T. R., and Ochs, H. D. (2008) Novel signal transducer and activator of transcription 3 (STAT3) mutations, reduced T(H)17 cell numbers, and variably defective STAT3 phosphorylation in hyper-IgE syndrome. *The Journal of allergy and clinical immunology* **122**, 181-187
143. Jiao, H., Toth, B., Erdos, M., Fransson, I., Rakoczi, E., Balogh, I., Magyarics, Z., Derfalvi, B., Csorba, G., Szaflarska, A., Megarbane, A., Akatcherian, C., Dbaibo, G., Rajnavolgyi, E., Hammarstrom, L., Kere, J., Lefranc, G., and Marodi, L. (2008) Novel and recurrent STAT3 mutations in hyper-IgE syndrome patients from different ethnic groups. *Molecular immunology* **46**, 202-206
144. Chandesris, M. O., Melki, I., Natividad, A., Puel, A., Fieschi, C., Yun, L., Thumerelle, C., Oksenhendler, E., Boutboul, D., Thomas, C., Hoarau, C., Lebranchu, Y., Stephan, J. L., Cazorla, C., Aladjidi, N., Micheau, M., Tron, F., Baruchel, A., Barlogis, V., Palenzuela, G., Mathey, C., Dominique, S., Body, G.,

Munzer, M., Fouyssac, F., Jaussaud, R., Bader-Meunier, B., Mahlaoui, N., Blanche, S., Debre, M., Le Bourgeois, M., Gandemer, V., Lambert, N., Grandin, V., Ndaga, S., Jacques, C., Harre, C., Forveille, M., Alyanakian, M. A., Durandy, A., Bodemer, C., Suarez, F., Hermine, O., Lortholary, O., Casanova, J. L., Fischer, A., and Picard, C. (2012) Autosomal dominant STAT3 deficiency and hyper-IgE syndrome: molecular, cellular, and clinical features from a French national survey. *Medicine* **91**, e1-19

145. Woellner, C., Gertz, E. M., Schaffer, A. A., Lagos, M., Perro, M., Glocker, E. O., Pietrogrande, M. C., Cossu, F., Franco, J. L., Matamoros, N., Pietrucha, B., Heropolitanska-Pliszka, E., Yeganeh, M., Moin, M., Espanol, T., Ehl, S., Gennery, A. R., Abinun, M., Breborowicz, A., Niehues, T., Kilic, S. S., Junker, A., Turvey, S. E., Plebani, A., Sanchez, B., Garty, B. Z., Pignata, C., Cancrini, C., Litzman, J., Sanal, O., Baumann, U., Bacchetta, R., Hsu, A. P., Davis, J. N., Hammarstrom, L., Davies, E. G., Eren, E., Arkwright, P. D., Moilanen, J. S., Viemann, D., Khan, S., Marodi, L., Cant, A. J., Freeman, A. F., Puck, J. M., Holland, S. M., and Grimbacher, B. (2010) Mutations in STAT3 and diagnostic guidelines for hyper-IgE syndrome. *The Journal of allergy and clinical immunology* **125**, 424-432 e428
146. Schimke, L. F., Sawalle-Belohradsky, J., Roesler, J., Wollenberg, A., Rack, A., Borte, M., Rieber, N., Cremer, R., Maass, E., Dopfer, R., Reichenbach, J., Wahn, V., Hoenig, M., Jansson, A. F., Roesen-Wolff, A., Schaub, B., Seger, R., Hill, H. R., Ochs, H. D., Torgerson, T. R., Belohradsky, B. H., and Renner, E. D. (2010) Diagnostic approach to the hyper-IgE syndromes: immunologic and clinical key findings to differentiate hyper-IgE syndromes from atopic dermatitis. *The Journal of allergy and clinical immunology* **126**, 611-617 e611
147. Milner, J. D., Brenchley, J. M., Laurence, A., Freeman, A. F., Hill, B. J., Elias, K. M., Kanno, Y., Spalding, C., Elloumi, H. Z., Paulson, M. L., Davis, J., Hsu, A., Asher, A. I., O'Shea, J., Holland, S. M., Paul, W. E., and Douek, D. C. (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* **452**, 773-776
148. Ma, C. S., Chew, G. Y., Simpson, N., Priyadarshi, A., Wong, M., Grimbacher, B., Fulcher, D. A., Tangye, S. G., and Cook, M. C. (2008) Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *The Journal of experimental medicine* **205**, 1551-1557
149. Ma, C. S., Avery, D. T., Chan, A., Batten, M., Bustamante, J., Boisson-Dupuis, S., Arkwright, P. D., Kreins, A. Y., Averbuch, D., Engelhard, D., Magdorf, K., Kilic, S. S., Minegishi, Y., Nonoyama, S., French, M. A., Choo, S., Smart, J. M., Peake, J., Wong, M., Gray, P., Cook, M. C., Fulcher, D. A., Casanova, J. L., Deenick, E. K., and Tangye, S. G. (2012) Functional STAT3 deficiency compromises the generation of human T follicular helper cells. *Blood* **119**, 3997-4008
150. Saito, M., Nagasawa, M., Takada, H., Hara, T., Tsuchiya, S., Agematsu, K., Yamada, M., Kawamura, N., Ariga, T., Tsuge, I., Nonoyama, S., Karasuyama, H., and Minegishi, Y. (2011) Defective IL-10 signaling in hyper-IgE syndrome results in impaired generation of tolerogenic dendritic cells and induced regulatory T cells. *The Journal of experimental medicine* **208**, 235-249
151. Siegel, A. M., Heimall, J., Freeman, A. F., Hsu, A. P., Brittain, E., Brenchley, J. M., Douek, D. C., Fahle, G. H., Cohen, J. I., Holland, S. M., and Milner, J. D.

(2011) A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. *Immunity* **35**, 806-818
152. Speckmann, C., Enders, A., Woellner, C., Thiel, D., Rensing-Ehl, A., Schlesier, M., Rohr, J., Jakob, T., Oswald, E., Kopp, M. V., Sanal, O., Litzman, J., Plebani, A., Pietrogrande, M. C., Franco, J. L., Espanol, T., Grimbacher, B., and Ehl, S. (2008) Reduced memory B cells in patients with hyper IgE syndrome. *Clinical immunology* **129**, 448-454
153. Avery, D. T., Deenick, E. K., Ma, C. S., Suryani, S., Simpson, N., Chew, G. Y., Chan, T. D., Palendira, U., Bustamante, J., Boisson-Dupuis, S., Choo, S., Bleasel, K. E., Peake, J., King, C., French, M. A., Engelhard, D., Al-Hajjar, S., Al-Muhsen, S., Magdorf, K., Roesler, J., Arkwright, P. D., Hissaria, P., Riminton, D. S., Wong, M., Brink, R., Fulcher, D. A., Casanova, J. L., Cook, M. C., and Tangye, S. G. (2010) B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *The Journal of experimental medicine* **207**, 155-171
154. Siegel, A. M., Stone, K. D., Cruse, G., Lawrence, M. G., Olivera, A., Jung, M. Y., Barber, J. S., Freeman, A. F., Holland, S. M., O'Brien, M., Jones, N., Nelson, C. G., Wisch, L. B., Kong, H. H., Desai, A., Farber, O., Gilfillan, A. M., Rivera, J., and Milner, J. D. (2013) Diminished allergic disease in patients with STAT3 mutations reveals a role for STAT3 signaling in mast cell degranulation. *The Journal of allergy and clinical immunology* **132**, 1388-1396
155. Leonard, G. D., Posadas, E., Herrmann, P. C., Anderson, V. L., Jaffe, E. S., Holland, S. M., and Wilson, W. H. (2004) Non-Hodgkin's lymphoma in Job's syndrome: a case report and literature review. *Leukemia & lymphoma* **45**, 2521-2525
156. Kumanovics, A., Perkins, S. L., Gilbert, H., Cessna, M. H., Augustine, N. H., and Hill, H. R. (2010) Diffuse large B cell lymphoma in hyper-IgE syndrome due to STAT3 mutation. *Journal of clinical immunology* **30**, 886-893
157. Krzewski, K., and Coligan, J. E. (2012) Human NK cell lytic granules and regulation of their exocytosis. *Frontiers in immunology* **3**, 335
158. Orange, J. S. (2008) Formation and function of the lytic NK-cell immunological synapse. *Nature reviews. Immunology* **8**, 713-725
159. Mace, E. M., Dongre, P., Hsu, H. T., Sinha, P., James, A. M., Mann, S. S., Forbes, L. R., Watkin, L. B., and Orange, J. S. (2014) Cell biological steps and checkpoints in accessing NK cell cytotoxicity. *Immunology and cell biology* **92**, 245-255
160. Romee, R., Leong, J. W., and Fehniger, T. A. (2014) Utilizing cytokines to function-enable human NK cells for the immunotherapy of cancer. *Scientifica* **2014**, 205796
161. Liang, S., Wei, H., Sun, R., and Tian, Z. (2003) IFNalpha regulates NK cell cytotoxicity through STAT1 pathway. *Cytokine* **23**, 190-199
162. Imada, K., Bloom, E. T., Nakajima, H., Horvath-Arcidiacono, J. A., Udy, G. B., Davey, H. W., and Leonard, W. J. (1998) Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *The Journal of experimental medicine* **188**, 2067-2074
163. Fortin, C., Huang, X., and Yang, Y. (2013) Both NK cell-intrinsic and -extrinsic STAT1 signaling are required for NK cell response against vaccinia virus. *Journal of immunology* **191**, 363-368

164. Lee, C. K., Rao, D. T., Gertner, R., Gimeno, R., Frey, A. B., and Levy, D. E. (2000) Distinct requirements for IFNs and STAT1 in NK cell function. *Journal of immunology* **165**, 3571-3577
165. Lesinski, G. B., Anghelina, M., Zimmerer, J., Bakalakos, T., Badgwell, B., Parihar, R., Hu, Y., Becknell, B., Abood, G., Chaudhury, A. R., Magro, C., Durbin, J., and Carson, W. E., 3rd. (2003) The antitumor effects of IFN-alpha are abrogated in a STAT1-deficient mouse. *The Journal of clinical investigation* **112**, 170-180
166. Vairo, D., Tassone, L., Tabellini, G., Tamassia, N., Gasperini, S., Bazzoni, F., Plebani, A., Porta, F., Notarangelo, L. D., Parolini, S., Giliani, S., and Badolato, R. (2011) Severe impairment of IFN-gamma and IFN-alpha responses in cells of a patient with a novel STAT1 splicing mutation. *Blood* **118**, 1806-1817
167. Kaplan, M. H., Sun, Y. L., Hoey, T., and Grusby, M. J. (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* **382**, 174-177
168. Thierfelder, W. E., van Deursen, J. M., Yamamoto, K., Tripp, R. A., Sarawar, S. R., Carson, R. T., Sangster, M. Y., Vignali, D. A., Doherty, P. C., Grosveld, G. C., and Ihle, J. N. (1996) Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* **382**, 171-174
169. Frank, D. A., Robertson, M. J., Bonni, A., Ritz, J., and Greenberg, M. E. (1995) Interleukin 2 signaling involves the phosphorylation of Stat proteins. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7779-7783
170. Kim, T. J., Kim, N., Kang, H. J., Kim, E. O., Kim, S. T., Ahn, H. S., Bluestone, J. A., and Lee, K. M. (2010) FK506 causes cellular and functional defects in human natural killer cells. *Journal of leukocyte biology* **88**, 1089-1097
171. Strengell, M., Matikainen, S., Siren, J., Lehtonen, A., Foster, D., Julkunen, I., and Sareneva, T. (2003) IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. *Journal of immunology* **170**, 5464-5469
172. Kalina, U., Kauschat, D., Koyama, N., Nuernberger, H., Ballas, K., Koschmieder, S., Bug, G., Hofmann, W. K., Hoelzer, D., and Ottmann, O. G. (2000) IL-18 activates STAT3 in the natural killer cell line 92, augments cytotoxic activity, and mediates IFN-gamma production by the stress kinase p38 and by the extracellular regulated kinases p44erk-1 and p42erk-21. *Journal of immunology* **165**, 1307-1313
173. Yu, C. R., Lin, J. X., Fink, D. W., Akira, S., Bloom, E. T., and Yamauchi, A. (1996) Differential utilization of Janus kinase-signal transducer activator of transcription signaling pathways in the stimulation of human natural killer cells by IL-2, IL-12, and IFN-alpha. *Journal of immunology* **157**, 126-137
174. Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Darnell, J. E., Jr. (1999) Stat3 as an oncogene. *Cell* **98**, 295-303
175. Yue, P., and Turkson, J. (2009) Targeting STAT3 in cancer: how successful are we? *Expert opinion on investigational drugs* **18**, 45-56
176. Somanchi, S. S., Senyukov, V. V., Denman, C. J., and Lee, D. A. (2011) Expansion, purification, and functional assessment of human peripheral blood NK cells. *Journal of visualized experiments : JoVE*

177. Bryant, J., Day, R., Whiteside, T. L., and Herberman, R. B. (1992) Calculation of lytic units for the expression of cell-mediated cytotoxicity. *Journal of immunological methods* **146**, 91-103
178. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics* **13**, 134
179. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature methods* **9**, 671-675
180. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408
181. Liadi, I., Roszik, J., Romain, G., Cooper, L. J., and Varadarajan, N. (2013) Quantitative high-throughput single-cell cytotoxicity assay for T cells. *Journal of visualized experiments : JoVE*, e50058
182. Romain, G., Senyukov, V., Rey-Villamizar, N., Merouane, A., Kelton, W., Liadi, I., Mahendra, A., Charab, W., Georgiou, G., Roysam, B., Lee, D. A., and Varadarajan, N. (2014) Antibody Fc engineering improves frequency and promotes kinetic boosting of serial killing mediated by NK cells. *Blood* **124**, 3241-3249
183. Merouane, A., Rey-Villamizar, N., Lu, Y., Liadi, I., Romain, G., Lu, J., Singh, H., Cooper, L. J., Varadarajan, N., and Roysam, B. (2015) Automated profiling of individual cell-cell interactions from high-throughput time-lapse imaging microscopy in nanowell grids (TIMING). *Bioinformatics*
184. Banerjee, P. P., and Orange, J. S. (2010) Quantitative measurement of F-actin accumulation at the NK cell immunological synapse. *Journal of immunological methods* **355**, 1-13
185. Banerjee, P. P., Pandey, R., Zheng, R., Suhoski, M. M., Monaco-Shawver, L., and Orange, J. S. (2007) Cdc42-interacting protein-4 functionally links actin and microtubule networks at the cytolytic NK cell immunological synapse. *The Journal of experimental medicine* **204**, 2305-2320
186. Mentlik, A. N., Sanborn, K. B., Holzbaur, E. L., and Orange, J. S. (2010) Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Molecular biology of the cell* **21**, 2241-2256
187. Roger, R., Issaad, C., Pallardy, M., Leglise, M. C., Turhan, A. G., Bertoglio, J., and Breard, J. (1996) BCR-ABL does not prevent apoptotic death induced by human natural killer or lymphokine-activated killer cells. *Blood* **87**, 1113-1122
188. Park, Y. W., Kee, S. J., Cho, Y. N., Lee, E. H., Lee, H. Y., Kim, E. M., Shin, M. H., Park, J. J., Kim, T. J., Lee, S. S., Yoo, D. H., and Kang, H. S. (2009) Impaired differentiation and cytotoxicity of natural killer cells in systemic lupus erythematosus. *Arthritis and rheumatism* **60**, 1753-1763
189. Voskens, C. J., Watanabe, R., Rollins, S., Campana, D., Hasumi, K., and Mann, D. L. (2010) Ex-vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity. *Journal of experimental & clinical cancer research : CR* **29**, 134
190. Bryceson, Y. T., Fauriat, C., Nunes, J. M., Wood, S. M., Bjorkstrom, N. K., Long, E. O., and Ljunggren, H. G. (2010) Functional analysis of human NK cells by flow cytometry. *Methods in molecular biology* **612**, 335-352

191. Sierich, H., and Eiermann, T. (2013) Comparing individual NK cell activity in vitro. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **Chapter 14**, Unit 14 32
192. Thielens, A., Vivier, E., and Romagne, F. (2012) NK cell MHC class I specific receptors (KIR): from biology to clinical intervention. *Current opinion in immunology* **24**, 239-245
193. Cooper, M. A., Fehniger, T. A., and Caligiuri, M. A. (2001) The biology of human natural killer-cell subsets. *Trends in immunology* **22**, 633-640
194. Nielsen, C. M., White, M. J., Goodier, M. R., and Riley, E. M. (2013) Functional Significance of CD57 Expression on Human NK Cells and Relevance to Disease. *Frontiers in immunology* **4**, 422
195. Vossen, M. T., Matmati, M., Hertoghs, K. M., Baars, P. A., Gent, M. R., Leclercq, G., Hamann, J., Kuijpers, T. W., and van Lier, R. A. (2008) CD27 defines phenotypically and functionally different human NK cell subsets. *Journal of immunology* **180**, 3739-3745
196. Clausen, J., Vergeiner, B., Enk, M., Petzer, A. L., Gastl, G., and Gunsilius, E. (2003) Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells. *Immunobiology* **207**, 85-93
197. Dancker, P., Low, I., Hasselbach, W., and Wieland, T. (1975) Interaction of actin with phalloidin: polymerization and stabilization of F-actin. *Biochimica et biophysica acta* **400**, 407-414
198. Lin, L., Amin, R., Gallicano, G. I., Glasgow, E., Jogunoori, W., Jessup, J. M., Zasloff, M., Marshall, J. L., Shetty, K., Johnson, L., Mishra, L., and He, A. R. (2009) The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-beta signaling. *Oncogene* **28**, 961-972
199. Liongue, C., and Ward, A. C. (2013) Evolution of the JAK-STAT pathway. *Jak-Stat* **2**, e22756
200. Tuli, A., Thiery, J., James, A. M., Michelet, X., Sharma, M., Garg, S., Sanborn, K. B., Orange, J. S., Lieberman, J., and Brenner, M. B. (2013) Arf-like GTPase Arl8b regulates lytic granule polarization and natural killer cell-mediated cytotoxicity. *Molecular biology of the cell* **24**, 3721-3735
201. Kanwar, N., and Wilkins, J. A. (2011) IQGAP1 involvement in MTOC and granule polarization in NK-cell cytotoxicity. *European journal of immunology* **41**, 2763-2773
202. Wang, X., Lee, D. A., Wang, Y., Wang, L., Yao, Y., Lin, Z., Cheng, J., and Zhu, S. (2013) Membrane-bound interleukin-21 and CD137 ligand induce functional human natural killer cells from peripheral blood mononuclear cells through STAT-3 activation. *Clinical and experimental immunology* **172**, 104-112
203. Siddiquee, K., Zhang, S., Guida, W. C., Blaskovich, M. A., Greedy, B., Lawrence, H. R., Yip, M. L., Jove, R., McLaughlin, M. M., Lawrence, N. J., Sebti, S. M., and Turkson, J. (2007) Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 7391-7396
204. Rook, A. H., Hooks, J. J., Quinnan, G. V., Lane, H. C., Manischewitz, J. F., Macher, A. M., Masur, H., Fauci, A. S., and Djeu, J. Y. (1985) Interleukin 2 enhances the natural killer cell activity of acquired immunodeficiency syndrome patients through a gamma-interferon-independent mechanism. *Journal of immunology* **134**, 1503-1507

205. Ogbomo, H., Michaelis, M., Kreuter, J., Doerr, H. W., and Cinatl, J., Jr. (2007) Histone deacetylase inhibitors suppress natural killer cell cytolytic activity. *FEBS letters* **581**, 1317-1322
206. Chiossone, L., Vitale, C., Cottalasso, F., Moretti, S., Azzarone, B., Moretta, L., and Mingari, M. C. (2007) Molecular analysis of the methylprednisolone-mediated inhibition of NK-cell function: evidence for different susceptibility of IL-2- versus IL-15-activated NK cells. *Blood* **109**, 3767-3775
207. Meazza, R., Azzarone, B., Orengo, A. M., and Ferrini, S. (2011) Role of common-gamma chain cytokines in NK cell development and function: perspectives for immunotherapy. *Journal of biomedicine & biotechnology* **2011**, 861920
208. Eckelhart, E., Warsch, W., Zebedin, E., Simma, O., Stoiber, D., Kolbe, T., Rulicke, T., Mueller, M., Casanova, E., and Sexl, V. (2011) A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. *Blood* **117**, 1565-1573
209. Hervas-Stubbs, S., Perez-Gracia, J. L., Rouzaut, A., Sanmamed, M. F., Le Bon, A., and Melero, I. (2011) Direct effects of type I interferons on cells of the immune system. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 2619-2627
210. Robbins, S. H., Tessmer, M. S., Van Kaer, L., and Brossay, L. (2005) Direct effects of T-bet and MHC class I expression, but not STAT1, on peripheral NK cell maturation. *European journal of immunology* **35**, 757-765
211. Robertson, M. J., Soiffer, R. J., Wolf, S. F., Manley, T. J., Donahue, C., Young, D., Herrmann, S. H., and Ritz, J. (1992) Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *The Journal of experimental medicine* **175**, 779-788
212. Gately, M. K., Warrier, R. R., Honasoge, S., Carvajal, D. M., Faherty, D. A., Connaughton, S. E., Anderson, T. D., Sarmiento, U., Hubbard, B. R., and Murphy, M. (1994) Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-gamma in vivo. *International immunology* **6**, 157-167
213. Gately, M. K., Wolitzky, A. G., Quinn, P. M., and Chizzonite, R. (1992) Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cellular immunology* **143**, 127-142
214. Chehimi, J., Starr, S. E., Frank, I., Rengaraju, M., Jackson, S. J., Llanes, C., Kobayashi, M., Perussia, B., Young, D., Nickbarg, E., and et al. (1992) Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus-infected patients. *The Journal of experimental medicine* **175**, 789-796
215. Trzonkowski, P., Szmit, E., Mysliwska, J., Dobyszuk, A., and Mysliwski, A. (2004) CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. *Clinical immunology* **112**, 258-267
216. Ghiringhelli, F., Menard, C., Terme, M., Flament, C., Taieb, J., Chaput, N., Puig, P. E., Novault, S., Escudier, B., Vivier, E., Lecesne, A., Robert, C., Blay, J. Y., Bernard, J., Caillat-Zucman, S., Freitas, A., Tursz, T., Wagner-Ballon, O., Capron, C., Vainchencker, W., Martin, F., and Zitvogel, L. (2005) CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth

factor-beta-dependent manner. *The Journal of experimental medicine* **202**, 1075-1085
217. Caligiuri, M. A., Murray, C., Robertson, M. J., Wang, E., Cochran, K., Cameron, C., Schow, P., Ross, M. E., Klumpp, T. R., Soiffer, R. J., and et al. (1993) Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. *The Journal of clinical investigation* **91**, 123-132
218. Yu, T. K., Caudell, E. G., Smid, C., and Grimm, E. A. (2000) IL-2 activation of NK cells: involvement of MKK1/2/ERK but not p38 kinase pathway. *Journal of immunology* **164**, 6244-6251
219. Vitale, M., Bassini, A., Secchiero, P., Mirandola, P., Ponti, C., Zamai, L., Mariani, A. R., Falconi, M., and Azzali, G. (2002) NK-active cytokines IL-2, IL-12, and IL-15 selectively modulate specific protein kinase C (PKC) isoforms in primary human NK cells. *The Anatomical record* **266**, 87-92
220. Yu, C. R., Young, H. A., and Ortaldo, J. R. (1998) Characterization of cytokine differential induction of STAT complexes in primary human T and NK cells. *Journal of leukocyte biology* **64**, 245-258
221. Carow, B., and Rottenberg, M. E. (2014) SOCS3, a Major Regulator of Infection and Inflammation. *Frontiers in immunology* **5**, 58
222. Braunschweig, A., Poehlmann, T. G., Busch, S., Schleussner, E., and Markert, U. R. (2011) Signal transducer and activator of transcription 3 (STAT3) and Suppressor of Cytokine Signaling (SOCS3) balance controls cytotoxicity and IL-10 expression in decidual-like natural killer cell line NK-92. *American journal of reproductive immunology* **66**, 329-335
223. Iannello, A., Boulassel, M. R., Samarani, S., Tremblay, C., Toma, E., Routy, J. P., and Ahmad, A. (2010) IL-21 enhances NK cell functions and survival in healthy and HIV-infected patients with minimal stimulation of viral replication. *Journal of leukocyte biology* **87**, 857-867
224. Fujita, M., Zhu, X., Sasaki, K., Ueda, R., Low, K. L., Pollack, I. F., and Okada, H. (2008) Inhibition of STAT3 promotes the efficacy of adoptive transfer therapy using type-1 CTLs by modulation of the immunological microenvironment in a murine intracranial glioma. *Journal of immunology* **180**, 2089-2098
225. Dai, B., Meng, J., Peyton, M., Girard, L., Bornmann, W. G., Ji, L., Minna, J. D., Fang, B., and Roth, J. A. (2011) STAT3 mediates resistance to MEK inhibitor through microRNA miR-17. *Cancer research* **71**, 3658-3668
226. Blaskovich, M. A., Sun, J., Cantor, A., Turkson, J., Jove, R., and Sebti, S. M. (2003) Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. *Cancer research* **63**, 1270-1279
227. Shi, X., Franko, B., Frantz, C., Amin, H. M., and Lai, R. (2006) JSI-124 (cucurbitacin I) inhibits Janus kinase-3/signal transducer and activator of transcription-3 signalling, downregulates nucleophosmin-anaplastic lymphoma kinase (ALK), and induces apoptosis in ALK-positive anaplastic large cell lymphoma cells. *British journal of haematology* **135**, 26-32
228. Stankiewicz, T. R., Loucks, F. A., Schroeder, E. K., Nevalainen, M. T., Tyler, K. L., Aktories, K., Bouchard, R. J., and Linseman, D. A. (2012) Signal transducer and activator of transcription-5 mediates neuronal apoptosis induced by

inhibition of Rac GTPase activity. *The Journal of biological chemistry* **287**, 16835-16848
229. Kortylewski, M., Kujawski, M., Wang, T., Wei, S., Zhang, S., Pilon-Thomas, S., Niu, G., Kay, H., Mule, J., Kerr, W. G., Jove, R., Pardoll, D., and Yu, H. (2005) Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nature medicine* **11**, 1314-1321
230. Gotthardt, D., Putz, E. M., Straka, E., Kudweis, P., Biaggio, M., Poli, V., Strobl, B., Muller, M., and Sexl, V. (2014) Loss of STAT3 in murine NK cells enhances NK cell-dependent tumor surveillance. *Blood* **124**, 2370-2379
231. Mestas, J., and Hughes, C. C. (2004) Of mice and not men: differences between mouse and human immunology. *Journal of immunology* **172**, 2731-2738
232. Vaidya, S. V., and Mathew, P. A. (2006) Of mice and men: different functions of the murine and human 2B4 (CD244) receptor on NK cells. *Immunology letters* **105**, 180-184
233. Chen, X., Trivedi, P. P., Ge, B., Krzewski, K., and Strominger, J. L. (2007) Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 6329-6334
234. Bae, D. S., Hwang, Y. K., and Lee, J. K. (2012) Importance of NKG2D-NKG2D ligands interaction for cytolytic activity of natural killer cell. *Cellular immunology* **276**, 122-127
235. Billadeau, D. D., Brumbaugh, K. M., Dick, C. J., Schoon, R. A., Bustelo, X. R., and Leibson, P. J. (1998) The Vav-Rac1 pathway in cytotoxic lymphocytes regulates the generation of cell-mediated killing. *The Journal of experimental medicine* **188**, 549-559
236. Orange, J. S., Ramesh, N., Remold-O'Donnell, E., Sasahara, Y., Koopman, L., Byrne, M., Bonilla, F. A., Rosen, F. S., Geha, R. S., and Strominger, J. L. (2002) Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 11351-11356
237. Riteau, B., Barber, D. F., and Long, E. O. (2003) Vav1 phosphorylation is induced by beta2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization. *The Journal of experimental medicine* **198**, 469-474
238. Butler, B., and Cooper, J. A. (2009) Distinct roles for the actin nucleators Arp2/3 and hDia1 during NK-mediated cytotoxicity. *Current biology : CB* **19**, 1886-1896
239. Orange, J. S., Roy-Ghanta, S., Mace, E. M., Maru, S., Rak, G. D., Sanborn, K. B., Fasth, A., Saltzman, R., Paisley, A., Monaco-Shawver, L., Banerjee, P. P., and Pandey, R. (2011) IL-2 induces a WAVE2-dependent pathway for actin reorganization that enables WASp-independent human NK cell function. *The Journal of clinical investigation* **121**, 1535-1548
240. James, A. M., Hsu, H. T., Dongre, P., Uzel, G., Mace, E. M., Banerjee, P. P., and Orange, J. S. (2013) Rapid activation receptor- or IL-2-induced lytic granule convergence in human natural killer cells requires Src, but not downstream signaling. *Blood* **121**, 2627-2637
241. Li, C., Ge, B., Nicotra, M., Stern, J. N., Kopcow, H. D., Chen, X., and Strominger, J. L. (2008) JNK MAP kinase activation is required for MTOC and

granule polarization in NKG2D-mediated NK cell cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 3017-3022
242. Segovis, C. M., Schoon, R. A., Dick, C. J., Nacusi, L. P., Leibson, P. J., and Billadeau, D. D. (2009) PI3K links NKG2D signaling to a CrkL pathway involved in natural killer cell adhesion, polarity, and granule secretion. *Journal of immunology* **182**, 6933-6942
243. Liu, D., Bryceson, Y. T., Meckel, T., Vasiliver-Shamis, G., Dustin, M. L., and Long, E. O. (2009) Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. *Immunity* **31**, 99-109
244. Liu, D., Meckel, T., and Long, E. O. (2010) Distinct role of rab27a in granule movement at the plasma membrane and in the cytosol of NK cells. *PloS one* **5**, e12870
245. Liu, D., Xu, L., Yang, F., Li, D., Gong, F., and Xu, T. (2005) Rapid biogenesis and sensitization of secretory lysosomes in NK cells mediated by target-cell recognition. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 123-127
246. Waldhauer, I., and Steinle, A. (2008) NK cells and cancer immunosurveillance. *Oncogene* **27**, 5932-5943
247. Levy, E. M., Roberti, M. P., and Mordoh, J. (2011) Natural killer cells in human cancer: from biological functions to clinical applications. *Journal of biomedicine & biotechnology* **2011**, 676198
248. Gorin, L. J., Jeha, S. C., Sullivan, M. P., Rosenblatt, H. M., and Shearer, W. T. (1989) Burkitt's lymphoma developing in a 7-year-old boy with hyper-IgE syndrome. *The Journal of allergy and clinical immunology* **83**, 5-10
249. Nester, T. A., Wagnon, A. H., Reilly, W. F., Spitzer, G., Kjeldsberg, C. R., and Hill, H. R. (1998) Effects of allogeneic peripheral stem cell transplantation in a patient with job syndrome of hyperimmunoglobulinemia E and recurrent infections. *The American journal of medicine* **105**, 162-164
250. Belada, D., Smolej, L., Stepankova, P., Kralickova, P., and Freiberger, T. (2010) Diffuse large B-cell lymphoma in a patient with hyper-IgE syndrome: Successful treatment with risk-adapted rituximab-based immunochemotherapy. *Leukemia research* **34**, e232-234
251. Goussetis, E., Peristeri, I., Kitra, V., Traeger-Synodinos, J., Theodosaki, M., Psarra, K., Kanariou, M., Tzortzatou-Stathopoulou, F., Petrakou, E., Fylaktou, I., Kanavakis, E., and Graphakos, S. (2010) Successful long-term immunologic reconstitution by allogeneic hematopoietic stem cell transplantation cures patients with autosomal dominant hyper-IgE syndrome. *The Journal of allergy and clinical immunology* **126**, 392-394
252. Clark, T. J., Herod, J. J., Kehoe, S., and Luesley, D. M. (1998) The development of invasive vulvar cancer in a patient with Job's syndrome, a rare immunodeficient condition. *British journal of obstetrics and gynaecology* **105**, 468-470
253. Oztop, I., Demirkan, B., Tarhan, O., Kayahan, H., Yilmaz, U., Kargi, A., and Alakavuklar, M. (2004) The development of pulmonary adenocarcinoma in a patient with Job's syndrome, a rare immunodeficiency condition. *Tumori* **90**, 132-135
254. Tursz, T., Dokhelar, M. C., Lipinski, M., and Amiel, J. L. (1982) Low natural killer cell activity in patients with malignant lymphoma. *Cancer* **50**, 2333-2335

255. Konjevic, G., Jurisic, V., Banicevic, B., and Spuzic, I. (1999) The difference in NK-cell activity between patients with non-Hodgkin's lymphomas and Hodgkin's disease. *British journal of haematology* **104**, 144-151
256. Reiners, K. S., Kessler, J., Sauer, M., Rothe, A., Hansen, H. P., Reusch, U., Hucke, C., Kohl, U., Durkop, H., Engert, A., and von Strandmann, E. P. (2013) Rescue of impaired NK cell activity in hodgkin lymphoma with bispecific antibodies in vitro and in patients. *Molecular therapy : the journal of the American Society of Gene Therapy* **21**, 895-903
257. Putz, E. M., Hoelzl, M. A., Baeck, J., Bago-Horvath, Z., Schuster, C., Reichholf, B., Kern, D., Aberger, F., Sexl, V., and Hoelbl-Kovacic, A. (2014) Loss of STAT3 in Lymphoma Relaxes NK Cell-Mediated Tumor Surveillance. *Cancers* **6**, 193-210
258. Roda, J. M., Parihar, R., Lehman, A., Mani, A., Tridandapani, S., and Carson, W. E., 3rd. (2006) Interleukin-21 enhances NK cell activation in response to antibody-coated targets. *Journal of immunology* **177**, 120-129
259. Eriksen, K. W., Sondergaard, H., Woetmann, A., Krejsgaard, T., Skak, K., Geisler, C., Wasik, M. A., and Odum, N. (2009) The combination of IL-21 and IFN-alpha boosts STAT3 activation, cytotoxicity and experimental tumor therapy. *Molecular immunology* **46**, 812-820
260. Takaki, R., Hayakawa, Y., Nelson, A., Sivakumar, P. V., Hughes, S., Smyth, M. J., and Lanier, L. L. (2005) IL-21 enhances tumor rejection through a NKG2D-dependent mechanism. *Journal of immunology* **175**, 2167-2173
261. Jamieson, A. M., Isnard, P., Dorfman, J. R., Coles, M. C., and Raulet, D. H. (2004) Turnover and proliferation of NK cells in steady state and lymphopenic conditions. *Journal of immunology* **172**, 864-870
262. Sun, J. C., Beilke, J. N., Bezman, N. A., and Lanier, L. L. (2011) Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection. *The Journal of experimental medicine* **208**, 357-368
263. Zhang, Y., Wallace, D. L., de Lara, C. M., Ghattas, H., Asquith, B., Worth, A., Griffin, G. E., Taylor, G. P., Tough, D. F., Beverley, P. C., and Macallan, D. C. (2007) In vivo kinetics of human natural killer cells: the effects of ageing and acute and chronic viral infection. *Immunology* **121**, 258-265
264. Lutz, C. T., Karapetyan, A., Al-Attar, A., Shelton, B. J., Holt, K. J., Tucker, J. H., and Presnell, S. R. (2011) Human NK cells proliferate and die in vivo more rapidly than T cells in healthy young and elderly adults. *Journal of immunology* **186**, 4590-4598
265. Lee, S. H., Kim, K. S., Fodil-Cornu, N., Vidal, S. M., and Biron, C. A. (2009) Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. *The Journal of experimental medicine* **206**, 2235-2251
266. Welsh, R. M., Brubaker, J. O., Vargas-Cortes, M., and O'Donnell, C. L. (1991) Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency. The stimulation of NK cells and the NK cell-dependent control of virus infections occur independently of T and B cell function. *The Journal of experimental medicine* **173**, 1053-1063
267. Guma, M., Angulo, A., Vilches, C., Gomez-Lozano, N., Malats, N., and Lopez-Botet, M. (2004) Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* **104**, 3664-3671

268. Guma, M., Budt, M., Saez, A., Brckalo, T., Hengel, H., Angulo, A., and Lopez-Botet, M. (2006) Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* **107**, 3624-3631
269. Williams, H., McAulay, K., Macsween, K. F., Gallacher, N. J., Higgins, C. D., Harrison, N., Swerdlow, A. J., and Crawford, D. H. (2005) The immune response to primary EBV infection: a role for natural killer cells. *British journal of haematology* **129**, 266-274
270. Mavilio, D., Lombardo, G., Benjamin, J., Kim, D., Follman, D., Marcenaro, E., O'Shea, M. A., Kinter, A., Kovacs, C., Moretta, A., and Fauci, A. S. (2005) Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 2886-2891
271. De Maria, A., Fogli, M., Mazza, S., Basso, M., Picciotto, A., Costa, P., Congia, S., Mingari, M. C., and Moretta, L. (2007) Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. *European journal of immunology* **37**, 445-455
272. Lee, S. H., and Biron, C. A. (2010) Here today--not gone tomorrow: roles for activating receptors in sustaining NK cells during viral infections. *European journal of immunology* **40**, 923-932
273. French, A. R., Sjolin, H., Kim, S., Koka, R., Yang, L., Young, D. A., Cerboni, C., Tomasello, E., Ma, A., Vivier, E., Karre, K., and Yokoyama, W. M. (2006) DAP12 signaling directly augments proproliferative cytokine stimulation of NK cells during viral infections. *Journal of immunology* **177**, 4981-4990
274. Geurs, T. L., Zhao, Y. M., Hill, E. B., and French, A. R. (2009) Ly49H engagement compensates for the absence of type I interferon signaling in stimulating NK cell proliferation during murine cytomegalovirus infection. *Journal of immunology* **183**, 5830-5836
275. Alter, G., Rihn, S., Walter, K., Nolting, A., Martin, M., Rosenberg, E. S., Miller, J. S., Carrington, M., and Altfeld, M. (2009) HLA class I subtype-dependent expansion of KIR3DS1+ and KIR3DL1+ NK cells during acute human immunodeficiency virus type 1 infection. *Journal of virology* **83**, 6798-6805
276. Andre, P., Castriconi, R., Espeli, M., Anfossi, N., Juarez, T., Hue, S., Conway, H., Romagne, F., Dondero, A., Nanni, M., Caillat-Zucman, S., Raulet, D. H., Bottino, C., Vivier, E., Moretta, A., and Paul, P. (2004) Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors. *European journal of immunology* **34**, 961-971
277. Wang, K. S., Ritz, J., and Frank, D. A. (1999) IL-2 induces STAT4 activation in primary NK cells and NK cell lines, but not in T cells. *Journal of immunology* **162**, 299-304
278. Pillet, A. H., Theze, J., and Rose, T. (2011) Interleukin (IL)-2 and IL-15 have different effects on human natural killer lymphocytes. *Human immunology* **72**, 1013-1017
279. Fukada, T., Hibi, M., Yamanaka, Y., Takahashi-Tezuka, M., Fujitani, Y., Yamaguchi, T., Nakajima, K., and Hirano, T. (1996) Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity* **5**, 449-460
280. Bowman, T., Broome, M. A., Sinibaldi, D., Wharton, W., Pledger, W. J., Sedivy, J. M., Irby, R., Yeatman, T., Courtneidge, S. A., and Jove, R. (2001) Stat3-mediated Myc expression is required for Src transformation and PDGF-induced

mitogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 7319-7324
281. Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S., and Jove, R. (1999) Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* **10**, 105-115
282. Gritsko, T., Williams, A., Turkson, J., Kaneko, S., Bowman, T., Huang, M., Nam, S., Eweis, I., Diaz, N., Sullivan, D., Yoder, S., Enkemann, S., Eschrich, S., Lee, J. H., Beam, C. A., Cheng, J., Minton, S., Muro-Cacho, C. A., and Jove, R. (2006) Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12**, 11-19
283. Quah, B. J., Warren, H. S., and Parish, C. R. (2007) Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nature protocols* **2**, 2049-2056
284. Parish, C. R., Glidden, M. H., Quah, B. J., and Warren, H. S. (2009) Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **Chapter 4**, Unit4 9
285. Zhang, G., Gurtu, V., Kain, S. R., and Yan, G. (1997) Early detection of apoptosis using a fluorescent conjugate of annexin V. *BioTechniques* **23**, 525-531
286. Pillet, A. H., Bugault, F., Theze, J., Chakrabarti, L. A., and Rose, T. (2009) A programmed switch from IL-15- to IL-2-dependent activation in human NK cells. *Journal of immunology* **182**, 6267-6277
287. Dunne, J., Lynch, S., O'Farrelly, C., Todryk, S., Hegarty, J. E., Feighery, C., and Doherty, D. G. (2001) Selective expansion and partial activation of human NK cells and NK receptor-positive T cells by IL-2 and IL-15. *Journal of immunology* **167**, 3129-3138
288. Huntington, N. D., Legrand, N., Alves, N. L., Jaron, B., Weijer, K., Plet, A., Corcuff, E., Mortier, E., Jacques, Y., Spits, H., and Di Santo, J. P. (2009) IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *The Journal of experimental medicine* **206**, 25-34
289. Leonard, W. J., and Spolski, R. (2005) Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nature reviews. Immunology* **5**, 688-698
290. Toomey, J. A., Gays, F., Foster, D., and Brooks, C. G. (2003) Cytokine requirements for the growth and development of mouse NK cells in vitro. *Journal of leukocyte biology* **74**, 233-242
291. Lee, J. K., Won, C., Yi, E. H., Seok, S. H., Kim, M. H., Kim, S. J., Chung, M. H., Lee, H. G., Ikuta, K., and Ye, S. K. (2013) Signal transducer and activator of transcription 3 (Stat3) contributes to T-cell homeostasis by regulating pro-survival Bcl-2 family genes. *Immunology* **140**, 288-300
292. Perdreau, H., Mortier, E., Bouchaud, G., Sole, V., Boublik, Y., Plet, A., and Jacques, Y. (2010) Different dynamics of IL-15R activation following IL-15 cis- or trans-presentation. *European cytokine network* **21**, 297-307

293. Huntington, N. D. (2014) The unconventional expression of IL-15 and its role in NK cell homeostasis. *Immunology and cell biology* **92**, 210-213
294. Kim, T. J., Kim, M., Kim, H. M., Lim, S. A., Kim, E. O., Kim, K., Song, K. H., Kim, J., Kumar, V., Yee, C., Doh, J., and Lee, K. M. (2014) Homotypic NK cell-to-cell communication controls cytokine responsiveness of innate immune NK cells. *Scientific reports* **4**, 7157
295. Zhao, W., Jaganathan, S., and Turkson, J. (2010) A cell-permeable Stat3 SH2 domain mimetic inhibits Stat3 activation and induces antitumor cell effects in vitro. *The Journal of biological chemistry* **285**, 35855-35865
296. Takeda, K., Hayakawa, Y., Smyth, M. J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H., and Okumura, K. (2001) Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nature medicine* **7**, 94-100
297. Steward-Tharp, S. M., Laurence, A., Kanno, Y., Kotlyar, A., Villarino, A. V., Sciume, G., Kuchen, S., Resch, W., Wohlfert, E. A., Jiang, K., Hirahara, K., Vahedi, G., Sun, H. W., Feigenbaum, L., Milner, J. D., Holland, S. M., Casellas, R., Powrie, F., and O'Shea, J. J. (2014) A mouse model of HIES reveals pro- and anti-inflammatory functions of STAT3. *Blood* **123**, 2978-2987

VITA

Prasad V. Phatarpekar was born in Savantwadi, Maharashtra. He is the son of Ms. Padma Phatarpekar and Mr. Vijaykumar Phatarpekar. He completed his B.Sc. in Zoology from Ruparel College, University of Mumbai, India in 1993. He thereafter earned a M.Sc. degree in Marine Sciences from Goa University, India in 1995.He worked at the National Institute of Oceanography (NIO) in Goa, India from 1995-2000 as a Marine Biologist.

In 2001, he moved to United States and joined the College of William & Mary, Virginia to pursue a Ph.D. in Marine Sciences at the Virginia Institute of Marine Sciences (VIMS). However, he decided to change his career from a Marine Biologist to a Cell Biologist. In 2002, he joined the M.S. program in Biology at Texas State University-San Marcos. After graduating from the program, he worked as a Research Assistant in the Free Radical and Aging Biology department at the Oklahoma Medical Research Foundation (OMRF), Oklahoma City, Oklahoma until 2005.

He joined the Graduate School of Biomedical Sciences at the University of Texas Health Science Center in August 2005. He joined the laboratory of Dr. Dean Anthony Lee in January 2010.