A new role for NKG2D signaling in $CD8^+$ T cells and autoimmune diabetes

By Andrew P Trembath

Submitted to the graduate degree program in Microbiology, Molecular Genetics and Immunology, and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Chair: Mary A. Markiewicz Ph.D

Wolfram R. Zückert Ph.D.

Patrick E. Fields, Ph.D.

Maria Kalamvoki Ph.D.

Joe Lutkenhaus Ph.D

Date Defended: 11/13/2019

The dissertation committee for Andrew Trembath certifies that this is the approved version of the following dissertation:

A new role for NKG2D signaling in $CD8^+$ T cells and autoimmune diabetes

Chair: Mary A. Markiewicz, Ph.D.

Date Approved: 12/19/2019

Abstract

The demands placed on the immune system are immense and highly complex. It must protect the body against untold threats while maintaining a balance between immune defense and autoimmune damage. One major player in immune recognition is the receptor Natural-Killer-Group-2-Member-D (NKG2D), best known for its expression on natural killer (NK) cells and $CD8^+$ T cells, where it recognizes NKG2D ligands expressed by stressed cells following viral infection or cancerous transformation. NKG2D is most well studied for its role in tumor immunity, for which NKG2D based therapies are currently being developed clinically. Despite this, it is apparent that NKG2D has other poorly understood immune regulating functions, such as its implicated involvement in type 1diabetes and other autoimmune disorders. However, the mechanism by which NKG2D signaling affects diabetes has been unclear. We therefore sought to further clarify the role NKG2D plays in autoimmune diabetes development.

Canonically, NKG2D engaging NKG2D ligand results in immune killing of the infected or damaged ligand-bearing cell by NK cells, and costimulation of $CD8^+$ cytotoxic lymphocytes (CTL) augmenting CTL responses and target cell killing. However, we and others have observed robust expression of NKG2D ligands by seemingly healthy immune cells. In the work presented in this dissertation, I show the work I performed to investigate how this expression by seemingly healthy cells affects the immune response. In particular I focused on $CD8^+$ T cells, which express both NKG2D and NKG2D ligands after activation, and play a key role in the development of autoimmune diabetes.

Using the non-obese diabetic (NOD) mouse model of autoimmune diabetes, I found that engagement of the NKG2D ligand H60a, expressed by NOD T cells, during $CD8^+$ T cell differentiation resulted in decreased cytokine production upon later antigen stimulation. This correlated with other findings from our lab showing decreased incidence of autoimmune diabetes in microbiota-depleted NKG2D sufficient versus NKG2D deficient NOD mice. Further, I showed that this correlated with NKG2D signaling driving an increase in $CD8^+$ T cells with a central memory phenotype in both mouse and human cells. I then found significantly reduced transfer of NOD diabetes by these central memory phenotype $CD8^+$ T cells compared to effector/effector memory $CD8^+$ T cells. NKG2D on human $CD8^+$ T cells increases generation of a subset of $CD8^+$ central memory phenotype T cells that match the surface phenotype of a described regulatory $CD8^+$ T cell population. Finally, I found that NKG2D stimulation increased expression of the inhibitory receptor PD-1 by NOD $CD8^+$ T cells. I therefore suggest a previously undescribed role for NKG2D signaling between healthy cells in immunity and immune regulation, and take steps towards answering critical questions to determine whether modulation of NKG2D signaling could be used in intervention strategies in type 1 diabetes.

Acknowledgments

Foremost, I would like to thank my family, without whom none of this would be possible. Their support and encouragement through all stages of my academic journey is the only reason I have made it to this point. Second, I would like to thank my research mentor Dr. Mary Markiewicz, for her scientific guidance, and for putting up with all of the stress I at times surely caused her. I must also thank all of the administrative staff in microbiology and the office of graduate studies, who are the unseen hands making the graduate process work. Next I would like to acknowledge my committee for their input, support and guidance throughout the graduate process. Of special note are my past members, Dr. Tom Yankee, Dr. Steve Benedict, and Dr. Michael Parmely, whose names do not appear on the cover sheet, but have contributed equally and invaluably to my development as both an academic and a person. Finally, I must thank all of my friends. Graduate school is not easy, but good friends make all the difference.

Table of Contents

List of Figures

List of Tables

Abbreviations

ATM – ataxia telangiectasia mutated

ATR – ataxia telangiectasia mutated- and Rad3-related protein

CAR – chimeric antigen receptor

CD – cluster of differentiation

CTL – cytotoxic lymphocyte

DAP10 - DNAX-activating protein of 10 kDa

DAP12 - DNAX-activating protein of 12 kDa

IFNγ – interferon gamma

Klrk1 – killer cell lectin like receptor K1

LPS – lipopolysaccharide

MHC – major histocompatibility complex

MICA – MHC class I polypeptide-related sequence A

MICB – MHC class I polypeptide-related sequence B

MULT1 – murine ULBP-like transcript 1

NK – natural killer

NKG2D – natural killer group 2 member

NOD – non-obese diabetic

PBMC – peripheral blood mononuclear cell

RAE-1 – retinoic acid early inducible 1

RAET – retinoic acid early transcript 1

TNF-α – tumor necrosis factor alpha

ULBP – UL16-binding protein

Chapter 1: Introduction

Parts of this chapter were previously published as an open access article (CC-BY) and are reprinted here with modification. Trembath AP and Markiewicz MA (2018) More than Decoration: Roles for Natural Killer Group 2 Member D Ligand Expression by Immune Cells. *Front. Immunol.* 9:231. doi: 10.3389/fimmu.2018.00231.

The demands placed on the immune system are immense and highly complex. Tasked with protecting the body against untold external threats, while maintaining a balance between immune defense and autoimmune damage, the stakes are literally life and death. Fortunately, millions of years of evolution have resulted in immunological systems which are equally complex and necessarily efficient. Increasingly, we are coming to appreciate that few immune mechanisms are 'single use,' with many systems having distinct functions dependent upon setting and context. While this immunological multi-purposing leads to a capable and nuanced immune response, it puts the onus on us to tease out the different roles played by many immune system components. A prime example is presented in the activating immune receptor Natural Killer Group 2 Member D (NKG2D) and its ligands.

NKG2D, which is encoded by the gene *Killer Cell Lectin Like Receptor K1* (*Klrk1*) and designated CD314, is one of the best-studied activating immune receptors. NKG2D is expressed by all human and mouse natural killer (NK) cells, all human $CD8^+$ T cells, activated mouse $CD8^+$ T cells, NKT cells, subsets of γδ T cells, and rare $CD4^+$ T cells in both human and mouse (Bauer, Groh et al. 1999, Jamieson, Diefenbach et al. 2002, Groh, Bruhl et al. 2003, Dai, Turtle et al. 2009). The expression and general characteristics of NKG2D have been extensively

reviewed (Lanier 2015, Zhang, Basher et al. 2015, Wensveen, Jelencic et al. 2018, Zingoni, Molfetta et al. 2018). In brief, NKG2D is a C-type lectin-like receptor expressed on the cell surface as a disulfide linked homodimer with a short cytoplasmic tail that does not contain any signaling motifs. To signal, NKG2D associates with one of two adapter proteins, DNAX-activating protein of 10 kDa (DAP10) or DNAX-activating protein of 12 kDa (DAP12). In human and mouse T cells and NK cells, NKG2D associates with DAP10, which has a YINM motif that induces PI3-kinase and Grb2-Vav signaling (Wu, Song et al. 1999, Lanier 2015). In mouse NK cells, NKG2D also associates with DAP12, which is an immunotyrosine-based activation motif (ITAM)-bearing signaling molecule that signals through Syk and Zap70 (Wu, Cherwinski et al. 2000, Gilfillan, Ho et al. 2002, Rosen, Araki et al. 2004) (Fig. 1). On NK cells, NKG2D is a primary activating receptor, triggering NK cell cytotoxicity and cytokine production in response to ligand-expressing cells. The function of NKG2D on $CD8^+$ T cells is less well defined with both co-stimulatory and T cell receptor independent functions being described (Bauer, Groh et al. 1999, Meresse, Chen et al. 2004, Ehrlich, Ogasawara et al. 2005, Markiewicz, Carayannopoulos et al. 2005, Markiewicz, Wise et al. 2012) (Fig. 1)

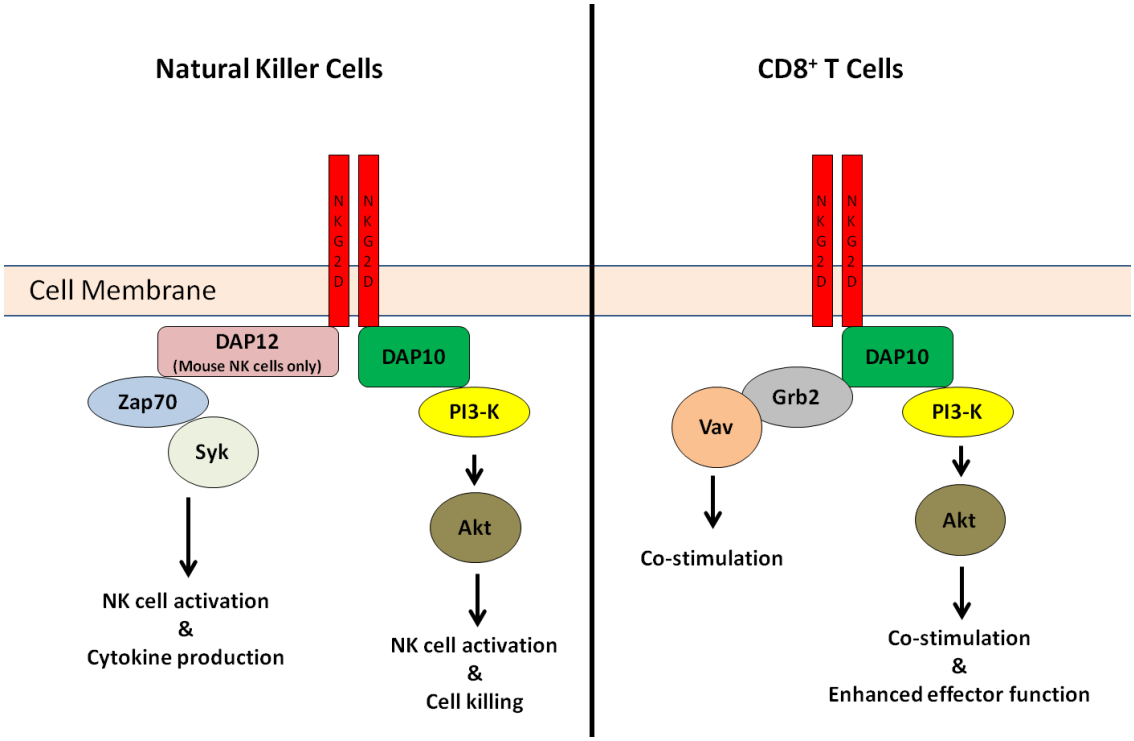

Figure 1: Schematic of NKG2D signaling in NK cells and CD8$^+$ T cells. NKG2D is expressed as a homodimer which associates with intracellular adaptor proteins for signaling. In human NK cells, CD8$^+$ T cells, and mouse CD8$^+$ T cells, this adaptor is the YXXM motif containing protein DAP10 which subsequently signals through PI3-kinase Grb2-Vav1. Mouse NK cells express a shorter NKG2D isoforms which can also associate with the adaptor protein DAP12, which contains an ITAM motif, and signals through Zap70.

NKG2D binds to a number of endogenous ligands (table 1) that are induced by cellular stress and were originally believed to be absent from healthy cells (Champsaur and Lanier 2010, Raulet, Gasser et al. 2013). There are 8 known human NKG2D ligands. These are MHC class I polypeptide-related sequence A (MICA) and B (MICB), and the Retinoic acid early transcript 1 (RAET1) family of proteins, which are better known as the UL16-binding proteins (ULBP1-6). There are 9 known ligands for NKG2D in mouse. These are RAE1α-ε, H60a-c, and Murine

ULBP-like transcript 1 (Mult1), which are all orthologs of human RAET1. NKG2D ligands are all distantly related to MHC class I molecules, but do not associate with β2 microglobulin or bind peptide, and are tethered to the cell membrane via a GPI anchor or transmembrane domain (Lanier 2015). Specifically, MICA, MICB, ULBP4, H60a, H60b and MULT1 have transmembrane domains, while ULBP1, ULBP3, and ULBP6, Rae1α-ε and H60c, are attached to the cell surface via GPI anchors. Interestingly, ULBP2 and ULBP5 can be inserted into the membrane via a transmembrane domain or a GPI anchor (Lanier 2015). NKG2D ligands can be shed from the cell surface via proteolytic cleavage, alternative splicing, phosphoinositide phospholipase C, or exosome release (Chitadze, Bhat et al. 2013). While the ligands do have different binding affinities with NKG2D, all NKG2D ligands are believed to signal similarly through NKG2D (Champsaur and Lanier 2010, Raulet, Gasser et al. 2013). NKG2D ligands have generally been considered markers of 'altered self,' being induced by stress, such as cellular transformation or infection, and acting as a distress signal to target affected cells for immune killing. NKG2D ligands in host defense, as well as the mechanisms regulating ligand expression are the subject of a number of reviews (Raulet, Gasser et al. 2013, Lanier 2015, Zingoni, Molfetta et al. 2018). In addition to this well studied role directing immune killing of ligand-expressing cells, a growing body of evidence suggests that NKG2D-NKG2D ligand interactions play other important roles in shaping the immune response. This idea came about after the appreciation of the importance of NKG2D ligand expression by otherwise healthy tissues (Eagle, Jafferji et al. 2009). Numerous reports show expression of NKG2D ligands by healthy tissues, but until relatively recently, the effects of this NKG2D ligand expression was not explored in depth. The expression of NKG2D ligands by healthy cells is the focus of a review by Eagle et al., wherein the authors address the potential significance of NKG2D ligand expression by both

healthy hematopoietic and non-hematopoietic cells and discuss the need for more systematic study of the role of NKG2D-NKG2D ligand signaling on apparently healthy cells (Eagle, Jafferji et al. 2009). In the years since this review, further evidence has accumulated that NKG2D ligand expression by healthy cells has distinct functions beyond targeting cells for immune killing. One major type of healthy cells, which evidence suggests routinely express NKG2D ligands, is cells of the hematopoietic lineage, specifically leukocytes. Many types of immune cells have been found to express NKG2D ligands with various immunological functions being reported, reviewed in Trembath and Markiewicz 2018 (Trembath and Markiewicz 2018)(Fig. 2). For my dissertation work, I focused on the expression of NKG2D ligands by T cells and the roles this expression plays in modulating the immune response by $CD8^+$ T cells.

Table 1: NKG2D ligands in humans and mice

Human NKG2D Ligand	**Gene Name**	**Membrane Tether**
MICA	*MICA*	Transmembrane
MICB	*MICB*	Transmembrane
ULBP1	*RAET1I*	GPI anchor
ULBP2	*RAET1H*	GPI anchor/Transmembrane
ULBP3	*RAET1N*	GPI anchor
ULBP4	*RAETE*	Transmembrane
ULBP5	*RAET1G*	Transmembrane/GPI anchor
ULBP6	*RAET1L*	GPI anchor
Mouse NKG2D Ligand	**Gene Name**	**Membrane Tether**
H60a	*H60a*	Transmembrane

H60b	*H60b*	Transmembrane
H60c	*H60c*	GPI anchor
MULT1	*Ulbp1*	Transmembrane
Rae1α	*Raet1a*	GPI anchor
Rae1β	*Raet1b*	GPI anchor
Rae1γ	*Raet1c*	GPI anchor
Rae1δ	*Raet1d*	GPI anchor
Rae1ε	*Raet1e*	GPI anchor

In their 1998 paper first describing the human NKG2D ligand MICA, Zwirner and colleagues showed that MICA was weakly expressed by freshly isolated $CD4^+$ and $CD8^+$ T cells, but that expression could be strongly induced in culture by addition of the polyclonal T cell activator phytohemagglutinin (PHA) (Zwirner, Fernandez-Vina et al. 1998). Further investigation showed that MICA was induced on human T cells upon activation with anti-CD3 and anti-CD28 or PMA stimulation, and this induction could be inhibited in a dose dependent manner by the NF-κB inhibitor sulfasalazine (Molinero, Fuertes et al. 2004). In these studies, the authors suggest that MICA expression by T cells could participate in the maintenance of immune homeostasis through NKG2D-mediated NK cell killing of activated T cells (Molinero, Fuertes et al. 2002). Indeed, a number of studies in both human and mouse have since observed expression of NKG2D ligands by activated T cells, and found that this expression makes T cells susceptible to NKG2D-mediated killing. In mice, a study by Rabinovich et al. showed that upon activation, T cells from either C57BL/6 or Balb/c mice became susceptible to syngeneic killing by NK cells or lymphokine-activated killer cells (Rabinovich, Li et al. 2003). In Balb/c mice, this killing was

mediated by NKG2D and was due to upregulation of an NKG2D ligand, most likely H60a (Rabinovich, Li et al. 2003). Curiously however, no NKG2D ligands were detected on activated C57BL/6 T cells, suggesting that recognition and killing of activated syngeneic C57BL/6 T cells is mediated through a different receptor (Rabinovich, Li et al. 2003). In a model of graft-versus-host disease, Noval Rivas and colleagues found that transferred host-specific $CD4^+$ T cells were limited by NKG2D-dependent killing by host NK cells (Noval Rivas, Hazzan et al. 2010). They found that upon antigen stimulation, monoclonal antigen specific $CD4^+$ T cells upregulated mRNA encoding the NKG2D ligands MULT1 and H60. It should be noted, however, that surface expression of MULT1 was not observed by flow cytometry, and surface expression of H60 proteins was not investigated (Noval Rivas, Hazzan et al. 2010). In humans, a similar finding was reported by Cerboni et al., who found that primarily MICA, but also ULBP1-3, were expressed by activated human $CD4^+$ and $CD8^+$ T cells upon antigen stimulation in an ataxia telangiectasia mutated (ATM)/ataxia telangiectasia mutated- and Rad3-related protein (Molfetta, Quatrini et al.)-dependent manner. In addition, expression of these ligands by activated T cells resulted in NKG2D-mediated NK cell lysis, again suggesting a potential mechanism for limiting T cell responses (Cerboni, Zingoni et al. 2007). Nielsen et al. also found that activated $CD4^+$ T cells expressed MICA, MICB, and ULBP1-3, and were susceptible to NK cell lysis (Nielsen, Odum et al. 2012). Further evidence supporting this role comes from a recent study that showed expression of MICA and MICB by liver infiltrating T cells in patients with chronic hepatitis B correlated with enhanced NK cell activation and NKG2D-dependent depletion of $CD4^+$ T cells upon short term *ex vivo* culture (Huang, Easom et al. 2017). It appears, however, that NKG2D-mediated T cell killing does not always result in a reduced immune response. For instance, during *Mycobacterium tuberculosis* infection, NK cells were shown to control regulatory T cell

(Treg) numbers through NKG2D-mediated lysis of NKG2D ligand-expressing Tregs (Roy, Barnes et al. 2008).

As discussed above, multiple studies demonstrate that NKG2D ligand expression by human and murine T cells has an important function in regulating T cell responses by directing the elimination of activated T cells. However there is also evidence of additional functions for NKG2D ligands expressed on apparently healthy T cells. Li et al. found that NKG2D ligands were expressed by double positive Balb/c thymocytes prior to fate determination, suggesting a role for NKG2D ligand expression in thymocyte development (Li, Rabinovich et al. 2005). It was also shown that human PBMCs, and in particular $CD4^+$ T cells, can release soluble NKG2D ligands in response to superantigen stimulation. These soluble ligands were found to downregulate NKG2D expression by $CD8^+$ T cells, which showed impaired proliferation, cytokine production, and cytotoxic activity (Cerboni, Ardolino et al. 2009). Finally, emerging evidence suggests that expression of NKG2D ligands by T cells directly affects the production of cytokines. A recent article reported that expression of ULBP proteins by $CD4^+$ T cells from inflamed Crohn's Disease intestine positively correlated with release of IL-10, while the frequency of $\gamma\delta^+$ and $CD56^+$ cells expressing NKG2D negatively correlated with inflammation and pro-inflammatory cytokine release (Vadstrup, Galsgaard et al. 2017).

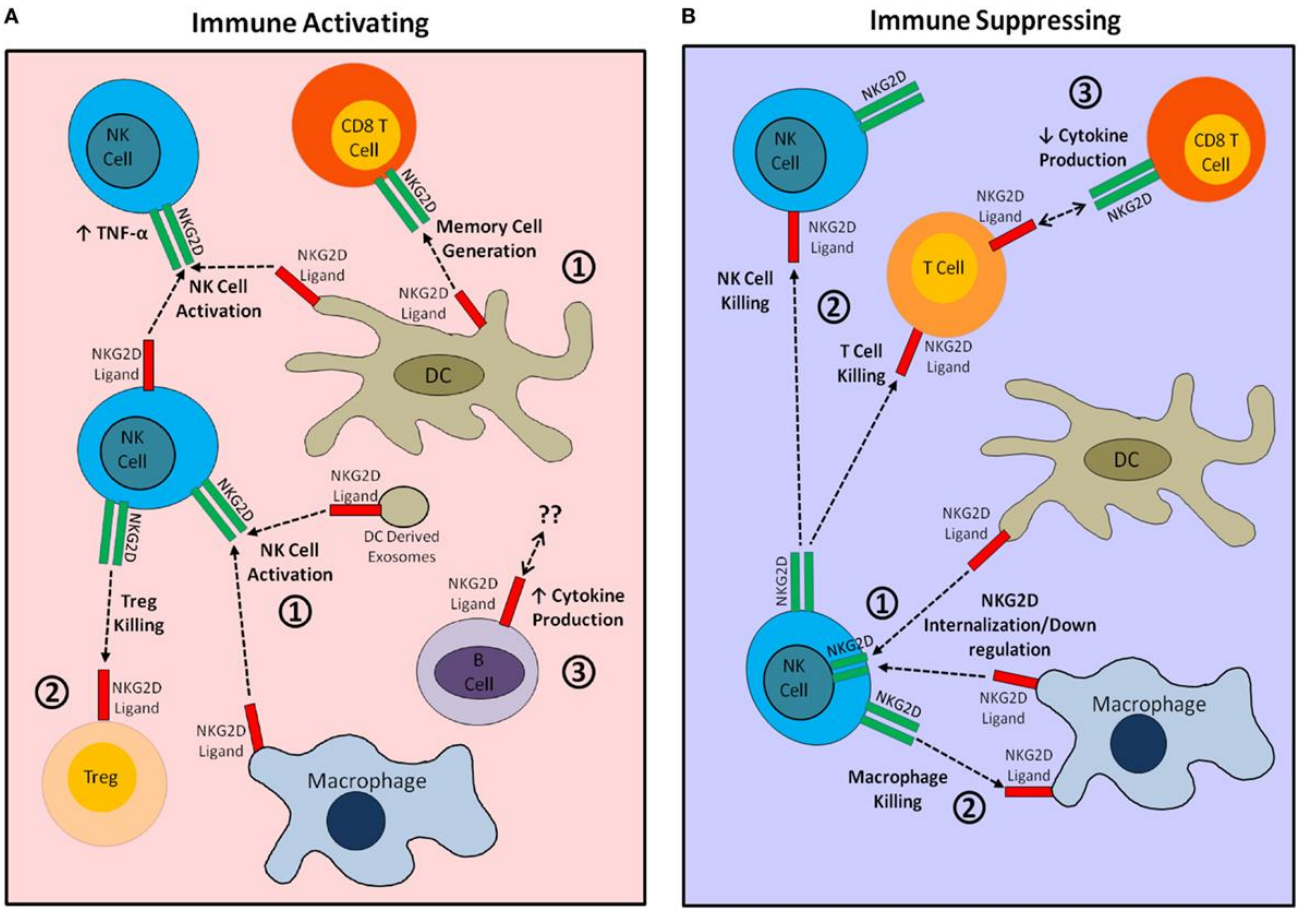

Figure 2: Visual summary of immunostimulatory and immunosuppressive effects of natural killer group 2 member D (NKG2D) ligand expression by cells of the immune system. (A) The immunostimulatory effects of NKG2D ligand expression by immune cells. (1) NKG2D ligand expression by dendritic cells (DCs) and macrophages provides activating and differentiation signals to NKG2D-bearing natural killer (NK) cells and $CD8^+$ T cells. (Granados, Draghi et al.) Expression of NKG2D ligand by regulatory T cells (Tregs) targets these cells for killing by NK cells, thereby increasing the overall immune response. (Campbell, Duggan et al.) NKG2D ligand expression may affect B cell cytokine production. **(B)** The immunosuppressive effects of NKG2D ligand expression by immune cells. (1) Widespread expression of NKG2D ligands by DCs or macrophages causes internalization of NKG2D, impairing NKG2D-mediated immune activation. (Granados, Draghi et al.) NKG2D ligand-bearing immune cells are directly

targeted for killing by autologous NKG2D-bearing NK and T cells. (Campbell, Duggan et al.) NKG2D–ligand interaction during CTL generation may decrease CTL cytokine production. For further review of NKG2D ligand expression by immune cell type, see Trembath and Markiewicz 2018.

Given that a major role of NKG2D and NKG2D ligands is directing the immune killing of infected or cancerous cells, it is logical to think that NKG2D may play a role in autoimmunity when this system aberrantly directs killing of healthy tissues. Indeed, NKG2D has been reported to be involved in a number of autoimmune conditions including multiple sclerosis (Fernandez-Morera, Rodriguez-Rodero et al. 2008, Guerra, Pestal et al. 2013), colitis (Kjellev, Haase et al. 2007), rheumatoid arthritis (Groh, Bruhl et al. 2003, Andersson, Sumariwalla et al. 2011), and autoimmune diabetes (Ogasawara, Hamerman et al. 2004, Qin, Lee et al. 2011, Van Belle, Ling et al. 2013, Trembath, Sharma et al. 2017), which is the focus of this dissertation. NKG2D is implicated to play a role in the development of autoimmune diabetes in both humans and mice. However reports describing what role NKG2D plays in disease development have been conflicting. An early report by Ogasawara and colleagues observed expression of the NKG2D ligand Rae-1 by cells of the pancreatic islets of NOD mice, and found that treatment with anti-NKG2D blocking antibody prevented NOD diabetes development (Ogasawara, Hamerman et al. 2004). Subsequent investigations, including our own , did not find Rae-1 mRNA (Angstetra, Graham et al. 2012, Carrero, Calderon et al. 2013) or Rae-1 protein expression in the NOD pancreas (Angstetra, Graham et al. 2012, Trembath, Sharma et al. 2017), and did not detect expression of NKG2D ligands on non-immune cells within the pancreatic islets of NOD mice (Trembath, Sharma et al. 2017). Using a RIP-LCMV model of diabetes, a study by Van Bell et

al. also concluded that antibody blockade of NKG2D had a beneficial effect, and reduced diabetes development in conjunction with antigen specific Treg therapy (Van Belle, Ling et al. 2013). Contrary to these findings, a different study reported minimal effect of either NKG2D deficiency or anti-NKG2D antibody blockade on autoimmune diabetes development (Guerra, Pestal et al. 2013). In this dissertation, I describe a protective role for NKG2D signaling in NOD diabetes development (Trembath, Sharma et al. 2017). In this same study, work from the Markiewicz lab found that NKG2D signaling exerts opposing effects on NOD diabetes development in different anatomical locations (Trembath, Sharma et al. 2017), likely explaining the lack of consensus in previous studies. In humans, children with type I diabetes have been observed to have defects in NKG2D signaling (Qin, Lee et al. 2011), and decreased NKG2D expression by NK cells has been observed in those with type 1 diabetes (Rodacki, Svoren et al. 2007). A genetic linkage study has also associated polymorphisms in the human NKG2D ligand MICA with diabetes susceptibility (Nikitina-Zake, Rajalingham et al. 2004).

A major finding during my graduate work is that T cells from NOD mice express H60a upon activation. Because NOD $CD8^+$ T cells also express NKG2D, I therefore set out to answer the question, how expression of NKG2D ligand by healthy T cells, affects $CD8^+$ T cell response. I then further asked how this NKG2D signaling affects the development of autoimmune diabetes. This investigation led to the finding that interaction between NKG2D and H60a expressed by T cells during cytotoxic T lymphocyte (CTL) differentiation reduces NOD CTL effector cytokine production both *in vitro* and *in vivo* (Trembath, Sharma et al. 2017). I also investigated the effects of NKG2D-NKG2D ligand interactions between human $CD8^+$ T cells, and found that this interaction also altered the $CD8^+$ T cell response. I found that NKG2D signaling causes

increased generation of central memory phenotype (T_{cm}) $CD8^+$ T cells and an increased ratio of T_{cm} to effector/effector memory phenotype (T_{eff+em}) $CD8^+$ T cell populations. Further, it is known that the $CD8^+$ T_{cm} population contains a population of potent regulatory $CD8^+$ T cells (Li, Xie et al. 2014) which I was able to observe. Finally, we show a significantly increased transfer of NOD diabetes by T_{eff+em} versus T_{cm} $CD8^+$ T cells. Together, these findings describe a new role for NKG2D signaling in $CD8^+$ T cells whereby signaling during differentiation changes the resulting $CD8^+$ T cell populations, favoring those which are less diabetogenic.

Chapter 2: A protective role for NKG2D-H60a interaction via homotypic T cell contact in NOD autoimmune diabetes pathogenesis

This chapter was previously published as an open access article (CC-BY) excerpts of which are reprinted here in modified form. Trembath, A. P., N. Sharma, R. Saravanan, B. Polic and M. A. Markiewicz (2017). "A Protective Role for NKG2D-H60a Interaction via Homotypic T Cell Contact in Nonobese Diabetic Autoimmune Diabetes Pathogenesis." Immunohorizons 1(9) 198-212.

Abstract

The NKG2D immune receptor is implicated in both human and mouse autoimmune diabetes. However, the significance of NKG2D in diabetes pathogenesis has been unclear due to conflicting reports as to the importance of this receptor in the non-obese diabetic (NOD) mouse model. Findings from our lab demonstrate that NKG2D expression affects NOD diabetes development by at least two previously undescribed, and opposing mechanisms. I then show that the NKG2D ligand H60a is induced on activated NOD T cells, and that NKG2D-H60a interaction during $CD8^+$ T cell differentiation into CTLs generally decreases the subsequent CTL effector cytokine response. This corresponds to data generated by others in the lab showing an increase in diabetes development in NKG2D-deficient compared with wild type NOD mice under microbiota-depleted conditions. Other lab members also demonstrated that NKG2D promotes NOD diabetes development through interaction with the microbiota. Together these findings reveal a previously undescribed role for NKG2D ligand expression by activated T cells

in CTL development, and demonstrate that NKG2D has both diabetogenic and antidiabetogenic roles in NOD diabetes development.

Introduction

In a previous study with the non-obese diabetic (NOD) mouse model, expression of RAE1 was observed in the pancreatic islets, and antibody blockade of NKG2D was found to inhibit $CD8^+$ T cell infiltration and prevent diabetes development (Ogasawara, Hamerman et al. 2004). It was subsequently shown that transgenic expression of RAE1ε by pancreatic β cells in non-autoimmune C57BL/6 mice caused recruitment of activated $CD8^+$ T cells to pancreatic islets (Markiewicz, Wise et al. 2012). This suggested a mechanism by which NKG2D ligand expression in the islets enhances diabetes development in NOD mice. However, RAE1 gene expression was not detected at any time during diabetes development in NOD pancreatic islets in two other studies (Angstetra, Graham et al. 2012, Carrero, Calderon et al. 2013). Additionally, diabetes development in NOD mice genetically deficient in NKG2D was reported to be similar to that of wild type NOD mice (Guerra, Pestal et al. 2013). These conflicting results suggest that NKG2D may play a more complex role in autoimmune diabetes development than originally proposed, affecting disease development through multiple mechanisms.

Previous work in the Markiewicz lab compared diabetes development between $Klrk1^{-/-}$ and wild type NOD littermates housed under SPF conditions. They found that in both sexes, the $Klrk1^{-/-}$ mice exhibited slower diabetes development compared to wild type mice. Because NOD diabetes

is sensitive to alterations in the microbiota (Pozzilli, Signore et al. 1993, King and Sarvetnick 2011, Mathis and Benoist 2011), they also assessed diabetes development in SPF housed Klrk1$^{-/-}$ and wild type NOD littermates treated with a broad spectrum antibiotic cocktail. In antibiotic treated animals, diabetes development was enhanced in Klrk1$^{-/-}$ compared with wild type NOD mice.

This pointed to at least two separate and opposing effects of NKG2D signaling, one resulting from interactions between NKG2D and the microbiota, and the other independent of microbiota interactions. My work presented here, investigates the effects of NKG2D signaling which is protective, and independent of microbiota interactions. Specifically, I focus on the role of NKG2D signaling on shaping the CD8^{+} T cell response.

A previous report described the expression of RAE1 family members in the pancreatic β-islet cells of NOD mice and suggested that this expression targeted β-islet cells for autoimmune destruction (Ogasawara, Hamerman et al. 2004). Previous work from the Markiewicz lab looked for RAE1 mRNA expression in the pancreatic islets of our NOD mice, and did not detect RAE1 mRNA in the pancreatic islets of wild type NOD mice in our colony (Trembath, Sharma et al. 2017). Other lab members also looked for expression of the other NKG2D ligand expressed in NOD mice, H60a. These data showed significant expression of H60a mRNA in both the pancreas and spleen of NOD mice by 12 weeks of age (Trembath, Sharma et al. 2017). In addition, these data compared H60a mRNA levels between immune (CD45^{+}) and non-immune (CD45^{-}) cells present within isolated islets from 12 week-old mice and revealed H60a mRNA was expressed in the CD45^{+} immune cells, but not the CD45^{-} islet cells (Trembath, Sharma et al.

2017). Flow cytometry revealed that this expression was not on islet cells, but on infiltrating T cells. We further found that both H60a and NKG2D were expressed on NOD $CD8^+$ T cells following activation, and that NKG2D-H60a interaction via homotypic $CD8^+$ T cell contact during NOD CTL differentiation decreased effector cytokine production by the CTL *in vitro*. This corresponded with increased effector cytokine production by *Klrk1*$^{-/-}$ NOD CTL *in vivo* and with previous data showing increased diabetes development in microbiota-depleted *Klrk1*$^{-/-}$ NOD mice. These results demonstrate that NKG2D-ligand interaction during CTL generation dampens the diabetogenic CTL response, corresponding to reduced NOD diabetes development *Klrk1*$^{-/-}$ NOD mice.

Results

The NKG2D ligand H60a is expressed on infiltrating T cells within the pancreas of NOD mice

Finally, I determined by flow cytometric analysis that the major cells expressing H60a were pancreas-infiltrating T cells (Fig. 3).

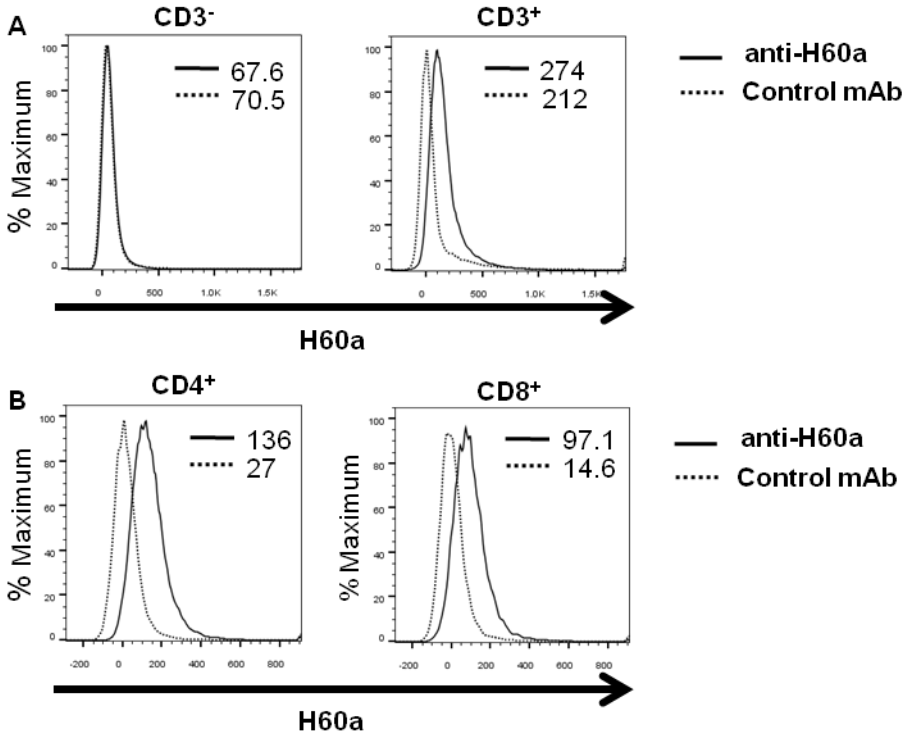

Figure 3: T Cells within the pancreas of NOD mice express the NKG2D ligand H60a A) H60a expression on $CD3^-$ and $CD3^+$ cells within the pancreas of a 12-week-old NOD mouse. **B)** H60a expression on $CD4^+$ and $CD8^+$ T cells within the pancreas of a 12-week-old NOD mouse. The mean fluorescence intensity (MFI) of staining is shown. Data are representative of at least three independent experiments.

NOD $CD8^+$ T cells express NKG2D and H60a upon activation

I next set out to determine the role of H60a expression by NOD T cells. I first characterized the expression of H60a on splenic T cells in young (6-8 week-old) NOD mice. I observed low, but detectable H60a expression on freshly isolated splenic T cells (Fig. 4). I tested whether activation would increase this expression by stimulating NOD splenocytes *in vitro* with anti-CD3 antibody. H60a expression increased substantially on both $CD4^+$ and $CD8^+$ T cells following activation, peaked on day 2 to 3 and remained elevated through day 5 of culture (Fig. 4).

In addition to H60a, activated NOD $CD8^+$ T cells express NKG2D (Ogasawara, Hamerman et al. 2004). Therefore, I determined the time course of both H60a and NKG2D expression after initial TCR activation and differentiation of NOD $CD8^+$ T cells into CTL. I purified $CD8^+$ T cells from the spleens and lymph nodes of 6-8 week old *Klrk1*$^{-/-}$ and wild type NOD mice. NKG2D and H60a expression was assessed at the time of cell isolation and daily following activation with anti-CD3 and anti-CD28 antibodies. Similar to what I observed with whole splenocytes (Fig. 5), a low level of H60a was detected prior to activation, this expression peaked 3 days after activation, and continued through day 5 (Fig. 5). This expression was unaffected by *Klrk1* (Fig. 5). In wild type mice, NKG2D became detectable at a low level on day 2, peaked at day 3, and persisted at a low level through day 5 (Fig. 5).

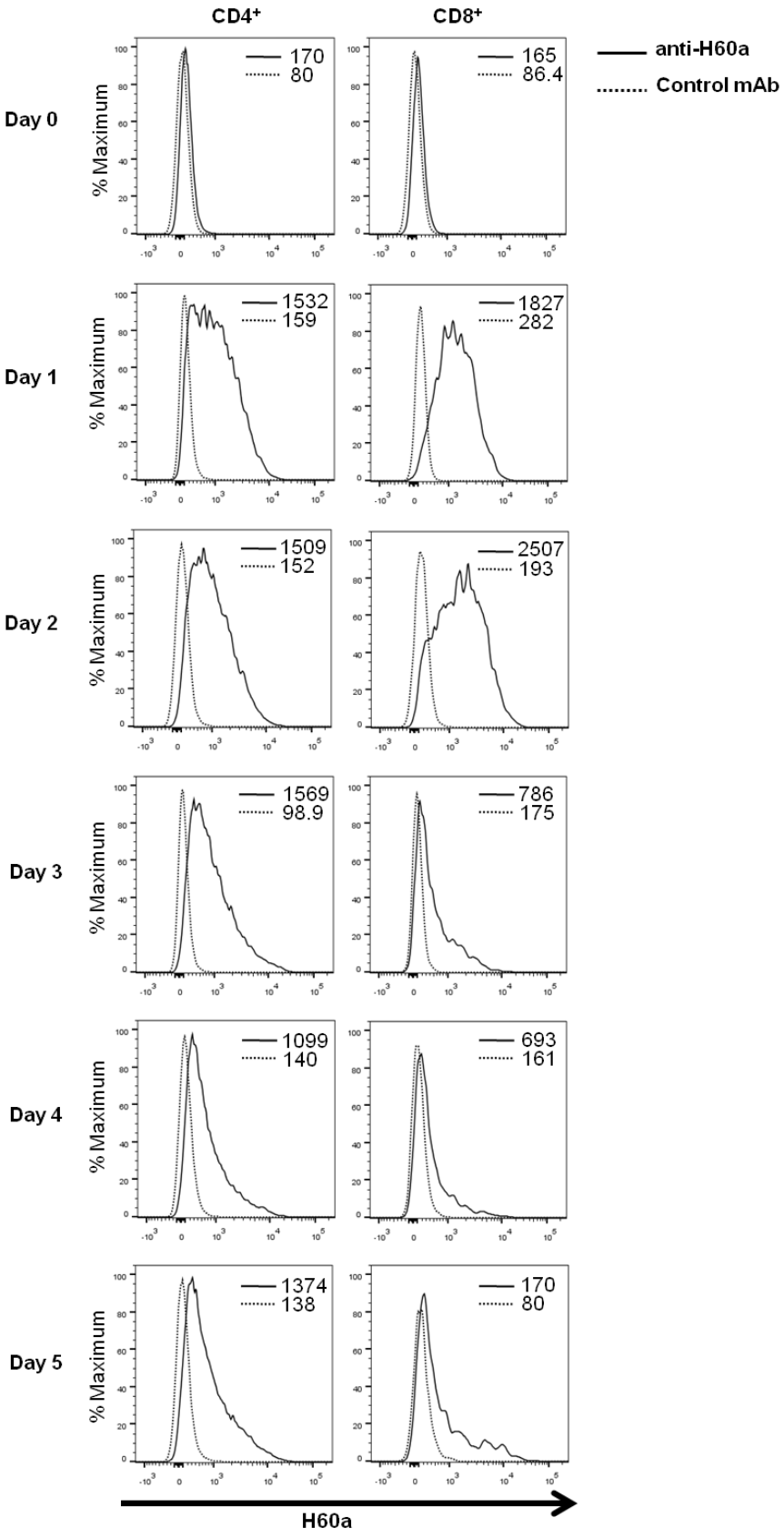
CD4+
CD8+
anti-H60a
Control mAb
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
% Maximum
170
80
165
86.4
1532
159
1827
282
1509
152
2507
193
1569
98.9
786
175
1099
140
693
161
1374
138
170
80
H60a

Figure 4: NOD T cells express H60a upon activation. H60a expression on $CD4^+$ and $CD8^+$ T cells following activation of NOD splenocytes with anti-CD3ε antibody. The MFI of staining is shown. H60a expression was assessed on $CD4^+$ and $CD8^+$ gated live cells. Data are representative of at least 4 independent experiments.

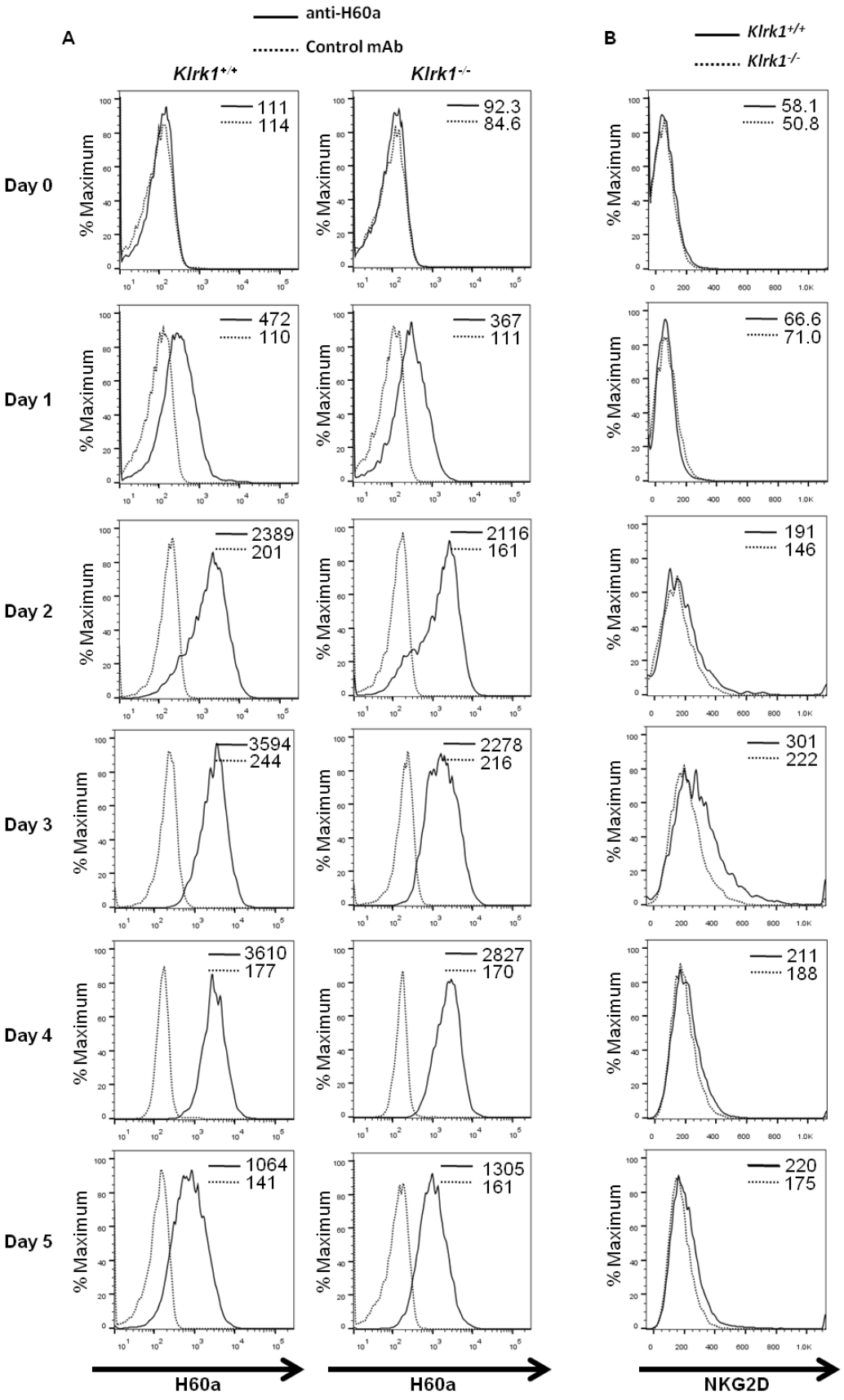
A
anti-H60a
Control mAb
Klrk1+/+
Klrk1-/-
B
Klrk1+/+
Klrk1-/-
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
% Maximum
111
114
92.3
84.6
58.1
50.8
472
110
367
111
66.6
71.0
2389
201
2116
161
191
146
3594
244
2278
216
301
222
3610
177
2827
170
211
188
1064
141
1305
161
220
175
H60a
H60a
NKG2D

Figure 5: NOD CD8$^+$ T cells express both H60a and NKG2D upon activation. Expression of **(A)** H60a and **(B)** NKG2D on CD8$^+$ T cells purified from 6-8 week-old *Klrk1*$^{-/-}$ and wild type NOD mice activated with anti-CD3ε and anti-CD28 antibodies. MFI of staining is shown. Data are representative of at least 4 independent experiments.

NKG2D expression alters NOD CTL effector cytokine responses *in vitro* and *in vivo*.

NKG2D engagement by ligands expressed on target cells enhances CTL effector function induced by T cell receptor engagement by antigen expressed on the same target cells (Bauer, Groh et al. 1999, Markiewicz, Carayannopoulos et al. 2005). Given my finding that NOD CTL co-express NKG2D and the NKG2D ligand H60a, I hypothesized NKG2D-H60a interaction via homotypic CD8$^+$ T cell contact could similarly alter NOD CTL effector function. To test this, I activated purified *Klrk1*$^{-/-}$ and wild type NOD CD8$^+$ T cells with anti-CD3 and anti-CD28 antibodies *in vitro* and allowed them to differentiate for 5 days. I assessed cytokine production and lytic granule release by the CTL after re-stimulation with various concentrations of plate bound anti-CD3 antibody to mimic target cell recognition.

In the absence of stimulation, or with a low concentration of anti-CD3 antibody, *Klrk1*$^{-/-}$ CTL produced significantly more IFNγ, TNFα, and IL-10 compared with wild type CTL (Fig. 6 and 7A). With a higher concentration of anti-CD3 antibody, *Klrk1*$^{-/-}$ CTL also produced greater amounts of TNFα and IL-10. At this higher level of stimulation, IFNγ production by *Klrk1*$^{-/-}$ CTL was lower in some experiments compared with wild type CTL, however this did not reach statistical significance when data from multiple experiments were combined. (Fig. 6 and 7A). In contrast to this effect on cytokine production, no change in lytic granule release was observed

(Fig. 7B). To determine if NKG2D expression similarly affected NOD $CD8^+$ T cell cytokine production *in vivo*, I purified $CD8^+$ T cells from *Klrk1*$^{-/-}$ or wild type NOD mice, and labeled them with eFluor 670 or CFSE, respectively. Without providing exogenous TCR activation, I adoptively transferred a 1:1 mixture of these cells into wild type NOD recipient mice (Fig. 8A). After 1 week, *Klrk1*$^{-/-}$ cells recovered from the spleen produced more TNFα and IFNγ than wild type cells recovered from the same spleen (Fig. 8B and C). Together, these results demonstrate that that expression of NKG2D by NOD $CD8^+$ T cells generally reduces effector cytokine production by these cells.

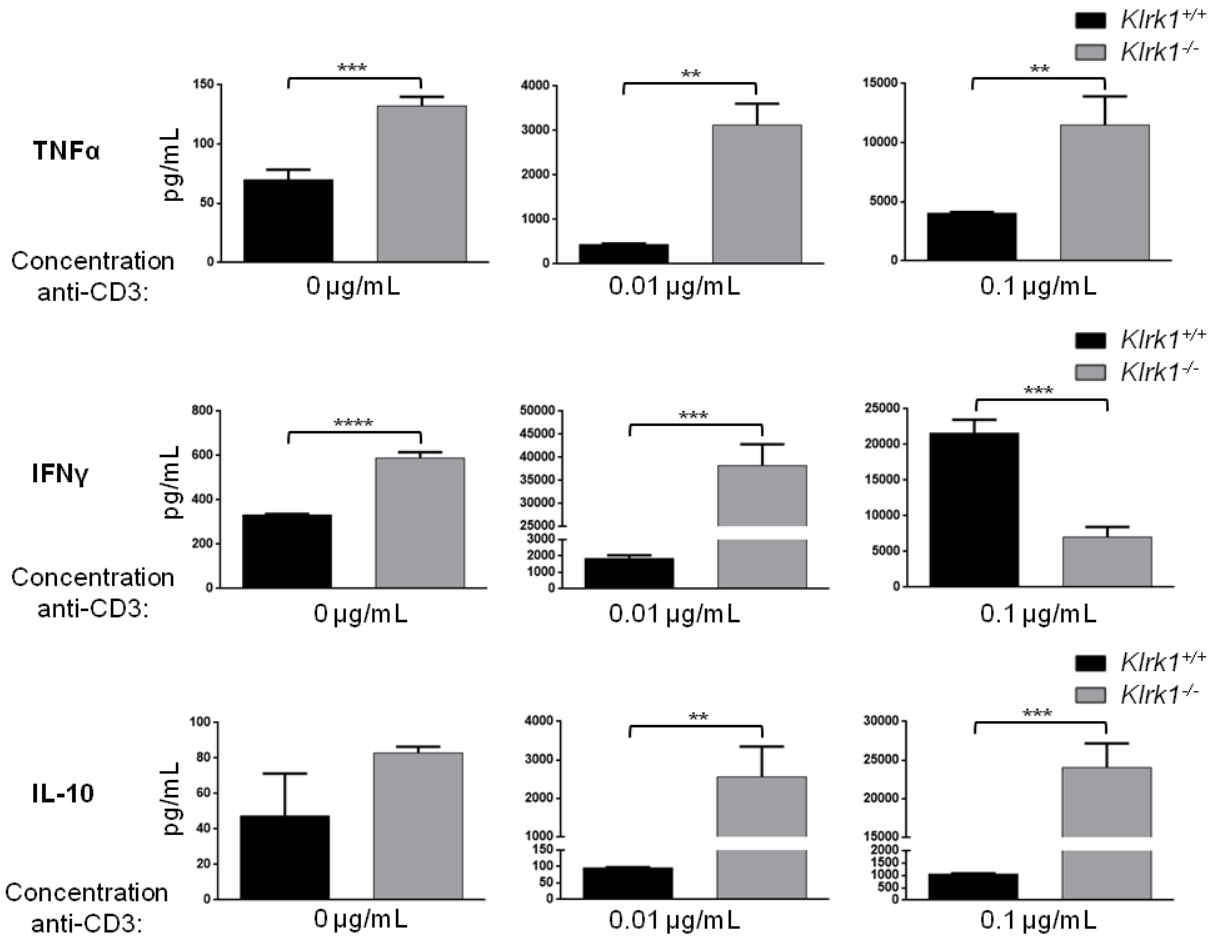

Figure 6: Altered cytokine production by *Klrk1*$^{-/-}$ NOD CTL *in vitro*. Secretion of TNFα, IFNγ, and IL-10 (mean+/-STD) by *in vitro* generated *Klrk1*$^{-/-}$ and wild type CTL stimulated with the indicated concentrations of anti-CD3ε antibody. Data are representative of at least 6 independent experiments. **p ≤ .01, ***p ≤ .001, ****p ≤ .0001 in two-tailed unpaired Mann-Whitney test.

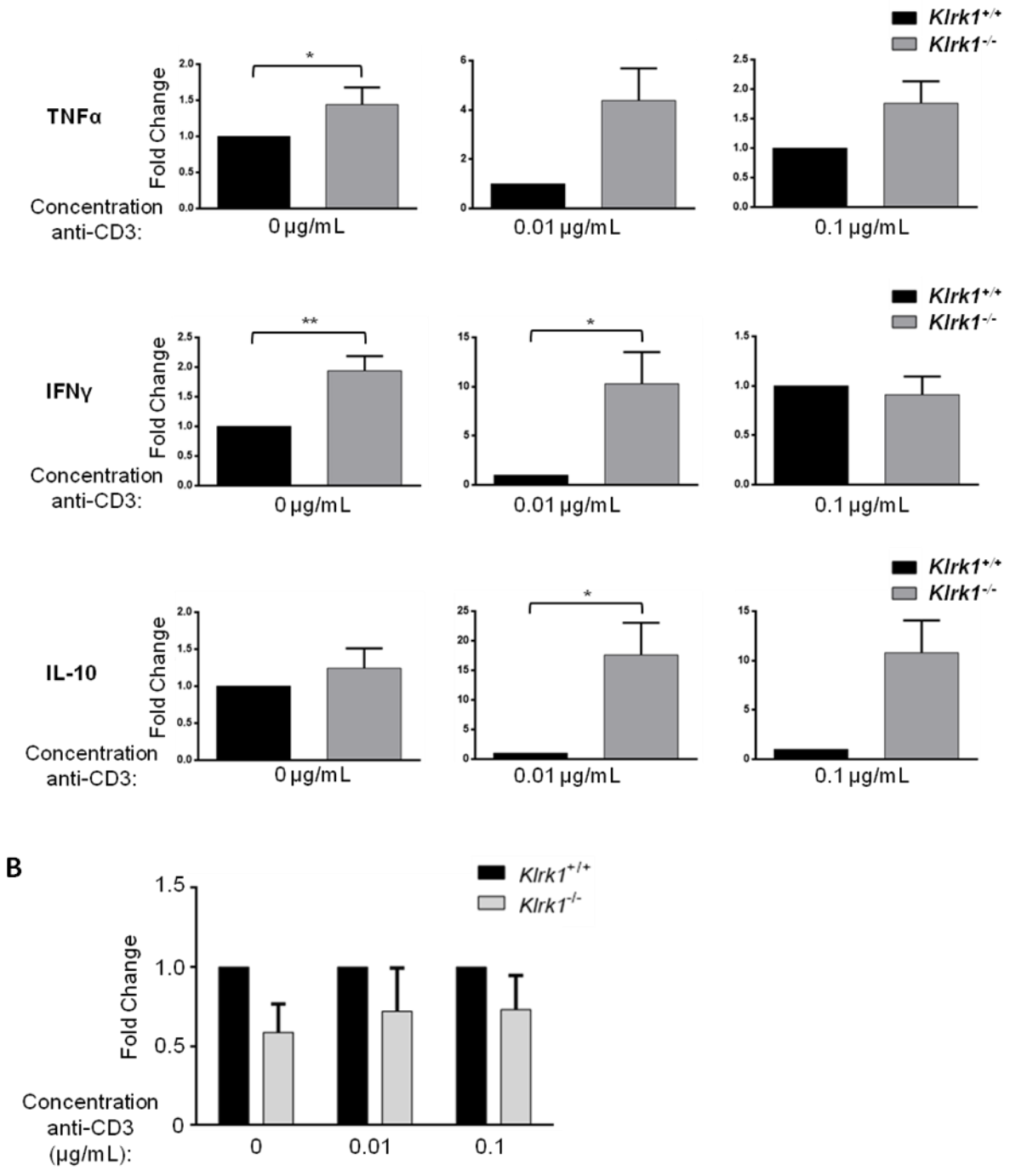

Figure 7: NKG2D deficiency alters NOD CTL cytokine production but does not significantly alter lytic granule release. (A) Fold change (mean +/- SEM) in TNFα, IFNγ and IL-10 secretion by *Klrk1*$^{-/-}$ CTL compared with wild type CTL. Data are combined from at least 4 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$. (B) Fold change (mean +/- SEM) in percent of

CD107a+ *Klrk1*$^{-/-}$ CTL compared with percent of CD107a+ wild type CTL. Data are combined from at least 4 independent experiments.

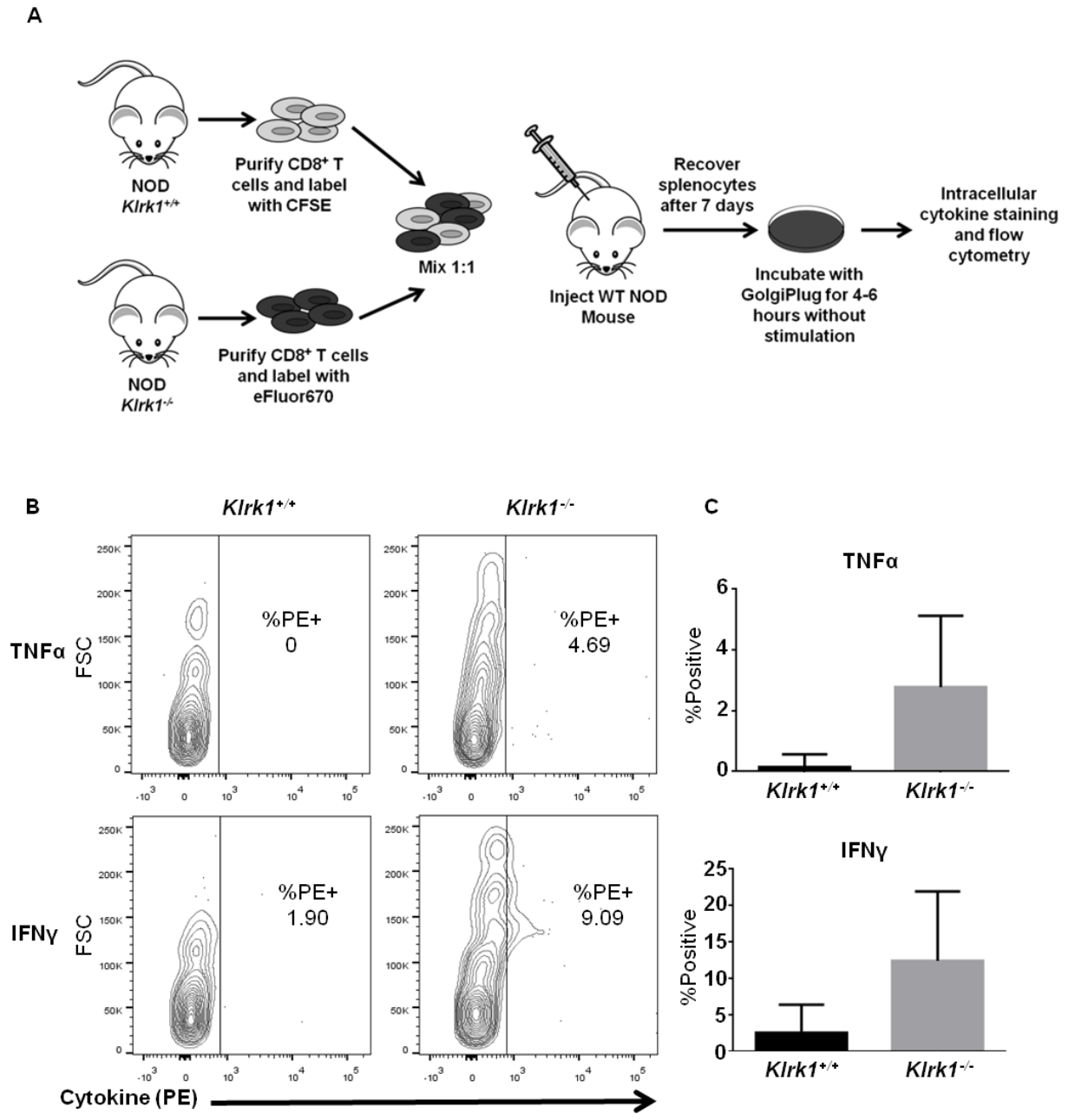

Figure 8: Altered cytokine production by *Klrk1*$^{-/-}$ NOD CTL *in vivo*. (A) Schematic of adoptive transfer and intracellular cytokine staining of labeled CD8^{+} T cells from wild type and *Klrk1*$^{-/-}$ mice**.** (B) Representative flow cytometry plots showing intracellular staining of TNFα and IFNγ in *Klrk1*$^{-/-}$ and wild type NOD CD8^{+} T cells 1 week after co-transfer into a wild type NOD adoptive transfer recipient mouse. (C) Combined results (mean+/-SEM) from transfers into

8 mice from three independent experiments showing the percent TNFα or IFNγ positive *Klrk1*$^{-/-}$ and wild type NOD CD8^{+} T cells 1 week after co-transfer into wild type NOD adoptive transfer recipient mice. **p ≤ .01, ***p ≤ .001, ****p ≤ .0001 in two-tailed unpaired Mann-Whitney test.

NKG2D-H60a interaction during NOD CTL differentiation dampens diabetogenic cytokine production.

To assess whether the increased cytokine production by *Klrk1*$^{-/-}$ CTL was the result of a loss of NKG2D interaction with H60a during the effector response, I included a blocking antibody against H60a during CTL stimulation. However, this blockade did not affect cytokine secretion by any of the CTLs (Fig. 9). This led us to hypothesize that NKG2D-H60a interaction during CTL differentiation, rather than during the effector CTL response, was responsible for the altered cytokine production by *Klrk1*$^{-/-}$ CTL. To test this, we added anti-H60a blocking antibody at the beginning of the T cell culture. This resulted in increased TNFα, IL-10, and IFNγ by wild type CTL when the cells were stimulated with a low concentration of anti-CD3 antibody (Fig. 10 and 11). With a higher concentration of anti-CD3 antibody, H60a blockade also resulted in greater production of TNFα and IL-10, but lower or similar production of IFNγ, by wild type CTLs (Fig. 10 and 11). Confirming this effect of H60a blockade was dependent on NKG2D expression, no difference in cytokine production was observed between CD3-stimulated anti-H60a- and isotype control- treated *Klrk1*$^{-/-}$ CTL (Fig. 10 and 11).

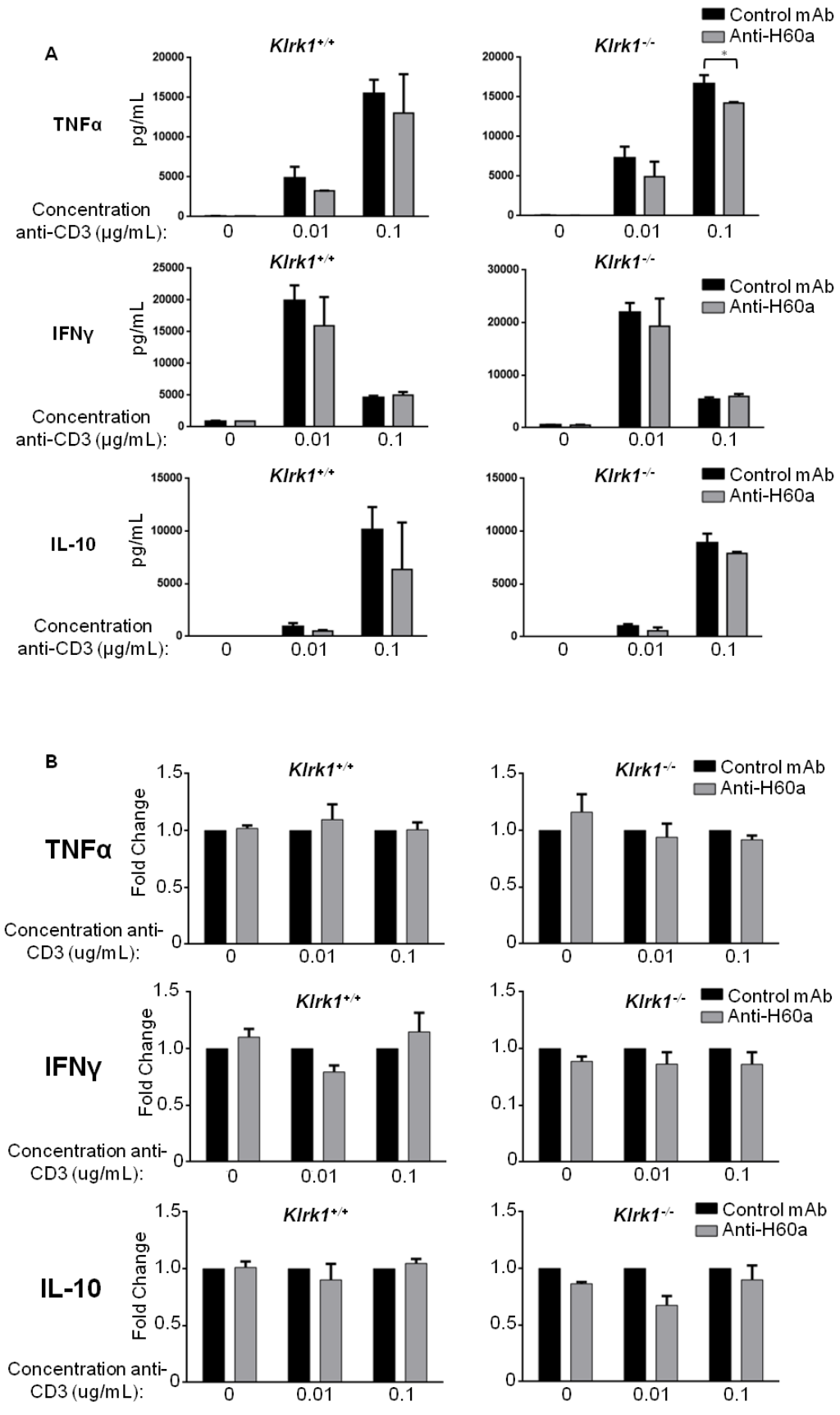
A
TNFα
IFNγ
IL-10
pg/mL
Klrk1+/+
Klrk1-/-
Control mAb
Anti-H60a
*
Concentration anti-CD3 (µg/mL):
0
0.01
0.1
B
Fold Change
Concentration anti-CD3 (ug/mL):

Figure 9: CTL effector cytokine production is not affected by blockade of NKG2D-H60a interaction during NOD CTL effector response. (A) Representative experiment showing TNFα, IFNγ and IL-10 secretion (mean +/- STD) by *Klrk1*-/- and wild type CTL stimulated with anti-CD3ε in the presence of an anti-H60a or isotype control antibody. Data are representative of at least 4 independent experiments. *$p \leq 0.05$ in one-tailed unpaired Mann-Whitney test. (B) Fold change (mean +/- SEM) in TNFα, IFNγ and IL-10 secretion by CTL stimulated with anti-CD3ε in the presence of anti-H60a compared with isotype control antibody. Data are combined from at least 4 independent experiments.

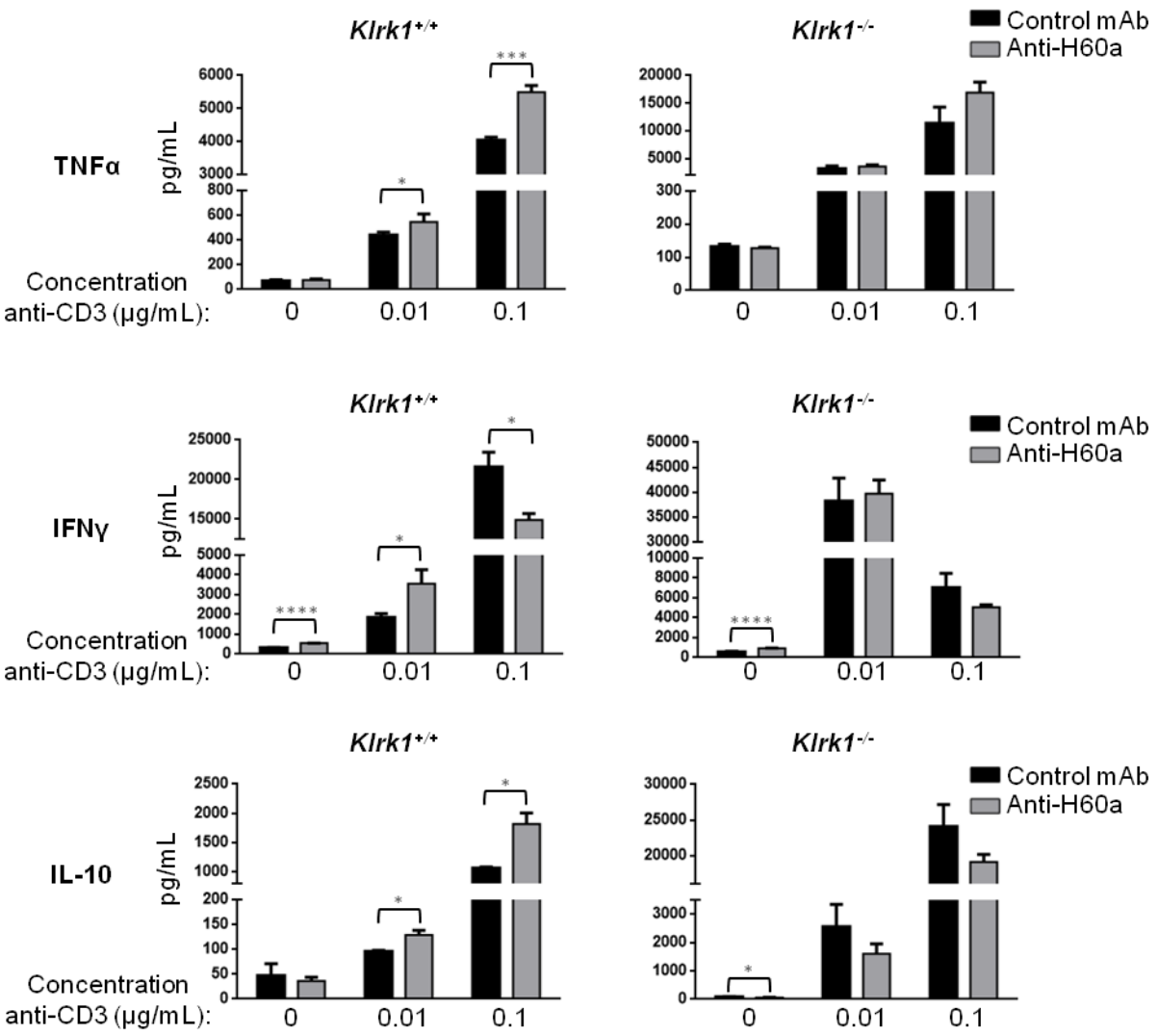

Figure 10: Blockade of NKG2D-H60a interaction during NOD CTL differentiation results in increased CTL effector cytokine production. Secretion of TNFα, IFNγ, and IL-10 by *in vitro* generated *Klrk1*$^{-/-}$ and wild type CTL stimulated with the indicated concentrations of anti-CD3ε antibody in the presence of an anti-H60a or isotype control antibody. Data are representative of at least 6 independent experiments. *$p \leq 0.05$, ***$p \leq .001$, ****$p \leq .0001$ in two-tailed unpaired Mann-Whitney test.

The similar effect of NKG2D deficiency (Fig. 6) and H60a blockade during CTL differentiation (Fig. 10 and 11) demonstrates that NKG2D-H60a interaction via homotypic $CD8^+$ T cell contact during CTL differentiation alters the NOD CTL effector cytokine response, generally reducing production.

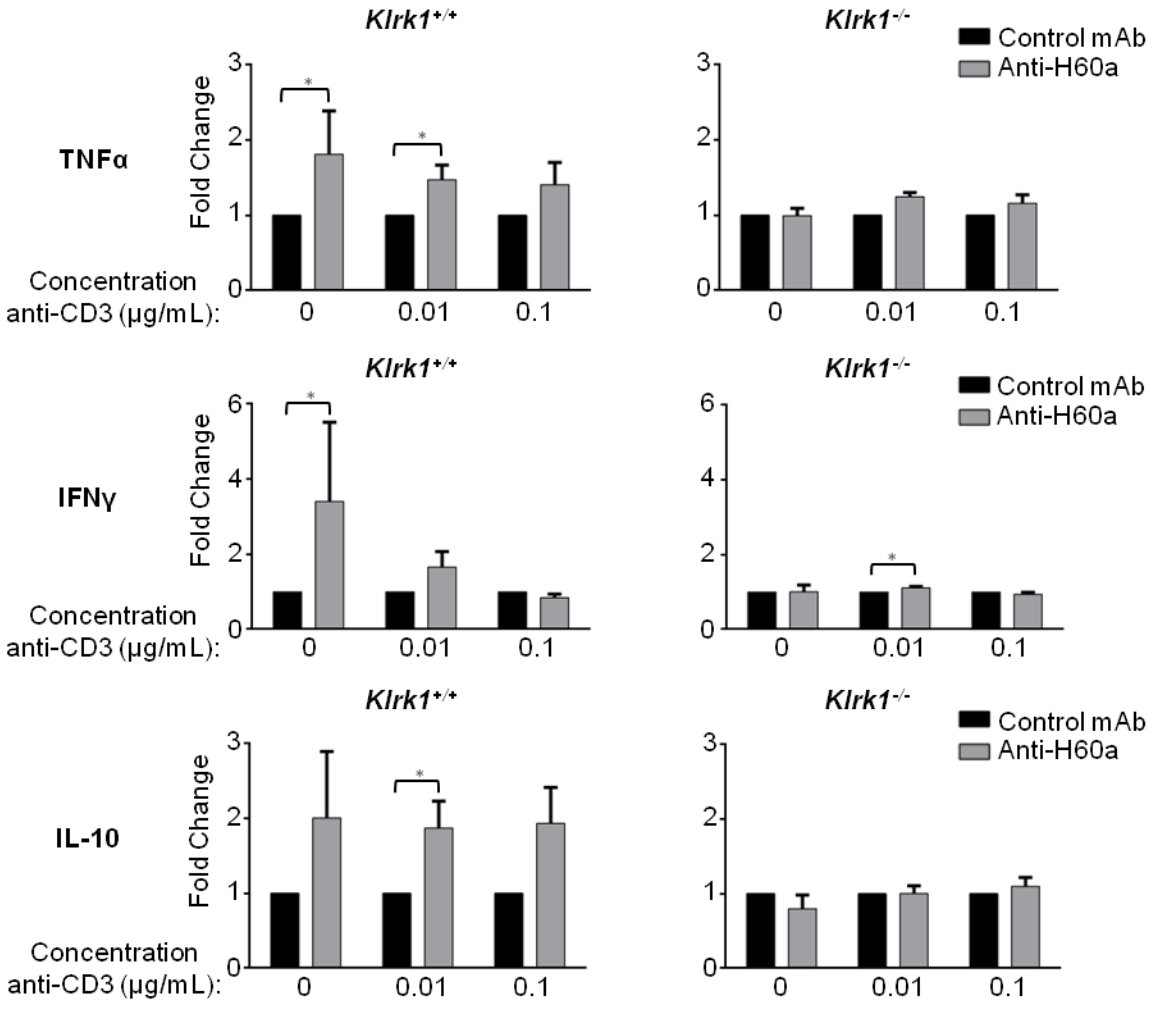

Figure 11: Figure S4. Blockade of NKG2D-H60a interaction during NOD CTL differentiation increases CTL effector cytokine production. Fold change (mean+/-SEM) in TNFα, IFNγ and IL-10 secretion by *Klrk1*$^{-/-}$ and wild type CTL stimulated with anti-CD3ε

generated in the presence of an H60a blocking compared with isotype control antibody. Data are combined from at least 6 independent experiments. *$p \leq 0.05$ in one-tailed Wilcoxon test.

Different pancreatic and splenic immune cell populations in specific pathogen free (SPF)-housed NOD mice vs. NOD mice treated with broad-spectrum antibiotics.

Expression of TNFα, IFNγ and IL-10 within the islets all enhance NOD insulitis and diabetes development (Rabinovitch 1998). Given the increased production of these cytokines by Klrk1$^{-/-}$ CTL, and the protective role of NKG2D in microbiota-depleted mice in NOD diabetes development, we hypothesized that increased cytokine production by CD8$^+$ T cells in Klrk1$^{-/-}$ mice may contribute to the increased incidence of diabetes. We therefore sought to determine the immune cell populations present in the spleen and pancreas of wild type and *Klrk1*$^{-/-}$ mice under both SPF and antibiotic-treated conditions. Antibiotic treatment did not significantly change expression of H60a (Fig. 12F and 13F) or NKG2D (Fig. 12G and 13G), and there was no difference in CD69 expression on immune cells in the pancreas or spleen between wild type and *Klrk1*$^{-/-}$ mice under either condition (Fig. 12E and 13E). Antibiotic treatment did result in a decrease in CD4$^+$ T cells in both the pancreas and spleen, and altered the ratio of effector to central memory CD4$^+$ T cells in the pancreas (Fig. 12B, 12C, 13B and 13C). Specifically, with antibiotics there were fewer effector CD4$^+$ T cells (CD44$^+$CD62L$^-$) and more central memory CD4$^+$ T cells (CD44$^+$CD62L$^+$) in the pancreas in both wild type and *Klrk1*$^{-/-}$ mice (Fig. 13C). There was a similar increase in central memory CD8$^+$ T cells in the pancreas; however, unlike the CD4$^+$ T cell population, there was not a corresponding decrease in effector CD8$^+$ T cells (Fig. 13D). These data demonstrate that there were fewer effector T cells in the pancreas of the

antibiotic-treated mice. This corresponds with the reduced diabetes development in these mice compared with SPF-housed wild type mice, as well as the increased ability to observe the protective role of NKG2D in antibiotic-treated mice (Trembath et al. 2017).

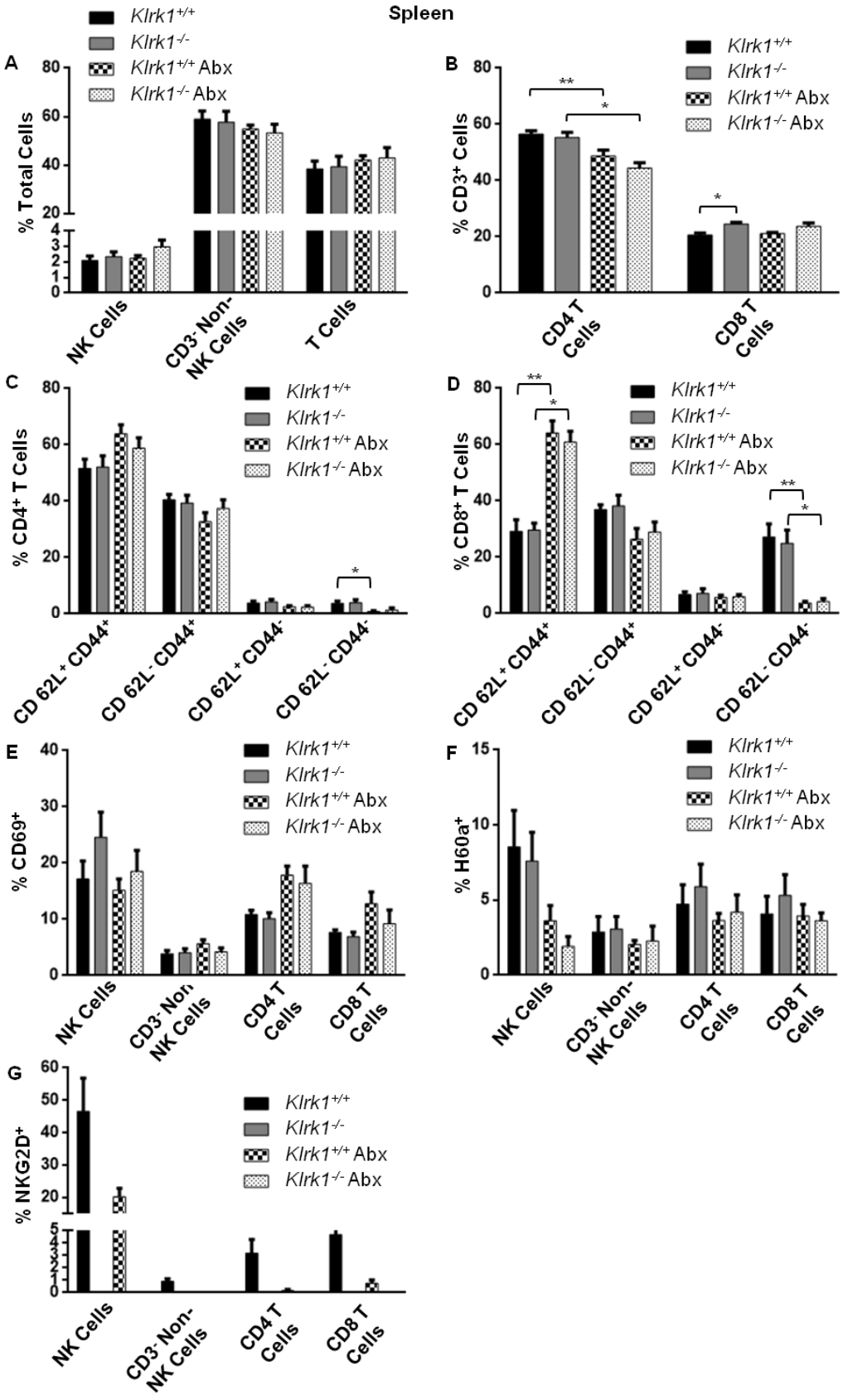
Spleen
A
% Total Cells
Klrk1+/+
Klrk1-/-
Klrk1+/+ Abx
Klrk1-/- Abx
NK Cells
CD3- Non-NK Cells
T Cells
B
% CD3+ Cells
CD4 T Cells
CD8 T Cells
C
% CD4+ T Cells
CD 62L+ CD44+
CD 62L- CD44+
CD 62L+ CD44-
CD 62L- CD44-
D
% CD8+ T Cells
E
% CD69+
F
% H60a+
G
% NKG2D+

Figure 12: Immune cell populations, activation, and NKG2D and H60a expression in the spleen of untreated and antibiotic-treated Klrk1+/+ and Klrk12/2 NOD mice. (A) Percentage of total splenocytes in 12 wk old wild-type and Klrk12/2 NOD mice with or without antibiotic that were CD32 CD49b+ (NK cells), CD32 CD49b2 (non-T or NK cells), or CD3+ (T cells). (B) Percentage of splenic T cells in (A) that were CD4+ and CD8+. (C) CD62L and CD44 expression by splenic CD4+ T cells in (A). (D) CD62L and CD44 expression by splenic CD8+ T cells in (A). (E–G) Percentage of splenic populations in (A) and (B) that expressed (E) CD69, (F) H60a, or (G) NKG2D. Data are combined results (mean 6 SEM) of at least four mice per group. *p # 0.05, **p # 0.01 in two-tailed unpaired Mann–Whitney U test.

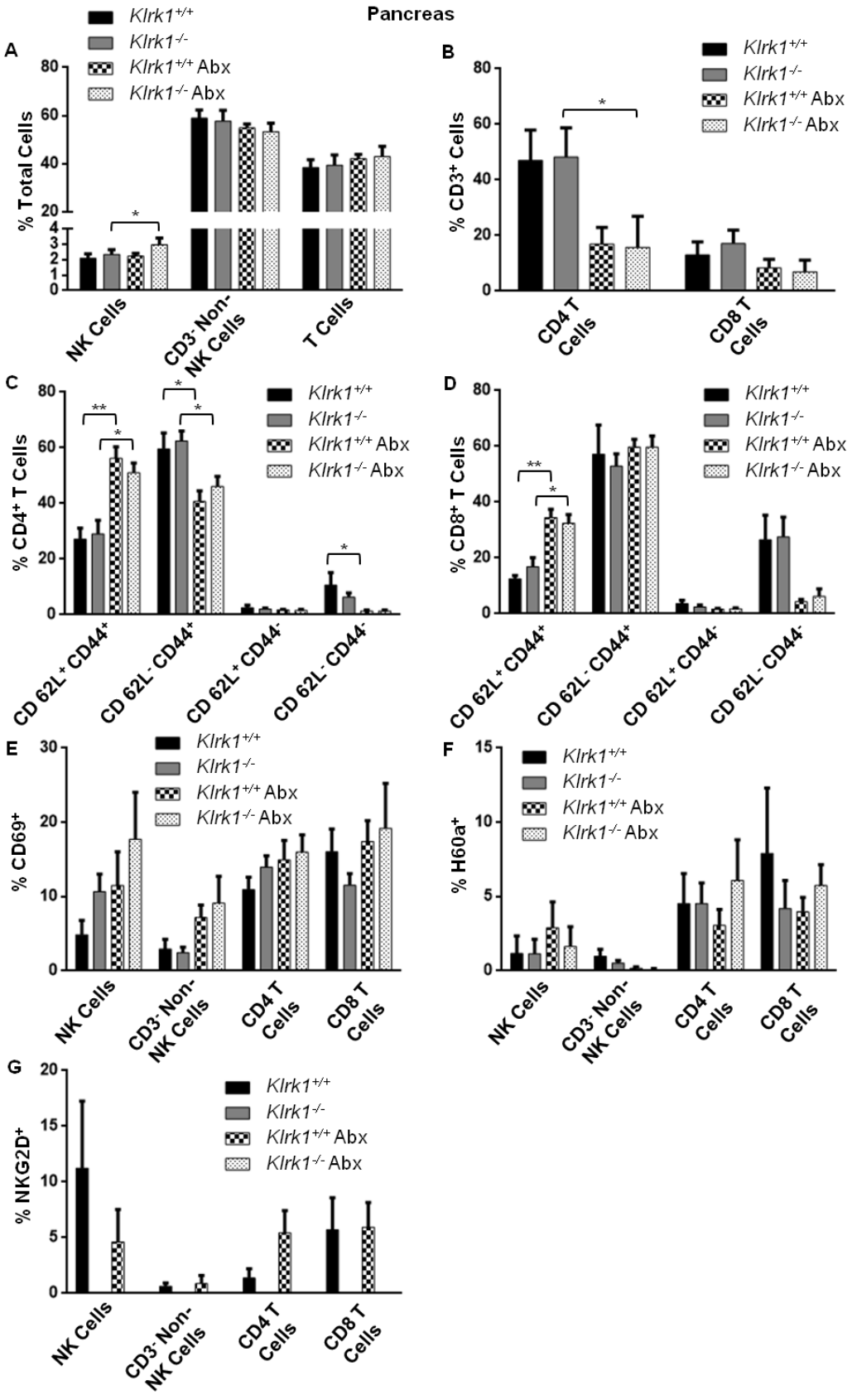
Pancreas
A
Klrk1+/+
Klrk1-/-
Klrk1+/+ Abx
Klrk1-/- Abx
% Total Cells
NK Cells
CD3- Non-NK Cells
T Cells
B
% CD3+ Cells
CD4 T Cells
CD8 T Cells
C
% CD4+ T Cells
CD 62L+ CD44+
CD 62L- CD44+
CD 62L+ CD44-
CD 62L- CD44-
D
% CD8+ T Cells
E
% CD69+
F
% H60a+
G
% NKG2D+

Figure 13: Immune cell populations, activation, and NKG2D and H60a expression in the pancreata of untreated and antibiotic-treated Klrk1+/+ and Klrk12/2 NOD mice. (A) Percentage of cells isolated from the pancreata of 12 wk old wild-type and Klrk12/2 NOD mice with or without antibiotic that were CD32 CD49b+ (NK cells), CD32 CD49b2 (non-T or NK cells), or CD3+ (T cells). (B) Percentage of T cells in (A) that were CD4+ and CD8+. (C) CD62L and CD44 expression by CD4+ T cells in (A). (D) CD62L and CD44 expression by CD8+ T cells in (A). (E–G) Percentage of populations in (A) and (B) that expressed (E) CD69, (F) H60a, or (G) NKG2D. Data are combined results (mean 6 SEM) of at least four mice per group. *p # 0.05, **p # 0.01 in two-tailed unpaired Mann–Whitney U test.

Discussion

I demonstrated that interaction between NKG2D and H60a, both expressed on differentiating CTL, reduces the production of effector cytokines by NOD CTL. This corresponds with decreased diabetes development in antibiotic-treated *Klrk1*$^{-/-}$ NOD mice.

NKG2D is implicated in the development of autoimmune diabetes in both humans and mice (Ogasawara, Hamerman et al. 2004, Gambelunghe, Brozzetti et al. 2007, Markiewicz, Wise et al. 2012, Van Belle, Ling et al. 2013), however a definitive role for NKG2D in this disease has not been established. Much of this uncertainty has been due to seemingly conflicting results in the NOD mouse model. Previous reports have demonstrated that NKG2D either promotes NOD diabetes (Ogasawara, Hamerman et al. 2004) or has little effect (Guerra, Pestal et al. 2013). In particular, Ogasawara et. al. (Ogasawara, Hamerman et al. 2004) suggested that expression of the NKG2D ligand RAE1 in pancreatic islets targets islet β cells for NKG2D-mediated destruction.

Dr. Markiewicz's later finding that transgenic expression of RAE1 by islet β cells drove immune cell infiltration of pancreatic islets in the non-autoimmune C57BL/6 background seemed to support the feasibility of this model (Markiewicz, Wise et al. 2012). However, the Markiewicz lab did not detect expression of RAE1 mRNA, or any other NKG2D ligand, by $CD45^-$ cells in pancreatic islets. This finding is consistent with other reports that found no RAE1 mRNA (Angstetra, Graham et al. 2012, Carrero, Calderon et al. 2013), or protein (Angstetra, Graham et al. 2012) expressed by NOD islet cells. These differing reports of RAE1 expression may be a result of variance between NOD mouse colonies due to gut microbial influence or genetic drift (Pozzilli, Signore et al. 1993, Yurkovetskiy, Burrows et al. 2013, Simecek, Churchill et al. 2015). A report by Adlercreutz et. al. (Adlercreutz, Weile et al. 2014) did show expression of H60a mRNA in the pancreatic islets of 13 week old NOD and Balb/c mice, but did not distinguish between immune and non-immune cells. This is consistent with the H60a mRNA expression observed in the Markiewicz lab.

My observation of robust H60a expression by activated NOD $CD8^+$ T cells was not anticipated, but is consistent with earlier reports. H60a mRNA was previously shown to be expressed in healthy NOD tissues and by NOD NK cells (Ogasawara, Hamerman et al. 2003, Adlercreutz, Weile et al. 2014). Similar to the NOD mouse, Balb/c immune cells express H60a (Cerwenka, Bakker et al. 2000, Ogasawara and Lanier 2005, Takada, Yoshida et al. 2008), and mRNA encoding this NKG2D ligand was shown to be upregulated in Balb/c T cells upon activation (Rabinovich, Li et al. 2003). Likewise, human T cells also express NKG2D ligands upon activation (Zwirner, Fernandez-Vina et al. 1998, Molinero, Fuertes et al. 2002, Cerboni, Zingoni et al. 2007).

Constitutive NKG2D ligand expression decreases NKG2D surface expression on NK and $CD8^+$ T cells (Coudert, Zimmer et al. 2005, Wiemann, Mittrucker et al. 2005, Champsaur and Lanier 2010). Poor NKG2D expression has been previously reported on NOD NK cells (Ogasawara, Hamerman et al. 2003), which correlated with RAE1 expression by these cells (Ogasawara, Hamerman et al. 2003). Similarly, the high level of H60a expression on NOD $CD8^+$ T cells is likely responsible for the low surface expression of NKG2D we observed on these cells. However, our results clearly show that this low NKG2D expression has functional relevance to CTL differentiation.

To date, the function of NKG2D in $CD8^+$ T cells has largely been studied in differentiated CTLs. These studies have generally shown a co-stimulatory role for NKG2D, enhancing TCR-stimulated effector function (Bauer, Groh et al. 1999, Groh, Rhinehart et al. 2001, Markiewicz, Carayannopoulos et al. 2005) or memory formation (Zloza, Kohlhapp et al. 2012, Wensveen, Lenartic et al. 2013). The effects of NKG2D signaling in the absence of TCR engagement is not well understood, but has been shown to induce immune synapse formation by mouse CTLs (Markiewicz, Carayannopoulos et al. 2005) and MHC-unrestricted killing by cytokine-activated human CTLs (Verneris, Karimi et al. 2004). Therefore, my finding that there is a role for NKG2D-H60a interaction during $CD8^+$ T cell differentiation into CTL describes a new mode of NKG2D-ligand interaction, which dampens, rather than enhances, later CTL responses.

In my *in vitro* experiments, there was a consistent decrease in TNFα, IL-10 and IFNγ production by CTL with either genetic knockout of NKG2D or antibody blockade of H60a with low levels

of stimulation. At the highest level of stimulation, IFNγ production was significantly decreased in a number of experiments. This decrease could potentially reflect a different cytokine response at very high levels of TCR stimulation. However, because this decrease was not significant when data from all experiments were combined,I did not investigate this further. Additionally, the consistently enhanced production of IFNγ by *Klrk1*$^{-/-}$ adoptively transferred CD8^{+} T cells *in vivo* suggests that low to medium levels of TCR stimulation more closely reflect *in vivo* conditions.

When the Markiewicz lab assessed diabetes incidence in *Klrk1*$^{-/-}$ and wild type NOD mice, the results indicated a different effect of NKG2D genotype on diabetes incidence depending upon whether or not the mice were treated with microbiota-depleting antibiotics. Under SPF housing conditions, there was a detrimental role for NKG2D in NOD diabetes development. By contrast, when animals were treated with antibiotics, wild type NOD mice had delayed disease compared to *Klrk1*$^{-/-}$ mice. This differential effect revealed a separate protective role for NKG2D. In addition, the lack of an effect of antibiotic treatment on disease incidence in *Klrk1*$^{-/-}$ mice provides evidence for NKG2D genotype-dependent changes in the microbiota affecting NOD diabetes incidence. Antibiotic treatment resulted in a decrease in effector T cells in the pancreas. This is a probable mechanism by which diabetes development was decreased in antibiotic-treated compared with SPF-housed wild type mice. Further, it is likely that this decrease in effector T cells is what is responsible for the increased ability to observe the protective role NKG2D in diabetes development with antibiotic treatment. Taken together, these results indicate a detrimental influence of NKG2D on diabetes development in the gut, which involves the microbiota, and a separate effect independent of microbiota in which NKG2D dampens the NOD CTL cytokine response.

The Markiewicz lab observed the well-established sexual dimorphism in NOD diabetes incidence, with increased rates of diabetes in females compared to males (Makino, Kunimoto et al. 1981, Kikutani and Makino 1992, Pozzilli, Signore et al. 1993). Within SPF housed or antibiotic treated groups however, the effect of NKG2D was consistent between males and females. The mechanism by which NKG2D promotes NOD diabetes through interactions with microbiota was not explored in the current study, but warrants further investigation. This may be due to a lack of B1a cells in these mice (Lenartic, Jelencic et al. 2017), or interaction of other immune cells with NKG2D ligands, which are constitutively expressed by intestinal epithelial cells (Hue, Mention et al. 2004, Allez, Tieng et al. 2007, Eagle, Jafferji et al. 2009, Hansen, Holm et al. 2013). Additionally, further work remains to conclusively attribute the protective effect of NKG2D genotype in antibiotic treated animals to the effects of NKG2D-H60a interactions between $CD8^+$ T cells. While my data do support this hypothesis, a definitive answer awaits the generation of a NOD $H60a^{-/-}$ mouse, as well as $CD8^+$ T cell conditional NOD $NKG2D^{-/-}$ and NOD $H60a^{-/-}$ animals. Finally, the model of opposing pro- and anti-diabetic effects of NKG2D suggests that a lack of consensus from previous studies concerning the role of NKG2D in NOD diabetes may be the result of variation in co-housing practices and differences in microbiota between mouse colonies.

In summary, I found that NKG2D-H60a interaction between $CD8^+$ T cells during NOD CTL differentiation *in vitro* resulted in decreased production of diabetogenic cytokines upon subsequent CTL activation. To my knowledge, this is the first report of a role for NKG2D-NKG2D ligand interaction during differentiation of mouse $CD8^+$ T cells into CTL rather than during the effector phase of differentiated CTL. Furthermore, these results describe a functional

role for NKG2D ligand expression by healthy murine $CD8^+$ T cells. Finding that NKG2D is protective in animals with depleted microbiota correlates with NKG2D signaling resulting in reduced cytokine production by NOD CTL. The additional observation that microbiota-depleting antibiotics profoundly affected NOD diabetes development in wild type, but not *Klrk1*$^{-/-}$ animals demonstrates a separate effect in which NKG2D-dependent alterations in the microbiota promote disease development. Additionally, these data may have broader implications for the role of NKG2D-ligand interaction between T cells in modulating $CD8^+$ T cell responses in both mice and humans.

Chapter 3: NKG2D signaling within the pancreatic islets reduces NOD diabetes by enhancing CD8+ central memory cell generation

Abstract

NKG2D is implicated in autoimmune diabetes, with NKG2D ligand expression in pancreatic islets believed to play a detrimental role. Work from the Markiewicz lab has found that NKG2D and its ligands are present in human pancreata. The expression of NKG2D and its ligands are increased in the islets of patients with type 1 diabetes. To directly assess the role of NKG2D in the pancreas, we generated NOD mice that express an NKG2D ligand in islet cells. Diabetes was not enhanced, but rather was reduced in these mice. The reduction corresponded with a decrease in the effector to central memory $CD8^+$ T cell ratio. Further, NKG2D signaling enhanced central memory $CD8^+$ T cell generation in vitro and diabetes protection by central memory $CD8^+$ T cells in vivo. Taken together, these studies demonstrate that there is a protective role for central memory $CD8^+$ T cells in autoimmune diabetes and that this protection is enhanced with NKG2D signaling.

Introduction

Early studies with the non-obese diabetic (NOD) mouse model led to the hypothesis that NKG2D signaling, induced by NKG2D ligands expressed on β-islet cells, enhances diabetes development (Ogasawara 2004, Markiewicz 2012). However, conflicting results were later reported which brought into question the importance of NKG2D to spontaneous autoimmune diabetes in this pre-clinical model (Guerra, Pestal et al. 2013, Trembath, Sharma et al. 2017). Our recent data implicate differential effects of NKG2D signaling induced by microbiota or islet

antigen (Trembath, Sharma et al. 2017) as a source of this controversy. Therefore, new experimental approaches are required to determine the various roles of NKG2D in diabetes.

Previous work in the Markiewicz lab analyzed expression of the mRNAs encoding NKG2D and NKG2D ligands within human pancreatic islets from tissue sections donated to the Network for Pancreatic Organ Donors with Diabetes (nPOD) by people with type 1 diabetes, people with islet antigen-specific autoantibodies but not diabetic (likely pre-diabetic), or non-diabetic age and sex matched controls. Increased transcripts encoding both NKG2D and NKG2D ligands were detected in pancreatic islets harvested from type 1 diabetes donors, but not individuals positive for islet-specific antibody, compared with non-diabetic donors. These data indicate that increased NKG2D-ligand interactions within the pancreas are present in patients with type 1 diabetes, and demonstrate that NKG2D signaling in the pancreas is likely relevant to type 1 diabetes pathogenesis.

Because both NKG2D and NKG2D ligands are expressed on immune cells in NOD mice (Zwirner, Fernandez-Vina et al. 1998, Molinero, Fuertes et al. 2002, Rabinovich, Li et al. 2003, Trembath and Markiewicz 2018), these proteins could not be eliminated specifically within the pancreas as a means to test the effects of NKG2D signaling in the pancreas on autoimmune diabetes development. Instead, NOD mice with constitutive expression of the mouse NKG2D ligand RAE1ε on β-islet cells, NOD.RIP-RAE1ε mice, were generated. These mice were generated by moving a cre-inducible RAE1ε transgene generated previously (RIP-RAE1ε) (Markiewicz, Wise et al. 2012) and a rat insulin promoter (RIP)-cre transgene onto the NOD background, followed by interbreeding of these two strains to generate NOD mice with

constitutive expression of RAE1ε in the pancreatic islets (NOD.RIP-RAE1ε). It was subsequently found that despite earlier infiltration of immune cells into the pancreas, these mice developed significantly less diabetes compared to wild type NOD mice. In addition, this difference was lost in NKG2D knockout NOD.RIP-RAE1 mice, demonstrating a dependence upon NKG2D.

Here, I find a reduced ratio of effector and effector memory $CD8^+$ T cells ($T_{eff + em}$) to central memory $CD8^+$ T cells (T_{cm}), corresponding with this previously observed reduction in diabetes. Correlating with these in vivo data, we found that stimulation of NKG2D on human and mouse $CD8^+$ T cells during in vitro activation increased the generation of $CD8^+$ T_{cm}. Finally, we found that $CD8^+$ T_{cm} actively suppress NOD diabetes development in vivo. Taken together, these results indicate that NKG2D ligand expression in pancreatic islets protects against autoimmune diabetes development by enhancing the generation of a protective $CD8^+$ T_{cm} population.

Results

Reduced $CD8^+$ $T_{eff + em}$:T_{cm} ratio in RIP-RAE1ε NOD mice

I compared the immune infiltrate in the pancreas of NOD.RIP-RAE1ε and RAE1ε- littermates (NOD.PCCALL and NOD. RIP-Cre) mice at 12 weeks of age, a time at which both of these strains have similar insulitis but have not yet developed diabetes. I focused on T cells and NK cells, as these are the cells that express NKG2D in the wild type NOD islets (Trembath, Sharma et al. 2017). I found similar numbers of T cells, including $CD4^+$ T and $CD8^+$ T, as well as NK cells, in the pancreas and spleen of NOD.RIP-RAE1ε and RAE1ε- littermates (NOD. PCCALL and NOD.RIP-cre) mice. However, NOD.RIP-RAE1ε mice had a lower percentage of $CD8^+$ T

cells with an effector (T_{eff}) or effector memory (T_{em}) phenotype ($CD44^{hi}CD62L^{lo}$) and a greater percentage of $CD8^+$ T cells with a central memory (T_{cm}) phenotype ($CD44^{hi}CD62^{hi}$) in the pancreas (Fig. 14A-C), resulting in a decreased CD8+ $T_{eff + em}$:T_{cm} ratio compared to RAE1ε- littermates (NOD. PCCALL and NOD.RIP-cre) mice (Fig. 14D). The $CD8^+$ $T_{eff + em}$:T_{cm} ratio was even lower in the spleen of NOD.RIP-RAE1ε mice (Fig. 14H-K), and this difference was consistently observed in both male and female mice. Demonstrating NKG2D signaling was required for this effect on $CD8^+$ T cell populations, no difference was observed between NOD.RIP-RAE1ε-*Klrk1*$^{-/-}$ versus RAE1ε- littermate NOD.*Klrk*1$^{-/-}$ (PCCALL/*Klrk*1$^{-/-}$ and RIP-cre/*Klrk*1$^{-/-}$) mice (Fig. 14E,F, L, and M). In contrast to these $CD8^+$ T cell population alterations, we did not observe a difference in the $CD4^+$ $T_{eff + em}$:T_{cm} ratio when comparing NOD.RIP-RAE1ε and RAE1ε- littermates (NOD. PCCALL and NOD.RIP-cre) (Fig. 14G and 14N) These data were collected by other lab members as well as myself.

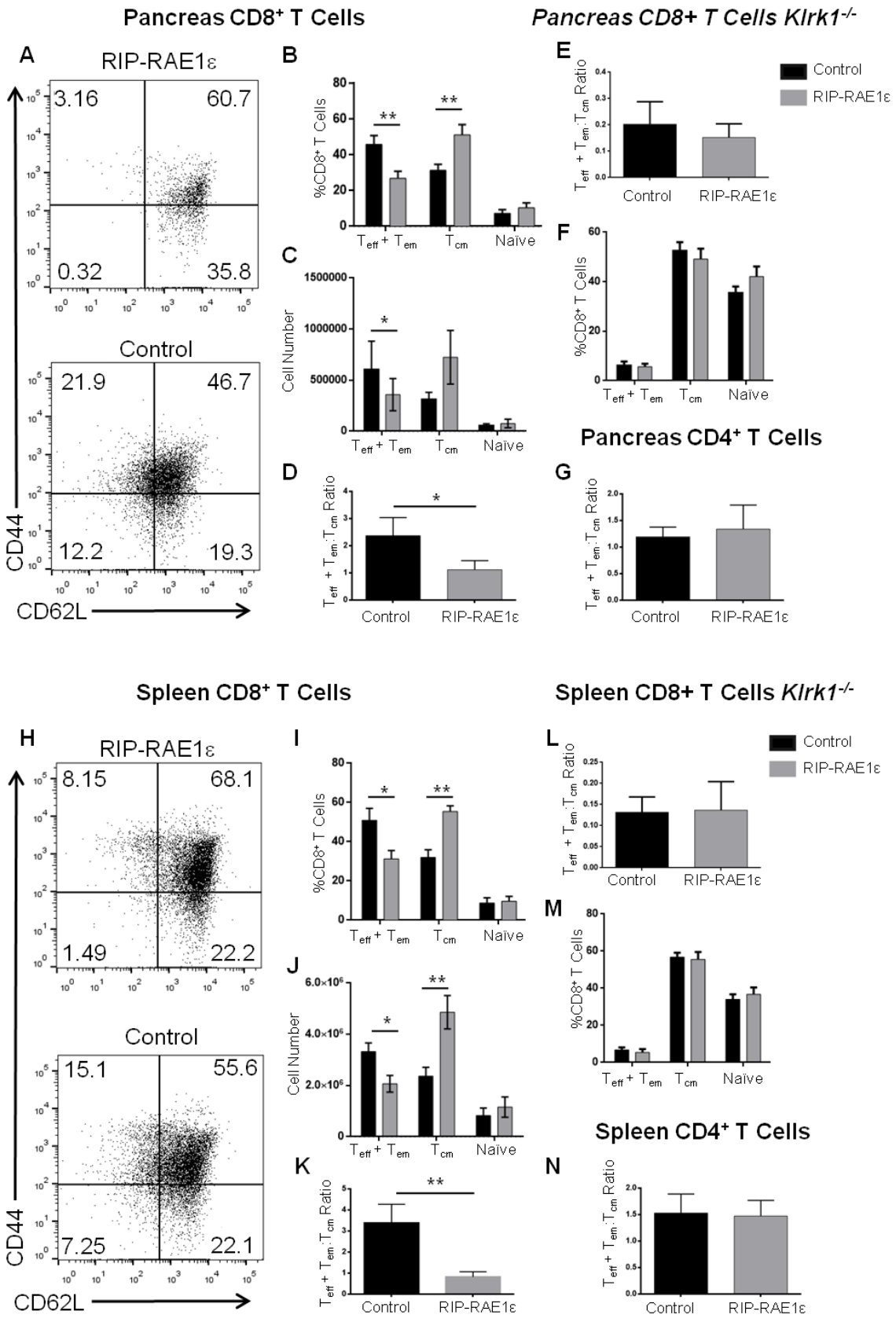
Pancreas CD8+ T Cells
Pancreas CD8+ T Cells Klrk1-/-
A
RIP-RAE1ε
3.16
60.7
0.32
35.8
Control
21.9
46.7
12.2
19.3
CD44
CD62L
B
%CD8+ T Cells
**
**
Teff + Tem
Tcm
Naïve
C
Cell Number
*
D
Teff + Tem:Tcm Ratio
*
Control
RIP-RAE1ε
E
Control
RIP-RAE1ε
Teff + Tem:Tcm Ratio
F
%CD8+ T Cells
Pancreas CD4+ T Cells
G
Teff + Tem:Tcm Ratio
Spleen CD8+ T Cells
Spleen CD8+ T Cells Klrk1-/-
H
RIP-RAE1ε
8.15
68.1
1.49
22.2
Control
15.1
55.6
7.25
22.1
CD44
CD62L
I
%CD8+ T Cells
*
**
J
Cell Number
*
**
K
Teff + Tem:Tcm Ratio
**
L
Control
RIP-RAE1ε
Teff + Tem:Tcm Ratio
M
%CD8+ T Cells
Spleen CD4+ T Cells
N
Teff + Tem:Tcm Ratio

Figure 14: Decreased ratio of $CD8^+$ T_{eff} + T_{em} to T_{cm} in RIP-RAE1ε NOD mice. (**A and H**) Representative flow cytometry data gated on $CD3^+CD8^+$ cells from the **(A)** pancreas or **(H)** spleen of a RIP-RAE1ε (top panel) or control (bottom panel) mouse showing CD44 and CD62L expression. The numbers shown are the percentage of cells present in each quadrant of the dot plot. (**B and I**) The percentage of $CD8^+CD3^+$ cells that were $CD44^+CD62L^-$ (T_{eff} + T_{em}), $CD44^+CD62L^+$ (T_{cm}), or $CD44^-CD62L^-$ (naïve) in the **(B)** pancreas or **(I)** spleen of RIP-RAE1ε mice (n=22) and control (RIP-Cre and PCCALL) mice (n=19). (**C and J**) The number of cells that were $CD44^+CD62L^-$(T_{eff} + T_{em}), $CD44^+CD62L^+$ (T_{cm}), or $CD44^-CD62L^-$ (naïve) in the **(C)** pancreas and **(J)** spleen of RIP-RAE1ε mice (n=22) and control (RIP-Cre and PCCALL) mice (n=19). **(D and K)** The ratio of $CD8^+$ T_{eff} + T_{em}:T_{cm} in the **(D)** pancreata and **(K)** spleen of RIP-RAE1ε and control (RIP-Cre and PCCALL) mice. **(E and L)** The ratio of CD4+ T_{eff} + T_{em}:T_{cm} in the **(E)** pancreata and **(L)** spleen of RIP-RAE1ε and control (RIP-Cre and PCCALL) *Klrk1*$^{-/-}$ mice. **(F and M)** The percentage of cells that were $CD44^+CD62L^-$ (T_{eff} + T_{em}), $CD44^+CD62L^+$ (T_{cm}), or $CD44^-CD62L^-$ (naïve) in the **(F)** pancreas and **(M)** spleen of RIP-RAE1ε/ *Klrk1*$^{-/-}$ (n=15) and control (RIP-Cre and PCCALL)/*Klrk1*$^{-/-}$ (n=20) mice. **(G and N)** The ratio of $CD4^+$ T_{eff} + T_{em}:T_{cm} in the **(G)** pancreata and **(M)** spleen of RIP-RAE1ε (n=22) and control (RIP-Cre and PCCALL) (n=29) mice. *p<0.05, **p<0.01 in a two-sided Mann-Whitney U test.

<u>$CD8^+$ T_{cm} delay NOD diabetes development</u>

I hypothesized that the greater number of $CD8^+$ T_{cm} in the NOD.RIP-RAE1ε was directly responsible for the decreased diabetes development in these mice. To test this, I purified $CD8^+$ $T_{eff + em}$ ($CD44^+CD62L^-$) and T_{cm} ($CD44^+CD62L^+$) populations from non-diabetic NOD mice. I then adoptively transferred $T_{eff + em}$, T_{cm}, or $T_{eff + em}$ and T_{cm}, along with $CD8^+$ T cell-depleted

NOD splenocytes, into NOD.*Scid* recipient mice (Fig. 15A). I measured blood glucose levels weekly, and time determined time to diabetes within each experiment relative to the first mouse to become diabetic. The mice that received the $CD8^+$ $T_{eff\ +}$ T_{em} cells developed diabetes significantly earlier compared with the ones that received T_{cm} or T_{cm}+ $T_{eff\ +\ em}$ (Fig. 15B). These results demonstrate that NOD $CD8^+$ T_{cm} not only are less diabetogenic compared $CD8^+$ $T_{eff\ +\ Tem}$, but actively suppress diabetes development.

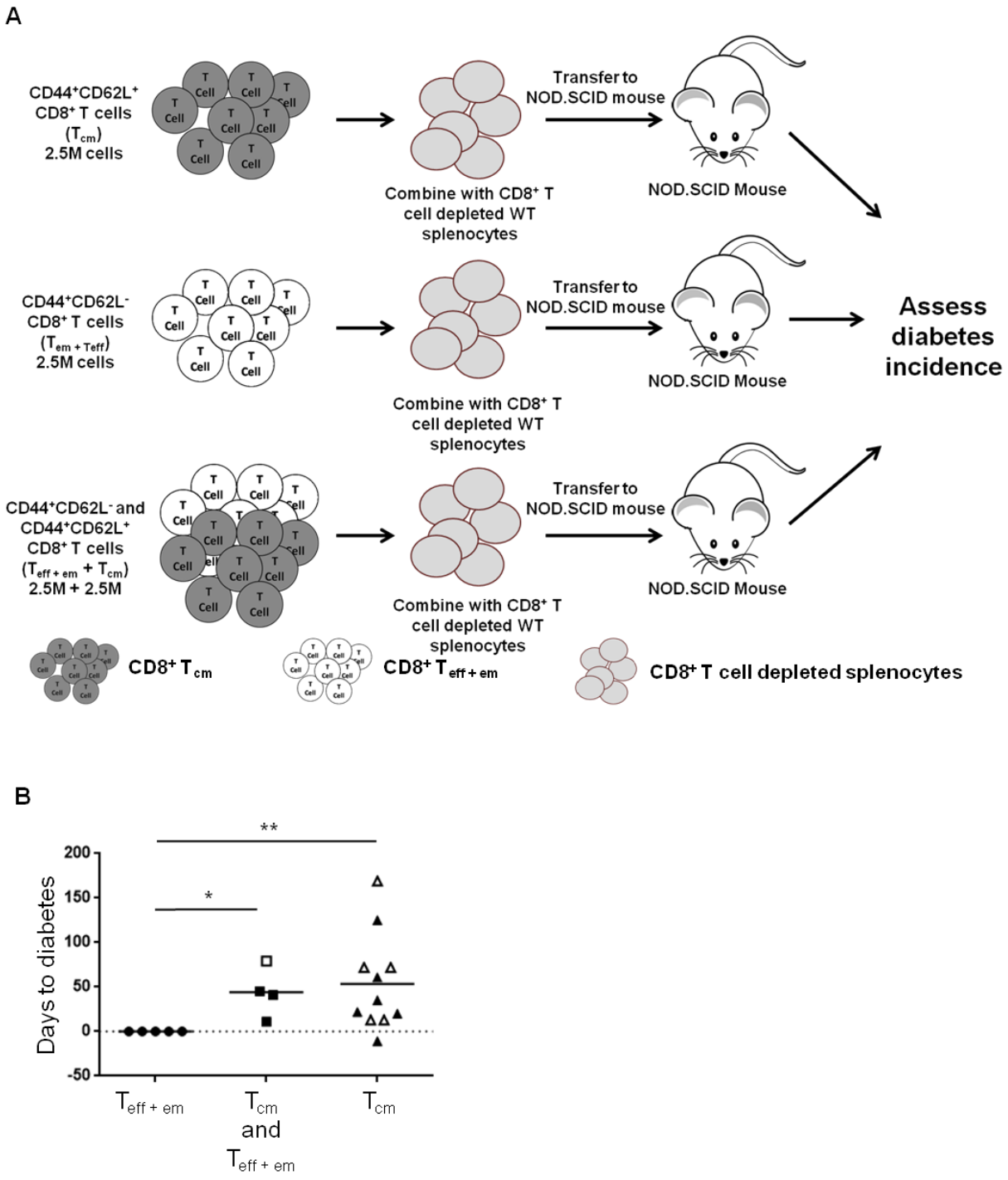

Figure 15: CD8+ T_{cm} delay NOD diabetes development. (**A**) Schematic of adoptive transfer experimental design. M: million. (**B**) The number of days to diabetes in NOD.SCID recipient mice. The day the first mouse in each experiment developed diabetes was called "day 0". The

data shown are combined data from five independent experiments. Open points denote mice that were not yet diabetic at the time of submission. *p< 0.05 and **p< 0.001 in Log-Rank test.

NKG2D signaling during NOD $CD8^+$ T cell activation *in vitro* drives T_{cm} generation and increases expression of PD-1

NKG2D has been shown to be important in $CD8^+$ T cell memory formation in other mouse strains, especially $CD8^+$ T_{cm} (Zloza, Kohlhapp et al. 2012, Wensveen, Lenartic et al. 2013, Perez, Prajapati et al. 2019). I therefore hypothesized that NKG2D signaling directly in NOD $CD8^+$ T cells was responsible for the increase in $CD8^+$ T_{cm} generation in the NOD.RIP-RAE1ε mice. To test this, I purified $CD8^+$ T cells from NOD mice and activated them in vitro with splenocytes from B6.RAE1ε, which have ubiquitous transgenic expression of RAE1ε (Cheney, Wise et al. 2012), or control B6 splenocytes. Similar to our observation with $CD8^+$ T cell populations in vivo in the NOD.RIP-RAE1ε mice (Fig. 12 and 13), NKG2D stimulation during in vitro activation with the B6.RAE1ε splenocytes resulted in a significantly reduced ratio of $T_{eff + em}$ to T_{cm} $CD8^+$ T cells (Fig. 16).

I also assessed PD-1 expression by the resulting $CD8^+$ T cells after stimulation on splenocytes expressing Rae-1ε or control. The immune inhibitory receptor PD-1 plays an important role in suppressing autoimmune diabetes development (Ansari, Salama et al. 2003, Clotman, Janssens et al. 2018), and has been found to be decreased in children with new onset type I diabetes (Granados, Draghi et al. 2017). I found that NKG2D stimulation significantly increased the expression of PD-1 on NOD $CD8^+$ T cells alone (Fig. 17 A and B) or with the addition of IL-15

(Fig. 17 C and D). Analysis of PD-1 expression by the $CD8^+$ T_{cm} population showed a similar increase with NKG2D stimulation.

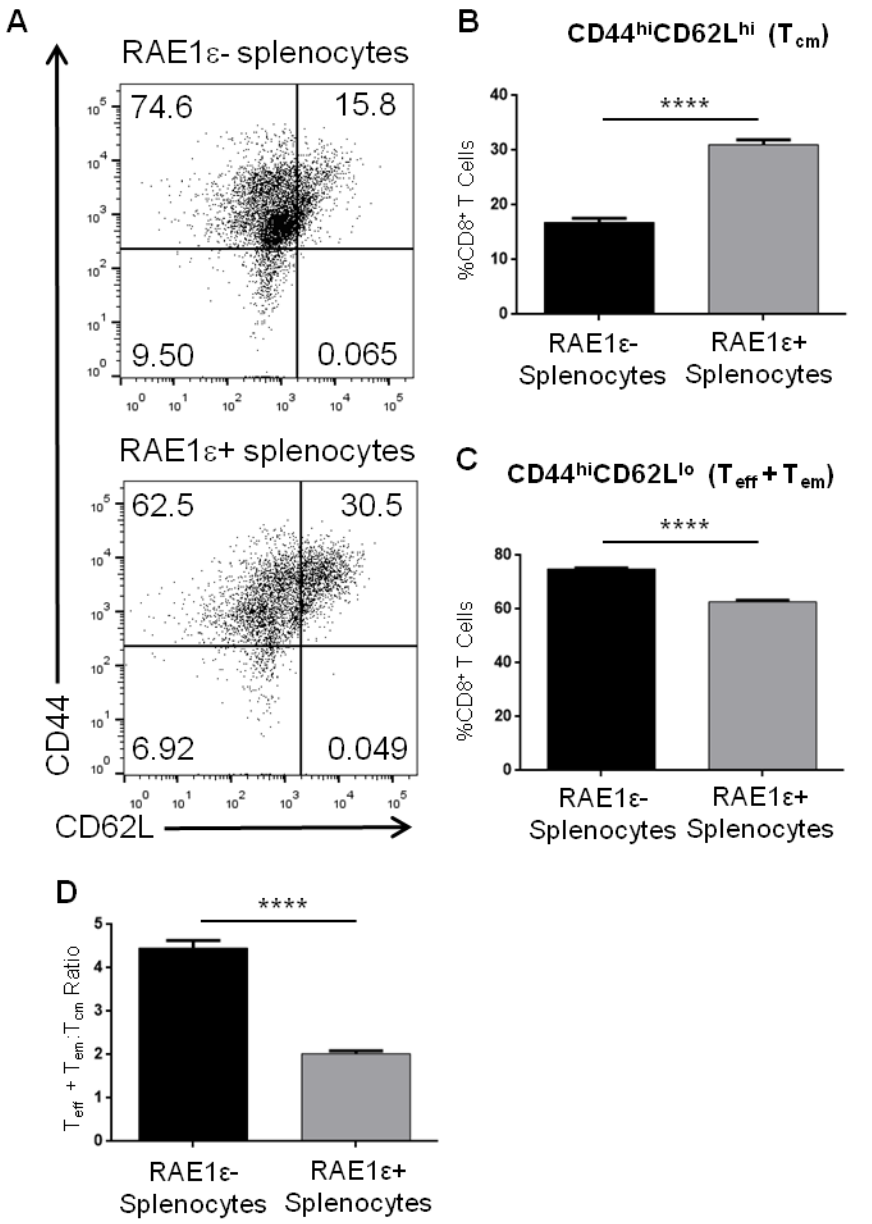

Figure 16: NKG2D signaling in NOD CD8+ T cells increases $CD8^+$ T_{cm} generation. (A) Representative flow cytometry data gated on in vitro activated NOD $CD3^+CD8^+$ cells showing CD44 and CD62L expression. The numbers shown are the percentage of cells present in each quadrant of the dot plot. (**B and C**) The percentage (mean +/- STD) of NOD $CD8^+CD3^+$ cells that were **(B)** $CD44^+CD62L^+$ (T_{cm}) or **(C)** $CD44^+CD62L^-$ ($T_{eff + em}$) after activation in vitro in the presence of RAE1ε+ or RAE1ε- splenocytes. (**D**) The ratio (mean +/- STD) of NOD CD8+ $T_{eff +}$

$_{em}$:T_{cm} generated after activation in vitro in the presence of RAE1ε+ or RAE1ε- splenocytes. These data are representative of 3 independent experiments. ****p<0.0001 in two-sided t-test.

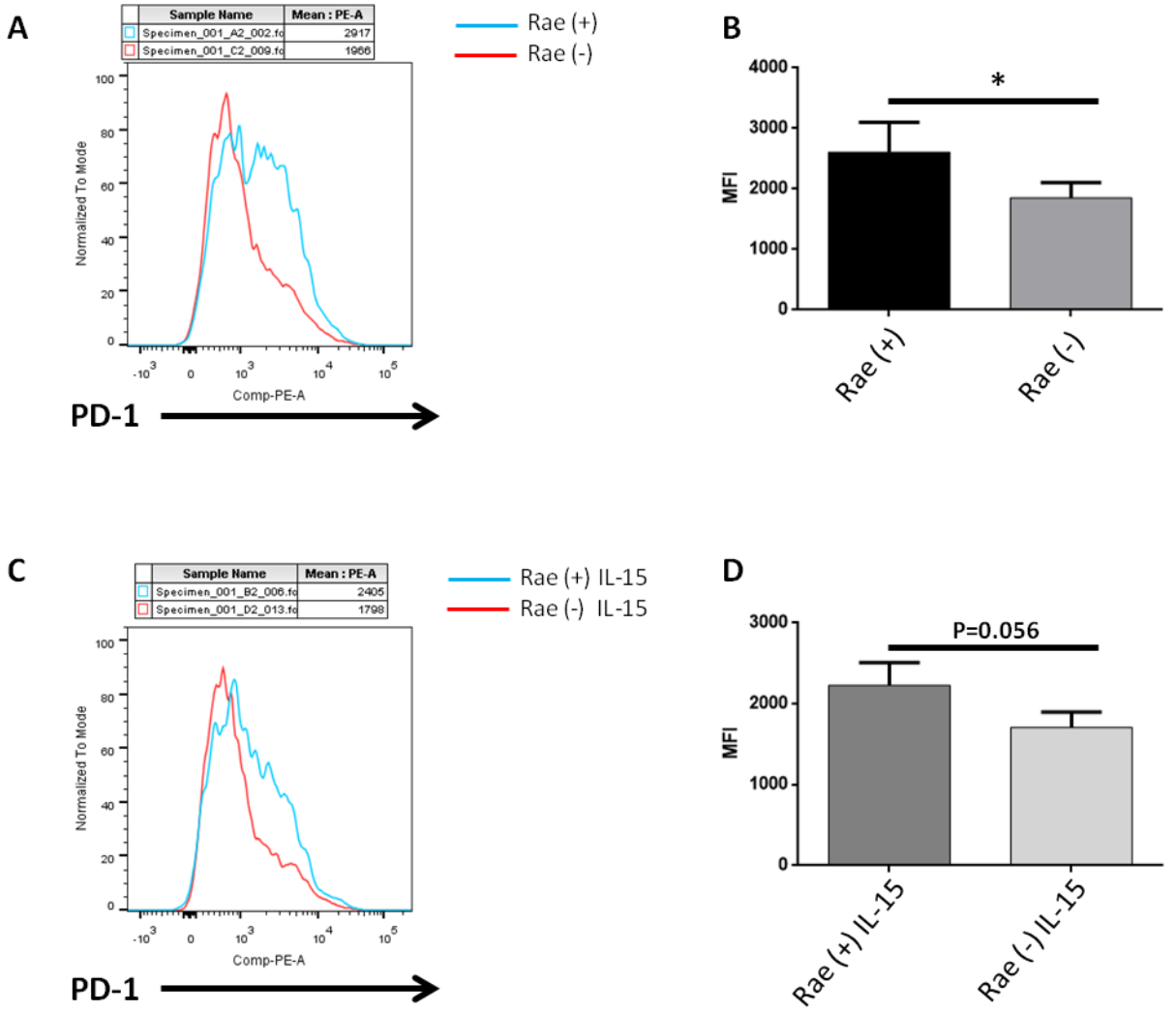

Figure 17: NKG2D stimulation in NOD CD8$^+$ T cells increases PD-1 expression. NOD CD8$^+$ T cells activated with anti-CD3 and irradiated Rae-1ε expressing B6 splenocytes or Rae-1ε (-) control with (A and B) or without (C and D) the addition of 20ng/mL IL-15. (A and B) Representative flow histograms showing PD-1 expression on CD8$^+$ T cells on day 5 following

activation with the indicated splenocytes. (B and D) mean fluorescence index (MFI) of three technical replicates. These results are representative of 3 independent experiments. *$p<0.05$.

NKG2D signaling during human $CD8^+$ T cell activation drives T_{cm} generation

I wanted to determine if NKG2D stimulation of human $CD8^+$ T cells similarly decreases the ratio of T_{eff+em} to T_{cm} $CD8^+$ T cells. To do this, I purified total $CD8^+$ T cells from human peripheral blood and activated these cells in vitro for 5 days in the presence of activating anti-NKG2D or control antibody. Similar to the mouse $CD8^+$ T cells, I found that NKG2D stimulation during in vitro activation significantly reduced the ratio of human $CD8^+$ T_{eff+em} to T_{cm} (Fig. 18 A and B). I next sought to determine whether NKG2D stimulation was driving $CD8^+$ T_{cm} generation during the initial activation of naïve $CD8^+$ T cells, or if it was expanding and enhancing the survival of a previously activated population. To do this, I repeated the experiment with naïve human $CD8^+$ T cells. I found that NKG2D stimulation similarly increased the generation of T_{cm} cells from naïve $CD8^+$ T cells (Fig. 18 C and F), significantly decreasing the T_{eff+em}:T_{cm} ratio (Fig. 18F).

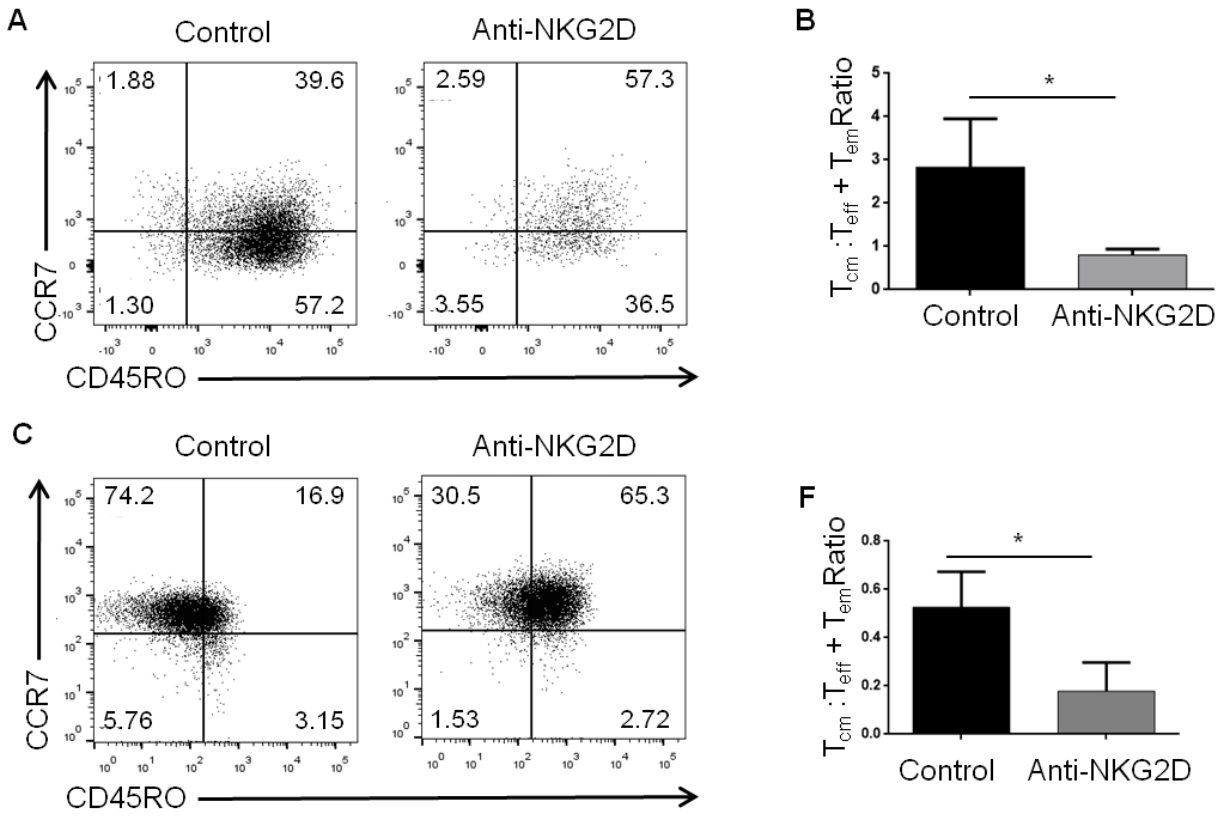

Figure 18: NKG2D signaling in human CD8+ T cells increases CD8+ Tcm generation. (A and C) Representative flow cytometry data gated on in vitro-activated human **(A)** total or **(C)** naïve CD3+CD8+ cells showing CCR7 and CD45RO expression. The numbers shown are the percentage of cells present in each quadrant of the dot plot. **(B)** The ratio of human $CD8^+$ T_{eff+em} ($CD45RO^+CCR7^-$):T_{cm} ($CD45RO^+CCR7^+$) generated after activation of human total $CD3^+CD8^+$ in vitro in the presence or absence of an activating anti-NKG2D antibody. These data are the combined results of five independent experiments. **(D)** The ratio of human $CD8^+$ $T_{eff\,+\,em}$:T_{cm} generated after activation of naïve CD8+CD3+ cells in vitro in the presence of or absence of an activating anti-NKG2D antibody. These data are the combined results of five independent experiments. *$p<0.05$ in one-tailed Mann-Whitney test.

NKG2D signaling during human $CD8^+$ T cell activation increases generation of regulatory $CD8^+$ T cells *in vitro*

The $CD8^+$ T_{cm} population is heterogeneous, and contains sub-populations if $CD8^+$ Tregs (Li, Xie et al. 2014). To determine if NKG2D stimulation specifically increased $CD8^+$ T cells with a regulatory phenotype, I activated human $CD8^+$ T cells *in vitro* with plate bound anti-CD3 and anti-CD28, in the presence of plate bound activating anti-NKG2D antibody or control. On day 5 I assessed the $CD8^+$ T_{cm} positive population, as well as expression of $CXCR3^+PD\text{-}1^+$ cells within the T_{cm} population. This population of $CXCR3^+PD\text{-}1^+$ T_{cm} phenotype cells has been shown to be analogous to the murine $CD122^+PD\text{-}1^+$ $CD8^+$ Tregs (Li, Xie et al. 2014) which have been shown to be beneficial in autoimmune diabetes (Tsai, Shameli et al. 2010). As before, I found that NKG2D stimulation caused a trend of increased $CD8^+$ T_{cm} population (Fig. 19A). In addition, I found that within the $CD8^+$ T_{cm} population, the population of $CXCR3^+PD\text{-}1^+$ $CD8^+$ Tregs also trended higher with NKG2D stimulation (Fig. 19B). I then asked if NKG2D stimulation was increasing generation of $CXCR3^+PD\text{-}1^+$ $CD8^+$ Tregs, or if NKG2D stimulation was enhancing survival or expansion of an existing population. I therefore activated naïve human $CD8^+$ T cells *in vitro* with plate bound activating anti-NKG2D antibody, or control. Because IL-15 has been shown to increase memory $CD8^+$ T cell populations (Schluns, Williams et al. 2002, Weng, Liu et al. 2002, Berard, Brandt et al. 2003), we activated cells with or without IL-15. As shown previously, NKG2D stimulation enhanced generation of $CD8^+$ T_{cm} cells (Fig. 18). Interestingly both NKG2D and IL-15 increased T_{cm} generation, and appeared to act in a combinatorial fashion with NKG2D and IL-15 together showing a trend towards increased T_{cm} generation (Fig. 19 C and D).

We found that $CXCR3^{+}PD\text{-}1^{+}$ $CD8^{+}$ Tregs represented a relatively consistent percentage of the T_{cm} population treatment groups (fig 19 E and F), but increased in total number proportionally with the increase in T_{cm} population observed with IL-15 or NKG2D stimulation (Fig. 19 G and H).

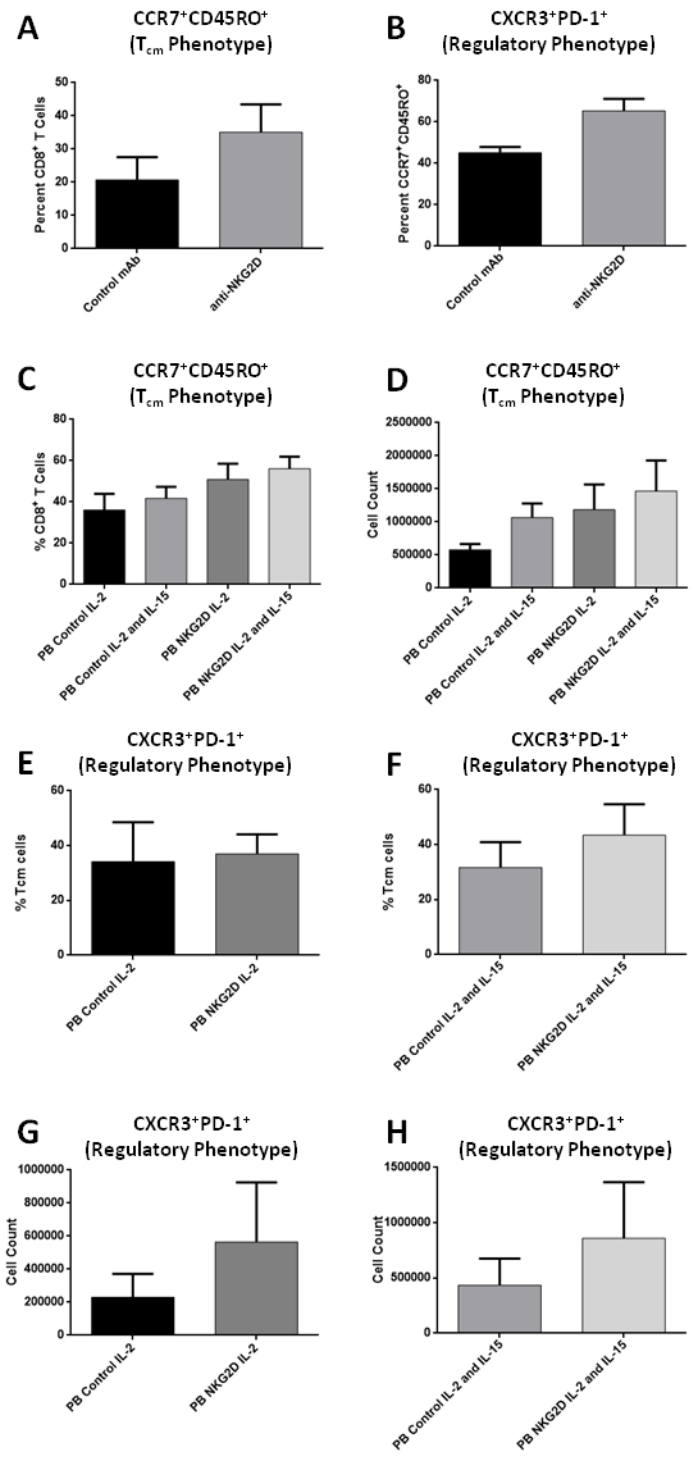

Figure 19: NKG2D stimulation increases CD8$^+$ Treg generation *in vitro*.

(A and B) Total human CD8$^+$ T cells isolated from PBMCs by negative selection and activated *in vitro* with plate bound anti-CD3 and anti-CD28 with plate bound activating anti-hNKG2D or

control. (A) Combined data from 4 experiments showing percentage of $CD8^+CD44^+CD62L^+$ T_{cm} phenotype cells after 5 days of culture. (B) The percentage of T_{cm} cells from (A) that are $CXCR3^+PD\text{-}1^+$.(C-H) Combined data from at least 5 independent experiments with naïve human $CD8^+$ T cells isolated from PBMCs by negative selection activated as in (A and B) with or without the addition of 20ng/uL IL-15. (C and D) The percentage and number of $CD8^+CD44^+CD62L^+$ T_{cm} phenotype cells after 5 days of culture. (E-H) The percentage and number of $CXCR3^+PD\text{-}1^+$ cells within the T_{cm} populations from (D and D).

Discussion

NKG2D and NKG2D ligand expression has been reported in the pancreas of NOD mice (Ogasawara, Hamerman et al. 2004, Trembath, Sharma et al. 2017). However, the expression of NKG2D and NKG2D ligands in the human pancreas had not been previously assessed. The Markiewicz lab demonstrated that mRNAs encoding NKG2D and NKG2D ligands are detectable in human pancreatic islets, with increased expression in patients with type 1 diabetes compared with age and sex matched non-diabetic controls. These findings establish that NKG2D signaling likely occurs in the pancreas during type 1 diabetes, supporting continued study of NKG2D signaling in the pancreas in diabetes pathogenesis.

NKG2D has been implicated in the development of type 1 diabetes. How it affects disease development, and whether these effects are positive or negative has been a point of uncertainty. As discussed in Chapter whatever, I showed a protective effect of NKG2D signaling on $CD8^+$ T cells in NOD diabetes, but the Markiewicz lab also found evidence that NKG2D signaling in the gut may have detrimental effects on disease development (Trembath, Sharma et al. 2017). These

findings suggest that NKG2D signaling plays different roles in autoimmune diabetes progression in different anatomical locations. In this study, I endeavored to more closely assess the role played by NKG2D signaling within the pancreas in autoimmune diabetes.

Because both NKG2D and its ligands are expressed on immune cells (Trembath and Markiewicz 2018), we could not eliminate these proteins specifically within the pancreas. Therefore, to test the effect of NKG2D signaling in the pancreas, we instead increased NKG2D signaling specifically within the pancreatic islets. In the NOD.RIP-RAE1ε model, NKG2D signaling is selectively increased in the islets by transgenic expression of RAE1ε using the rat insulin promoter. In this model, it was the Markiewicz lab previously showed reduced diabetes development by NOD.RIP-RAE1ε mice. This diabetes reduction correlated with a decreased ratio of $CD8^+$ $T_{eff + em}$ cells to $CD8^+$ $T_{cm.}$ Given the central role $CD8^+$ T_{eff} cells play in autoimmune diabetes pathology (Tsai, Shameli et al. 2008, Burrack, Martinov et al. 2017), it is not surprising that reduction in these cells correlated with the previously observed reduction in diabetes development. In contrast, the increase in $CD8^+$ T_{cm} in NOD.RIP-RAE1ε mice was somewhat surprising. This increase is unlikely simply a reflection of the lower percentage of NOD.RIP-RAE1ε mice on their way to developing diabetes. If this was the case, we would have observed a similar increase in $CD8^+$ T_{cm} in male NOD mice, which have a lower diabetes incidence compared with female NOD mice, but we did not. Therefore, our data support the hypothesis that this increase in $CD8^+$ T_{cm} does not just correspond to the reduced diabetes in both female and male NOD.RIP-RAE1ε compared with NOD, but is the cause for the RAE1ε-mediated reduction. This hypothesis is further supported by the results of the NOD.*Scid* adoptive cell transfer studies that demonstrated suppression of diabetes development by $CD8^+$ T_{cm}.

The cell adoptive transfer studies revealed that $CD8^+$ T_{cm} not only blocked diabetes onset but also delayed disease transfer by $CD8^+$ T_{eff+em}. $CD8^+$ T_{eff+em} cells correlate with islet pathology (Chee, Ko et al. 2014), and $CD8^+$ T_{eff} cells play a central role in autoimmune diabetes pathology (Tsai, Shameli et al. 2008, Burrack, Martinov et al. 2017). In contrast, $CD8^+$ T_{cm} have reduced cytotoxicity and effector functions (Farber, Yudanin et al. 2014), correlating with reduced diabetes transfer by these cells. However, the delay in diabetes I observed with co-transfer of $CD8^+$ T_{cm} cells with $CD8^+$ T_{eff+em} demonstrates a regulatory effect of the $CD8^+$ T_{cm} population. Such a regulatory role for NOD $CD8^+$ T_{cm} was suggested by previous studies performed by Santamaria and colleagues using transgenic T cell systems (Khadra, Tsai et al. 2010, Tsai, Shameli et al. 2010, Shameli, Clemente-Casares et al. 2011), however this had not been investigated in parental NOD mice. T_{cm} can also be precursors to T_{eff} cells (Sallusto, Geginat et al. 2004). Therefore, the protective cells are likely a subset of the $CD8^+$ T_{cm} population. $CD8^+$ T_{cm} are a heterogeneous population, which have been shown in other mouse strains to contain a subpopulation of immune regulatory $CD8^+$ T cells (Tregs) (Li, Xie et al. 2014). In humans, one of these T_{cm} $CD8^+$ Treg populations has been identified as $CXCR3^+PD\text{-}1^+$ (Li, Xie et al. 2014), the population and number of which trended strongly upwards with NKG2D stimulation *in vitro*. This observation is consistent with the significant increase in PD-1 expression by mouse T cells with NKG2D stimulation *in vitro*. PD-1 has been shown to be important in suppressing NOD diabetes (Ansari, Salama et al. 2003, Clotman, Janssens et al. 2018), as well as type 1 diabetes in humans (Schroder, Khattar et al. 2015) Future studies will need to be performed to determine the identity of the protective NOD $CD8^+$ T_{cm} population, but our results demonstrate this population is expanded by NKG2D signaling.

My results indicate that the reduced T_{eff+em} to T_{cm} ratio in NOD.RIP-RAE1ε mice is due to enhanced NKG2D signaling directly in $CD8^+$ T cells. I found that NKG2D stimulation during in vitro activation of NOD $CD8^+$ T cells drove a similar decrease in the ratio of $T_{eff} + T_{em}$ to T_{cm} as observed in NOD.RIP-RAE1ε mice. This finding that NKG2D stimulation drives a shift towards $CD8^+$ T_{cm} generation in the NOD mouse is consistent with a role for NKG2D in promoting memory cell development, particularly T_{cm}, demonstrated by others with C57BL/6 mice (Zloza, Kohlhapp et al. 2012, Wensveen, Lenartic et al. 2013, Wensveen, Jelencic et al. 2018, Perez, Prajapati et al. 2019). Importantly, demonstrating the translatability of the findings of these mouse studies to the human, I showed that NKG2D signaling during the activation of human $CD8^+$ T cells also favors T_{cm} generation over T_{em+eff}.

Initial reports demonstrated that RAE-1 family members were expressed by β-islet cells in NOD mice (Ogasawara, Hamerman et al. 2004), that RAE1ε expression by β-islet cells enhanced the recruitment of $CD8^+$ T cells to the islets (Markiewicz, Wise et al. 2012), and that NKG2D inhibition reduced NOD diabetes (Ogasawara, Hamerman et al. 2004). This led to the hypothesis that inappropriate NKG2D ligand expression by β-islet cells contributed to autoimmune diabetes development or progression. However, the Markiewicz lab and others have been unable to detect RAE1 expression in the pancreas of NOD mice, and do not detect NKG2D ligand expression by β-islet cells themselves in our NOD colony (Angstetra, Graham et al. 2012, Carrero, Calderon et al. 2013, Trembath, Sharma et al. 2017). The reason for these differing reports of RAE1 expression in islets of NOD mice from different mouse colonies is unclear, but may be the result of variation due to genetic drift or differences in microbiota composition. Regardless, the lack of

RAE1 expression in the islets of mice in our NOD colony afforded us a model with which we could directly test the hypothesis that NKG2D ligand expression on β-islet cells promotes NOD diabetes. In contrast to the original hypothesis, our data demonstrate that RAE1ε expression by islet cells is protective against NOD diabetes and acts via interaction with NKG2D. These data may seem surprising given that NKG2D ligand expression canonically targets cells for killing (Lanier 2015). However, this delay in diabetes in NOD.RIP-RAE1ε mice is consistent with my studies demonstrating a protective role for NKG2D signaling in NOD mice (Trembath et al. 2017), and a role for NKG2D signaling in memory $CD8^+$ T cell generation (Zloza, Kohlhapp et al. 2012, Wensveen, Lenartic et al. 2013, Perez, Prajapati et al. 2019). These results indicate that the diabetes promoting effect of NKG2D signaling observed in some NOD colonies (Ogasawara, Hamerman et al. 2004, Van Belle, Ling et al. 2013) occurs outside of the pancreas, which other studies in the Markiewicz lab suggest is in response to microbiota antigen (Trembath, Sharma et al. 2017). Further studies will be required to determine the identity of such disease-promoting NKG2D signaling. In addition, the Markiewicz lab reported that the NKG2D ligand H60a is endogenously expressed by infiltrating activated T cells in the NOD pancreas (Trembath, Sharma et al. 2017). Future studies will be required to determine if this endogenous NKG2D ligand expression in the pancreas also induces $CD8^+$ T_{cm} generation and diabetes protection.

Although constitutive exposure to ligand can lead to NKG2D downregulation and dysfunction (Oppenheim, Roberts et al. 2005, Wiemann, Mittrucker et al. 2005), the Markiewicz lab data are consistent with enhanced, not reduced, NKG2D signaling in pancreatic islets results in reduced diabetes in the NOD.RIP-RAE1ε mice. While reduced NKG2D expression on $CD8^+$ T cells of NOD.RIP-RAE1ε mice in the pancreas was observed, NKG2D expression by $CD8^+$ T cells in the

spleen was unaffected. This is indicative of increased local NKG2D signaling, as NKG2D is internalized upon ligand engagement and signaling (Molfetta, Quatrini et al. 2016), and recovers upon removal of ligand stimulation (Oppenheim, Roberts et al. 2005). Additionally, the reduced diabetes in NOD.RIP-RAE1ε mice correlates with the increased rate of diabetes in NKG2D-deficient NOD mice observed with treatment with gut microbiota-depleting antibiotics (Trembath, Sharma et al. 2017). Finally, my in vitro data demonstrate that NKG2D signaling in $CD8^+$ T cells enhances T_{cm} generation, which correlates with the increased $CD8^+$ T_{cm} generation observed in NOD.RIP-RAE1ε mice. NKG2D is not expressed on mouse $CD8^+$ T cells until 3-4 days following initial activation (Markiewicz, Carayannopoulos et al. 2005, Trembath, Sharma et al. 2017). This is a time when these cells start to migrate to the site of antigen (Kinjyo, Qin et al. 2015). Taken together, these data support the conclusion that NKG2D signaling in islet-specific $CD8^+$ T cells present in the islets during activation enhances T_{cm} generation. Interestingly, a previous study demonstrated that expression of a high level of soluble RAE1ε to the NOD pancreas led to reduced diabetes (Joo, Jeong et al. 2012). Given the data demonstrating inhibition of NKG2D signaling by soluble ligand (Zingoni, Molfetta et al. 2018), this was hypothesized to be due to reduced NKG2D signaling within the pancreas on islet-specific T cells. However, no mechanistic studies were performed. Taken in light of our data with cell-bound RAE1ε, it is interesting to speculate that this soluble RAE1 may have instead been carried outside of the pancreas and inhibited NKG2D signaling in response to microbiota antigen.

In conclusion, we found an increased presence of NKG2D and NKG2D mRNA within the pancreatic islets of patients with type 1 diabetes and developed a novel mouse model, NOD.RIP-RAE1ε, to test the effects of NKG2D signaling specifically within the pancreas. We showed that

increased NKG2D signaling within the pancreas of NOD mice had a protective effect, delaying diabetes in NOD.RIP-RAE1ε mice. This correlated with a decrease in the ratio of $CD8^+$ $T_{eff + em}$ cells to T_{cm} cells in NOD.RIP-RAE1ε mice. We demonstrated that NKG2D signaling on $CD8^+$ T cells drives a similar shift in both human and NOD $CD8^+$ T cell populations in vitro. Finally, we found that $CD8^+$ T_{cm} cells transferred significantly less NOD diabetes than $CD8^+$ $T_{eff + em}$ cells, and had a protective effect, delaying diabetes when co-transferred with $CD8^+$ $T_{eff + em}$ cells. We therefore propose a protective role for NKG2D signaling within the pancreas in autoimmune diabetes, by increasing a protective $CD8^+$ T_{cm} population relative to the more pathogenic $CD8^+$ $T_{eff + em}$ population. These findings reiterate the importance of NKG2D in autoimmune diabetes, and further stress the importance of anatomical location when determining the role NKG2D signaling plays in disease development.

Chapter 4: Further Investigations into NKG2D ligand expression by $CD8^+$ T cells

Expression profile of H60a differs between NOD and Balb/c $CD8^+$ T cells upon activation *in vitro*.

I previously showed expression of H60a by NOD $CD8^+$ T, which is increased upon activation *in vitro*. In addition, I showed that engagement of H60a by NKG2D on $CD8^+$ T cells during differentiation alters the populations and cytokine production of mature $CD8^+$ T cells. I therefore hypothesized that NKG2D stimulation on $CD8^+$ T cells during differentiation may be an important mechanism for regulating $CD8^+$ T cell function. The H60a gene is not functionally expressed by C57BL/6 mice (Takada, Yoshida et al. 2008), however expression of the mouse NKG2D ligand Rae-1ε has been found to be expressed by endothelial cells within lymph nodes. This expression may provide similar NKG2D stimulation during $CD8^+$ T cell differentiation in the C57BL/6 strain. Balb/cJ mice do however possess a functional H60a gene. I therefore wanted to see if H60a was similarly expressed by Balb/cJ $CD8^+$ T cells. To do this, I harvested splenocytes from NOD or Balb/cJ mice and activated them *in vitro* with soluble anti-CD3 antibody in the presence of absence of 2ug/mL anti-NKG2D blocking antibody. Cells were then assessed daily for expression of NKG2D ligands by flow cytometry gating on $CD8^+$ T cells. The only NKG2D ligand we readily observed on both NOD and Balb/c $CD8^+$ T cells was H60a. In the absence of anti-NKG2D antibody blockade, both NOD and Balb/c $CD8^+$ T cells showed low but detectable expression of H60a on day 0, which increased upon activation, and peaked at day 2 (Fig. 20). While H60a expression decreased through day 5 on both NOD and Balb/c $CD8^+$ T cells, NOD $CD8^+$ T cells retained markedly higher H60a expression compared Balb/c $CD8^+$ T cells whose expression of H60a decreased to levels near those seen on day 0 (Fig. 20). Antibody

blockade of NKG2D during culture resulted in less down regulation of H60a after day 2 by both NOD and Balb/c $CD8^+$ T cells, with the effects being most pronounced on $CD8^+$ T cells from Balb/c mice (Fig. 20). While we did not further investigate this differential regulation of H60a by NOD vs. Balb/c $CD8^+$ T cells, it remained consistent over three experiments.

In mice, NKG2D is not expressed by naïve $CD8^+$ T cells, and becomes expressed 2-3 days after activation (Markiewicz, Wise et al. 2012, Lanier 2015, Trembath, Sharma et al. 2017). Therefore, the decreased in H60a expression we observed coincides with the time when NKG2D becomes present on the cell surface. A decreased reduction in H60a after with NKG2D blockade further suggests that NKG2D signaling contributes to the regulation of H60a expression by $CD8^+$ T cells. Further, it has been shown that NOD mice have a defect in NKG2D signaling (Ogasawara, Hamerman et al. 2003), which may play a role in the increased expression of H60a by NOD vs. Balb/c $CD8^+$ T cells by day 5 following activation *in vitro*. Whether this observation is a result of defective NKG2D signaling in NOD mice, or may contribute to NOD NKG2D dysfunction has yet to be determined. It has been found that H60a mRNA is negatively regulated by IFNγ via the 3'UTR (Zhang et al. 2010). While we did not assess IFNγ production while conducting these experiments, this could further contribute to changes in H60a expression. The functional implications of this differential H60a expression pattern were not studied, but may lead to further insights into the importance of NKG2D-NKG2D ligand signaling on $CD8^+$ T cell function.

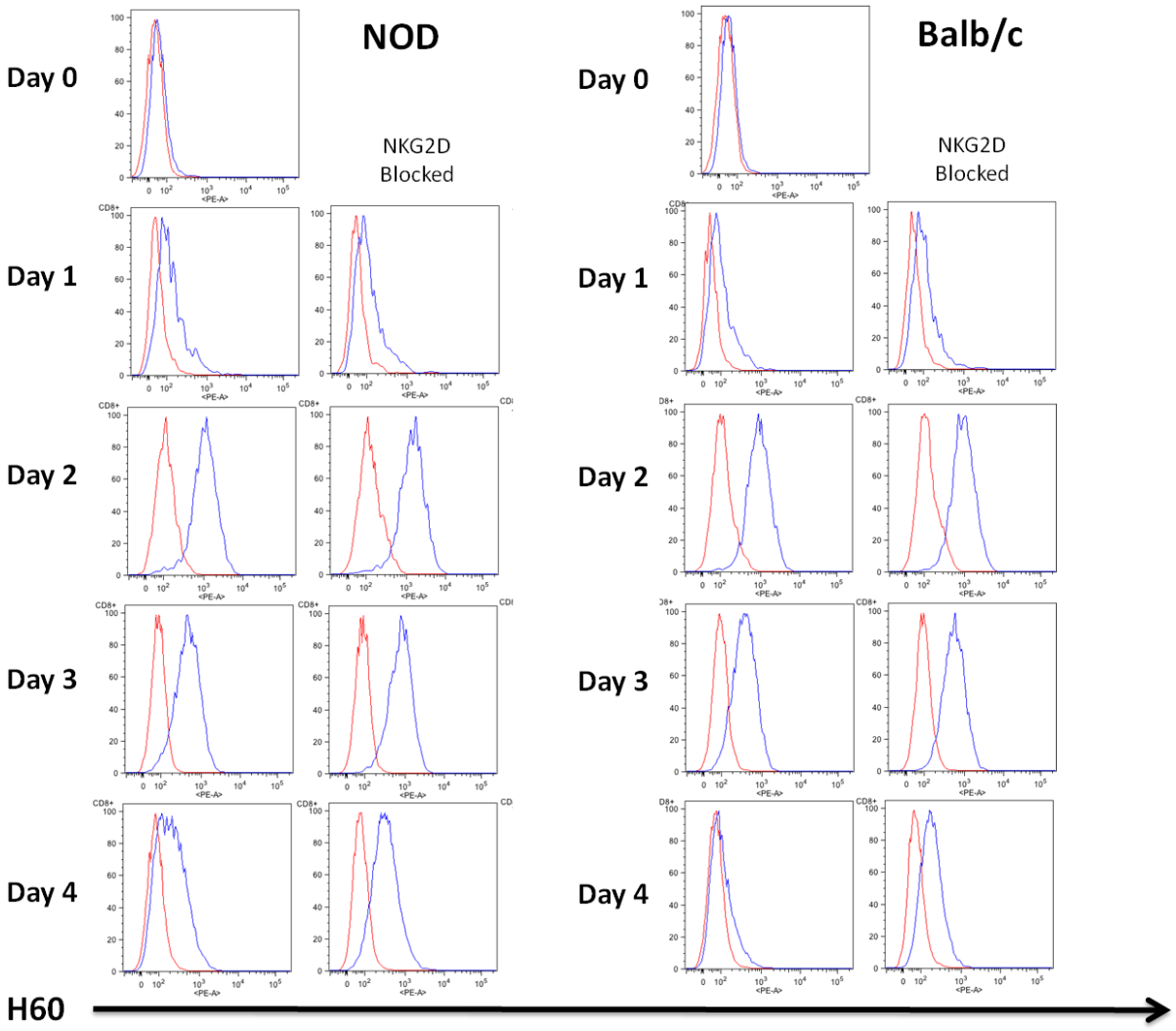

Figure 20: Expression of H60a differs between NOD and Balb/c CD8$^+$ T cells upon activation *in vitro*. CD8$^+$ T cells from NOD and Balb/c mice purified by negative selection and activated *in vitro* using plate bound anti-CD3 and anti-CD28 in the presence of soluble anti-NKG2D blocking antibody or control. Cells were assessed daily, through day 4, for H60a expression via flow cytometry.

The NKG2D ligand H60a is expressed by NOD thymocytes

My study of H60a expression on NOD $CD8^+$ T cells was primarily focused on cells harvested from spleens and lymph nodes. In doing so I captured $CD8^+$ T cell populations ranging from naïve to memory. It is worth noting that I also observed expression of H60a by NOD thymocytes. I dissociated thymi from 6-8 week old NOD mice and analyzed immune cell populations by flow cytometry. I found expression of H60a NOD thymocytes. H60a was primarily expressed by $CD3^+$ T cells, though there was some expression of H60a by the $CD3^-$ population (Fig. 21). NKG2D ligands have been previously described in the thymus of Balb/c mice (Diefenbach, Jamieson et al. 2000, Li, Rabinovich et al. 2005), however this expression was observed with an NKG2D tetramer, and these studies did not identify a particular NKG2D ligand. mRNA for the mouse NKG2D ligand MULT1 was found to be expressed by thymocytes (Nice, Coscoy et al. 2009), but the protein was not found to be expressed on the cell surface, and the mouse strain assessed was not reported.

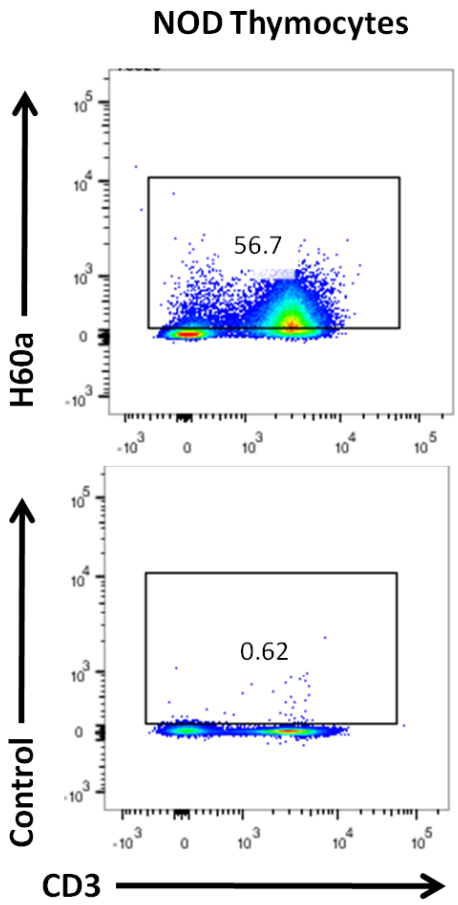

Figure 21: NOD Thymocytes express the NKG2D ligand H60a. Representative flow plots showing thymocytes from 6-8 week old NOD mice stained for CD3 and H60a or control. Representative of staining from two experiments and 10 different mice.

NOD diabetes incidence is not significantly different between NOD 8.3 NKG2D knockout vs. NOD 8.3 NKG2D wild type animals.

Data from the Markiewicz lab suggests that NKG2D has pro-diabetic and anti-diabetic effects on NOD diabetes, acting through at least two separate mechanisms. Previous data from our lab points to interactions between NKG2D bearing cells and the microbiota as playing a role in diabetes development. My work has described a role for NKG2D signaling specifically in $CD8^+$ T cells, reducing the CTL immune response. Because $CD8^+$ T cells are important mediators of pancreatic β-cell destruction, I sought to further determine the effects of NKG2D on $CD8^+$ T cells on NOD diabetes. To do this, I used NOD 8.3 mice, whose $CD8^+$ T cells have transgenic T cell receptors specific for a known auto antigen, and develop NOD diabetes at an accelerated rate (Verdaguer, Schmidt et al. 1997). Because auto reactive $CD8^+$ T cells are the main drivers of accelerated diabetes in this model, I hypothesized that this model would highlight the effects of NKG2D specifically in $CD8^+$ T cells in NOD diabetes. I therefore used NOD 8.3 NKG2D knockout animals which I generated by crossing NOD 8.3 mice with our NOD NKG2D knockout animals. Blood glucose was then monitored weekly. Diabetes incidence was not significantly different between NKG2D WT (n=23) and KO (n=19) males, or NKG2D WT (n=15) and KO (n=17) females (Fig. 22). These findings differ from our earlier observations in non-TCR transgenic NOD mice in which NKG2D influenced NOD diabetes development via multiple mechanisms (Trembath et al. 2017). It is possible that effects of NKG2D are not observed in this model due to the accelerated diabetes, and specific monoclonal $CD8^+$ T cell TCR signaling in 8.3 NOD mice.

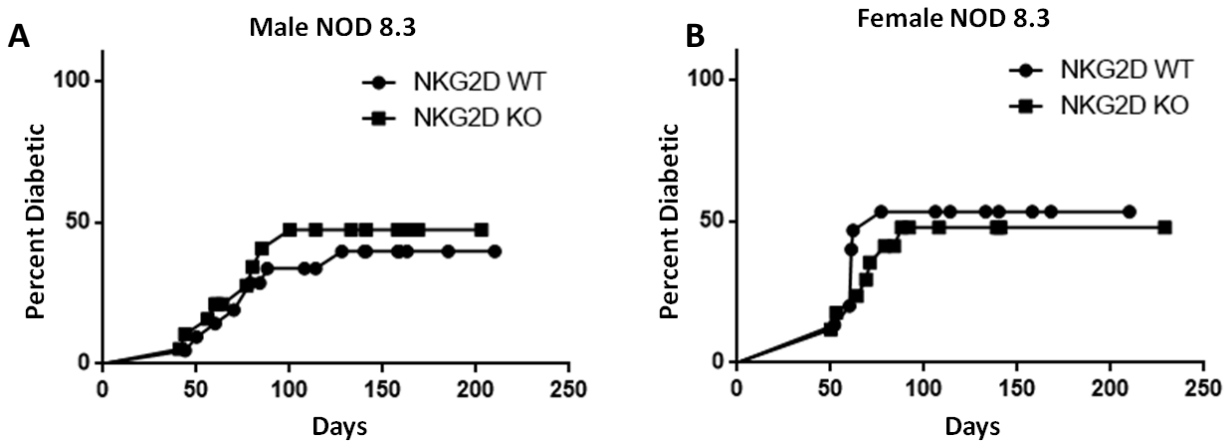

Figure 22: NOD diabetes is not significantly different between NKG2D deficient and wild type NOD 8.3 mice. Diabetes incidence in (A) male NOD 8.3 NKG2D KO (n=19) and WT (n=23) and (B) female NOD 8.3 NKG2D KO (n=17) and WT (n=15) animals.

Generation of NOD mice genetically deficient in H60a – establishing the NOD.B6 H60a mouse line

H60a was the only NKG2D ligand I observed expressed by NOD $CD8^+$ T cells. However, NOD mice do posses functional genes for other NKG2D ligands. In order to determine if the observed effects of NKG2D knockout on $CD8^+$ T cells and NOD diabetes were a result of NKG2D interactions with H60a, we sought to generate NOD mice deficient in H60a. Because of the potential for introducing unintended effects during the genetic manipulation of any mouse strain, the lab chose to generate two separate lines of H60a deficient NOD mice. One strain was generated using CRISPR gene editing technology, and the other through transfer of the C57BL/6

H60a pseudo gene (Zhang, Hardamon et al. 2011) onto the NOD background. Here I will discuss generation the latter, NOD.B6 H60a mouse line.

Expression of H60a has been observed on cells from various mouse strains, including Balb/b, Balb/c, and 129/Sv (Malarkannan et al. 1998; Samarakoon et al. 2009; Zhang et al. 2010). However, the widely used C57BL/6 mouse possesses a truncated H60a pseudo gene, which does not produce a functional protein (Malarkannan et al. 1998; Zhang et al. 2010). We took advantage of this naturally non-functional C57BL/6 H60a pseudo gene, and Transferred the C57BL/6 H60a pseudo gene to the NOD genetic background. The resultant mice are congenic mice that possess the C57BL/6 H60a pseudo gene which are at least 98.23% NOD based on SNP analysis (Fig. 23).

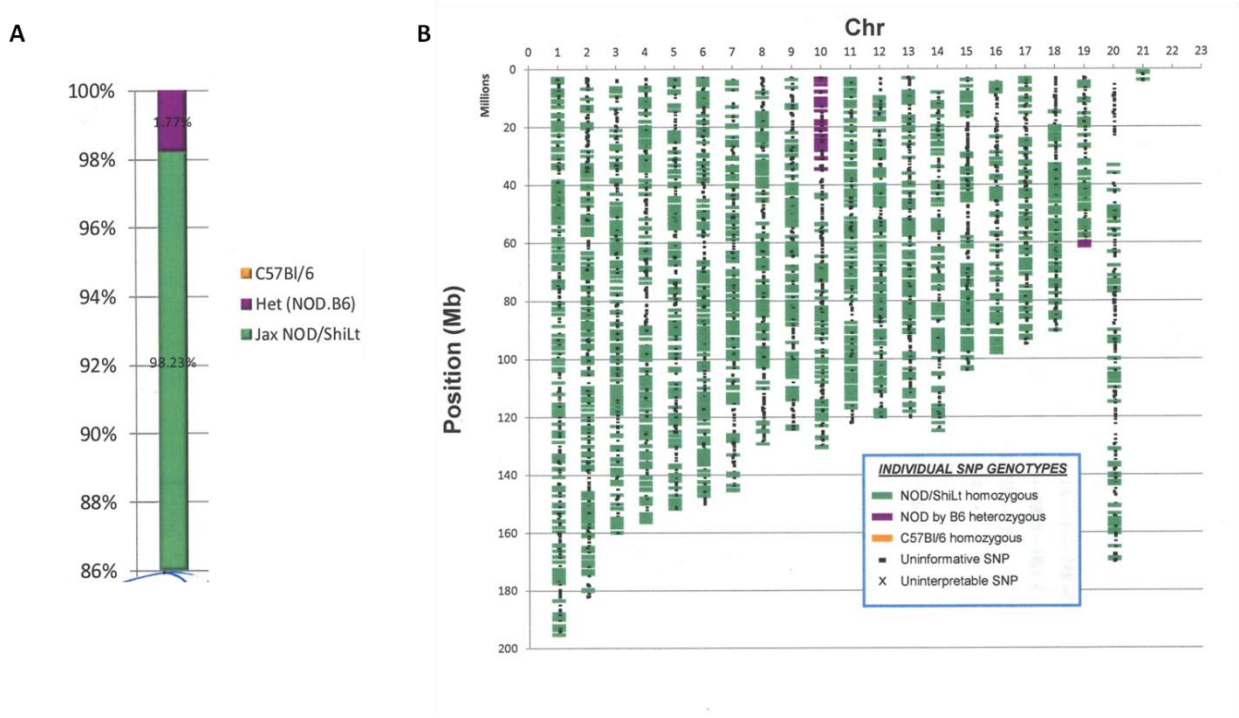

Figure 23: Establishing the C57B/6 H60a gene on the NOD background. (A) Summary of single nucleotide polymorphism (SNP) analysis of the mouse used to generate the NOD.B6 H60a mouse line. Green indicates percentage of SNPs homozygous for the NOD mouse background, with purple showing the percentage of SNPs heterozygous for both the NOD and C57BL/6 background. There were no SNPs which were homozygous for the B6 background. (B) Chormosome map of the same mouse showing distribution of SNPs with the same color schematic. SNP analysis was performed by DartMouse.

Preliminary characterization of NOD.B6 H60a CD8$^+$ T cells

Generation of the NOD.B6 H60a mouse allowed us to test whether loss of H60a affected CD8$^+$ T cell function in a similar manner to genetic knockout of NKG2D. To do this, we again isolated CD8$^+$ T cells from NOD.B6 H60a or control animals, and activated them *in vitro* with plate bound anti-CD3 and anti-CD28. After 5 days, cells were re-stimulated with varying

concentrations of plate bound anti-CD3. After 20 hours, supernatant cytokines were assessed using a BD cytometric bead array. We found that with the intermediate concentration of anti-CD3 for re-stimulation, $CD8^+$ T cells from NOD.B6 $H60a^{-/-}$ animals produced more TNFα and IFNγ compared to control. This trend was observed in 4 separate experiments, but in combined fold change data did not reach statistical significance (Fig. 24), which would likely be achieved with further repetition. Despite this, these data are consistent with our earlier results showing increased cytokine production by $CD8^+$ T cells deficient in NKG2D (Fig. 6), or with anti-H60a antibody blockade (Fig. 10)

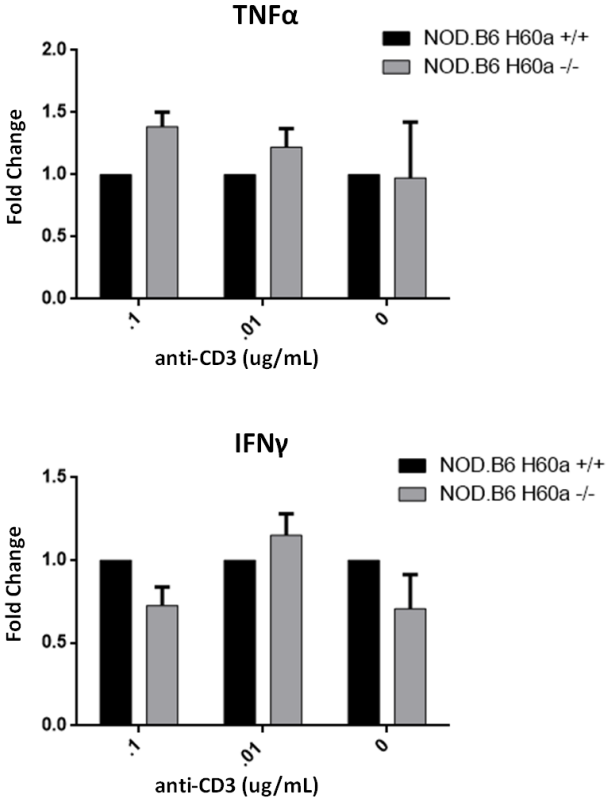

Figure 24: NOD.B6 $H60a^{-/-}$ $CD8^+$ T cells produce increased TNFα and IFNγ upon re-stimulation *in vitro*. Production of TNFα and IFNγ by NOD.B6 $H60a^{-/-}$ and NOD.B6 $H60a^{+/+}$ CTL after re-stimulation on varying concentrations of plate bound anti-CD3. Supernatant

cytokines were measured by BD cytometric bead array, and are presented as fold change vs. control (NOD.B6 H60a$^{+/+}$). These are combined data from at least 3 independent experiments.

Transcription factors AP2, KLF4, Prox1, and Foxo1 are candidates that may bind and regulate Rae-1 expression.

In their most studied role, NKG2D ligands are described as "stress ligands" and become expressed by cells undergoing cellular stress such as viral infection or cancerous transformation. Despite this, there is lack of consensus as to which signals and transcription factors lead to NKG2D ligand expression. Factors including DNA damage, LPS treatment, heat shock, IFNγ, and cellular proliferation, reviewed in (Champsaur and Lanier 2010, Raulet, Gasser et al. 2013), have been reported. Given the CD8^{+} T cell modulating role for NKG2D ligand expression by immune cells that I uncovered, I sought to determine what signals regulate NKG2D ligand expression. To do this, I used a commercial transcription factor binding assay, and quarried which transcription factors bound to the promoter region of the mouse Raet1 gene. Using the Raet1 promoter region I PCR-amplified from a Rae-1-expressing mouse embryonic fibroblast cell line, identified the transcription factors AP2, KLF4, Prox1, and Foxo1 as positive hits for binding the Rae-1 promoter (Table 2). Sequence analysis of the Rae-1 promoter region revealed exact matches to sequences known to be bound by AP2, KLF4, and Prox1 (Table 2). Despite positive hits for Foxo1 binding in both trials, the Rae-1ε promoter region did not contain a known Foxo1 binding sequence (Table 2). Further verification and investigation will be needed to determine if these transcription factors play a role in the expression of Rae-1ε and other NKG2D ligands.

Table 2: Candidate transcription factors positive for binding the Rae-1ε promotor

Transcription Factor	Known Sequence Specificity	Appearance in Rae-1ε Promoter
AP2 (TFAP2)	$GCCN_{3-4}GGC$	Yes – Exact match
KLF4	CACCC	Yes – Exact reverse complement match, and multiple close
Prox1	C[A/T][C/T]NNC[T/C] and TAAGACG	Yes – Multiple Exact
Foxo1	TT[G/A]TTT[T/A][G/C]	No

NKG2D stimulation of mouse $CD8^+$ CTL with Rae-1 alters mRNA expression profile.

NKG2D is best known studied for its role as an activating receptor on NK cells, where engagement of NKG2D ligand is sufficient to activate lytic granule release and target cell killing (Lanier 2015). The role of NKG2D on $CD8^+$ T cells has been primarily described as co-stimulatory, enhancing the response to TCR signaling (Wensveen, Jelencic et al. 2018). I therefore asked what effects NKG2D stimulation has in mouse $CD8^+$ T cells in the absence of TCR stimulation. To do this, I conducted microarray analysis of the mRNA from OT-1 CTL stimulated with beads coated with ICAM-1 and Rae-1ε, or ICAM-1 and Fc control. The purpose of ICAM-1 to these beads was to promote cell-bead contact. A Rae-1ε-Fc fusion protein was used to so that bead bound anti-Fc antibody could be used to attach the Rae-1ε-Fc to the bead surface. The same isotype antibody Fc, lacking Rae-1ε fusion, was therefore used as a control. OT-1 CTLs were then incubated with ICAM-1+ Rae-1ε or ICAM-1+Fc control beads at a 1:1 ratio for 5 hours. RNA was then extracted, and analyzed by the KUMC genomics core using a GeneChip 430 2.0. Microarray analysis identified 9 genes whose expression was down-regulated more than 2-fold, and 199 genes with expression down regulated greater than 1.5-fold in CTL stimulated with ICAM-1 + Rae-1ε vs those stimulated with ICAM-1 + Fc control. 163 genes had

expression up-regulated greater than 1.5-fold, with none greater than 2.0. The top up-regulated (Table 3) and top down regulated (Table 4) genes are reported here.

Based on their described function, I chose 11 genes of interest from the microarray, and confirmed expression changes with RT-qPCR of mRNA from ICAM-1 + Rae-1ε activated OT-1 CTL vs. those activated with ICAM-1 + Fc control. These genes were Notch3, Itpka, Scin, Ftsl1, Ptprj, Peli1, Etxbp4, Nedd4, INPP4B, TLR4, and KLF4. Of these, I found only Nedd4 and Fstl1 to be up-regulated. I did not further pursue this project, but given our subsequent findings of the importance of NKG2D signaling during $CD8^+$ T cell differentiation, analysis of $CD8^+$ T cell gene expression with or without NKG2D stimulation during CTL differentiation would be worthwhile.

Experimental Methods

Mice

NOD mice were purchased from Jackson Laboratory. *Klrk1*$^{-/-}$ mice on the C57BL/6 background have been previously described (Zafirova, Mandaric et al. 2009). The *Klrk1*$^{-/-}$ allele was moved from the C57BL/6 to the NOD genetic background using the speed congenics service of the Washington University School of Medicine to generate *Klrk1*$^{-/-}$ NOD mice. By SNP analysis (performed by DartMouse), these mice are 98% NOD, with the only observed genomic region containing a continuous interval of non-NOD-like SNPs found at the site of the expected site of the *Klrk1* knockout allele. Experiments were performed with *Klrk1*$^{+/+}$ (wild type) and *Klrk1*$^{-/-}$ NOD littermates from *Klrk1*$^{+/-}$ NOD interbreeding. Mice were housed under specific pathogen

free (SPF) conditions in the Washington University School of Medicine or University of Kansas Medical Center animal facilities in accordance with institutional guidelines.

Antibodies

Unconjugated anti-CD28 (Clone 37.51), anti-CD3ε (Clone 2C11), anti-mCD8-APC, anti-mCD4-BV786, anti-CD3ε-PE-Cy7, anti-mCD107 (LAMP1)-PE, anti-DX5-FITC and anti-mCD45 were purchased from BD Biosciences. Anti-H60-PE, anti-mNKG2D-PE, rat IgG2a-PE and rat IgG2b-PE were purchased from R&D Systems.

In vitro CTL generation

All cells were grown in IMDM (Cellgro) supplemented with 10% defined FBS (HyClone), penicillin-streptomycin-glutamine (Gibco) and β-mercaptoethanol (Sigma-Aldrich). For activation of total splenocytes, spleens were harvested from 6-8 week-old SPF housed NOD mice, passed through a 40μm cell strainer and plated in a 6-well dish at 1.5 X 10^7 cells/well in 3 mL media and 1μg/mL of plate-bound anti-CD3ε (2C11). For purified $CD8^+$ T cells, cells were harvested from spleens and lymph nodes of 6-8 week-old NOD mice, and enriched by negative selection using magnetic beads (BD Biosciences) according to the manufacturer's protocol. $CD8^+$ T cells were then plated at 3-5 X 10^6 cells/well in a 6-well dish with 1μg/mL plate-bound anti-C3ε (2C11) and soluble anti-CD28 (37.51). The cells were allowed to expand for 5 days and split as needed.

In vitro cytokine analysis

CTL were harvested and plated at 1 X 10^5 cells/well in 100 μL of fresh media in 96-well plates pre-coated with anti-CD3 (2C11) at the stated concentrations. Cells were incubated at 37° C for 20 hours. Supernatant was then collected and cytokines were analyzed using the Mouse Inflammation Cytometric Bead Array kit (BD Biosciences) and an LSR II (BD Biosciences). Data were quantified using FCAP array software (BD Biosciences).

In vivo cytokine analysis

Total $CD8^+$ T cells were purified from spleens and lymph nodes of 6-8 week old *Klrk1*$^{-/-}$ or wild type NOD mice by negative selection using magnetic beads (BD Biosciences) according to the manufacturer's protocol. Isolated *Klrk1*$^{-/-}$ and wild type $CD8^+$ T cells were then labeled with eFluor 670 (eBioscience) and CFSE (Thermo Scientific), respectively. The labeled cells were then mixed 1:1 and adoptively transferred via retro orbital injection into 6-8 week old wild type NOD recipient mice (Figure S2). Total splenocytes were harvested after 7 days, incubated for 4-6 hours in complete media with GolgiPlug protein transport inhibitor (BD Biosciences), followed by fixation and permeablization using BD Cytofix/Cytoperm (BD Biosciences) according to manufacture directions. Cells were then stained overnight at 4° C with anti-mouse TNFα (BD Pharmingen), anti-mouse IFNγ (BD Pharmingen), and anti-mouse CD8 (BioLegend), and analyzed by flow cytometry. Transferred $CD8^+$ T cells were analyzed by gating on live lymphocytes based on forward and side scatter, $CD8^+$ cells, then either eFluor 670^+ or $CFSE^+$ cells.

Lamp-1 granule release analysis

CTL were plated at 1 X 10^5 in 100 μL fresh media in 96-well plates pre-coated with anti-CD3ε (2C11) at the stated concentrations. Before plating, PE- conjugated anti-CD107a was added to the cells at 4uL/mL. Cells were incubated at 37° C for 3 hours before being washed, fixed in 2% paraformaldehyde, and assessed for LAMP1 staining by flow cytometry.

Antibiotic treatment of mice

A solution of 0.5 g/L Vancomycin (Sigma-Aldrich), 1 g/L Neomycin (Sigma-Aldrich), 1 g/L Ampicillin (Sigma-Aldrich), and 1 g/L Metronidazole (Sigma-Aldrich) was made in grape KoolAid. This solution was mixed with Dietgel Boost (ClearH_2O) and crushed food pellets. *Klrk1*$^{-/-}$ and wild type NOD littermates were exclusively fed this antibiotic mixture beginning at weaning until euthanasia. Sequencing of the 16S rRNA genes present in feces confirmed that the Firmicutes and Bacteroidetes populations, the two predominant bacteria phyla present in the intestine, were reduced 94-99% within 4 weeks of antibiotic treatment.

Diabetes determination

In all experiments age of diabetes development was defined as the age at which the first of two consecutive blood glucose measurements $\geq$250 mg/dl was obtained.

Statistical analysis

Data were analyzed using a two-tailed unpaired Mann-Whitney test, one-tailed Wilcoxon test, or two-way ANOVA as described in figure legends. All statistical analyses were performed using GraphPad Prism.

Creation of ICAM-1 + Rae1ε coated beads

MACS anti-biotin beads (Miltenyi Biotec) were loaded with biotinylated anti-Fc antibody for at least 2 hrs at 4°C. After washing, beads were then incubated with a 1:1 mixture of ICAM-1-Fc and Rae-1ε-Fc, or a 1:1 mixture of ICAM-1-Fc and IgG1-Fc as an isotype control. Beads were then incubated overnight at 4°C, and washed.

Microarray

OT-1 CTL were plated in a 6-well dish at $6x10^6$ cells/well. ICAM-1-Fc + Rae-1ε-Fc or ICAM-1-Fc + IgG1-Fc coated beads were added at a ratio of 1:1 ($6x10^6$ beads/well) and allowed to incubate at 37°C for 5 hours. Following incubation, RNA was extracted using Trizol reagent according to protocol. Purified RNA was diluted to 250ug/uL and submitted to the University of Kansas Medical Center Genomics Core for microarray analysis using a GeneChip type: 430 2.0.

Flow cytometry

Spleens and thymi were dissociated by pressing through a 40um cell strainer in isolation buffer containing PBS, 2% fetal calf serum, and 2mM EDTA. Pancreata were chopped into small sections, digested with 1mg/ml collagenase IV (Life Technologies) in IMDM for 10 minutes at 37 degrees with shaking, and a single cell suspension generated by pressing through a 40um cell strainer. The cells were then washed at least 5 times, followed by antibody staining at 4°C for at least 15 minutes. After staining, cells were washed and analyzed. Cultured cells were washed once in isolation buffer and stained before similar staining and analysis.

NOD diabetes transfer

Male and female mice were used, with all transfers containing male cells going only into male NOD.*Scid* recipients. Spleens and lymph nodes from non-diabetic 6-12 week old NOD mice were harvested and dissociated by passing through a 40um cell strainer into isolation buffer containing PBS, 2% fetal calf serum, and 2mM EDTA. $CD8^+$ T cells were then enriched by negative selection using magnetic bead separation (BD Biosciences Cat. No. 558471) according to the manufacturer's instructions. $CD8^+$ T cells were then stained with anti-mCD3-PE-Cy7, anti-mCD8-APC, anti-mCD44-BV650, and anti-mCD62L-BV510 antibodies (BD Biosciences). $CD3^+CD8^+CD44^+CD62L^-$ (T_{eff+em}) and $CD3^+CD8^+CD44^+CD62L^+$ (T_{cm}) populations were separated by fluorescence activated cell sorting by the University of Kansas Medical Center Flow Core using an AriaIIIu (BD Biosciences). Separately, splenocytes from non-diabetic 6-12 week old mice were depleted of $CD8^+$ T cells by positive selection using anti-mCD8-PE (BD Biosciences) and anti-PE magnetic bead separation (BD Biosciences Cat. No. 557899). $2.5X10^6$ T_{eff+em}, $2.5X10^6$ T_{cm}, or a mixture of $2.5X10^6$ T_{eff+em} and $2.5X10^6$ T_{cm} were each combined with $1.5X10^7$ $CD8^+$ T cell-depleted NOD splenocytes and adoptively transferred into 2-6 month old NOD.SCID mice by retro-orbital injection.

In vitro cell culture

All cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 0.1U/mL penicillin, 0.1U/mL streptomycin, 0.29ug/mL L-glutamine, 5.5uM 2-mercaptoethanol, and 10% fetal calf serum. Cells were grown at 37° C at 5% CO_2.

In vitro activation of mouse $CD8^+$ T cells with irradiated splenocytes

$CD8^+$ T cells were isolated from the spleens and lymph nodes of 6-8 week-old NOD mice by negative selection using magnetic bead separation (BD Biosciences Cat. No. 558471) according to the manufacturer's instructions. Separately, splenocytes from ubiquitous RAE1ε mice or control C57BL/6 mice were dissociated by passing through a 40um cell strainer and irradiated with 15 Gy using a Cesium-137 irradiator. 10^6 $CD8^+$ T cells were then plated at a 1:4 ratio with either irradiated C57BL/6 RAE-1ε or C57BL/6 control splenocytes ($3X10^6$) along with 1 □g/mL anti-mCD3 (clone 2C11) in a 24 well plate.

In vitro activation of B6 OT-1 $CD8^+$ T cells

Spleens were harvested from 6-12 week old SPF housed B6 OT-1 mice, passed through a 40μm cell strainer and plated in a 6-well dish at 1.5 X 10^7 cells/well in 3 mL media. 1uL of 2uM OVA peptide was then added to each well. Cells were allowed to grow and split as needed for 5 days. On day 5, cells were harvested and, and live cells were separated using Ficoll Paque gradient according to protocol.

In vitro activation of human $CD8^+$ T cells

Naïve or total $CD8^+$ T cells were isolated from human peripheral blood mononuclear cells (PBMCs) by negative selection using the Human Naïve CD8 T Cell enrichment Set (BD Biosciences) and Human CD8 T Lymphocyte Enrichment Set (BD Biosciences), respectively, according to manufacturer's instructions. 24 well plates were incubated with 3μg/mL anti-hCD3 (clone: OKT3) and anti-hCD28 (clone: 9.3), along with 20μg/mL anti-hNKG2D (clone: 149810), or isotype control in PBS overnight at 4 degrees. The plates were washed with PBS and then the CD8+ T cells were added to the plate (10^6 cells/well). After 3 days, cells were split 1:2 and

maintained in the presence of 20□g/mL plate bound anti-hNKG2D. After 5 days of culture, the cells were harvested for analysis.

Future Directions, and Conclusions

Going forward there are still many avenues of investigation stemming from the findings presented earlier. I have outlined some thoughts and remaining questions below.

Regulatory $CD8^+$ T cells

Our data suggest that NKG2D signaling reduces the T_{eff+em} : T_{cm} ratio, but also causes expansion of a regulatory subset of $CD8^+$ T cells within the T_{cm} compartment. Several different populations of regulatory $CD8^+$ T cells are known to express a T_{cm} phenotype (Yu, Ma et al. 2018). Here, we began to investigate one of these populations, $CD122^+PD\text{-}1^+$ T_{cm} $CD8^+$ T cells in mice, and CXCR3 expressing T_{cm} $CD8^+$ T cells in humans, with preliminary results showing an increase in these populations with *in vitro* NKG2D stimulation. However, more work needs to be done to determine if NKG2D signaling in $CD8^+$ T cells increases this regulatory population *in vivo*. In addition, more in depth characterization of the T_{cm} population will need to be done to identify other known $CD8^+$ Tregs, and assess the effects of NKG2D signaling on their generation and survival. These include $CD8^+CD25^+FOXP3^+$ cells (Pellegrino, Crino et al. 2019), $CD8^+CD25^-FOXP3^+$ cells (Yu, Ma et al. 2018), and Qa-1 restricted $CD8\alpha\alpha^+TCR\alpha\beta^+$ T cells (Tang, Maricic et al. 2006). This same work would also assess whether NKG2D signaling is selectively driving expansion of a regulatory $CD8^+$ T cell population within the memory compartment, or if regulatory $CD8^+$ T cells are being expanded as a result of the increased $CD8^+$ T_{cm} population as a whole.

NKG2D signaling and T cell metabolism

Metabolic changes are known to drive differentiation into effector vs memory T cells, with a more glycolytic program favoring T_{eff} and T_{em} generation, and increased oxidative phosphorylation (OXPHOS) and fatty acid metabolism favoring T_{cm} cells (Buck, Sowell et al. 2017). In our own *in vitro* experiments, we have repeatedly observed increase media acidification in NKG2D deficient $CD8^+$ T cells compared to wild type counterparts, and decreased relative media acidification in with NKG2D stimulation using plate bound anti-NKG2D antibody. While these observations are somewhat anecdotal, and based on color change of media pH indicator, they were consistent with mouse and human $CD8^+$ T cells and point to a change in metabolic profile with NKG2D signaling. Given our findings that NKG2D signaling in $CD8^+$ T cells decreased the $T_{eff + em}$: T_{cm} ratio, and the known metabolic differences between these two populations (Buck, Sowell et al. 2017), it is logical that we might see decreased acidification with NKG2D signaling. Conversely, NKG2D signaling may contribute to metabolic changes that in turn drive $CD8^+$ T_{cm} generation. Indeed, NKG2D has been shown to activate mTOR signaling at levels that may favor differentiation of memory $CD8^+$ T cells (McQueen, Trace et al. 2016), and mTOR is known to be a key regulator of cellular metabolism (Saxton and Sabatini 2017) and mitochondrial dynamics (Morita, Prudent et al. 2017). In T cells, mTOR is further known to direct differentiation and cell fate through metabolic regulation (Salmond 2018). A study by Buck et al. demonstrates how a metabolic shift towards increased OXPHOS resulting from mitochondrial fusion is capable of driving memory T cell formation, even in the presence of otherwise activating signals favoring effector T cell development (Buck, O'Sullivan et al. 2016). This finding suggests that altered metabolism favoring increased OXPHOS may not

result from memory T cell differentiation, but may instead drive it. Future work may therefore focus on elucidating how NKG2D signaling contributes to a shift in $CD8^+$ T cell metabolism favoring T_{cm} cell generation.

TCR signaling and memory generation

Work out of the lab of Dr. Pere Santamaria has shown that $CD8^+$ T cells with low avidity TCR against autoantigen differentiate into a regulatory $CD8^+$ T cell population which is protective in NOD mice (Tsai, Shameli et al. 2010, Shameli, Clemente-Casares et al. 2011). Here NKG2D could potentially play a role in increasing generation of memory phenotype regulatory $CD8^+$ T cells either by enhancing survival of these low avidity cells, or by providing differentiating signals in the absence of signaling by a higher avidity TCR.

Interesting Parallels with P2X7

P2X7, encoded by P2Rx7, is a ligand gated ion channel which recognizes extracellular ATP. It is expressed on many immune cells, and allows passage of cations in response to elevated levels of extracellular ATP. In response to prolonged exposure to high concentrations of extracellular ATP P2X7 can trigger apoptosis by the formation of large non-specific membrane pores (Savio, de Andrade Mello et al. 2018). The P2RX7 gene has been proposed as a T1D susceptibility gene in NOD mice. Interestingly, a polymorphism in the P2X7 receptor has been described in C57BL/6 and DBA/2 mice, which greatly decreases the sensitivity of the receptor to ATP (Adriouch, Dox et al. 2002). This polymorphism was not observed in Balb/c (Adriouch, Dox et al. 2002, Syberg, Schwarz et al. 2012), 129, or NOD mice (Syberg, Schwarz et al. 2012). P2X7 signaling has been well established in driving an inflammatory response by stimulating the

production of pro-inflammatory cytokines by immune cells, in particular acting as a major driver or NLRP3 inflammasome activation and IL-1β release (Di Virgilio, Dal Ben et al. 2017). However, P2X7 has also been shown to have anti-inflammatory effects, with P2X7 activation being found to control inflammatory Th1 and Th17 cells and suppress T cell induced colitis in mice (Hashimoto-Hill, Friesen et al. 2017). In experimental autoimmune encephalomyelitis, genetic knockout of P2X7 was also found to worsen disease (Chen and Brosnan 2006). P2X7 has been shown to be protective in a mouse model of rheumatoid arthritis as well by inducing apoptosis in Payers Patch T follicular helper cells (Felix, Teng et al. 2019). While suggested to be a T1D susceptibility locus, the role of P2X7 in autoimmune diabetes remains unclear. A study by Chen et al. found that P2X7 deficiency alone did not affect NOD diabetes incidence, but lead to decreased in disease incidence in P2X7 and CD38 double knockout animals (Chen, Scheuplein et al. 2011). In this study, the authors point out the pleiotropic effects of P2X7 on inflammation, and suggest that P2X7 may be acting with multiple pro-diabetic and anti-diabetic effects on different cells making the individual effects of P2X7 difficult to distinguish (Chen, Scheuplein et al. 2011).

It is likely that NKG2D signaling does not play a role in the regulation of immune cells through P2X7 induced apoptosis. However, P2X7 has recently been found to play an important role in the generation and maintenance of $CD8^+$ T_{cm} cells (Borges da Silva, Beura et al. 2018). In this report the authors show that P2X7 signaling in response to extracellular ATP both increases intracellular calcium and likely participates in a feed forward loop enhancing release of ATP by Pannexin 1. Further, they show that the resulting increase in intracellular calcium and decrease in intracellular ATP levels activate AMPK promoting enhanced mitochondrial function and biogenesis (Borges da Silva, Beura et al. 2018). The authors suggest that this increased

mitochondrial mass and corresponding increase in OXPHOS capacity leads to the increased survival of $CD8^+$ T_{cm} cell compartment (Borges da Silva, Beura et al. 2018). Indeed, increased mitochondrial biogenesis, and increased capacity for OXPHOS are known to be important for the formation and survival of memory T cells (Buck, Sowell et al. 2017). In their 2018 study, Borges Da Silva et al. did not extensively investigate the source of extracellular ATP, but did show that Pannexin 1 was likely the key mediator of ATP release, and posit that P2X7 activation of Pannexin 1 could be responsible for sustained ATP release and continued signaling (Borges da Silva, Beura et al. 2018). Interestingly, P2X7 is not the only activator of Pannexin 1, and it has been shown that Pannexin 1 can be directly activated by caspase 3 mediated cleavage of a C terminal inhibitory domain (Sandilos, Chiu et al. 2012). In T cells, caspases 3 and 7 have been shown to be activated, without initiation of apoptotic pathways, by chronic NKG2D signaling (Hanaoka, Jabri et al. 2010). Therefore, it is feasible to think that sustained NKG2D signaling in $CD8^+$ T cells by NKG2D ligand expressed by immune cells enhances or initiates Pannexin 1-ATP-P2X7 driven signaling leading to increased $CD8^+$ T_{cm} cell populations.

Conclusions

The beginning of this dissertation laid out the complex challenges faced by the immune system in identifying and defeating potential immunological threats while simultaneously regulating a response to minimize damage to healthy tissues. In autoimmune diabetes, this regulation has failed. However, the very complexity that allows for diverse and effective immunity also complicates our ability to understand which combinations of factors have gone awry leading to autoimmunity. Strong evidence supports the involvement of the immune receptor NKG2D in the development of autoimmune diabetes in both mice and humans. Indeed, it seems logical that a

receptor which canonically functions to identify and direct killing of ligand expressing targets cells may inadvertently be acting against healthy insulin producing β-islet cells in the pancreas. An initial investigation seemed to support this role for NKG2D in NOD diabetes development. However subsequent conflicting reports cast doubt on the role of NKG2D-mediated killing of pancreatic cells as a mechanism of autoimmunity, and suggest that NKG2D signaling plays a more complex role shaping an immune response.

The findings presented in this dissertation support the idea that NKG2D signaling in $CD8^+$ T cells plays a protective role in the development of autoimmune diabetes. We first demonstrate that NKG2D ligands are expressed by healthy immune cells, and that NKG2D-NKG2D ligand interactions between $CD8^+$ T cells during T cell differentiation reduced their later cytokine response. This effect correlated with a reduction in diabetes both between NKG2D sufficient vs. NKG2D deficient mice which were treated with microbiota depleting antibiotics, as well as NOD RIP-Rae1ε mice, which have increased NKG2D signaling within the pancreas, compared to control. It should however be noted that a major challenge yet remaining is to demonstrate that NKG2D signaling on $CD8^+$ T cells is responsible for the observed protective effect of NKG2D in antibiotic treated animals. Finding that the population of $CD8^+$ T cells in the spleen and pancreas of NOD RIP-Rae1ε mice contained a decreased ratio of T_{eff+em} to T_{cm} cells lead to the finding that NKG2D stimulation increased T_{cm} generation in both human and mouse $CD8^+$ T cells *in vitro*, and in both cases reduced the ratio of $CD8^+$ T_{eff+em} to T_{cm} cells. My finding that NKG2D stimulation *in vitro* also increased PD-1 expression by mouse $CD8^+$ T cells, and increased generation of a human $CD8^+$ Treg phenotype population, further suggests an immune regulatory role for NKG2D signaling between $CD8^+$ T cells.

In conclusion, I have described a novel role for NKG2D signaling on $CD8^+$ T cells, whereby NKG2D interacts with ligand expressed by healthy immune cells, and plays a regulatory role shaping the immune response. While there is still significant work to be done to further characterize this mechanism, the protective role we find for NKG2D in autoimmune diabetes may explain the conflicting reports regarding the role of NKG2D in the disease, when taken in conjunction with evidence for a separate detrimental effect of NKG2D signaling. Finally, I feel that these findings represent a general mechanism for NKG2D acting in directing $CD8^+$ T cell development with broader implication in other autoimmune conditions, anti-tumor responses, and viral infection.

Table 3: Top upregulated genes after Rae-1ε stimulation of OT-1 CTL Genes listed in bold are those which were chosen for verification via RT-qPCR.

Fold-Change	Representative Public ID	UniGene ID	Gene Title	Gene Symbol	Chromosomal Location
1.99042	BG244279	Mm.208919	angiopoietin-like 2	Angptl2	chr2 B|2
1.98848	BB204400	---	---	---	---
1.90351	BM249924	---	RIKEN cDNA 4733401A01 gene	4733401A01Rik	chr4|4
1.89487	AF064749	Mm.7562	collagen, type VI, alpha 3	Col6a3	chr1 D|1 53.9 cM
1.86917	AK005893	Mm.84927	allantoicase	Allc	chr12 A2|12 13.0 cM
1.86917	BB235708	Mm.7233	**cytoplasmic polyadenylation element binding protein 2**	**Cpeb2**	chr5|5 B
1.8129	BB298201	---	RIKEN cDNA 1600023N17 gene	1600023N17Rik	chr5 B3|5
1.80999	AK016828	---	RIKEN cDNA 4933416A02 gene	4933416A02Rik	chr15|15
1.80977	C79829	---	expressed sequence C80120	C80120	---
1.80567	BG079416	---	expressed sequence C78228	C78228	chr5 67.2 cM
1.80263	BB615057	Mm.78372	cDNA sequence BC048562	BC048562	chr9 F2|9
1.76615	BB451758	Mm.485420	contactin 2	Cntn2	chr1 E4|1

1.75395	NM_011040	Mm.2533	paired box gene 8	Pax8	chr2 B|2 13.5 cM
1.74227	BB462504	---	---	---	---
1.74017	NM_016669	Mm.9114	crystallin, mu	Crym	chr7 F2|7 55.0 cM
1.73552	BI155210	Mm.330393	**protein tyrosine phosphatase, receptor type, J**	**Ptprj**	chr2 E1-2|2 49.5 cM
1.73174	BM124834	---	---	---	---
1.72742	BG074758	Mm.27259	Nuclear mitotic apparatus protein 1	Numa1	chr7 E3|7
1.72742	NM_009132	Mm.2416	**scinderin**	**Scin**	chr12 B1|12
1.71949	AK015386	---	RIKEN cDNA 4930445B03 gene	4930445B03Rik	---
1.71849	BB561086	Mm.314618	RIKEN cDNA B230120H23 gene	B230120H23Rik	chr2 C3|2
1.71788	NM_013479	Mm.25988	Bcl2-like 10	Bcl2l10	chr9|9 D
1.71406	BB097040	Mm.143404	predicted gene 14204	Gm14204	chr2|2
1.71406	BG101505	Mm.757	Ras homolog gene family, member A	Rhoa	chr9 F2|9
1.70352	C77540	---	---	---	---
1.69739	BB858329	Mm.22776	eukaryotic translation initiation factor 3, subunit C	Eif3c	chr7 F4|7 69.14 cM
1.69527	AI452177	---	---	---	---

1.69362	AK017352	Mm.79700	amelotin	Amtn	chr5|5 E2
1.69147	BE653037	Mm.22891	coatomer protein complex, subunit gamma 2, antisense 2	Copg2as2	chr6 A3.3|6
1.69147	BM120952	---	RIKEN cDNA 8430408J09 gene	8430408J09Rik	chr5|5
1.68643	BC027291	Mm.65337	**inositol 1,4,5-trisphosphate 3-kinase A**	**Itpka**	chr2 E5|2
1.68324	BB447551	Mm.388880	GATA binding protein 5	Gata5	chr2 H4|2 106.0 cM
1.68121	BB076298	Mm.160124	phosphatase and actin regulator 1	Phactr1	chr13 A4|13
1.67604	BB656515	Mm.102970	SET binding factor 2	Sbf2	chr7|7 F2
1.67526	BF301241	Mm.321293	immunoglobulin kappa chain variable 28 (V28) /// immunoglobulin kappa variable 6-14 /// immunoglobulin kappa variable 6-23 /// immunoglobulin kappa variable 8-19	Igk-V28 /// Igkv6-14 /// Igkv6-23 /// Igkv8-19	chr6 C1|6 /// chr6 C1|6 30.0 cM
1.67288	BB404952	Mm.387187	RIKEN cDNA C330024D21 gene	C330024D21Rik	chr5 C3.1|5
1.67171	BB431371	Mm.34977	neuron navigator 1	Nav1	chr1 E4|1
1.67082	BM116765	---	---	---	---
1.66064	BE952918	Mm.369673	---	---	---

1.65737	AK009747	Mm.431343	RIKEN cDNA 2310042D19 gene	2310042D19Rik	chr4 E2|4
1.65558	AV351492	Mm.32840	acylglycerol kinase	Agk	chr6 B1|6
1.65482	BB280339	Mm.287100	nuclear receptor subfamily 2, group E, member 1	Nr2e1	chr10 B2|10 25.5 cM
1.65382	AW455994	Mm.459433	---	---	---
1.65161	AV339091	Mm.485420	contactin 2	Cntn2	chr1 E4|1
1.65122	NM_008763	Mm.377103	olfactory receptor 16	Olfr16	chr1|1 H4-H5
1.64488	BM225171	Mm.306256	---	---	---
1.64091	BB707145	Mm.446956	Predicted gene 15527	Gm15527	chr6|6 26.84 cM
1.63497	BB344763	Mm.401062	endothelin converting enzyme 1	Ece1	chr4 D3|4
1.63423	C79862	---	---	---	---
1.62641	AK008392	Mm.96997	RIKEN cDNA 2010110P09 gene	2010110P09Rik	chr7|7 F3
1.62641	AV272467	Mm.312623	membrane-associated ring finger (C3HC4) 3	3-Mar	chr18 D2|18
1.62037	AK016895	---	RIKEN cDNA 4933424L07 gene	4933424L07Rik	chr12|12
1.62037	NM_010253	Mm.4655	galanin	Gal	chr19 A|19 2.0 cM
1.61876	NM_008716	Mm.439741	**notch 3**	**Notch3**	chr17 B1|17 20.0 cM

1.61747	BB748743	Mm.412326 /// Mm.463362	---	---	---
1.61585	BG063470	---	---	---	---
1.60769	AV376713	---	RIKEN cDNA 9130214F15 gene	9130214F15Rik	chr8|8
1.60691	AW543242	Mm.132634	ras responsive element binding protein 1	Rreb1	chr13 A3.3|13
1.60445	BE951601	Mm.30602	ubiquitin specific peptidase 22	Usp22	chr11 B2|11
1.60134	AK017208	---	RIKEN cDNA 5033430J17 gene	5033430J17Rik	chr15|15
1.59749	NM_026108	Mm.158150	haloacid dehalogenase-like hydrolase domain containing 1A	Hdhd1a	chr18|18 C
1.59749	BC009165	Mm.28585	thyroid hormone responsive SPOT14 homolog (Rattus)	Thrsp	chr7|7 E3
1.59734	BB023293	Mm.489569	somatostatin receptor 5	Sstr5	chr17 A3.3|17 13.0 cM
1.59179	AK019700	Mm.293635	solute carrier family 25, member 37	Slc25a37	chr14 D2|14
1.59079	BI076806	---	---	---	---
1.59079	BC012653	Mm.44065	chemokine (C-X3-C) receptor 1	Cx3cr1	chr9 F4|9

1.58965	AK013806	Mm.259863	RIKEN cDNA 2900079G21 gene	2900079G21Rik	chr9 F3|9
1.58877	AK016735	Mm.488103	RIKEN cDNA 1700080N15 gene	1700080N15Rik	chr2 A1|2
1.58877	BB183509	Mm.150857	family with sequence similarity 163, member A	Fam163a	chr1 G3|1
1.58708	AK018109	---	RIKEN cDNA 6330403N20 gene	6330403N20Rik	chr7|7
1.58644	BG070700	Mm.261542	solute carrier family 19, member 3	Slc19a3	chr1 C5|1 51.0 cM
1.58591	AW488885	Mm.291595	Kruppel-like factor 9	Klf9	chr19|19 C1
1.58107	BM228795	Mm.199793	expressed sequence AU022751	AU022751	chrX A1.1|X
1.57868	BB246032	Mm.277665	calbindin 1	Calb1	chr4 A2|4 10.5 cM
1.57809	AK010109	---	RIKEN cDNA 2310068G24 gene	2310068G24Rik	chr12|12
1.57725	BQ033083	Mm.46161	RIKEN cDNA 1700011M02 gene	1700011M02Rik	chrX D|X
1.57616	BB535528	Mm.9478	endothelin 3	Edn3	chr2 H4|2 104.0 cM
1.57436	AV278035	Mm.444803	HYDIN, axonemal central pair apparatus protein	Hydin	chr8 E1|8 57.0 cM
1.56998	AW493584	---	---	---	---
1.56987	BB771227	---	---	---	---
1.56935	Z95477	Mm.304143	immunoglobulin kappa chain variable 1	Igk-V1	chr6 30.0 cM

			(V1)		
1.5656	BB209296	Mm.210125	BCL2 modifying factor	Bmf	chr2 E5|2
1.56319	AK003232	Mm.4512	carbonyl reductase 3	Cbr3	chr16 C4|16 67.2 cM
1.56247	BB485193	---	RIKEN cDNA D430030G11 gene	D430030G11Rik	chr19|19
1.56164	AK016200	Mm.159511	RIKEN cDNA 4930563E18 gene	4930563E18Rik	chr18 A1|18
1.56164	BG076078	Mm.445768	---	---	---
1.56051	D38146	Mm.15675	ephrin A1	Efna1	chr3 F1|3 48.5 cM
1.55769	BG069305	---	---	---	---
1.55722	BM943059	Mm.449164	---	---	---
1.55647	BB497580	Mm.146194	interleukin-1 receptor-associated kinase 3	Irak3	chr10 D2|10
1.55015	AK002365	Mm.28766	transmembrane protein 174	Tmem174	chr13 D1|13
1.54717	BB268139	Mm.295212	ring finger protein 217	Rnf217	chr10 A4|10
1.54559	BG070459	Mm.441552	---	---	---
1.5438	X66603	Mm.56946	POU domain, class 3, transcription factor 4	Pou3f4	chrX E1|X 48.4 cM
1.5438	AI462839	---	---	---	---

1.53982	BB533148	Mm.175176	RIKEN cDNA 1810033B17 gene	1810033B17Rik	chr8 A1|8 0.38 cM
1.53864	BM119405	---	---	---	---
1.53811	AW122376	Mm.397903	---	---	---
1.53756	NM_007986	Mm.41816	fibroblast activation protein	Fap	chr2 C1.3|2 36.0 cM
1.53756	AB000500	Mm.40615	potassium voltage-gated channel, subfamily Q, member 2	Kcnq2	chr2 H3-4|2 104.0 cM
1.53756	AK014609	---	RIKEN cDNA 4633401B06 gene	4633401B06Rik	chr3|3
1.53756	BE980854	Mm.448983	---	---	---
1.53756	BI452727	Mm.182434	**follistatin-like 1**	**Fstl1**	chr16 B3|16 27.3 cM

Table 4: Top down regulated genes after Rae-1ε stimulation of OT-1 CTL Genes listed in bold are those which were chosen for verification via RT-qPCR.

Fold-Change	UniGene ID	Representative Public ID	Gene Title	Gene Symbol	Chromosomal Location
-2.5688	Mm.215745	BB704706	rearranged L-myc fusion sequence	Rlf	chr4 D2.2|4
-2.24708	Mm.271555	NM_031390	expressed sequence AV320801 /// predicted gene 15023 /// predicted gene 5128 /// predicted gene 7903 /// preferentially expressed antigen in melanoma-like 3	AV320801 /// Gm15023 /// Gm5128 /// Gm7903 /// Pramel3	chrX F1|X
-2.24547	Mm.138091	BB427489	SECIS binding protein 2-like	Secisbp2l	chr2 F1|2
-2.12799	Mm.275869	NM_029417	TBC1 domain family, member 8B	Tbc1d8b	chrX F1|X
-2.10813	Mm.40331	BB253608	bromodomain adjacent to zinc finger domain, 1B	Baz1b	chr5 G2|5
-2.10538	---	AK011527	RIKEN cDNA 2610024D14 gene	2610024D14Rik	chr11|11
-2.08282	Mm.290922	NM_011409	schlafen 3	Slfn3	chr11 C|11
-2.07987	Mm.28957	BB542335	**Pellino 1**	**Peli1**	chr11 A3.2|11 13.5 cM

-2.02144	Mm.202518	BB818947	cancer susceptibility candidate 5	Casc5	chr2 E5|2
-1.99096	Mm.272548	BG066916	ethanolamine kinase 1	Etnk1	chr6 G3|6 74.0 cM
-1.90385	Mm.100273	BC020052	junction-mediating and regulatory protein	Jmy	chr13 C3|13
-1.88573	Mm.292102 /// Mm.488588 /// Mm.490005	AK019505	---	---	---
-1.86622	Mm.197630	BB829477	protein arginine methyltransferase 10 (putative)	Prmt10	chr8 C1|8
-1.85908	Mm.130362	BB166592	topoisomerase (Nowbakht, Ionescu et al.) II beta	Top2b	chr14|14 A3
-1.82184	---	AK009978	RIKEN cDNA 2310058F05 gene	2310058F05Rik	chrX|X
-1.80633	---	AV154597	RIKEN cDNA 2900064B18 gene	2900064B18Rik	chr16|16
-1.80565	---	AFFX-BioB-3	---	---	---
-1.79975	Mm.2845	BB832806	upstream binding transcription factor, RNA polymerase I	Ubtf	chr11|11 D
-1.79382	---	AFFX-r2-Ec-bioB-5	---	---	---
-1.79168	Mm.229107	BG060641	family with sequence similarity 20, member B	Fam20b	chr1 H1|1
-1.78083	Mm.207203	BG245414	**syntaxin binding protein 4**	**Stxbp4**	chr11|11 C

-1.77737	Mm.246713	BB661182	integrator complex subunit 8	Ints8	chr4 A1|4
-1.77643	Mm.379490	BB278528	predicted gene 12541	Gm12541	chr3 B|3
-1.77011	Mm.273350	AV144937	solute carrier family 35, member A4	Slc35a4	chr18|18 B3
-1.76615	Mm.290563	BB100151	centromere protein A	Cenpa	chr5 B1|5 18.0 cM
-1.76569	---	AFFX-BioB-M	---	---	---
-1.76148	Mm.137991	NM_007937	Eph receptor A5	Epha5	chr5 E1|5 43.0 cM
-1.75838	Mm.5127	BB395066	potassium inwardly-rectifying channel, subfamily J, member 3	Kcnj3	chr2 C1.1|2
-1.75838	Mm.28449	BG923812	RIKEN cDNA B230219D22 gene	B230219D22Rik	chr13 B1|13
-1.75625	Mm.279923	NM_010890	**neural precursor cell expressed, developmentally down-regulated 4**	**Nedd4**	chr9 D|9 40.0 cM
-1.75252	Mm.490252	AV220161	RAB6B, member RAS oncogene family	Rab6b	chr9 F1|9 56.0 cM
-1.74729	Mm.324468	BB540672	**inositol polyphosphate-4-phosphatase, type II**	**Inpp4b**	chr8 C2|8 38.0 cM
-1.7448	Mm.205266	BB210491	acetyl-Coenzyme A acyltransferase 1A	Acaa1a	chr9 F4|9 71.0 cM
-1.73941	Mm.441235	AA267307	---	---	---
-1.73684	Mm.425165	AF290198	TD and POZ domain containing 1	Tdpoz1	chr3 F2.1|3
-1.73051	Mm.399997	BG069505	solute carrier family 12, member 2	Slc12a2	chr18 D3|18 32.0

					cM
-1.72676	---	AFFX-r2-Ec-bioB-M	---	---	---
-1.72661	Mm.298256	AF146523	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	Malat1	chr19 A|19
-1.72252	---	C76273	---	---	---
-1.72151	Mm.245446	BB481861	proline-rich coiled-coil 2C	Prrc2c	chr1 H1|1
-1.71476	Mm.102470	BI737125	ankyrin repeat domain 33B	Ankrd33b	chr15|15 B3.2
-1.71197	---	BB698189	---	---	---
-1.70353	---	AU015645	---	---	---
-1.70191	Mm.33617	AA560280	RNA pseudouridylate synthase domain containing 2	Rpusd2	chr2 E5|2
-1.69452	---	BB389697	---	---	---
-1.6942	Mm.27804	BB439838	zinc finger CCCH-type containing 15	Zc3h15	chr2|2 E1
-1.69147	---	W91590	---	---	---
-1.68324	---	C77185	predicted gene 7969	Gm7969	chr13 B3|13
-1.68324	Mm.31275	BB454719	UBX domain protein 7	Ubxn7	chr16 A1|16

-1.68188	Mm.271988	AK015064	transmembrane and tetratricopeptide repeat containing 4	Tmtc4	chr14 E5|14
-1.67921	Mm.3794	BB706079	polo-like kinase 4	Plk4	chr3 B|3
-1.67841	Mm.298256	AF146523	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	Malat1	chr19 A|19
-1.67171	Mm.214755	NM_008843	prolactin induced protein	Pip	chr6 B2|6 20.5 cM
-1.67095	Mm.357792	AV310201	FK506 binding protein 15	Fkbp15	chr4 B3|4
-1.66669	Mm.241147	BB105325	solute carrier family 8 (sodium/calcium exchanger), member 2	Slc8a2	chr7 A2|7 15.3 cM
-1.66643	---	AFFX-BioB-5	---	---	---
-1.66334	---	BG069481	---	---	---
-1.66334	Mm.23872	AI843088	---	---	---
-1.66187	Mm.24738	BB702377	ribonucleotide reductase M2 B (TP53 inducible)	Rrm2b	chr15 B3.1|15
-1.65928	---	AK012088	acyl-CoA synthetase long-chain family member 3	Acsl3	chr1 C4|1 24.1 cM
-1.65812	Mm.400455	BB712315	---	---	---
-1.65638	Mm.207907	BB486179	RIKEN cDNA 4932438A13 gene	4932438A13Rik	chr3 B|3
-1.65621	---	AFFX-r2-Ec-	---	---	---

		bioB-3			
-1.65551	---	AFFX-18SRNAMur/X00686_5	---	---	---
-1.65178	Mm.393511	BB079339	---	---	---
-1.64809	---	AFFX-BioC-3	---	---	---
-1.64714	Mm.288980	BB450608	neural precursor cell expressed, developmentally down-regulated gene 9	Nedd9	chr13 A3.3-A4|13
-1.64091	---	BB395032	mitochondrial intermediate peptidase	Mipep	chr14 D1|14
-1.63891	Mm.464277	BM197200	predicted pseudogene 2035 /// predicted gene 2056 /// predicted gene 8300 /// predicted pseudogene 8332 /// eukaryotic translation initiation factor 1A-like /// eukaryotic translation initiation factor 1A-like	Gm2035 /// Gm2056 /// Gm8300 /// Gm8332 /// LOC100861898 /// LOC100861908	chr12 D2|12 /// chr12 D3|12
-1.63266	---	BG065719	---	---	---

-1.63085	---	AFFX-r2-Ec-bioC-5	---	---	---
-1.62635	Mm.196846	BB482313	RAD23b homolog (S. cerevisiae)	Rad23b	chr4|4 B3
-1.6249	---	AFFX-BioC-5	---	---	---
-1.62328	Mm.252213	AW910654	bromodomain adjacent to zinc finger domain, 2A	Baz2a	chr10 D3|10
-1.62037	Mm.228903	AK010162	ring finger protein 14	Rnf14	chr18 B3|18 17.0 cM
-1.61556	---	AV240479	---	---	---
-1.61556	Mm.391403	AK013418	RIKEN cDNA 2410018L13 gene	2410018L13Rik	chr12 A1.3|12
-1.61556	Mm.355327	AV297961	desmoplakin	Dsp	chr13 A3.3|13
-1.61556	---	BG067054	---	---	---
-1.61063	---	AFFX-r2-Ec-bioC-3	---	---	---
-1.60769	Mm.196424	NM_008930	prolactin family 7, subfamily a, member 1	Prl7a1	chr13 A3.1|13 14.0 cM
-1.59771	Mm.174044	BB480651	RIKEN cDNA 2700050L05 gene	2700050L05Rik	chr7 F3|7
-1.59528	Mm.71498	AV231984	ligand dependent nuclear receptor corepressor-	Lcorl	chr5 B3|5

			like		
-1.59289	Mm.128273	NM_022724	suppressor of variegation 3-9 homolog 2 (Drosophila)	Suv39h2	chr2 A|2 2.5 cM
-1.59289	Mm.32025	BB555268	RIKEN cDNA 4831440E17 gene	4831440E17Rik	chr5 A3|5
-1.59131	---	BB204671	---	---	---
-1.59079	Mm.385180	NM_010232	flavin containing monooxygenase 5	Fmo5	chr3 F2.2|3
-1.59079	---	AW121800	---	---	---
-1.58965	---	BE949277	RIKEN cDNA 6230424C14 gene	6230424C14Rik	chr13|13
-1.58877	---	BB208683	---	---	---
-1.58877	Mm.486378	AK012300	uncharacterized LOC100504854 /// uncharacterized LOC100862512	LOC100504854 /// LOC100862512	---
-1.58871	Mm.330496	BM238259	INO80 homolog (S. cerevisiae)	Ino80	chr2 E5|2
-1.58843	Mm.152987	BB198104	exportin 7	Xpo7	chr14 D2|14
-1.58843	---	BB051628	---	---	---
-1.58654	Mm.141235	AK016350	RIKEN cDNA 1700003P14 gene	1700003P14Rik	chr13|13
-1.58443	Mm.38049	AF185285	**toll-like receptor 4**	**Tlr4**	chr4 C1|4 33.0 cM

-1.58443	---	BB463790	RIKEN cDNA A830039H05 gene	A830039H05Rik	chr19|19
-1.58443	Mm.217216	AF027505	membrane associated guanylate kinase, WW and PDZ domain containing 1	Magi1	chr6|6 D3
-1.58443	Mm.280557	AV265508	cyclin-dependent kinase-like 3	Cdkl3	chr11 B1.3|11
-1.58338	Mm.14796	BI150149	microsomal glutathione S-transferase 1	Mgst1	chr6 G1|6
-1.58338	Mm.219648	AW107924	THO complex 1	Thoc1	chr18 A1|18
-1.58231	Mm.133623	NM_025747	RIKEN cDNA 4933411K20 gene	4933411K20Rik	chr8 B2|8 27.0 cM
-1.58152	Mm.358699	AK015001	potassium voltage-gated channel, subfamily G, member 4	Kcng4	chr8 E1|8
-1.58091	---	BG074604	---	---	---
-1.57868	Mm.93636	AK018275	Dmx-like 2	Dmxl2	chr9|9 C
-1.57411	Mm.259879	BF134200	X-linked inhibitor of apoptosis	Xiap	chrX|X A3-A5
-1.57366	Mm.234823	BE995635	armadillo repeat containing 8	Armc8	chr9|9 F1
-1.57121	Mm.206934	AK002809	membrane-bound transcription factor peptidase, site 1	Mbtps1	chr8 E1|8
-1.57071	---	BG069580	DNA segment, Chr 10, ERATO Doi 761, expressed	D10Ertd761e	chr10 43.0 cM
-1.56987	Mm.4325	BG069413	**Kruppel-like factor 4 (gut)**	**Klf4**	chr4 B3|4 19.7 cM

References

Adlercreutz, E. H., C. Weile, J. Larsen, K. Engkilde, D. Agardh, K. Buschard and J. C. Antvorskov (2014). "A gluten-free diet lowers NKG2D and ligand expression in BALB/c and non-obese diabetic (NOD) mice." Clin Exp Immunol **177**(2): 391-403.

Adriouch, S., C. Dox, V. Welge, M. Seman, F. Koch-Nolte and F. Haag (2002). "Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse P2X7 receptor." J Immunol **169**(8): 4108-4112.

Allez, M., V. Tieng, A. Nakazawa, X. Treton, V. Pacault, N. Dulphy, S. Caillat-Zucman, P. Paul, J. M. Gornet, C. Douay, S. Ravet, R. Tamouza, D. Charron, M. Lemann, L. Mayer and A. Toubert (2007). "CD4+NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions." Gastroenterology **132**(7): 2346-2358.

Andersson, A. K., P. F. Sumariwalla, F. E. McCann, P. Amjadi, C. Chang, K. McNamee, D. Tornehave, C. Haase, H. Agerso, V. W. Stennicke, D. Ahern, B. Urso, J. Trowsdale, M. Feldmann and F. M. Brennan (2011). "Blockade of NKG2D ameliorates disease in mice with collagen-induced arthritis: a potential pathogenic role in chronic inflammatory arthritis." Arthritis Rheum **63**(9): 2617-2629.

Angstetra, E., K. L. Graham, Y. Zhao, A. E. Irvin, L. Elkerbout, P. Santamaria, R. M. Slattery, T. W. Kay and H. E. Thomas (2012). "An indirect role for NK cells in a CD4(+) T-cell-dependent mouse model of type I diabetes." Immunol Cell Biol **90**(2): 243-247.

Ansari, M. J., A. D. Salama, T. Chitnis, R. N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S. J. Khoury, H. Auchincloss, Jr. and M. H. Sayegh (2003). "The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice." J Exp Med **198**(1): 63-69.

Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier and T. Spies (1999). "Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA." Science **285**(5428): 727-729.

Berard, M., K. Brandt, S. Bulfone-Paus and D. F. Tough (2003). "IL-15 promotes the survival of naive and memory phenotype CD8+ T cells." J Immunol **170**(10): 5018-5026.

Borges da Silva, H., L. K. Beura, H. Wang, E. A. Hanse, R. Gore, M. C. Scott, D. A. Walsh, K. E. Block, R. Fonseca, Y. Yan, K. L. Hippen, B. R. Blazar, D. Masopust, A. Kelekar, L. Vulchanova, K. A. Hogquist and S. C. Jameson (2018). "The purinergic receptor P2RX7 directs metabolic fitness of long-lived memory CD8(+) T cells." Nature **559**(7713): 264-268.

Buck, M. D., D. O'Sullivan, R. I. Klein Geltink, J. D. Curtis, C. H. Chang, D. E. Sanin, J. Qiu, O. Kretz, D. Braas, G. J. van der Windt, Q. Chen, S. C. Huang, C. M. O'Neill, B. T. Edelson, E. J.

Pearce, H. Sesaki, T. B. Huber, A. S. Rambold and E. L. Pearce (2016). "Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming." Cell **166**(1): 63-76.

Buck, M. D., R. T. Sowell, S. M. Kaech and E. L. Pearce (2017). "Metabolic Instruction of Immunity." Cell **169**(4): 570-586.

Burrack, A. L., T. Martinov and B. T. Fife (2017). "T Cell-Mediated Beta Cell Destruction: Autoimmunity and Alloimmunity in the Context of Type 1 Diabetes." Front Endocrinol (Lausanne) **8**: 343.

Campbell, A. R., M. C. Duggan, L. P. Suarez-Kelly, N. Bhave, K. S. Opheim, E. L. McMichael, P. Trikha, R. Parihar, E. Luedke, A. Lewis, B. Yung, R. Lee, D. Raulet, S. Tridandapani, V. Groh, L. Yu, V. Yildiz, J. C. Byrd, M. A. Caligiuri and W. E. Carson, 3rd (2017). "MICA-Expressing Monocytes Enhance Natural Killer Cell Fc Receptor-Mediated Antitumor Functions." Cancer Immunol Res **5**(9): 778-789.

Carrero, J. A., B. Calderon, F. Towfic, M. N. Artyomov and E. R. Unanue (2013). "Defining the transcriptional and cellular landscape of type 1 diabetes in the NOD mouse." PLoS One **8**(3): e59701.

Cerboni, C., M. Ardolino, A. Santoni and A. Zingoni (2009). "Detuning CD8+ T lymphocytes by down-regulation of the activating receptor NKG2D: role of NKG2D ligands released by activated T cells." Blood **113**(13): 2955-2964.

Cerboni, C., A. Zingoni, M. Cippitelli, M. Piccoli, L. Frati and A. Santoni (2007). "Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK- cell lysis." Blood **110**(2): 606-615.

Cerwenka, A., A. B. Bakker, T. McClanahan, J. Wagner, J. Wu, J. H. Phillips and L. L. Lanier (2000). "Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice." Immunity **12**(6): 721-727.

Champsaur, M. and L. L. Lanier (2010). "Effect of NKG2D ligand expression on host immune responses." Immunol Rev **235**(1): 267-285.

Chee, J., H. J. Ko, A. Skowera, G. Jhala, T. Catterall, K. L. Graham, R. M. Sutherland, H. E. Thomas, A. M. Lew, M. Peakman, T. W. Kay and B. Krishnamurthy (2014). "Effector-memory T cells develop in islets and report islet pathology in type 1 diabetes." J Immunol **192**(2): 572-580.

Chen, L. and C. F. Brosnan (2006). "Exacerbation of experimental autoimmune encephalomyelitis in P2X7R-/- mice: evidence for loss of apoptotic activity in lymphocytes." J Immunol **176**(5): 3115-3126.

Chen, Y. G., F. Scheuplein, J. P. Driver, A. A. Hewes, P. C. Reifsnyder, E. H. Leiter and D. V. Serreze (2011). "Testing the role of P2X7 receptors in the development of type 1 diabetes in nonobese diabetic mice." J Immunol **186**(7): 4278-4284.

Cheney, E. E., E. L. Wise, J. D. Bui, R. D. Schreiber, L. N. Carayannopoulos, D. Spitzer, B. Zafirova, B. Polic, A. S. Shaw and M. A. Markiewicz (2012). "A dual function of NKG2D ligands in NK-cell activation." Eur J Immunol **42**(9): 2452-2458.

Chitadze, G., J. Bhat, M. Lettau, O. Janssen and D. Kabelitz (2013). "Generation of soluble NKG2D ligands: proteolytic cleavage, exosome secretion and functional implications." Scand J Immunol **78**(2): 120-129.

Clotman, K., K. Janssens, P. Specenier, I. Weets and C. E. M. De Block (2018). "Programmed Cell Death-1 Inhibitor-Induced Type 1 Diabetes Mellitus." J Clin Endocrinol Metab **103**(9): 3144-3154.

Coudert, J. D., J. Zimmer, E. Tomasello, M. Cebecauer, M. Colonna, E. Vivier and W. Held (2005). "Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells." Blood **106**(5): 1711-1717.

Dai, Z., C. J. Turtle, G. C. Booth, S. R. Riddell, T. A. Gooley, A. M. Stevens, T. Spies and V. Groh (2009). "Normally occurring NKG2D+CD4+ T cells are immunosuppressive and inversely correlated with disease activity in juvenile-onset lupus." J Exp Med **206**(4): 793-805.

Di Virgilio, F., D. Dal Ben, A. C. Sarti, A. L. Giuliani and S. Falzoni (2017). "The P2X7 Receptor in Infection and Inflammation." Immunity **47**(1): 15-31.

Diefenbach, A., A. M. Jamieson, S. D. Liu, N. Shastri and D. H. Raulet (2000). "Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages." Nat Immunol **1**(2): 119-126.

Eagle, R. A., I. Jafferji and A. D. Barrow (2009). "Beyond Stressed Self: Evidence for NKG2D Ligand Expression on Healthy Cells." Curr Immunol Rev **5**(1): 22-34.

Ehrlich, L. I., K. Ogasawara, J. A. Hamerman, R. Takaki, A. Zingoni, J. P. Allison and L. L. Lanier (2005). "Engagement of NKG2D by cognate ligand or antibody alone is insufficient to mediate costimulation of human and mouse CD8+ T cells." J Immunol **174**(4): 1922-1931.

Farber, D. L., N. A. Yudanin and N. P. Restifo (2014). "Human memory T cells: generation, compartmentalization and homeostasis." Nat Rev Immunol **14**(1): 24-35.

Felix, K. M., F. Teng, N. A. Bates, H. Ma, I. A. Jaimez, K. C. Sleiman, N. L. Tran and H. J. Wu (2019). "P2RX7 Deletion in T Cells Promotes Autoimmune Arthritis by Unleashing the Tfh Cell Response." Front Immunol **10**: 411.

Fernandez-Morera, J. L., S. Rodriguez-Rodero, C. Lahoz, A. Tunon, A. Astudillo, O. Garcia-Suarez, J. Martinez-Borra, A. Lopez-Vazquez, L. Rodrigo, S. Gonzalez and C. Lopez-Larrea (2008). "Soluble MHC class I chain-related protein B serum levels correlate with disease activity in relapsing-remitting multiple sclerosis." Hum Immunol **69**(4-5): 235-240.

Gambelunghe, G., A. Brozzetti, M. Ghaderi, P. Candeloro, C. Tortoioli and A. Falorni (2007). "MICA gene polymorphism in the pathogenesis of type 1 diabetes." Ann N Y Acad Sci **1110**: 92-98.

Gilfillan, S., E. L. Ho, M. Cella, W. M. Yokoyama and M. Colonna (2002). "NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation." Nat Immunol **3**(12): 1150-1155.

Granados, H. M., A. Draghi, 2nd, N. Tsurutani, K. Wright, M. L. Fernandez, F. A. Sylvester and A. T. Vella (2017). "Programmed cell death-1, PD-1, is dysregulated in T cells from children with new onset type 1 diabetes." PLoS One **12**(9): e0183887.

Groh, V., A. Bruhl, H. El-Gabalawy, J. L. Nelson and T. Spies (2003). "Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis." Proc Natl Acad Sci U S A **100**(16): 9452-9457.

Groh, V., R. Rhinehart, J. Randolph-Habecker, M. S. Topp, S. R. Riddell and T. Spies (2001). "Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells." Nat Immunol **2**(3): 255-260.

Guerra, N., K. Pestal, T. Juarez, J. Beck, K. Tkach, L. Wang and D. H. Raulet (2013). "A selective role of NKG2D in inflammatory and autoimmune diseases." Clin Immunol **149**(3): 432-439.

Hanaoka, N., B. Jabri, Z. Dai, C. Ciszewski, A. M. Stevens, C. Yee, H. Nakakuma, T. Spies and V. Groh (2010). "NKG2D initiates caspase-mediated CD3zeta degradation and lymphocyte receptor impairments associated with human cancer and autoimmune disease." J Immunol **185**(10): 5732-5742.

Hansen, C. H., T. L. Holm, L. Krych, L. Andresen, D. S. Nielsen, I. Rune, A. K. Hansen and S. Skov (2013). "Gut microbiota regulates NKG2D ligand expression on intestinal epithelial cells." Eur J Immunol **43**(2): 447-457.

Hashimoto-Hill, S., L. Friesen, M. Kim and C. H. Kim (2017). "Contraction of intestinal effector T cells by retinoic acid-induced purinergic receptor P2X7." Mucosal Immunol **10**(4): 912-923.

Huang, W. C., N. J. Easom, X. Z. Tang, U. S. Gill, H. Singh, F. Robertson, C. Chang, J. Trowsdale, B. R. Davidson, W. M. Rosenberg, G. Fusai, A. Toubert, P. T. Kennedy, D. Peppa and M. K. Maini (2017). "T Cells Infiltrating Diseased Liver Express Ligands for the NKG2D Stress Surveillance System." J Immunol **198**(3): 1172-1182.

Hue, S., J. J. Mention, R. C. Monteiro, S. Zhang, C. Cellier, J. Schmitz, V. Verkarre, N. Fodil, S. Bahram, N. Cerf-Bensussan and S. Caillat-Zucman (2004). "A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease." Immunity **21**(3): 367-377.

Jamieson, A. M., A. Diefenbach, C. W. McMahon, N. Xiong, J. R. Carlyle and D. H. Raulet (2002). "The role of the NKG2D immunoreceptor in immune cell activation and natural killing." Immunity **17**(1): 19-29.

Joo, W. S., J. H. Jeong, K. Nam, K. S. Blevins, M. E. Salama and S. W. Kim (2012). "Polymeric delivery of therapeutic RAE-1 plasmid to the pancreatic islets for the prevention of type 1 diabetes." J Control Release **162**(3): 606-611.

Khadra, A., S. Tsai, P. Santamaria and L. Edelstein-Keshet (2010). "On how monospecific memory-like autoregulatory CD8+ T cells can blunt diabetogenic autoimmunity: a computational approach." J Immunol **185**(10): 5962-5972.

Kikutani, H. and S. Makino (1992). "The murine autoimmune diabetes model: NOD and related strains." Adv Immunol **51**: 285-322.

King, C. and N. Sarvetnick (2011). "The incidence of type-1 diabetes in NOD mice is modulated by restricted flora not germ-free conditions." PLoS One **6**(2): e17049.

Kinjyo, I., J. Qin, S. Y. Tan, C. J. Wellard, P. Mrass, W. Ritchie, A. Doi, L. L. Cavanagh, M. Tomura, A. Sakaue-Sawano, O. Kanagawa, A. Miyawaki, P. D. Hodgkin and W. Weninger (2015). "Real-time tracking of cell cycle progression during CD8+ effector and memory T-cell differentiation." Nat Commun **6**: 6301.

Kjellev, S., C. Haase, D. Lundsgaard, B. Urso, D. Tornehave and H. Markholst (2007). "Inhibition of NKG2D receptor function by antibody therapy attenuates transfer-induced colitis in SCID mice." Eur J Immunol **37**(5): 1397-1406.

Lanier, L. L. (2015). "NKG2D Receptor and Its Ligands in Host Defense." Cancer Immunol Res **3**(6): 575-582.

Lenartic, M., V. Jelencic, B. Zafirova, M. Ozanic, V. Marecic, S. Jurkovic, V. Sexl, M. Santic, F. M. Wensveen and B. Polic (2017). "NKG2D Promotes B1a Cell Development and Protection against Bacterial Infection." J Immunol **198**(4): 1531-1542.

Li, J., B. A. Rabinovich, R. Hurren, D. Cosman and R. G. Miller (2005). "Survival versus neglect: redefining thymocyte subsets based on expression of NKG2D ligand(s) and MHC class I." Eur J Immunol **35**(2): 439-448.

Li, S., Q. Xie, Y. Zeng, C. Zou, X. Liu, S. Wu, H. Deng, Y. Xu, X. C. Li and Z. Dai (2014). "A naturally occurring CD8(+)CD122(+) T-cell subset as a memory-like Treg family." Cell Mol Immunol **11**(4): 326-331.

Makino, S., K. Kunimoto, Y. Muraoka and K. Katagiri (1981). "Effect of castration on the appearance of diabetes in NOD mouse." Jikken Dobutsu **30**(2): 137-140.

Markiewicz, M. A., L. N. Carayannopoulos, O. V. Naidenko, K. Matsui, W. R. Burack, E. L. Wise, D. H. Fremont, P. M. Allen, W. M. Yokoyama, M. Colonna and A. S. Shaw (2005). "Costimulation through NKG2D enhances murine CD8+ CTL function: similarities and differences between NKG2D and CD28 costimulation." J Immunol **175**(5): 2825-2833.

Markiewicz, M. A., E. L. Wise, Z. S. Buchwald, A. K. Pinto, B. Zafirova, B. Polic and A. S. Shaw (2012). "RAE1epsilon ligand expressed on pancreatic islets recruits NKG2D receptor-expressing cytotoxic T cells independent of T cell receptor recognition." Immunity **36**(1): 132-141.

Mathis, D. and C. Benoist (2011). "Microbiota and autoimmune disease: the hosted self." Cell Host Microbe **10**(4): 297-301.

McQueen, B., K. Trace, E. Whitman, T. Bedsworth and A. Barber (2016). "Natural killer group 2D and CD28 receptors differentially activate mammalian/mechanistic target of rapamycin to alter murine effector CD8+ T-cell differentiation." Immunology **147**(3): 305-320.

Meresse, B., Z. Chen, C. Ciszewski, M. Tretiakova, G. Bhagat, T. N. Krausz, D. H. Raulet, L. L. Lanier, V. Groh, T. Spies, E. C. Ebert, P. H. Green and B. Jabri (2004). "Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease." Immunity **21**(3): 357-366.

Molfetta, R., L. Quatrini, B. Zitti, C. Capuano, R. Galandrini, A. Santoni and R. Paolini (2016). "Regulation of NKG2D Expression and Signaling by Endocytosis." Trends Immunol **37**(11): 790-802.

Molinero, L. L., M. B. Fuertes, M. V. Girart, L. Fainboim, G. A. Rabinovich, M. A. Costas and N. W. Zwirner (2004). "NF-kappa B regulates expression of the MHC class I-related chain A gene in activated T lymphocytes." J Immunol **173**(9): 5583-5590.

Molinero, L. L., M. B. Fuertes, G. A. Rabinovich, L. Fainboim and N. W. Zwirner (2002). "Activation-induced expression of MICA on T lymphocytes involves engagement of CD3 and CD28." J Leukoc Biol **71**(5): 791-797.

Morita, M., J. Prudent, K. Basu, V. Goyon, S. Katsumura, L. Hulea, D. Pearl, N. Siddiqui, S. Strack, S. McGuirk, J. St-Pierre, O. Larsson, I. Topisirovic, H. Vali, H. M. McBride, J. J. Bergeron and N. Sonenberg (2017). "mTOR Controls Mitochondrial Dynamics and Cell Survival via MTFP1." Mol Cell **67**(6): 922-935 e925.

Nice, T. J., L. Coscoy and D. H. Raulet (2009). "Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress." J Exp Med **206**(2): 287-298.

Nielsen, N., N. Odum, B. Urso, L. L. Lanier and P. Spee (2012). "Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A." PLoS One **7**(2): e31959.

Nikitina-Zake, L., R. Rajalingham, I. Rumba and C. B. Sanjeevi (2004). "Killer cell immunoglobulin-like receptor genes in Latvian patients with type 1 diabetes mellitus and healthy controls." Ann N Y Acad Sci **1037**: 161-169.

Noval Rivas, M., M. Hazzan, K. Weatherly, F. Gaudray, I. Salmon and M. Y. Braun (2010). "NK cell regulation of CD4 T cell-mediated graft-versus-host disease." J Immunol **184**(12): 6790-6798.

Ogasawara, K., J. A. Hamerman, L. R. Ehrlich, H. Bour-Jordan, P. Santamaria, J. A. Bluestone and L. L. Lanier (2004). "NKG2D blockade prevents autoimmune diabetes in NOD mice." Immunity **20**(6): 757-767.

Ogasawara, K., J. A. Hamerman, H. Hsin, S. Chikuma, H. Bour-Jordan, T. Chen, T. Pertel, C. Carnaud, J. A. Bluestone and L. L. Lanier (2003). "Impairment of NK cell function by NKG2D modulation in NOD mice." Immunity **18**(1): 41-51.

Ogasawara, K. and L. L. Lanier (2005). "NKG2D in NK and T cell-mediated immunity." J Clin Immunol **25**(6): 534-540.

Oppenheim, D. E., S. J. Roberts, S. L. Clarke, R. Filler, J. M. Lewis, R. E. Tigelaar, M. Girardi and A. C. Hayday (2005). "Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance." Nat Immunol **6**(9): 928-937.

Pellegrino, M., A. Crino, M. M. Rosado and A. Fierabracci (2019). "Identification and functional characterization of CD8+ T regulatory cells in type 1 diabetes patients." PLoS One **14**(1): e0210839.

Perez, C., K. Prajapati, B. Burke, L. Plaza-Rojas, N. J. Zeleznik-Le and J. A. Guevara-Patino (2019). "NKG2D signaling certifies effector CD8 T cells for memory formation." J Immunother Cancer **7**(1): 48.

Pozzilli, P., A. Signore, A. J. Williams and P. E. Beales (1993). "NOD mouse colonies around the world--recent facts and figures." Immunol Today **14**(5): 193-196.

Qin, H., I. F. Lee, C. Panagiotopoulos, X. Wang, A. D. Chu, P. J. Utz, J. J. Priatel and R. Tan (2011). "Natural killer cells from children with type 1 diabetes have defects in NKG2D-dependent function and signaling." Diabetes **60**(3): 857-866.

Rabinovich, B. A., J. Li, J. Shannon, R. Hurren, J. Chalupny, D. Cosman and R. G. Miller (2003). "Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells." J Immunol **170**(7): 3572-3576.

Rabinovitch, A. (1998). "An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus." Diabetes Metab Rev **14**(2): 129-151.

Raulet, D. H., S. Gasser, B. G. Gowen, W. Deng and H. Jung (2013). "Regulation of ligands for the NKG2D activating receptor." Annu Rev Immunol **31**: 413-441.

Rodacki, M., B. Svoren, V. Butty, W. Besse, L. Laffel, C. Benoist and D. Mathis (2007). "Altered natural killer cells in type 1 diabetic patients." Diabetes **56**(1): 177-185.

Rosen, D. B., M. Araki, J. A. Hamerman, T. Chen, T. Yamamura and L. L. Lanier (2004). "A Structural basis for the association of DAP12 with mouse, but not human, NKG2D." J Immunol **173**(4): 2470-2478.

Roy, S., P. F. Barnes, A. Garg, S. Wu, D. Cosman and R. Vankayalapati (2008). "NK cells lyse T regulatory cells that expand in response to an intracellular pathogen." J Immunol **180**(3): 1729-1736.

Sallusto, F., J. Geginat and A. Lanzavecchia (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annu Rev Immunol **22**: 745-763.

Salmond, R. J. (2018). "mTOR Regulation of Glycolytic Metabolism in T Cells." Front Cell Dev Biol **6**: 122.

Sandilos, J. K., Y. H. Chiu, F. B. Chekeni, A. J. Armstrong, S. F. Walk, K. S. Ravichandran and D. A. Bayliss (2012). "Pannexin 1, an ATP release channel, is activated by caspase cleavage of its pore-associated C-terminal autoinhibitory region." J Biol Chem **287**(14): 11303-11311.

Savio, L. E. B., P. de Andrade Mello, C. G. da Silva and R. Coutinho-Silva (2018). "The P2X7 Receptor in Inflammatory Diseases: Angel or Demon?" Front Pharmacol **9**: 52.

Saxton, R. A. and D. M. Sabatini (2017). "mTOR Signaling in Growth, Metabolism, and Disease." Cell **169**(2): 361-371.

Schluns, K. S., K. Williams, A. Ma, X. X. Zheng and L. Lefrancois (2002). "Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells." J Immunol **168**(10): 4827-4831.

Schroder, P. M., M. Khattar, C. E. Baum, Y. Miyahara, W. Chen, R. Vyas, S. Muralidharan, B. Mierzejewska and S. M. Stepkowski (2015). "PD-1-dependent restoration of self-tolerance in the NOD mouse model of diabetes after transient anti-TCRbeta mAb therapy." Diabetologia **58**(6): 1309-1318.

Shameli, A., X. Clemente-Casares, J. Wang and P. Santamaria (2011). "Development of memory-like autoregulatory CD8+ T cells is CD4+ T cell dependent." J Immunol **187**(6): 2859-2866.

Simecek, P., G. A. Churchill, H. Yang, L. B. Rowe, L. Herberg, D. V. Serreze and E. H. Leiter (2015). "Genetic Analysis of Substrain Divergence in Non-Obese Diabetic (NOD) Mice." G3 (Bethesda) **5**(5): 771-775.

Syberg, S., P. Schwarz, S. Petersen, T. H. Steinberg, J. E. Jensen, J. Teilmann and N. R. Jorgensen (2012). "Association between P2X7 Receptor Polymorphisms and Bone Status in Mice." J Osteoporos **2012**: 637986.

Takada, A., S. Yoshida, M. Kajikawa, Y. Miyatake, U. Tomaru, M. Sakai, H. Chiba, K. Maenaka, D. Kohda, K. Fugo and M. Kasahara (2008). "Two novel NKG2D ligands of the mouse H60 family with differential expression patterns and binding affinities to NKG2D." J Immunol **180**(3): 1678-1685.

Tang, X., I. Maricic, N. Purohit, B. Bakamjian, L. M. Reed-Loisel, T. Beeston, P. Jensen and V. Kumar (2006). "Regulation of immunity by a novel population of Qa-1-restricted CD8alphaalpha+TCRalphabeta+ T cells." J Immunol **177**(11): 7645-7655.

Trembath, A. P. and M. A. Markiewicz (2018). "More than Decoration: Roles for Natural Killer Group 2 Member D Ligand Expression by Immune Cells." Front Immunol **9**: 231.

Trembath, A. P., N. Sharma, R. Saravanan, B. Polic and M. A. Markiewicz (2017). "A Protective Role for NKG2D-H60a Interaction via Homotypic T Cell Contact in Nonobese Diabetic Autoimmune Diabetes Pathogenesis." Immunohorizons **1**(9): 198-212.

Tsai, S., A. Shameli and P. Santamaria (2008). "CD8+ T cells in type 1 diabetes." Adv Immunol **100**: 79-124.

Tsai, S., A. Shameli, J. Yamanouchi, X. Clemente-Casares, J. Wang, P. Serra, Y. Yang, Z. Medarova, A. Moore and P. Santamaria (2010). "Reversal of autoimmunity by boosting memory-like autoregulatory T cells." Immunity **32**(4): 568-580.

Vadstrup, K., E. D. Galsgaard, H. Jensen, L. L. Lanier, J. C. Ryan, S. Y. Chen, G. P. Nolan, M. K. Vester-Andersen, J. S. Pedersen, J. Gerwien, T. Jensen and F. Bendtsen (2017). "NKG2D ligand expression in Crohn's disease and NKG2D-dependent stimulation of CD8+ T cell migration." Exp Mol Pathol **103**(1): 56-70.

Van Belle, T. L., E. Ling, C. Haase, D. Bresson, B. Urso and M. G. von Herrath (2013). "NKG2D blockade facilitates diabetes prevention by antigen-specific Tregs in a virus-induced model of diabetes." J Autoimmun **40**: 66-73.

Verdaguer, J., D. Schmidt, A. Amrani, B. Anderson, N. Averill and P. Santamaria (1997). "Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice." J Exp Med **186**(10): 1663-1676.

Verneris, M. R., M. Karimi, J. Baker, A. Jayaswal and R. S. Negrin (2004). "Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells." Blood **103**(8): 3065-3072.

Weng, N. P., K. Liu, M. Catalfamo, Y. Li and P. A. Henkart (2002). "IL-15 is a growth factor and an activator of CD8 memory T cells." Ann N Y Acad Sci **975**: 46-56.

Wensveen, F. M., V. Jelencic and B. Polic (2018). "NKG2D: A Master Regulator of Immune Cell Responsiveness." Front Immunol **9**: 441.

Wensveen, F. M., M. Lenartic, V. Jelencic, N. A. Lemmermann, A. ten Brinke, S. Jonjic and B. Polic (2013). "NKG2D induces Mcl-1 expression and mediates survival of CD8 memory T cell precursors via phosphatidylinositol 3-kinase." J Immunol **191**(3): 1307-1315.

Wiemann, K., H. W. Mittrucker, U. Feger, S. A. Welte, W. M. Yokoyama, T. Spies, H. G. Rammensee and A. Steinle (2005). "Systemic NKG2D down-regulation impairs NK and CD8 T cell responses in vivo." J Immunol **175**(2): 720-729.

Wu, J., H. Cherwinski, T. Spies, J. H. Phillips and L. L. Lanier (2000). "DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells." J Exp Med **192**(7): 1059-1068.

Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier and J. H. Phillips (1999). "An activating immunoreceptor complex formed by NKG2D and DAP10." Science **285**(5428): 730-732.

Yu, Y., X. Ma, R. Gong, J. Zhu, L. Wei and J. Yao (2018). "Recent advances in CD8(+) regulatory T cell research." Oncol Lett **15**(6): 8187-8194.

Yurkovetskiy, L., M. Burrows, A. A. Khan, L. Graham, P. Volchkov, L. Becker, D. Antonopoulos, Y. Umesaki and A. V. Chervonsky (2013). "Gender bias in autoimmunity is influenced by microbiota." Immunity **39**(2): 400-412.

Zafirova, B., S. Mandaric, R. Antulov, A. Krmpotic, H. Jonsson, W. M. Yokoyama, S. Jonjic and B. Polic (2009). "Altered NK cell development and enhanced NK cell-mediated resistance to mouse cytomegalovirus in NKG2D-deficient mice." Immunity **31**(2): 270-282.

Zhang, H., C. Hardamon, B. Sagoe, J. Ngolab and J. D. Bui (2011). "Studies of the H60a locus in C57BL/6 and 129/Sv mouse strains identify the H60a 3'UTR as a regulator of H60a expression." Mol Immunol **48**(4): 539-545.

Zhang, J., F. Basher and J. D. Wu (2015). "NKG2D Ligands in Tumor Immunity: Two Sides of a Coin." Front Immunol **6**: 97.

Zingoni, A., R. Molfetta, C. Fionda, A. Soriani, R. Paolini, M. Cippitelli, C. Cerboni and A. Santoni (2018). "NKG2D and Its Ligands: "One for All, All for One"." Front Immunol **9**: 476.

Zloza, A., F. J. Kohlhapp, G. E. Lyons, J. M. Schenkel, T. V. Moore, A. T. Lacek, J. A. O'Sullivan, V. Varanasi, J. W. Williams, M. C. Jagoda, E. C. Bellavance, A. L. Marzo, P. G. Thomas, B. Zafirova, B. Polic, L. Al-Harthi, A. I. Sperling and J. A. Guevara-Patino (2012). "NKG2D signaling on CD8(+) T cells represses T-bet and rescues CD4-unhelped CD8(+) T cell memory recall but not effector responses." Nat Med **18**(3): 422-428.

Zwirner, N. W., M. A. Fernandez-Vina and P. Stastny (1998). "MICA, a new polymorphic HLA-related antigen, is expressed mainly by keratinocytes, endothelial cells, and monocytes." Immunogenetics **47**(2): 139-148.