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Regulation and Heterogeneity of Pancreatic Natural Killer Cells During Type 1 Diabetes

A dissertation presented by

Jonathan Ryan Sitrin

To

The Division of Medical Sciences

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Immunology

Harvard University

Cambridge, Massachusetts

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Regulation and Heterogeneity of Pancreatic Natural Killer Cells During Type 1 Diabetes

Abstract

The vertebrate immune system contains a diverse inventory of genetic, epigenetic, molecular and cellular mechanisms dedicated to distinguishing and to determining appropriate responsiveness to "self" and "non-self." Autoimmune diseases such as type 1 diabetes (T1D), caused by immune destruction of insulin-producing β cells in the pancreas, are the result of breakdowns in these mechanisms. Recent T1D research efforts have uncovered the opposing functions of pancreatic Foxp3+ regulatory T (T_{reg}) cells and natural killer (NK) cells as critical determinants of tolerance versus autoimmunity. Here, we examine the extrinsic adaptive regulation of NK cells by T_{reg} cells, and profile the tissue-specific heterogeneity of NK cells for intrinsic mediators of NK cell tolerance.

Depletion of T_{reg} cells in the BDC2.5/NOD model resulted in destruction of pancreatic islets and a high penetrance of T1D. Prior to the activation of T cells, there was a rapid and localized activation of pancreatic NK cells, including their proliferation and production of diabetogenic IFN-γ. How T_{reg} cells exerted their dominant tolerance on NK cells in this setting was unclear. We explored the molecular mechanisms underlying this NK/T_{reg} cell axis, following leads from a kinetic exploration of gene-expression changes early after punctual perturbation of T_{reg} cells. Our data supported a scenario in which T_{reg} cells controlled NK cell functions by limiting the bioavailability of T cell-derived IL-2 in the islets, representing a novel intertwining of innate and adaptive immunity.

Cell intrinsic regulatory mechanisms, such as the expression of the Ly49 receptor family during NK cell education, tune NK cells to be functionally self-tolerant. However, the transcriptional repertoire of Ly49 receptors had never been comprehensively explored. We performed RNAseq-based profiling of the Ly49 receptors, and uncovered a subtle difference in the expression of Ly49E and Ly49H on pancreatic NK cells compared to spleen. We also expanded the phenotypic profiling of pancreatic NK cells using high-dimensional mass cytometry and uncovered greater diversity than had previously been described.

Taken together, these studies highlighted a new degree of heterogeneity in pancreatic NK cells and uncovered a novel regulatory mechanism, originating from the adaptive immune system, responsible for maintaining NK cell tolerance.

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.
-Marie Curie

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Glossary of Abbreviations

BCR, B cell receptor
TCR, T cell receptor
T_{reg} cell, Regulatory T cell
IL, Interleukin
IPEX, Immune dysfunction, polyendocrinopathy, enteropathy, X-linked inheritance
DTR, Diphtheria toxin receptor
NK cell, Natural killer cell
DC, Dendritic cells
T1D, Type 1 diabetes
HLA, Human leukocyte antigen
NOD, Nonobese diabetic
PLN, Pancreatic lymph node
MHC, Major histocompatibility complex
APC, Antigen presenting cell
TNF-α, Tumor necrosis factor-α
CRIg, Complement receptor of the immunoglobulin superfamily
NKT cell, Natural killer T cell
pDC, Plasmacytoid dendritic cell
NKCR, Natural killer cell receptor
KIR, Killer immunoglobulin-like receptor
T_{conv}, Conventional T cell
CFA, Complete freunds adjuvant
B6, C57BL/6 mouse
IFN-γ, Interferon-γ
$Foxp^{sf}$, *scurfy*
T_{eff} cell, Effector T cell
TGF-β, Transforming growth factor-β
MCMV, Mouse cytomegalovirus
CyTOF, Time-of-flight mass cytometry
ITIM, Immunoreceptor tyrosine-based inhibitory motifs
ITAM, Immunoreceptor tyrosine-based activating motif
RNASeq, High throughput RNA sequencing
MFI, Mean fluorescence intensity
SPADE, Spanning-tree progression analysis of density-normalized events
MGD panel, Myeloid-granulocyte-degranulation panel
BMDC, Bone-marrow-derived dendritic cell
TLR, Toll-like receptor
ILC, Innate lymphoid cell
cAMP, cyclic AMP

Chapter 1: Adaptive and Innate Contributions to Autoimmunity

1.1 Immune tolerance and autoimmunity

The vertebrate immune system contains a diverse inventory of genetic, epigenetic, molecular and cellular mechanisms dedicated to distinguishing and to determining appropriate responsiveness to "self" and "non-self". The evolutionary necessity for such a system is a direct manifestation of our best model of adaptive immunity termed "clonal selection theory." This model was first postulated by Burnett and Jerne in the 1950s and has since been supported by decades of evidence (Hodgkin et al., 2007). In an effort to explain the immune system's ability to generate lymphocytes capable of combating a diverse repertoire of pathogens, the authors hypothesized that a "spontaneous production of random specificities must take place" (Jerne, 1955). In brief, their model proposed an immune system which generated a relatively low frequency of each "random specificity," but that this low frequency expanded upon recognition of its target antigen. The "randomness" that Burnett and Jerne first described was later confirmed to occur during the generation of B and T cell receptors (BCR and TCR, respectively) on developing lymphocytes. The evolutionary fitness of such a system is clear in that it grants organisms with recognition capabilities against novel foreign pathogens. The theory, however, also implies an obvious fitness consequence in that randomness within the immune response creates the potential for collateral or direct targeting of self antigens. The elimination or disablement of these self-reactive lymphocytes following their genesis, designated as central tolerance, partially explains how such a system remains viable. However, it is clear that central tolerance is an imperfect system that allows for auto-reactive lymphocytes to escape into the

periphery. Once in the periphery, these autoreactive lymphocytes are free to encounter their cognate self-antigen and to cause autoimmunity (Mueller, 2010; Mathis and Benoist, 2010). Because of this imperfect system, mechanisms of peripheral tolerance are necessary to control aberrant immune responses.

Although a formidable backup plan, peripheral tolerance can also breakdown. The many consequences of a collective failure in central or peripheral tolerance are exhibited by the numerous clinical manifestations collectively termed "autoimmune disease." Experimental evidence supporting a complementary role for each system is best illustrated by the discovery that mice double deficient for *Aire* (a critical mediator of thymic central tolerance) and *Foxp3* (required for the development of Regulatory T (T_{reg}) cells, further described in detail below as a dominant mediator of peripheral tolerance) develop significantly worse disease than either individual genetic perturbation (Chen et al., 2005a; Mathis and Benoist, 2010). Interestingly, even in the double-deficient animals, not all organs are affected. This heterogeneous disease pattern supports a model for peripheral tolerance in which a breakdown in multiple regulatory pathways, including anatomical barriers, are required for tissue-specific diseases.

1.2 Foxp3: a dominant mediator of peripheral tolerance required for T_{reg} cells

T_{reg} cells are now widely accepted as primary controllers of immune-responsiveness and peripheral immunological tolerance (Rudensky, 2011). Initially, the existence of an auto-regulatory "suppressor" population was wrought with controversy, prior to the identification of a rare (5-10%) population of CD25-expressing CD4+ T cells capable of preventing neonatal-

thymectomy-induced multi-organ autoimmunity (Sakaguchi et al., 1995) and gastritis (Suri-Payer et al., 1998). Due to its controversial past, it was not until follow-up studies were published identifying specific T_{reg} cell functional immuno-modulatory cytokines such as interleukin (IL)-10 (Papiernik et al., 1997), and receptors such as CTLA-4, that the general consensus shifted towards acceptance (Sakaguchi, 2000). A landmark finding came in a series of genetic studies identifying mutant *Foxp3*/*FOXP3* as the cause of the *scurfy* disease in mice and IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked inheritance) disorder in humans – each characterized by X-linked inheritance, systemic lymphoproliferation as well as autoimmune-mediated wasting. (Chatila et al., 2000; Brunkow et al., 2001; Bennett et al., 2001; Wildin et al., 2001). Multiple follow-up studies confirmed that *Foxp3* uniquely identified and was required for the development of the CD4+CD25+ suppressive cells, and was partially sufficient to promote regulatory function in otherwise non-suppressive cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). The specific depletion of T_{reg} cells using transgenic animals, generated by expressing diphtheria toxin receptor (DTR) under the promoter elements of *Foxp3,* confirmed the absolute requirement for T_{reg} cells throughout the lifespan of mice to control systemic peripheral tolerance (Kim et al., 2007; Lahl et al., 2007). Notably, Kim and colleagues also described the expansion of multiple populations of immune cells downstream of T_{reg} cell ablation besides T cells, including B cells, dendritic cells (DCs), neutrophils, macrophages, and natural killer (NK) cells. These findings supported a broad suppression profile for T_{regs} cells that play a critical role in the maintenance of peripheral tolerance, by suppressing an otherwise autoreactivity-prone immune system.

1.3 Type 1 diabetes (T1D): the autoimmune manifestation of a break in tissue-tolerance for innate and adaptive immunity

1.3.1 T1D overview: mouse models and the role of adaptive immunity

T1D is caused by destruction of insulin-producing β cells within the pancreatic islets of Langerhans. As the biomass of β cells in the islets is diminished, lack of insulin production leads to impaired glucose homeostasis and hyperglycemia (Tisch and McDevitt, 1996). T1D is a complex polygenic disease with the strongest genetic influence, like many autoimmune diseases, coming from the human leukocyte antigen (HLA) region, and contributions from over 50 other genomic loci (Xie et al., 2014). However, genetic predisposition is not sufficient to cause T1D, illustrated by the fact that monozygotic twins have only 50% disease concordance rates (Barnett et al., 1981). The remaining disease susceptibility presumably stems from epigenetic and environmental influences (although the potential contribution of stochasticity such as during the generation of the BCR and the TCR remains unclear). Perhaps the most alarming evidence for an environmental influence on T1D is the dramatically increased prevalence of disease over the last several decades (Vehik and Dabelea, 2011) – significantly faster than could be accounted for by alterations in population-level genetics.

Due to both the scarcity of human pancreatic tissue samples, and tools capable of effectively predicting clinical disease, much of the mechanistic understanding of T1D etiology comes from animal models, particularly the nonobese diabetic (NOD) mouse. In brief, autoreactive T cells to numerous T1D-related antigens escape selection in the thymus and migrate to the pancreatic lymph node (PLN), where they encounter and are activated by their

cognate antigen beginning around 2-3 wks of age (Mathis and Benoist, 2012). Upon activation, these autoreactive T (and B) cells invade the pancreatic tissue and establish residence near and within islets. B and T cells are both clear roleplayers in T1D pathogenesis as experimental elimination of either cell type in mice ameliorate disease (Christianson et al., 1993; Yang et al., 1997; Noorchashm et al., 1997). This stage is often denoted as "checkpoint 1" in mice. Strong evidence supports the notion that the establishment of a robust insulitis does not necessitate the eventual progression to clinical diabetes ("checkpoint 2").

In any given NOD colony, although 100% of mice usually develop insulitis, many animals will remain disease-free, including both males and females. The lack of disease determinism following insulitis points to tissue-level peripheral tolerance mechanisms protecting the animals from local inflammation and islet destruction. Around this stage, NOD mice present with serum autoantibodies, which are considered clinical predictors of diabetes susceptibility. Interestingly, in humans, not all new-onset patients have serum autoantibodies (Bingley, 2010), and not all autoantibody-positive individuals present with T1D (Knip et al., 2010). Together, these phenomenon support a model in which multiple immuno-regulatory checkpoints exist downstream of the generation of autoreactive lymphocytes, especially in the local tissue environment. Whether or not the same checkpoints exist in humans as they do in mice, and more broadly, whether or not T1D is the same disease in humans and mice, is currently under debate. Human T1D could be a set of disease etiologies all leading to the eventual destruction of β cells and hyperglycemia.

The strongest evidence for multiple checkpoints during disease came from transgenic derivatives of the NOD mouse model, such as transgene-encoded, cloned, islet-specific TCRs. These models were further useful in that they provided a reductionist view of the role of

individual autoreactive TCR specificities in disease. One such example was the BDC2.5 model, derived from a CD4+ T cell clone restricted to the NOD major histocompatibility complex (MHC)-class-II Ag7 molecule (Haskins et al., 1988; Gonzalez et al., 1997b). The BDC2.5 TCR clone is specific for the pancreatic antigen: chromagranin A (Stadinski et al., 2010). Supporting its utility as a mouse model in a more accurate representation of human disease, chromogranin A has also been confirmed as an autoantigen in human T1D (Gottlieb et al., 2014). The BDC2.5 transgenic line has proven a rich resource for the discovery of genes molecules and cells responsible for pathogenicity of autoreactive T cells (Luhder et al., 1998; Kanagawa et al., 2002; Poirot et al., 2004). When the BDC2.5 TCR transgenes are preferentially expressed on the NOD genetic background, a drastic increase in the rate of islet invasion occurs. Whereas in a normal NOD mouse, T cells stereotypically invade the islets at 15-18 days of age and progressively accumulate over the course of several months, the BDC2.5/NOD line has an immediate and massive infiltration – to the degree of invasion that a normal NOD islet, even in the later stages of disease, may never achieve. However, progression to diabetes occurs rarely (10-20%), and only months later, reflecting strong immunoregulation (Gonzalez et al., 1997a).

1.3.2 An emerging role for innate immunity during T1D

Conventional dogma implicates B and T cells, comprising adaptive immunity, as the primary cells responsible for autoimmunity. However, emerging research now supports a more complex cooperation of innate and adaptive immunity to mediate autoimmune disease. For example, canonical innate immune signaling was implicated in systemic autoimmune diseases

such as lupus and rheumatoid arthritis upon the discovery that these patients contained elevated peripheral blood type 1 interferon (IFN) signatures compared to healthy controls. (Ronnblom and Eloranta, 2013). Innate immunity was also implicated during the pathogenesis of organ-specific autoimmune diseases such as T1D through numerous immunological mechanisms. Antigen presenting cells (APCs), such as DCs, impacted disease through delivery (Turley et al., 2003) as well as presentation of islet antigens in the PLN (Hoglund et al., 1999). Furthermore, early work indicated that macrophage-derived tumor necrosis factor (TNF)-α and type 1 IFN signaling were involved in disease by blocking macrophage invasion into the islets (Hutchings et al., 1990). However, more recent work implicated local pancreatic macrophage in T1D protection via their expression of the complement receptor of the immunoglobulin superfamily (CRIg) (Fu et al., 2012). The diversity of macrophage in the pancreas as well as the respective roles each subsets plays during diabetogensis is still under debate. Innate-like natural killer T (NKT) cells are considered protective in T1D given that supplementation of NKT cells or transgenic expression of their TCR protected NOD mice from disease (Hammond et al., 1998; Lehuen et al., 1998). A recent controversial study proposed that innate immunity (including neutrophils and plasmacytoid DCs [pDCs]) initiated T1D (Diana et al., 2013). Finally, an emerging literature points to a pathogenic role for NK cells, traditionally considered innate immune cells. In these studies, NK cells were correlated to the aggressivity of inflamed islet lesions in acute models of T1D (Alba et al., 2008; Tang et al., 2008; Feuerer et al., 2009b) and the onset of disease in NOD mice (Gur et al., 2010), discussed further, below.

The exact role of innate immunity in T1D is still under investigation. However, a common theme from these studies is that innate immune function often results in the modulation of adaptive immune responses. Given these findings, a current model for T1D necessitates the

inclusion of crosstalk between innate and adaptive immunity. In this dissertation, we explore the role and regulation of NK cells during the pathogenesis of T1D – with a particular focus on NK cell crosstalk with the adaptive immune system.

1. 4 NK cells and T1D

1.4.1 NK cell biology

NK cells represent the third major arm of the lymphocyte lineage (along with B and T cells) and are historically described as having two primary effector functions: cytokine production and cytotoxicity, encompassing a critical share of the body's defense against microbial infection and cancer (Vivier et al., 2008). NK cells are traditionally described as members of "innate" immunity given their propensity for rapid immune responses and lack of randomized receptor rearrangement. However, a growing body of evidence supports the notion that NK cells have immunological "memory" (Paust and von Andrian, 2011; Sun et al., 2014), placing NK cells as a bridge between innate and adaptive immunity (discussed further, below). Furthermore, often overlooked in the field of immune tolerance, NK cells are critical players in the recognition of self, non-self as well as "altered self."

The primary cell-intrinsic form of self-recognition by NK cells is mediated by a variety of activating and inhibitory receptors, collectively known as the NK cell receptors (NKCRs). The biological significance of the NKCR system is illustrated by the fact that the NKCR system evolved convergently in multiple species (mainly the Ly49 family in mice, the killer cell

immunoglobulin-like receptor (KIR) gene family in humans and the CD94/NKG2 family in both) (Lanier, 2008). These receptors recognize cellular markers of stress as well as MHC:peptide molecules. Depending on the context of an MHC molecule-NK cell interaction, NK cells can be activated or inhibited via these receptors. Cell extrinsic regulation also guides the activation state of NK cells. Like most immune cells, there is a significant amount of crosstalk that takes place between NK cells and the rest of the immune system. This communication network includes DCs and macrophages as well as conventional T (T_{conv}) cells, T_{reg} cells, and NKT cells (Lehuen et al., 2010). The combination of intrinsic and extrinsic stimuli leads to NK cell activation, including inflammatory cytokine production and cytotoxicity, or NK cell inhibition (a continuation of the tolerant state).

1.4.2 NK cells in autoimmunity and T1D

Given their well-described inflammatory and immunomodulatory role, as well as their propensity to kill autologous cells in rare contexts (Hansson et al., 1981; King, 2000; Della Chiesa et al., 2003), NK cells have been hypothesized as mediators of autoimmunity (Johansson et al., 2005). In reality, very few biological scenarios where NK cells kill healthy self-tissue have been discovered. NK cells were first directly linked to T1D since early studies indicated that rat NK cells killed islet cells in culture (MacKay et al., 1986). However, the earlier studies focusing on the role of NK cells in diabetes were full of confounding data. For example, although depletion of NK cells in induced models of diabetes (streptozotocin and cyclophosphamide) indicated that they were required for the disease (Maruyama et al., 1991a;

Maruyama et al., 1991b), the earliest results in rat genetic models did not agree (Ellerman et al., 1993). Further confusion came with findings in NOD mice showing that diabetes progression can be prevented with injection of Complete Freunds Adjuvant (CFA) (McInerney et al., 1991), the effects of which were shown to be mediated by NK cells (Lee et al., 2004). It is important to note that many of the experimental tools (such as antibodies) used in these early studies were subsequently shown to not be NK cell-specific.

The strongest evidence supporting a role for NK cells in T1D came from more recent studies focusing on pancreatic NK cells. Multiple studies have agreed that NK cells are readily found in the pancreas of NOD mice, some reporting evidence of NK cells even in the absence of autoreactive B and T cells (Brauner et al., 2010; Beilke et al., 2012). In both of these studies, the phenotype of pancreatic NK cells was explored. Compared to spleen and PLN, pancreatic NK cells had a distinct mature phenotype and were highly proliferative. Despite their activated phenotype (including increased CD25, CD69, and KLRG1 expression), pancreatic NK cells were hyporesponsive to activating stimuli – which paralleled an "exhausted" T cell phenotype.

Poirot and colleagues explored the BDC2.5 T cell receptor transgenic mouse line in the NOD and C57BL/6.H-2^{g7} (B6.H-2^{g7}) backgrounds, which displayed innocuous and destructive forms of autoimmunity, respectively. Their efforts were aimed at differentiating these insulitic lesions to determine what makes an aggressive lesion unique. They described that the more aggressive form of insulitis contained higher proportions of NK cells and NK cell specific transcripts (specifically the Ly49 family) compared with the control NOD background (Poirot et al., 2004). Because the MHC haplotypes between these strains were identical (each contained the NOD haplotype of MHC), the influence of MHC ligands was ruled out. This finding has since been corroborated in numerous models of aggressive/acute T1D (Alba et al., 2008; Feuerer et al.,

2009b; Tang et al., 2008). Collectively, this correlation might support an immune-modulatory role for NK cells to impact the aggressiveness of the disease. Importantly, depletion of NK cells using anti-asialo GM1 or anti-NK1.1 monoclonal antibody treatment protected from disease and/or dampened T cell activation in these studies, supporting a functional role for NK cells during acute forms of disease. Almost no evidence exists describing the mechanistic role of NK cells in the pancreas. However, one study reported that depleting NK cells or neutralizing IFN-γ following T_{reg} cell ablation dampened pancreatic $CD4^{+}$ T cell activation and substantially delayed the onset of diabetes, respectively (Feuerer et al., 2009b).

The proposed functional role for NK cells recently came under fire by Beilke and colleagues who showed little effect when NK cells were depleted in the NODNK1.1 mouse model (Beilke et al., 2012). However, it is noteworthy that their treatment protocol lasted only from the 3-10 week age range and was cut off before the onset of disease (>12 weeks). The age range is noteworthy because the treatment protocol effectively missed two critical stages in T1D. First, the initial activation of autoreactive T cells which occur between day 14 through 18 following birth. Second, the treatment missed the later stages of insulitis maturation and disease onset. Because of these deficiencies, whether or not NK cells participate in activation of autoreactive T cells in the PLN or the onset of disease remains unclear. Importantly, given that NK cell frequency best correlates with the onset of disease in NOD mice (Gur et al., 2010), depleting NK cells during this timeframe is critical to drawing any conclusions about their function.

In contrast, Angstetra and colleagues studied the NOD4.1 model which preferentially expressed the transgene-encoded NOD4.1 TCR, restricted to the NOD MHC-class-II Ag7 molecule (Verdaguer et al., 1997). They assessed whether or not NK cells acted as the functional

killing arm in this model using NK cell depletion over 20wks, and discovered significant protection by doing so. However, they also transferred BDC2.5 T cells into an NK cell-deficient NOD host and found that the absence of NK cells did not change the disease occurrence. Furthermore, *in vitro* studies indicated that NK cells were incapable of killing β cells from MHC sufficient or deficient animals. Their data, (similar to Fuerer *et.al.*) supported an immuno-modulating role for NK cells in T1D, rather than a direct killing of islet cells (Angstetra et al., 2012).

NK cells were reported to have a unique phenotype in human T1D patients. One study compared recent-onset and long-standing blood samples and found a small but distinct reduction in peripheral blood NK cell numbers of patients with recent-onset disease. These cells also displayed the most wide-ranging expression of IFN-γ. In contrast, the long-standing diabetic patients had the lowest levels of activating receptors NKG2D, NKp46 and NKp30 (Rodacki et al., 2007). These data supported earlier work that indicated reduced NK cell cytotoxicity in patients with long-standing T1D (Lorini et al., 1994). Whether or not the "altered" NK cell phenotype in human T1D is a result of the disease or a potential cause is still unknown. One explanation might be that NK cells in long-standing T1D patients have an "exhausted" phenotype due to an undocumented hyperactivation in the pancreas at the onset of insulitis or β cell destruction. Another hypothesis is that the phenotype of NK cells in T1D patients are an inconsequential result of a dysfunctional immune system in an autoimmune-prone individual.

The preponderance of evidence points to NK cells playing a role in T1D. Despite their name, NK cells likely do not kill β cells. Rather, NK cells might support or cause an inflammatory environment in the pancreas that influences disease onset. The small line of evidence supporting a protective role for NK cells during CFA treatment is noteworthy, but

represents a highly contrived setting not representative of the normal spontaneous disease in NOD mice. However, this confounding data might support a context-dependent role for NK cells in T1D – subject to where and how NK cells are activated

.

Chapter 2: How Regulatory T Cells Control Pancreas-Infiltrating NK Cells to Prevent Islet Inflammation and Autoimmunity

2.1 Publication Reference (see appendix A for a full original reprint)

Jonathan Sitrin, Aaron Ring, K. Christopher Garcia, Christophe Benoist and Diane Mathis (2013). Tregs Control NK Cells in an Insulitic Lesion by Depriving them of IL-2. The Journal of Experimental Medicine 93:2260. doi:10.1084/jem.20122248

2.2 Additional Introduction

The immune system requires a complex set of central and peripheral tolerance mechanisms in order to appropriately respond to foreign pathogenic insult while simultaneously maintaining self tolerance. T_{reg} cells are a critical player in the lifelong maintenance of that tolerance and utilize a variety of mechanisms in order to suppress inappropriate immune responses. When T_{reg} cells fail, autoimmune diseases such as T1D develop. Many immune cells are subject to T_{reg} cell control, including NK cells (Kim et al., 2007; Feuerer et al., 2009c), but the molecular basis for this regulation was unknown. A widely-accepted paradigm in immunology is that innate immunity instructs adaptive immunity via inflammatory cytokine secretion, and activation of antigen presentation machinery. The discovery that T_{reg} cells control the innate immune compartment was one of the findings that altered that model. A more accurate model for immune tolerance includes innate and adaptive immune-cell cross-talk. However, the exact mechanisms used by innate and adaptive immune cells to signal to one

another were not well understood. Specifically, how T_{reg} cells control NK cells in the pancreas was unclear, and whether or not there were tertiary cells involved was unknown. In this chapter, we explored this molecular interaction and uncovered a novel mechanism for innate and adaptive immune-cell cross-talk involved in diabetes protection.

2.2.1 The protective role of T_{reg} cells in T1D

Diminished T_{reg} cell function has been implicated in numerous human autoimmune diseases, including T1D (Buckner, 2010). Supporting this concept, there is a drastic overrepresentation of T1D in patients with mutations in *FOXP3* (Wildin et al., 2002; Torgerson and Ochs, 2007; Moraes-Vasconcelos et al., 2008). While the association of T_{reg} cells and autoimmune diseases is clear, the specific role T_{reg} cells play in each autoimmune setting, and how T_{reg} cell suppressive function is diminished remains unclear.

The earliest line of evidence supporting a protective role for T_{reg} cells in NOD mice was the discovery that the accelerated disease found in CD28-deficient NOD mice could be explained by the lack of CD4+CD25+ cells expressing CTLA-4 (Salomon et al., 2000). In this study, adoptive transfer of (what were later confirmed to be Foxp3-expressing) T_{reg} cells delayed or completely protected these mice. This finding supported earlier studies implicating CTLA-4 in the pathogenesis of T1D using monoclonal antibody blockade (Luhder et al., 1998)– although at the time, the effect of anti-CTLA-4 treatment on T_{reg} cells was unknown.

While the protective effect of T_{reg} cell adoptive transfer was confirmed in numerous studies, many of these studies also noted that transfer of T_{reg} cells did not affect the formation of

robust insulitic lesions (Tang et al., 2004; Tarbell et al., 2004; Herman et al., 2004). These findings implicated local pancreatic suppression as the primary mechanism supporting T_{reg} cell-mediated protection from T1D.

Supporting this concept, efforts to locally expand or boost T_{reg} cells have also proven fruitful in the prevention of T1D in NOD mice. Numerous signaling pathways and strategies involved in T_{reg} cell function, activation and homeostasis were invoked to accomplish this task. These pathways included treatment with IL-2 (Rabinovitch et al., 2002; Tang et al., 2008; Grinberg-Bleyer et al., 2010), retrovirus-induced local production of IL-2 (Johnson et al., 2013), alternative expression of CTLA-4 splice variants (Vijayakrishnan et al., 2004), and islet-specific pulsed TGF-β (Peng et al., 2004). In all of these studies, boosting T_{reg} cell number, frequency or function protected T1D models from disease.

2.2.2 Exploring where and how T_{reg} cells protect from T1D

Where and how T_{reg} cells exert there protective effect during diabetogenisis has become clearer over the last several years. Two experimental strategies were crucial for this determination. First, the BDC2.5 TCR transgenic model was crossed to the Foxp3-deficient *scurfy* mutant mouse (Foxp^{sf}.BDC2.5/NOD) to generate T_{reg} cell-deficient T1D-prone animals. In this model, the absence of T_{reg} cells did not change the kinetics or phenotype of the initial T effecter (T_{eff}) cell priming phase and did not alter the trafficking of T_{eff} cells to the islets. However, these animals developed a highly penetrant and aggressive form of T1D, indicating that the insulitis was immediately destructive compared with the normally longstanding

protection in a Foxp3-sufficent setting. These data supported a model in which T_{reg} cells impinged on the local activation of T cells at the site of inflammation and had little effect on priming in the PLN (Chen et al., 2005b). However, a major critique of this study was the constitutive absence of T_{reg} cells.

The second crucial study that uncovered a functional role for T_{reg} cells during T1D protection entailed a mouse expressing the DTR under the promoter/enhancer elements of *Foxp3,* in conjunction with the BDC2.5 TCR transgenic mouse line (BDC2.5/NOD.Foxp3^{DTR} mice). This model combined normal (T_{reg} cell-sufficient) immune system hematopoiesis, T cell activation in the PLN and insulitis formation with the ability to perturb T_{reg} cells in a punctual and specific manner. In doing so, this model also allowed for the careful molecular delineation of the downstream effects of T_{reg} cell loss because genes that changed immediately following T_{reg} cell ablation were presumed to be indicative of the molecular programs that T_{reg} cells were continually suppressing. T_{reg} cell ablation provoked nearly 100% penetrance of diabetes within days (Feuerer et al., 2009c), confirming the absolute and continual requirement for T_{reg} cells to guard against T1D. Analysis of the insulitic lesion revealed, surprisingly, that the earliest detectable responders to the loss of T_{reg} cells were NK cells, which accumulated to a higher fraction of the infiltrate and produced IFN-γ within hours. Subsequently, there was increased activation of and IFN-γ production by diabetogenic $CD4^+$ T cells. Neutralizing IFN-γ or depleting NK cells dampened pancreatic $CD4^+$ T cell activation and substantially delayed the onset of diabetes. Thus, there seemed to be a direct and continual requirement for T_{reg} cells to keep NK cells, and ultimately diabetes, in check. However, it remained unclear exactly how T_{reg} cells controlled NK cell function.

2.2.3 Mechanisms of T_{reg} cell suppression and the control of NK cell functions in other contexts

T_{reg} cells utilize a wide variety of mechanisms to maintain peripheral tolerance. First and foremost, the very nature of commitment to the T_{reg} cell lineage is widely accepted as an immunosuppressive mechanism given the trend for autoreactive thymocytes to become T_{reg} cells (Hsieh et al., 2006). *Foxp3* expression inhibits the production of IL-2 while amplifying the production of CTLA-4 and CD25 in a cell-intrinsic fashion. The former has potent immunosuppressive function and the latter is critical for the maintenance, stability and function of the cell. Other basic mechanisms of suppression include inhibitory cytokine secretion, direct cytolysis, indirect targeting of antigen presentation, metabolic disruption and competition for IL-2 (Vignali et al., 2008). Whether these described mechanisms, or new mechanisms were relevant for the control of NK cell functions in the BDC2.5/NOD.Foxp3^{DTR} model was unclear.

T_{reg} cells produce a number of inhibitory cytokines including IL-10, transforming growth factor (TGF)-β and IL-35 that are potent inhibitors of immune cell activation *in vitro* and *in vivo*. TGF-β is particularly relevant given its described role in the suppression of NK cells in the Foxp^{sf} model (Ghiringhelli et al., 2005). This report also described an inverse correlation between T_{reg} cells and NK cell frequency and function in cancer patients. Similar correlations were seen in numerous other clinical settings, especially during other cancer treatments (Ghiringhelli et al., 2006) and during blood cell transplantation (Trzonkowski et al., 2004). Furthermore, transfer of T_{reg} cells into tumor models prevented NK cell anti-tumor activity (Shimizu et al., 1999; Smyth et al., 2006) and transplant rejection (Barao et al., 2006). In these studies, when modeled *in vitro,* T_{reg} cells were potent suppressors of NK cell cytotoxicity but T_{reg}

cell function was contact dependent, blocked using anti-TGF-β monoclonal antibodies and was insensitive to formaldehyde fixation (Ghiringhelli et al., 2005). While these data supported a suppressive mechanism involving direct contact between T_{reg} cells and NK cells, one further study showed that impairment of T_{reg} cells resulted in the activation of NK cells through indirect activation of DCs and increased expression of IL15Rα (Terme et al., 2008). Whether or not the control of NK cells by T_{reg} cells was mediated through direct TGF-β signaling or indirectly through APCs was unclear, but explored further in our study.

2.3 Additional Discussion

We described a model in which T_{reg} cells, through expression of CD25 and sequestration of IL-2, act as gatekeepers of tolerance and immune function in the pancreas. From this model, we conclude that the level of IL-2, as well as its availability to certain IL-2R-expressing cells of different affinities, is paramount to the determination of autoimmunity versus tolerance. In our proposed scenario, an active competition for limiting cytokine acts as a buffer between local T_{reg} cells and NK cells, but also likely for local CD4+ and CD8+ T cells, DCs and B cells. Whether or not these cell types, or others, are active players in this competition should be explored in further detail.

The expression of CD25, required for functional T_{reg} cell generation, also enables T_{reg} cells to bind IL-2 with high affinity and sequester IL-2 away from other cells (de la Rosa et al., 2004; Barthlott et al., 2005; Scheffold et al., 2005; Pandiyan et al., 2007). This suppression-via-sequestration mechanism has been shown to dampen IL-2 production and proliferation, and to lead to apoptosis in T_{eff} cells (Wing and Sakaguchi, 2012). Notably, this mechanism does not require the presence of an APC or costimulation, and thus has the capacity to act directly and broadly on immune cells in many contexts.

The CD25-deficient mouse model develops a systemic lymphoproliferative syndrome similar to that of both the Foxp3-deficient and CTLA-4-deficient animals (Willerford et al., 1995). Initially, this finding was thought to be due to the lack of T_{reg} cells; however, follow-up studies using a Foxp3-GFP reporter highlighted a pool of Foxp3-expressing cells in these animals that could suppress effector cells *in vitro* (Fontenot et al., 2005). It remains unclear how much of the phenotype of CD25-deficiency can be explained by the lack of sequestration of IL-2

versus the lack of overall fitness and protective functions of T_{reg} cells. Our findings add a level of complexity to the growing body of work focused on T_{reg} cell-expressed CD25. As Fontenot and colleagues described, the lack of CD25 on T_{reg} cells is associated with reduced *Foxp3* expression and yields cells that are functionally “unfit” in the periphery (Fontenot et al., 2005). However, we and others have shown that CD25 itself acts in a functional capacity to limit immune responses. Whether or not this mechanism acts in a broader set of organs and disease settings remains to be explored. Also unclear is to what degree the wild-type *in vivo* suppressive capacity of T_{reg} cells is attributable to their sequestration of IL-2 compared with other suppressive mechanisms. Given that CD25 is highly expressed by T_{reg} cells in most settings, these cells likely act as IL-2 buffers wherever they reside, supporting the concept that CD25 acts as a “core module” of T_{reg} cell function as described by Wing and Sakaguchi (Wing and Sakaguchi, 2012).

In an effort to explain the systemic control of IL-2, one group proposed a “quorum sensing” model (Amado et al., 2013). In their study, deficiency in IL-2, and therefore T_{reg} cells, led to increased IL-2 production in T cells (as read out using IL-2 reporter mice). However, administration of IL-2 decreased the number of IL-2-producing T cells, diminished the IL-2 production in those cells and increased T_{reg} cell numbers. These findings experimentally supported a systemic auto-regulatory feedback loop controlling IL-2 availability. In brief, decreased IL-2 eventually yielded more IL-2 and vice versa. However, it is clear from our work that many other cell types can participate in this feedback loop or be subject to its imbalances (as an “input” or “output”) – notably NK cells that are subject to IL-2 availability and produce IL-2-promoting cytokines such as IFN-γ.

Our findings emphasize an intriguing cross-talk between innate and adaptive immunity with parallels to models of infection. NK cell production of IFN-γ was critical to the proper clearance of Leishmania major infection in one study (Bihl et al., 2010). In this model, it was discovered that CD4-deficient animals showed improper NK cell activation that was not the case for CD8-depleted animals. The origin of the NK cell-activating stimulus during infection was antigen-specific CD4+ T_{conv} cells that produced IL-2. An indirect role for APCs was also described via the engagement of the CD40/CD40L pathway upstream of IL-12 production. Neutralization of IL-2, together with IL-12, was the strongest inhibitor of this response. These findings offered support for a scenario similar to the one we proposed in that antigen-specific CD4+ T_{conv} cells created an IL-2-rich environment, with secondary extrinsic stimuli from DCs.

There are two major differences between our findings and the findings in the Leishmania major infection model. First, Bihl and colleagues found no change in the activation status of NK cells in their model when CD25+ T_{reg} cells were blocked and/or depleted (albeit with anti-CD25). Although activating IL-2 is a molecular mechanism shared between the two studies, the primary limiting factor controlling IL-2 in their system was likely the production of IL-2 rather than the post-secreted competition for IL-2. The second major difference involved the role of IL-12 produced by local myeloid cells. In Feuerer *et.al.*, preliminary data indicated a requirement for IL-12 for the normal response of NK cells to T_{reg} cell ablation. More precisely, anti-IL-12 treatment in conjunction with T_{reg} cell ablation decreased the production of IFN-γ by NK cells but did not alter the levels of granzyme B transcript. However, the kinetics did not match as a primary regulatory mechanism, because IL-12 production by DCs was slower than IFN-γ production by NK cells. Therefore, although an indirect role for IL-12 could have been partially responsible, it must have been secondary compared with control via competition for IL-

2. The difference in interpretation and conclusion might be due to the time points being analyzed in each study. In our study, the very earliest transcriptional responses (7-8hrs) were examined in order to make an isolated determination of primary mechanism without confounding effects from other activated cells. In the infection system, 12hrs and further were analyzed. This might seem like a small discrepancy but evidence from Feuerer *et. al* supported the notion that DCs did not produce IL-12 until after the 12hr timeframe. Importantly, pancreatic NK cells did showed signs of transcriptional activation prior to T_{reg} cell ablation in the BDC2.5/NOD model (unpublished observations). This mild stimulatory state could have provided the priming needed for NK cell responsiveness, including the induction of CD25 on NK cells. Supporting this concept, IL-12 was required for the upregulation of CD25 on NK cells during mouse cytomegalovirus (MCMV) infection (Lee et al., 2012). Together, T_{reg} cell ablation, in an environment of prior local inflammation, might act as the brake-release on an already "primed" cell.

Supporting a role for local inflammation for the full responsiveness of NK cells to the loss of T_{reg} cells, Beaulieu and colleagues recently reported that the induction of *Zbtb32* drives NK cell proliferation in an inflammatory cytokine-dependent manner (Beaulieu et al., 2014). In their study, IL-12, IL-18 and type 1 IFN induced *Zbtb32* expression – rather than activating receptor engagement. The combination of IL-12 and IL-18 was the most potent *Zbtb32*-inducing condition, which implied that a strong inflammatory setting was needed to drive maximal NK cell proliferation. Their findings might explain the variable kinetics differentiating pancreatic and splenic NK cells responding to T_{reg} cell ablation from our study or the differences between the BDC2.5/NOD.Foxp3^{DTR} and Foxp3^{DTR} models' rates of NK cell expansion (hours versus days) (Kim et al., 2007; Feuerer et al., 2009c). Those differences may be due to differential expression of *Zbtb32*. NK cells residing in an already-stimulated state, such as they are in the

inflamed pancreas, would be much more proliferative following a secondary driving stimulus such as the loss of T_{reg} cells, and newly available IL-2. Supporting this hypothesis, although *Zbtb32* was not significantly different in pancreatic versus splenic NK cells at baseline (unpublished observations), it was uniquely induced in the pancreas following the loss of T_{reg} cells (Sitrin et al., 2013).

Taken together, our studies and the study by Bihl and colleagues emphasized a tight molecular, cellular, and tissue-level control of IL-2, which may need to be kept within a tolerogenic balance to prevent systemic or tissue-specific autoimmunity. Reminiscent of a sieve with dynamic inputs and outputs, as long as IL-2 remains in a balanced input-output state, the tissue remains tolerant. However, a severe IL-2 decrease or increase pushes the system into an autoimmunity-prone state (the former due to an effect on T_{reg} cells and the latter due to NK and T_{eff} cells). These inputs/outputs could include transcriptional control of IL-2, the number of cells secreting IL-2, its secretion rate, the number of cells able to "experience" the IL-2 via IL-2R (including non-immune cells), the type of IL-2R complexes on those cells as well as the need for any co-activating cytokines or cell types. Future research efforts must take careful consideration into studying the local IL-2 availability in a given tissue in order to gain a better understanding about how a tissue can mount an appropriate immune response versus developing autoimmunity. The pancreas served as an instructive example in which low- and high-dose IL-2 had opposing effects on T1D. The former boosted T_{reg} cells and protected from disease while the latter caused mouse and human T1D (Tang et al., 2008; Long et al., 2012; Sitrin et al., 2013). To that point, T1D clinical trials dedicated to appropriate dosing of IL-2 were very recently undertaken (Waldron-Lynch et al., 2014).

Our study does not implicate a role for the T_{reg} cell contact-dependent TGF-β suppressive mechanism previously described in numerous cancer models. Why specific T_{reg} cell mechanisms are utilized under certain conditions (such as TGF-β in cancer) and not others (T1D) remains unclear. T_{reg} cells are now appreciated to be highly context-dependent immune suppressors. The mechanism explaining this contextualized function seems to be the co-opting of transcription factors previously identified to polarize T_{eff} cells based upon their stimulation and local environment. For example, T_{reg} cell conditional ablation of IRF4 (a transcription factor required for T_H2 polarization), STAT3 (required for T_H17 polarization) or Tbet (required for T_H1 polarization) led to aberrant immune control centered around each transcription factor's respective T_H cell polarization profile (T_H2, T_H17 and T_H1, respectively) (Zheng et al., 2009; Chaudhry et al., 2009; Koch et al., 2009). It remains possible that tumor T_{reg} cells and pancreatic T_{reg} cells prior to T1D-onset have distinct transcriptional or functional identities. Further analysis of the transcriptional programs from each population would yield a better grasp on how each cell suppresses the local immune compartment in a unique fashion.

Gasteiger and colleagues confirmed T_{reg} cell control of NK cells via an IL-2-dependent mechanism in two accompanying publications to our own (Gasteiger et al., 2013a; Gasteiger et al., 2013b). In their study, T_{reg} cells restrained the splenic expansion of a phenotypically immature NK cell due to the NK cell's preferential expression of CD25 – similar to what we described in the pancreas. With regards to NK cell tolerance, they presented compelling data showing that T_{reg} cell ablation did not enhance NK cell responsiveness to general activating self- and non-self ligands. However, depletion of T_{reg} cells did enhance NK cell targeting of "missing-self" ligands such as MHC class 1-deficient target cells – paralleling the increased cytotoxicity against MHC class 1-deficient targets in our model. Importantly, they mechanistically explained

these findings by showing that IL-2 enhanced NK cell conjugation with weaker target cells and increased Ca^{2+} flux upon target cell engagement.

The increased recognition of weaker target cells is particularly intriguing because it could explain the usefulness of IL-2-based cancer therapies. Despite the more recent body of work outlining the tolerogenic function of IL-2, it was initially described as an immuno-stimulatory molecule and has been used in stimulatory immunotherapy for years. Recombinant IL-2 was first approved for renal cell carcinoma and metastatic melanoma in 1992 and 1998, respectively (Rosenberg, 2014). There are two important clinical implications for the effects of IL-2 on NK cells. First, given that T_{reg} cell ablation could not provoke direct NK cell autoreactivity, even in the presence of a strong activating ligand, NK cells may be poor responders to some cancers (especially if those cancerous cells contain features of self-tissue). However, given that IL-2 enhanced NK cell killing of weaker (previously-tolerated) target cells, the opposite possibility could also be true: certain cancers might be great targets for IL-2-based NK cell therapies, even if NK cells are naturally non-responsive. Understanding how to manipulate NK cell responsiveness to target cells will be critical for appropriately designing cancer therapies moving forward.

One major difference between the findings from our study and that of Gasteiger and colleagues was the interpretation of whether or not the IL-2-based suppressive mechanism was direct or indirect. Rather than a semantic argument, these interpretations imply different, albeit not mutually exclusive findings. In our direct model, the NK cell-activating IL-2 was already available in the tissue and did not need to be newly produced by T cells. In the indirect model, T_{reg} cell loss provoked new IL-2 expression from T cells, which implied that T cell help was the limiting factor in this regulatory axis. Similar to Leishmania major infection with IL-12, the

differences for IL-2 might come down to timing. As previously discussed, the very immediate downstream effects of T_{reg} cell depletion included an immediate burst of IL-2 availability that was responsible for NK cell activation in the BDC2.5/NOD setting. Evidence from transcriptional profiling supported the notion that T cells produced new IL-2 sometime between 15 and 24 hrs (Feuerer et al., 2009c) but that the new protein was not yet apparent at 24hrs. The IL-2-induced NK cell activation in the Gasteiger model was likely due to new IL-2, produced after the 24hr timeframe (or the combination of the initial burst and secondary production). Both sources of IL-2 could contribute to the immune response following the loss or dysfunction of T_{reg} cells.

The sequestration of tissue-available IL-2, as we report, represents a novel mechanism explaining T_{reg} cell control of NK cell function. More broadly, this regulatory mechanism also adds to the growing body of literature describing the complexity of innate and adaptive immune-cell crosstalk. In the future, it would be advantageous to determine whether or not this regulatory axis functions in other contexts, as well as to determine if this mechanism is similarly utilized by the human immune system.

Chapter 3: Profiling of the Ly49 Receptors and NK Cell Heterogeneity in T1D

3.1 Introduction

3.1.1 Historical definitions and heterogeneity of NK cells

Unlike T and B cells with randomly generated receptors, NK cell heterogeneity is currently accepted as germline encoded, implying a theoretical "maximum" heterogeneity. The question of NK cell heterogeneity has been historically complex. Part of the lack of understanding can be attributed to the variable historical definition of NK cells. NK cells in mice have been defined by the lack of expression of CD3 combined with the expression of the Ly49 family, the NKG family (such as NKG2D), the NKR family (such as NK1.1) and most recently by the NKp46 receptor. Human NK cells have traditionally been defined by their expression of CD56 and CD16. NK cells have also been defined by their anatomical locations, including the bone marrow, thymus, spleen, liver, lung, lymph nodes, pancreas and placenta, as well as by their dependency on certain cytokines, such as IL-15. More recently, NK cell definitions have included dependency on certain transcription factors. For example, thymic NK cells depend on *Gata-3* for differentiation, whereas blood and splenic NK cells do not (Vosshenrich et al., 2006). Circulating liver and splenic NK cells depend on *Nfil3,* whereas resident liver, skin and uterine NK cells remain present in *Nfil3*-deficiency. Resident liver and skin NK cells depended on *Tbet* whereas uterine NK cells remained present in the *Tbet*-knockout animals (Sojka et al., 2014a). A better understanding of the molecular cues required for NK cell subset differentiation is required for understanding the true heterogeneity of this population.

3.1.2 A modern view of NK cell heterogeneity using mass cytometry

A recent technological advancement enabled a much clearer picture for the phenotypic diversity of NK cells. Horowitz and colleagues utilized the novel time-of-flight mass cytometry (CyTOF) technology on human peripheral blood, including 5 pairs of monozygotic twins. Their analysis included 28 different receptors commonly expressed by NK cells (with a theoretical maximum of 2^{28} possible combinations of markers). Their data indicated a vastly greater phenotypic heterogeneity than had ever been described before. No single NK cell phenotype could account for more than 7% of total NK cells. Furthermore, the top 50 phenotypes represented only ~15% of total NK cells. The study also found a high concordance of inhibitory receptor expression between identical twins, not true of the activating receptors. This finding invoked a proposed model in which genetics were strongly deterministic for inhibitory receptor patterns (and tolerance to self) whereas environmental cues impacted the expression of activating receptors (and recognition of non-self). The total diversity was estimated to be between 6,000 and 30,000 distinct human NK cell phenotypes within an individual and 108,000-125,000 at a population level (Horowitz et al., 2013). These data vastly expand our view of NK cell phenotypic diversity and emphasize an environmental responsiveness in NK cell phenotypes.

3.1.3 NK cell education and tolerance through expression of Ly49 receptors

NK cells are sensitive to their environment, especially through interactions of the Ly49 family of receptors and cells expressing MHC class I molecules. The inhibitory Ly49 receptors,

containing transmembrane immunoreceptor tyrosine-based inhibitory motifs (ITIMs), are expressed in a variegated fashion on single NK cells and can show great variability between individual cells (Nash et al., 2014). This variegated pattern is thought to arise during bone marrow differentiation when NK cells first engage MHC class I molecules – the point at which they become "educated." This model, often called the "arming" model, postulates that the education process tunes NK cell reactivity in the periphery and prevents autologous cell killing. Once in the periphery, NK cells continually interact with local autologous cells in a cell-to-cell trans-fashion, at which point two major outcomes are possible. If the target cell has normal MHC class I expression, with a non-activating peptide, direct engagement of ITIM-bearing receptors leads to the intracellular recruitment of inhibitory tyrosine phosphatases and the prevention of immune synapse formation (Jaeger and Vivier, 2012). However, in a non-self (virus) or transformed self (cancer) setting, NK cells recognize either the altered MHC:peptide repertoire, the lack of MHC class I expression or an overexpression of activating ligands. The lack of inhibitory signal or the excess of activating signal through immunoreceptor tyrosine-based activating motif (ITAM)-containing receptors, such as CD3ξ or DAP10/12, leads to proliferation, cytotoxicity and often inflammatory cytokine production (Lanier, 2005). This balance of activating and inhibitory receptors, along with the ability of NK cells to respond to the inflammatory milieu in tissues, outlines the need for NK cells to be tuned in an effective way to remain tolerant to self.

The NK cell education process is best illustrated by two major findings. First was the discovery that NK cells from MHC-I-deficient mice were hyporesponsive in killing capacity (Bix et al., 1991). This finding can be partially explained by follow-up studies showing that full activation of NK cells to target cells required co-engagement of their activating receptors

(Cerwenka et al., 2001; Diefenbach et al., 2001; Arase et al., 2002; Smith et al., 2002; Sun, 2010). The second discovery identified a small fraction (~15%) of B6 NK cells that lacked any inhibitory Ly49 expression. Although these NK cells maintained a mature phenotype, they exhibited hyporesponsiveness when engaged through activating receptors (Fernandez et al., 2005).

Unlike B and T cell differentiation which is appreciated to be clonotypically restricted following proper BCR and TCR rearrangement and expression, NK cell education is considered to be highly dynamic. The best evidence supporting a non-terminal NK cell education process came from a set of studies describing NK cells from MHC-deficient animals regaining function and normal responsiveness following their transfer into MHC-sufficient hosts. Additionally, MHC-sufficient NK cells can be transferred into MHC-I-deficient hosts and eventually lose responsiveness (Joncker et al., 2010; Joncker et al., 2010; Sun, 2010). Interestingly, this tolerant state can be broken by a highly proinflammatory stimulus such as MCMV infection, in which previously tolerant NK cells rapidly reject the MHC-deficient cells of chimeric mice (Sun and Lanier, 2008). Collectively, these findings support a highly complex, dynamic and environment-dependent model for NK cell education and tolerance, in an effort to keep NK cell-responsiveness in check.

3.1.4 NK cell receptors in T1D: NKG2D and NKp46

NK cell receptors are critical cell intrinsic regulators of NK cell responsiveness that have been implicated in the control of viruses (Schenkel et al., 2013; Rahim et al., 2014), tumor

surveillance (Tu et al., 2014), allergies (Kusnierczyk, 2013) and autoimmunity (Rogner et al., 2001; Carnaud et al., 2001). Exactly where and how these receptors exert their disease influences is currently under investigation. With regards to T1D, two groups have identified potential mechanistic functions for NK cell receptors, including NKG2D and NKp46.

The discovery that NKG2D stress ligands are expressed by islet cells in the pancreas of prediabetic NOD mice opened up the possibility that NK cells directly target and kill these stress-ligand-expressing cells. In line with that hypothesis, blockade of NKG2D using non-depleting monoclonal antibody treatment in NOD mice significantly delayed diabetes. However, the authors attributed that protection to the inhibition of autoreactive CD8+ T cell activation rather than through an effect on NK cells (Ogasawara et al., 2004). In addition, the role of NKG2D in T1D recently came under fire from a study showing that anti-NKG2D treatment and NKG2D deficiency had no protective effect in either the BDC2.5/NOD or NOD.NK1.1 T1D models (Guerra et al., 2013). It remains unclear exactly how NKG2D participates in T1D. The discrepancy in NKG2D-focused research in T1D may point to environmental cues altering the levels of stress ligands (NKG2D targets) on β cells from different facilitates but more work is needed in this area.

A second line of research identified NKp46 ligands in the pancreas and showed that an NKp46 fusion protein "stained" β cells in the islets of prediabetic NOD mice. Furthermore, NKp46 deficiency was associated with protection from streptozotocin-mediated T1D (but keep in mind that the relevancy of this induced model to natural T1D is suspect). This study also confirmed a defect in NK cell killing of β cells *in vitro,* and less degranulation of pancreatic NK cells *in vivo* due to NKp46 deficiency. To support the relevance of NK cells in the natural NOD disease course, they correlated NK cell frequency with hyperglycemia onset (Gur et al., 2010).

The authors did not identify the ligand relevant to NKp46 binding, but did identify the specific regions (membrane proximal domain) and residues (Thr^{125} and Asn^{216}) of NKp46 required for binding to β cells in a follow-up study (Gur et al., 2011). From a functional standpoint, injection of an NKp46-Fc fusion protein protected NOD mice from disease. The investigators assigned this protection to the endogenous generation of anti-NKp46 blocking antibodies. Whether or not their assumption is true remains unclear. In disagreement with their proposed mechanism of action, anti-NKp46 monoclonal antibody treatment had no effect on T1D in BDC2.5/NOD or NOD mice in our hands (unpublished observations).

3.1.5 NK cell receptors in T1D: Ly49 and KIR

A key open question in the T1D field is how established genetic risk loci contribute to disease in the NOD mouse. One locus, *idd6*, is situated at the NK cell gene complex on chromosome 6 near the Ly49 receptor family (Rogner et al., 2001). In support of this finding, NOD mice congenic for the B6 NK cell receptor gene complex (including the B6 allele of the *idd6* region) showed increased NK cell functionality as well as protection from disease (Carnaud et al., 2001). How, mechanistically, the B6 *idd6* region and the Ly49 receptors confer protection from disease remains unclear. Genomic sequencing of this region indicated that the NOD *idd6* region contained the largest set of Ly49 receptors, especially the activating Ly49 receptors, compared with all other sequenced strains (Belanger et al., 2008). This study reported some strain-specific Ly49 expression differences between B6 and NOD mice, for Ly49D, G and H/I, as measured by flow cytometry, corroborating a prior study that found differences in the

expression of Ly49D, C/F/H and A/P (Poirot et al., 2004). Given that monoclonal antibodies are not available for many Ly49 receptors, and because the ones that do exist tend to be cross-reactive (between Ly49 receptors) or specific for B6 alleles, little was known about their comprehensive expression patterns in NOD mice (especially in the pancreas).

Studies on multiple human populations implicated the KIR genes in susceptibility to diabetes. One study found a significant overrepresentation in inhibitory KIR2DL2 and 2DL5 and activating 2DS1, 2DS2 and 3DS1 in Latvian T1D patients compared with controls (Nikitina-Zake et al., 2004). Furthermore, they stratified for the high risk HLA-DR3 and DR4 alleles and found that the DR3 or DR4 genotype combined with 2DL2 or 2DS2 were significantly more likely to be patients than controls. Similar results were reported from our lab describing an overrepresentation of KIR2DS3 in connection with the HLA-DRB1*04 and DQB1*0302 HLA complexes in patient cases but not controls (Rodacki et al., 2007). Conflicting data came from Park and colleagues who did similar work in Korean patients but found 2DL5 and 2DS2 to be underrepresented in T1D patients (Park et al., 2006). A Finnish study also showed an underrepresentation of 2DS5 in T1D patients (Middleton et al., 2006). However, there is a great deal of conflict in this field, potentially due to altered KIR haplotypes in different global communities, as emphasized by another study that found no association of KIR to T1D in a Basque population (Santin et al., 2006).

In subsequent years, more studies showed that polymorphisms of specific KIR genes were better predictors of T1D than KIR genotype alone (e.g. KIR2DL2 polymorphism rs2756923) (Ramos-Lopez et al., 2009). Although the complexity of these data make it hard to distill, one current model asserts that human diabetes patients have, on average, more activating KIR than non-diabetic individuals (van der Slik et al., 2003). This model notably matches the

genomic data from NOD mice as previously discussed (Belanger et al., 2008). Importantly, the connection of T1D to both activating and inhibitory receptors indicates a more complex mechanism than hyperactive/hypersensitive NK cells. Given that HLA DR3/DR4 and DRB1*04/DQB1*0302 increased the risk for T1D in certain KIR genotypes, and stratifications that include KIR ligands are better predictors than KIR alone, it is evident that NK cell receptor genotypes are part of a complex set of autoimmunity risks. Notably, some of the same KIR genes that predicted risk for T1D also did so for other diseases such as KIR2DS1 with psoriasis (Jobim et al., 2008).

3.1.6 Tissue-specific profiling of Ly49 receptors

Despite their long-standing connection to T1D in mice and humans, a functional association between the KIR and Ly49 receptors and diabetogenesis has never been established. This lack of connection is perhaps unsurprising given that little effort has been expended to comprehensively assess the expressed repertoire of these receptors, especially in contrast to the wealth of strain-to-strain genomic comparisons (Rahim et al., 2014).

In regards to tissue-specific NK cell expression differences, Sojka and colleagues profiled 8 Ly49 receptors by flow cytometry on tissue-resident versus conventional NK cells and found significant differences between the two populations. However, these tissues included liver, thymus, uterus, skin and spleen, but pancreas data were lacking. Only one study analyzed the differences between pancreas and spleen, but only for 2 Ly49 receptors (G2 and D). This study found a slight underrepresentation for both in the pancreas of NOD mice (Brauner et al., 2010).

Here, we describe our efforts to fill these many different knowledge gaps by profiling the diversity of NK cells and their receptors in multiple fashions: first, we assessed the collective Ly49 receptors expressed by NOD mice in diabetes-relevant and control organs using monoclonal antibody staining and flow cytometry. Second, we comprehensively assessed the Ly49 receptor expression profile using high-throughput RNA sequencing (RNAseq). To our knowledge, this represents the first comprehensive transcriptional repertoire assessment in any strain – including a quantitative comparison between multiple organs. Finally, we used the novel CyTOF platform to assess high-dimensional NK cell heterogeneity at the single cell level.

3.2 Materials and Methods

Mice

NOD/ShiltJ and BDC2.5/NOD TCR transgenic (Katz et al., 1993) mice were bred in our colony at the Jackson Laboratory, and were genotyped and maintained at Harvard Medical School (under specific-pathogen-free conditions). Females between 7-10 (Ly49 profiling) and 17-19 (CyTOF profiling) weeks of age were generally used. Protocols were approved by Harvard Medical School's Institutional Animal Care and Use Committee.

Cell Preparations and Monoclonal Ly49 Receptor Antibody Staining

For the purpose of collecting a highly purified sample of NK cells, the following steps were taken. Postmortem intracardial perfusion was performed with 30ml PBS. Following surgical removal of organs, cells were isolated from the pancreas, spleen and lung by mechanically separating with scissors before passing through a 40μm filter into DMEM supplemented with 2% fetal bovine serum (Omega Scientific). Bloody samples were treated with ACK Lysing Buffer (Lonza) for 5min on ice. Cells were Fc blocked (2G42, prepared in-laboratory) before surface or intracellular stains were performed using mAbs against CD3 (145-2C11 and 17A2, BioLegend), CD19 (6D5, BioLegend) and NKp46 (29A1.4, BioLegend). Flow cytometry was performed using an LSRII (BD), and data were analyzed using FlowJo (Tree Star) software. Fluorescent-coupled mAbs from Biolegend or Ebiosciences were used to identify Ly49 receptors (Ly49H clone 3D10; Ly49A clone YE1/48.10.6; Ly49E clone CM4; Ly49A-B6 clone A1; Ly49A/D clone ebio12A8; Ly49G clone AT8; Ly49I clone YLI90; LY49D clone 4E5; Ly49G2W clone ebio4D11; Ly49CFHI clone 14B11).

RNASeq

Cells were double-sorted to high purity (>99%) on a Moflo Cell Sorter (Beckman Coulter) directly into Trizol (Invitrogen). RNA was prepared as described by the Immunological Genome Project (www.immgen.org). Barcoded sequencing libraries were prepared using the Illumina TruSeq RNA Preparation v2 Low Sample protocol (Rev. D). Quality control for library read size was performed on the 2100 Bioanalyzer (Agilent Technologies) before normalizing and pooling. Sequencing was run on the Illumina HiSeq2500 with 100bp single-reads. The Makrigiannis lab (A Makrigiannis, S. Anderson) provided annotation of the *idd6* region as described in (Belanger et al., 2008), which was used as a scaffold to align sequencing using TopHat (Trapnell et al., 2009). Aligned sequencing data were visualized using IGV Genome Browser (https://www.broadinstitute.org/igv/home). HTSeq-count was used for counting reads. Strict criteria were used for aligning and counting – no basepair mismatches and only 1 alignment per read. Normalization was performed using the DESeq package in bioconductor. Normalized data were visualized and analyzed using Microsoft Excel.

Organ and Cell Preparation for CyTOF

Postmortem intracardial perfusion was performed with 30ml PBS. Following surgical removal of organs, cells were isolated from the pancreas and spleen by mechanical separation with scissors. The pancreas was bathed in a shaking water bath at 37°C in digestion buffer (1 mg/ml collagenase IV [Sigma-Aldrich], 10 U/ml DNaseI [Sigma-Aldrich], and 1% [Thermo Fischer Scientific] in DMEM [Invitrogen]) before passing through a 40μm filter into Cell Staining Buffer [CSB] (500mL $Ca^{2+}Mg^{2+}$-free PBS [Gibco], 2.5g (0.5%) BSA, 100mg (.02%)

Sodium Azide). Bloody samples were treated with ACK Lysing Buffer (Lonza) for 5min on ice. Cells were Fc blocked (2G42, prepared in-laboratory) before staining 1-2million cells in 1uM 103Rh viability dye intercalator (DVS Sciences) in CSB for 15min at 37°C. Cells were washed and then stained with surface metal-coupled monoclonal antibodies (provided by the Harvard Medical School CyTOF Consortium and Antibody Core: Joshua Keegan and Jim Lederer) in CSB for 30min at room temperature. Cells were then washed twice in CSB before diluted into fixation and permeabilization buffer (1.6% formaldehyde and 0.3% saponin in $Ca^{2+}Mg^{2+}$-free PBS) for 10min at room temperature. The cells were then washed in permeabilization buffer (0.3% saponin in CSB) before staining with intracellular metal-coupled antibodies for 30min at room temperature. Stained cells were washed twice in permeabilization buffer with the final dilution into fixation and permeabilization buffer overnight with DNA Intercalator (DVS Sciences). The samples were then washed three times in MilliQ water before dilution with EQTM four element calibration beads (DVS Sciences) and run on the CyTOF machine at the Dana Farber Cancer Institute Flow Cytometry Core.

CyTOF Data Analysis

.FCS files were generated for each sample during data acquisition on the CyTOF machine which were analyzed using Cytobank as previously described (Kotecha et al., 2010). Hierarchical, high-throughput, multi-dimensional analysis was performed using Spanning-tree Progression Analysis of Denisty-Normalized Events (SPADE) and visualized in Cytobank as previously described (Qiu et al., 2011). For SPADE analysis, settings included: 500 maximum nodes, 5% down-sampling with 3 biological replicates clustered in the same tree.

Statistical analyses

Data are presented as means ± S.D. Significance was assessed by the Student's t-test or Mann Whitney test using Prism 5 (GraphPad Software). A P value of <0.05 was deemed statistically significant.

3.3 Results

3.3.1 Identifying Ly49 receptor expression on splenic and pancreatic NK cells

The genomic region containing the Ly49 receptor family was recently sequenced in NOD mice. However, whether or not NOD-derived NK cells express these receptors was less clear. To better understand the protein-level Ly49 receptor profile of NK cells from BDC2.5/NOD mice, we assessed splenocytes using a full set of monoclonal antibodies to the Ly49 receptors (Figure 3.1). In brief, 8 of 9 Ly49 receptor antibodies from this panel had a clear positive signal on NK cells, with little to no background signal in non-NK cells. Ly49I (clone YLI90), reactive in B6 animals, had no signal in either population. Ly49A and D antibodies stained the majority of NK cells. Meanwhile, Ly49H antibodies identified about half of NK cells and the Ly49G2/W, A/P and E/F antibodies identified minority fractions of NK cells.

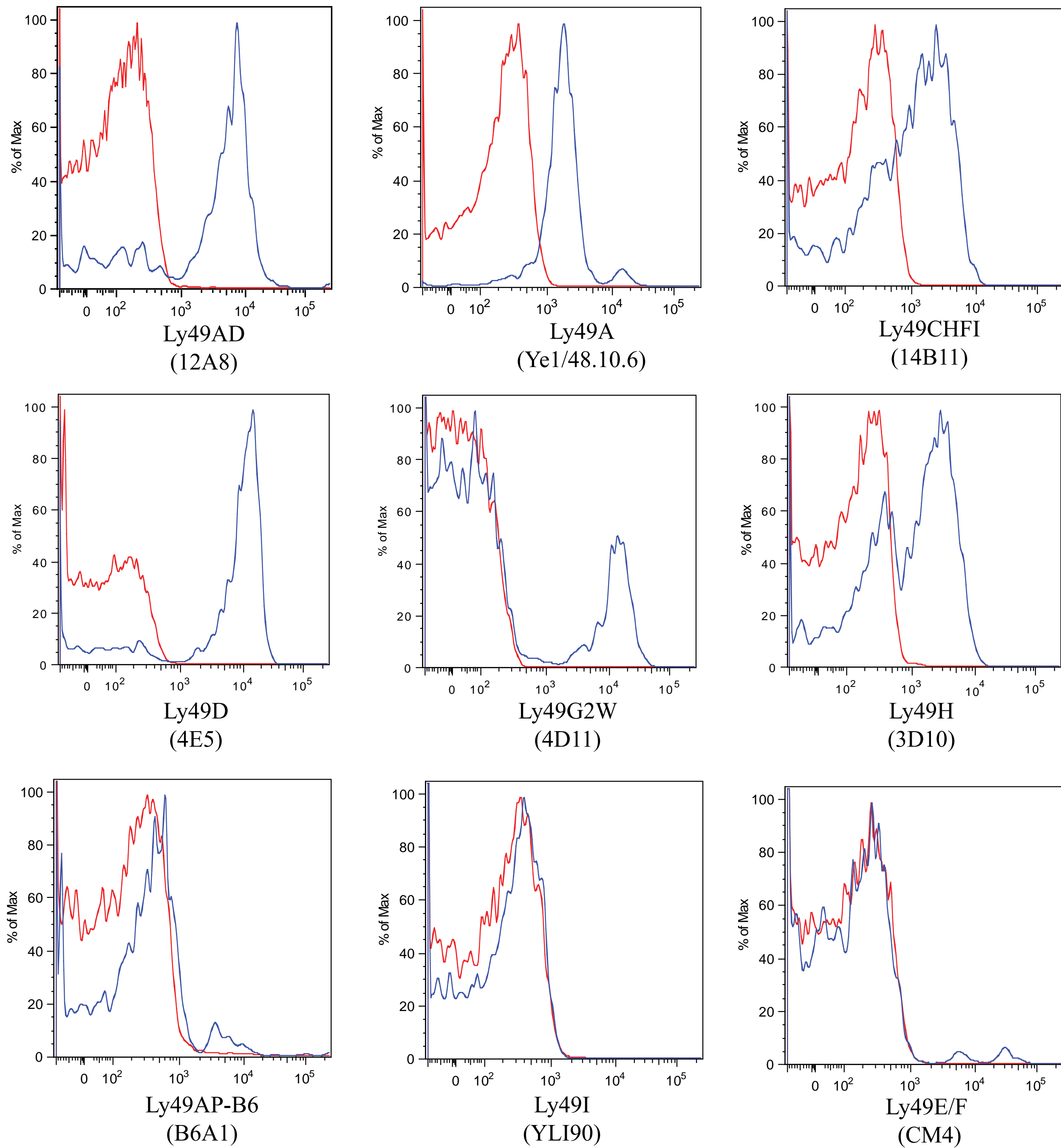


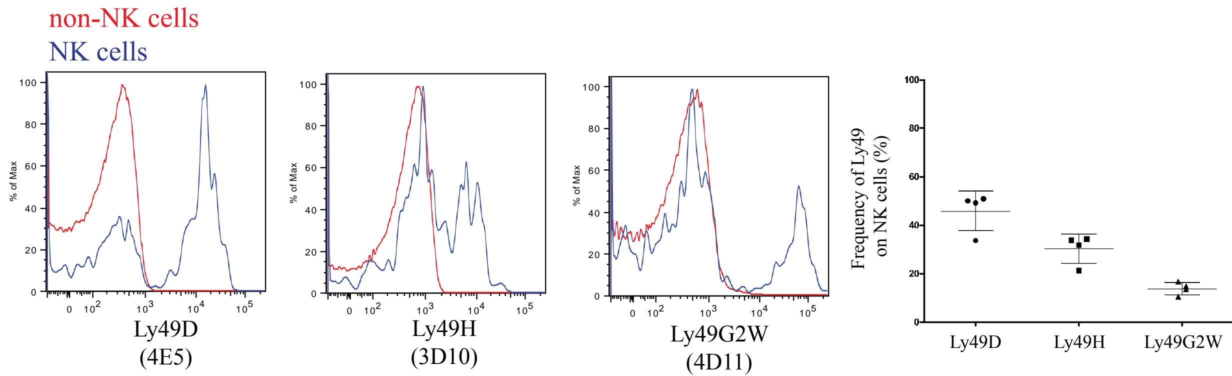
Figure 3.1: Ly49 Receptor Antibodies Identify Splenic NK Cells from NOD Mice. Representative overlayed Ly49 receptor monoclonal antibody flow cytometry plots for splenocytes from BDC2.5/NOD mice. Ly49 receptor antibodies and clones are listed below each plot. Blue histograms indicate $CD3^{-}NKp46^{+}$ NK cells. Red histograms indicate NKp46- cells.

A smaller set of Ly49 receptor antibodies (in the same staining panel as allowable by fluorescent signal overlap and compensation) was used to assess the expression of Ly49 on pancreatic NK cells (Figure 3.2A). As was the case for spleen, non-NK cells expressed very little or no Ly49 receptor antibody signal. In contrast, pancreatic NK cells readily stained with all 3 Ly49 receptors examined. Similar to what was seen in the splenic NK cells, anti-Ly49D recognized the largest fraction of NK cells, followed by Ly49H, then Ly49G2W.

Due to the variegated expression pattern of Ly49 receptors, the combined expression of Ly49 receptors on pancreatic NK cells was assessed with the smaller panel. All possible combinations of Ly49 receptors (2^3=8) were represented in the pancreas, to varying degrees (Figure 3.2B). Surprisingly, the largest fraction (representing about half of all pancreatic NK cells) was a Ly49-triple-negative population. Whether or not this population is similar to the hyporesponsive, non-Ly49-expressing population in B6 animals remains unclear. Pancreatic NK cells were described as hyporesponsive in one study (Brauner et al., 2010) and the lack of Ly49 expression might indicate an “uneducated” or “disarmed” NK cell phenotype.

Notably, all 4 combinations of Ly49D+ NK cells were the next 4 most represented populations following the triple-negative population.

A



B

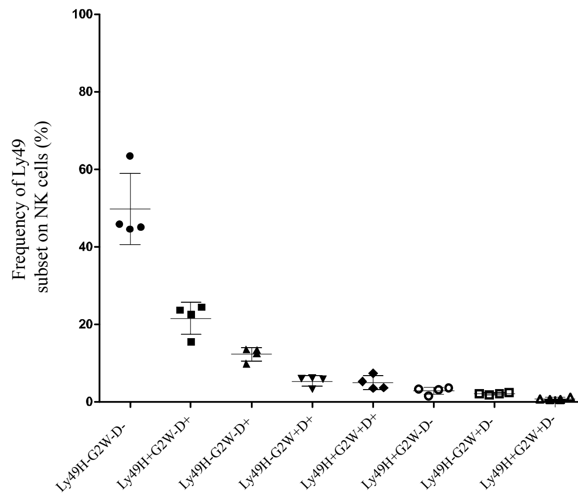


Figure 3.2: Variegated Expression Pattern of Ly49 Receptors on Pancreatic NK Cells.
(A) (Left) Representative overlayed flow cytometry plots for pancreatic infiltrate from non-NK cells (NKp46-, red histogram) and NK cells ($CD19^{-}CD3^{-}NKp46^{+}$, blue histogram). (Right) Summary data for individual Ly49 receptor expression from 4 replicates of 2 independent experiments. (B) The same data as in A, separated into the 8 combinatorial expression patterns of 3 different Ly49 receptors. Ranked in order from left to right of most expressed to least expressed. Mean ± SD presented for summary data.

3.3.2 Comprehensive, unbiased, and tissue-specific Ly49 receptor profiling

Monoclonal antibody profiling of the Ly49 receptor expression on NK cells ultimately had three major issues. First, many of the Ly49 receptors do not have antibodies available to identify them. Second, the antibodies that are available are highly cross-reactive. Finally, almost no validation of accurate Ly49-reactivity has been performed in the NOD mouse. Due to these issues, a more thorough and strain-specific analysis tool for Ly49 receptor identification is desirable.

RNAseq-based gene expression profiling was utilized as an unbiased and comprehensive approach. For the organ- and strain-specific analysis, the sequenced and annotated Ly49-receptor-containing region of NOD mice (*idd6*) was used to map reads. A first-pass visualization of the mapped reads showed no obvious exon-level differences in gene expression between organs (Ly49D shown for example in Figure 3.3), arguing against pre-translational modification of RNA species for these genes (including differential-splicing events). Of note, the “densest” read-pileups were consistently centered at exons but some intronic mapping was evident. Whether or not there is a functional consequence to intronic RNA expression at the Ly49 receptor loci remains unclear, but it could indicate higher levels of splicing for genes with more reads mapped to the intronic region.

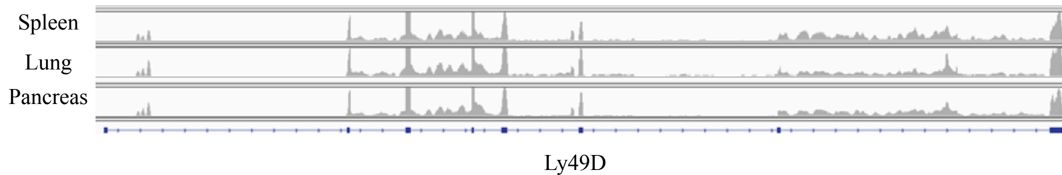


Figure 3.3: Visualization of the NOD-Mapped Ly49 Receptor Aligned Reads.
Representative Ly49 receptor transcript reads for Ly49D using IGV Genome Browser. For each row, the grey histogram indicates a summation of total mapped reads to that genomic region (for each organ listed on the left). Bottom: Blue horizontal line indicates the full genomic region for Ly49D with thick blue boxes indicating exons.

The sequenced genomic region, *idd6*, contains all but one known NOD Ly49 family member, Ly49W, which Belanger and colleagues predicted to be in a gap between Ly49M and Ly49P1, outside the sequenced region (Belanger et al., 2008). Therefore, the mapped library represented a near-comprehensive analysis of the transcribed repertoire of Ly49 receptors. All 20 of the Ly49 genes contained showed some level of expression, ranging from 39 (Ly49A, spleen) to 6859 aligned RNA reads (Ly49P3, pancreas) (Table 3.1). Notably, there was a range of expression between different Ly49 family members but predicted activating genes, in general, showed higher expression compared with inhibitory genes, which in turn showed higher expression than pseudo-genes (briefly summarized in Table 3.2).

Table 3.1: Organ-Specific Transcriptional Repertoire of the Ly49 Receptors
Left: Full list of Ly49 receptors as predicted by sequence. The color of highlight for each gene indicates activating (green), inhibitory (blue) or pseudogene (grey) as predicted by the genomic sequence. Middle: Total counts for the normalized aligned reads for each gene in each organ. Right: fold-change calculations to indicate where genes changed between organs. Fold-changes were then highlighted using conditional formatting (from (blue) low to equal (white) to high (red) based on numerical percentile).

Receptor	Spleen Aligned Reads	Lung Aligned Reads	Pancreas Aligned Reads	Pancreas vs. Spleen	Lung vs. Spleen	Pancreas vs. Lung
Ly49a	39	89	101	2.58	2.28	1.13
Ly49e	299	409	537	1.80	1.37	1.31
Ly49p2	1580	1534	2162	1.37	0.97	1.41
Ly49x	61	47	81	1.32	0.76	1.74
Ly49p3	5994	6180	6859	1.14	1.03	1.11
Ly49i1	470	742	528	1.12	1.58	0.71
Ly49p1	4962	4925	5540	1.12	0.99	1.12
Ly49q	77	102	84	1.09	1.32	0.83
Ly49g2	1072	965	1171	1.09	0.90	1.21
Ly49u	1151	1135	1256	1.09	0.99	1.11
Ly49c	190	302	199	1.05	1.59	0.66
Ly49d	3243	4038	3248	1.00	1.25	0.80
Ly49g1	139	136	139	1.00	0.98	1.02
Ly49m	2397	1840	2331	0.97	0.77	1.27
Ly49i3	538	537	488	0.91	1.00	0.91
Ly49i2	216	252	185	0.85	1.17	0.73
Ly49h	2368	2347	1930	0.81	0.99	0.82
Ly49f	142	120	106	0.74	0.85	0.88
Ly49pd2	384	244	202	0.53	0.63	0.83
Ly49pd1	394	232	174	0.44	0.59	0.75

Table 3.2: Summary Statistics for Table 3.1

	Pseudo	Activating	Inhibitory
Number of Genes	8	6	6
Minimum Counts	47	1135	39
Maximum Counts	2162	6859	1171
Average Counts	420.54	3430.22	409.78

The differences in Ly49 receptor expression between the pancreas (disease-relevant organ), spleen (systemic control) and lung (irrelevant organ control) were assessed using a fold-change calculation. Overall, there were relatively few differences between organs. Only 4 of 20 genes (encoding Ly49A, E, P2 and X) were >20% increased and a different set of 4 genes (encoding Ly49H, F, PD2 and PD1) were ~20% decreased. Of the 4 increased genes, only those encoding Ly49A (2.58-fold increase) and Ly49E (1.8-fold increase) produced proteins. Of the 4 decreased genes, only that encoding Ly49H (0.81x) produced a protein. Expression of Ly49A, and Ly49E to a smaller extent, were similar between the pancreas and the lung – potentially indicating their overrepresentation as a function of their "tissue" location. Ly49H, to the contrary, was also slightly underrepresented in the pancreas compared with the lung – indicating that this Ly49 was distinctly down in the pancreas compared with both spleen and lung.

With transcriptional evidence in hand as support, the protein-level expression of Ly49 receptors was carefully assessed using flow cytometry (Figure 3.4A). No change in the frequency of Ly49A+ cells was apparent, disagreeing with the over-representation in the RNAseq analysis. However, it was unclear if increases in Ly49 receptor transcription would be apparent at the level of cellular frequency rather than a change to mean fluorescence intensity (MFI) on a per-cell basis. Therefore, the relative MFI fold-change of pancreas compared to spleen was calculated for samples in which the pancreas and spleen were run on the same day in the same fluorescent compensation file (Figure 3.4B). Still, no clear change in MFI was apparent when comparing the pancreas versus the spleen for Ly49A. Ly49E, on the other hand, had a mild increase in frequency supporting the over-representation in the RNAseq analysis. However, no difference was apparent for Ly49E when comparing MFI between pancreas vs. spleen. A slight (~20%) underrepresentation of NK cells expressing Ly49H was apparent between pancreas and

spleen, as predicted by the RNASeq analysis. However, similar to Ly49E and A, no change in the MFI was clear (and if anything, trended in the direction of pancreas-enrichment).

Collectively, the protein-level expression pattern matched the predictions by RNAseq for an over-representation of Ly49E and under-representation of Ly49H (but not for Ly49A). However, the changes were rather subtle, including a shift in the Ly49E+ population from 4.66% ± 0.16 (spleen) to 8.23± 0.59 (pancreas) and a shift in the Ly49H+ population from 52.07% ± 6.43 (spleen) to 45.47% ± 5.35 (pancreas). Whether or not these minute differences yield some functional change in NK cell reactivity or the pathogenicity of the NOD *idd6* locus remains unclear.

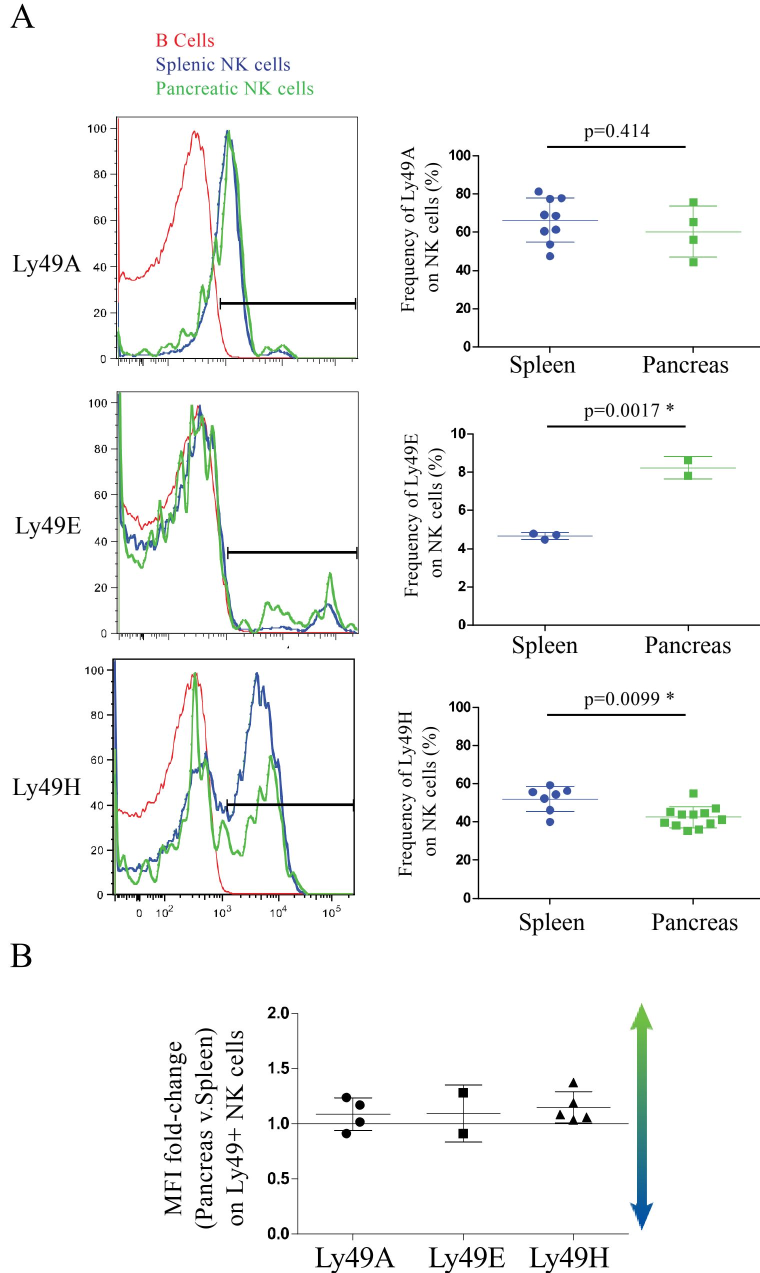


Figure 3.4: Organ-Specific Expression of the Ly49A, E and H Receptors
(A) Left: representative flow cytometry plots with negative control CD19+ B cells (red) used for gating, $CD3^{-}NKp46^{+}$ NK cells (shown in blue: spleen, and green: pancreas) indicate Ly49 expression. Right: summary statistics for the frequency of Ly49-expressing populations. (B) Summary statistics for the fold-change of pancreatic versus splenic MFI of Ly49 receptor expression. Pancreas-to-spleen comparisons were only calculated for samples run on the same flow cytometry compensation file on the same day. Fold-change=1 is indicated with a horizontal black line (implies no difference in MFI between organs). Vertical arrow on the right indicates the potential organ-centric gradient from pancreas (green) to spleen (blue). Mean ± S.D. of at least 2 independent experiments.

3.3.3 Profiling pancreatic NK cell heterogeneity using CyTOF

NK cells also express a range of non-Ly49 receptors that collectively differentiate their state of maturity, activation and functional capacity. However, very little effort has been placed on characterizing NK cell diversity at the single-cell level with a high dimensionality approach, especially in the pancreas. To study cell changes during insulitis, we developed two antibody panels for use with the CyTOF (Table 3.3). One panel focused on cells, markers and receptors typically found on lymphoid cells ("L" panel – 2^{31} possible combinations of markers) while the second focused on myeloid cells, granulocytes and a marker of degranulation ("MGD" panel - 2^{22} possible combinations of markers).

Table 3.3: List of Antibodies, Clones and Metal-Couplings for L and MGD Panels

Lymphoid - L - Panel		
Target	**Clone**	**Metal**
CD44	IM7	141Pr
IgM	RMM-1	142Nd
CD21	7E9	143Nd
CD5	53-7.3	144Nd
CD4	GK1.5	145Nd
CD62L	MEL-14	146Nd
CD138	281-2	147Sm
CD127	A7R34	148Nd
CD19	6D5	149Sm
TCRb	H57-597	150Nd
CD27	LG.3A10	151Eu
CD3e	145-2C11	152Sm
NKp46	298A1.4	153Eu
CD103	2E7	154Sm
CD69	H1.2F3	156Gd
Tbet	4B10	158Gd
CD23	B3B4	159Tb
Sca-1	D7	160Gd
Foxp3	FJK-16s	162Dy
CD8a	53-6.7	164Dy
CD45	30-F11	165Ho
GATA3	TWAJ	166Er
CD25	3C7	167Er
c-kit	ACK2	168Er
Thy1	G7	169Tm
RORγT	AFKJS-9	170Er
PD-1	29F.1A12	171Yb
TCRgd	GL3	172Yb
Ag7	g7	174Yb
IgD	11-26c.2a	175Lu
CCR7	4B12	176Yb

Myeloid Granulocyte Degranulation - MGD - Panel		
Target	**Clone**	**Metal**
CRIg	17C9	143Nd
CD4	GK1.5	145Nd
CD11c	N418	146Nd
PDCA-1	927	148Nd
CD19	6D5	149Sm
CD11b	MAC1	150Nd
Ly6C	HK1.4	151Eu
CD3e	145-2C11	152Sm
CD335	298A1.4	153Eu
CD103	2E7	154Sm
Ly6G	1A8	158Gd
NKp46	A2F10	162Dy
CD8	53-6.7	164Dy
CD45	30-F11	165Ho
CD86	GL-1	166Er
CD107a	1D4B	167Er
FcεR1a	41334	169Tm
CD80	16-10A	170Er
CD49b	Dx5	171Yb
Ag7	g7	174Yb
F4/80	BM8	175Lu
CCR7	4B12	176Yb

Spanning-tree progression analysis of density-normalized events (SPADE) was utilized to cluster pancreatic infiltrate from 18wk prediabetic female NOD mice into 445 (L panel) and 381 (MGD panel) populations of similar-marker expression (Figure 3.5). In a SPADE tree,

groups of cells are visualized in an inter-connected fashion based on their relative similarity to one another. The overall shape of the tree is irrelevant; rather the connectivity of these populations, also called nodes, indicates similarity between groups of cells. NK cells (defined by NKp46 expression) represented 4.2% of total cells, in line with previously reported frequencies for NOD mice of this age. NKp46-containing nodes included 33 out of 445 total nodes (representing 7.4% of total pancreatic diversity) for the L panel analysis. In the MGD panel analysis, NK cells were 4.6% of cells and 25/385 nodes (equating to 6.5% of diversity). The most over-represented NK cell phenotype accounted for only 12.6% of NK cells in the L panel analysis and 21% of the MGD panel analysis.

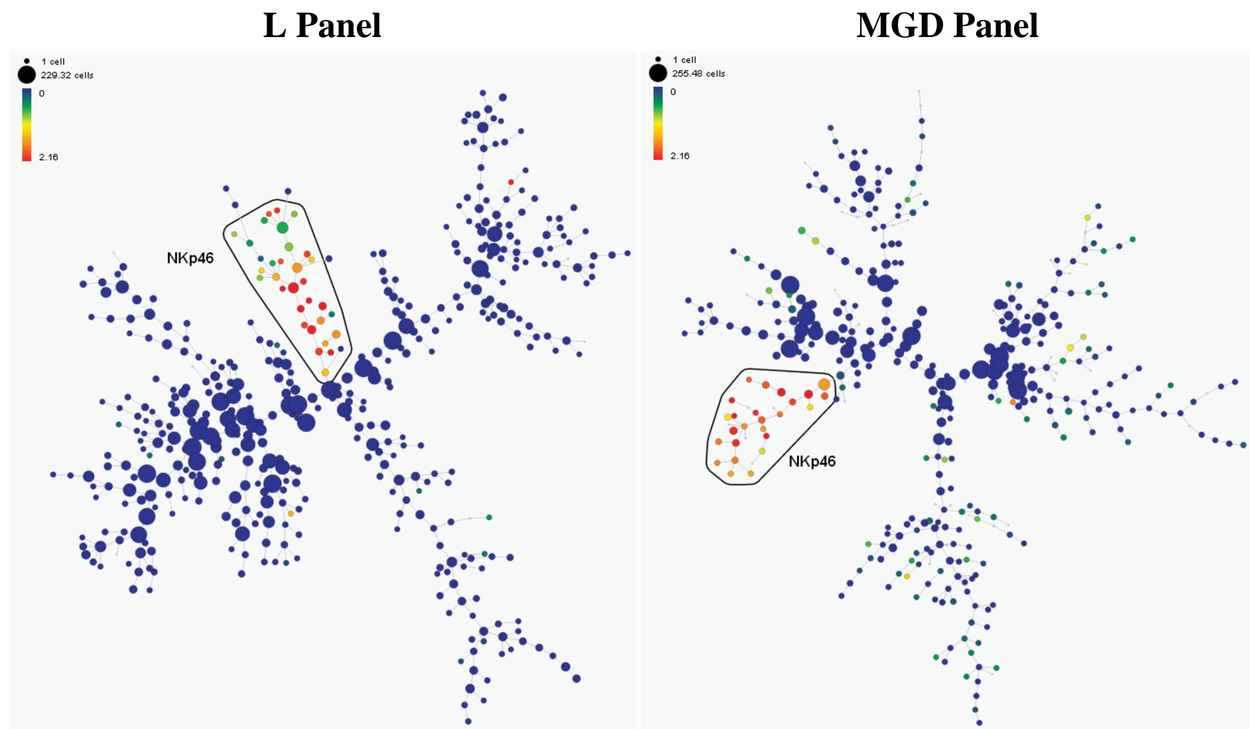


Figure 3.5: SPADE Analysis Reveals High-Dimensional Diversity for Pancreatic NK Cells. Representative SPADE trees of NKp46 expression in pancreatic infiltrate. Left: L panel. Right: MGD panel. Node color represents signal intensity (NKp46 in this figure) while the size of the node represents frequency (ranging from 1-229 cells for L panel and 1-255 cells for MGD panel). The black bubble labeled "NKp46" identifies the group of nodes most consistently and highly expressing NKp46.

In order to assess the underlying expression patterns driving the heterogeneity between nodes, the signal intensity for each antibody co-expressed in NKp46-containing nodes was visualized separately (Figure 3.6A and B for the L panel and 3.6C and D for the MGD panel). NKp46-containing nodes in the L panel co-expressed a wide variety of markers including CD44, CD62L, CD127, CD27, CCR7, PD1, CD69, Tbet, CD25 and RORγT (Figure 3.6A). Marker signal intensities presented in two general patterns. Tbet, CD44 and CD27, for example, were widely expressed at consistent levels. CD62L, CD127, PD1, CD69, CD25 and RORγT were

more punctuate (highly expressed in some nodes but not expressed in others). Markers were not shown if they were not expressed or if the antibody failed.

Highlighting T cell-identifying markers such as TCRγδ and TCRαβ revealed that some of the NKp46-containing nodes also contained T cell populations due either to phenotypic similarity (and limitations in the clustering methodology) or to expression of NKp46 on T cells (Figure 3.6B) that was previously reported on rare populations of T cells (Narni-Mancinelli et al., 2011). γδT cells, which are known to be phenotypically (and functionally) similar to NK cells, were the most prevalent in the NKp46-expressing collection of nodes (in terms of γδTCR signal). T cell marker-containing nodes tended to have lower NKp46 signal, supporting a mix of NK cells with contaminating non-NK cells, or lower NKp46-expressing T cells in these nodes. Curiously, CD4 and CD8 were not expressed above background signal levels in either panel on NKp46+ cells (unpublished observation), supporting representation from γδT cells and/or rare/controversial αβT cells lacking CD4 and CD8 (D'Acquisto and Crompton, 2011) in these nodes. Importantly, this overlap would skew the diversity representation, as those nodes might contain receptors expressed either partially or fully from T cells rather than NK cells. Therefore, this SPADE clustering analysis may have produced an overestimate for the diversity of markers expressed by NK cells, or cannot differentiate rare NKp46-expressing T cells from bona fide NK cells.

NKp46-containing nodes in the MGD panel contained 3 types of expression patterns (Figure 3.6C). CD11b, for example, was widespread at low levels in all nodes. CD49b was expressed highly in a large fraction of NKp46-containing nodes. Ly6C, CD103 and CD107α had a more punctate expression pattern. As was the case for the L panel, highlighting the T cell

receptor molecule CD3 revealed a contaminating T cell population in the NKp46-expressing nodes – also indicating a potential overestimation on NK cell diversity (Figure 3.6D).

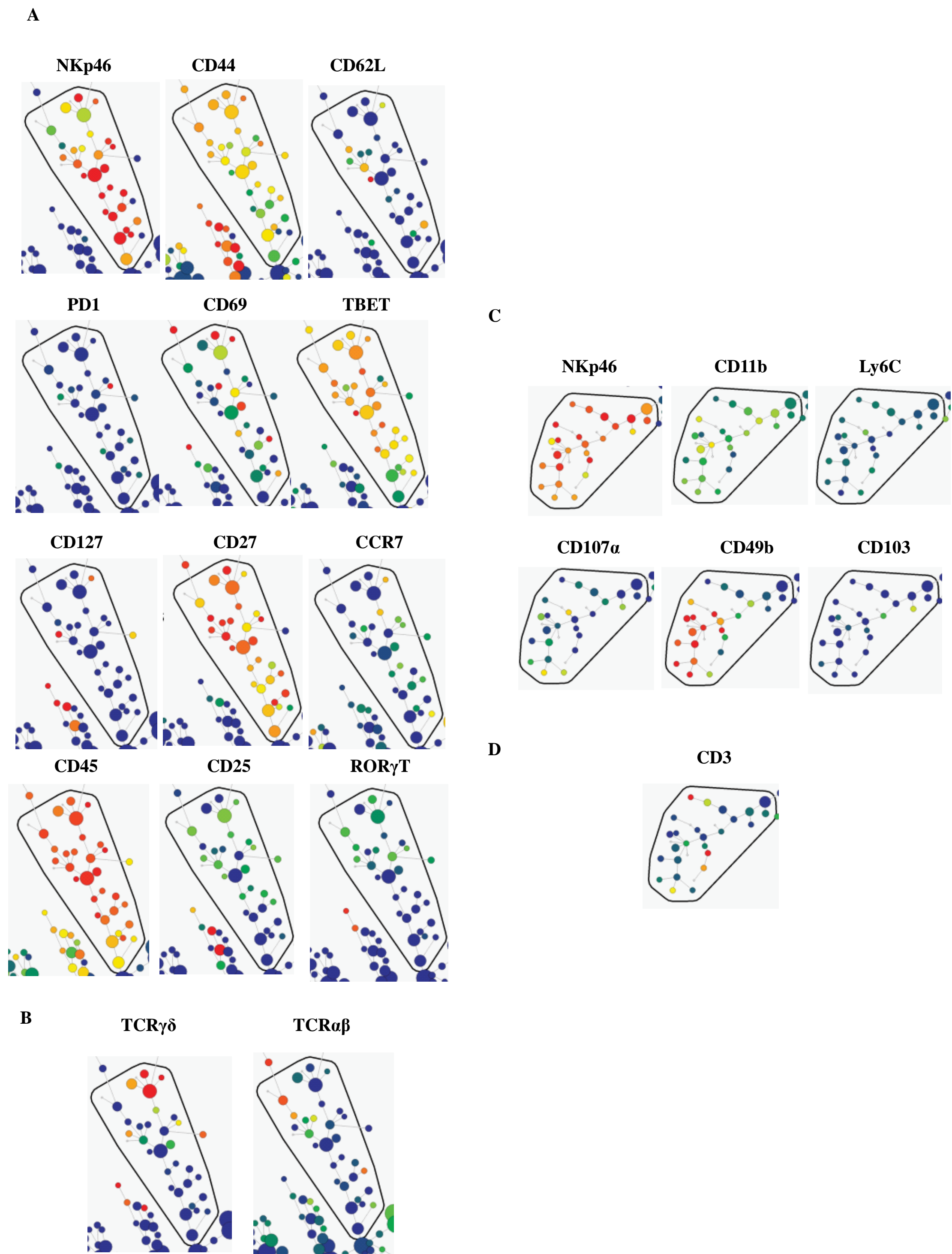


Figure 3.6: Individual Receptor Diversity for Pancreatic NK Cells. Representative NKp46-containing groups of nodes from the L panel (A and B) and MGD panel (C and D). Node color represents signal intensity (for each indicated marker above the group of nodes) while the size of the node represents relative population frequency. The images, including the black bubble surrounding the NKp46-expressing population, is the same as presented in Figure 3.5.

3.4 Discussion

Despite early claims to the contrary, NK cells are no longer considered a homogenous lineage (Lanier, 2014; Sojka et al., 2014b). Rather, subsets of NK cells are found in a wide variety of organs, depend on a wide variety of cytokines and transcription factors and express a wide variety of functional molecules. Here, we explored the heterogeneity of NK cells in NOD mice, specifically in the pre-diabetic pancreas. We uncovered an array of Ly49 receptors expressed by splenic and pancreatic NK cells in these mice. We also showed that pancreatic NK cells have a subtle difference in surface phenotype compared with NK cells from the spleen. Finally, we discovered a greater resolution of heterogeneity in pancreatic NK cells than had ever been previously described.

Most of the monoclonal antibodies used to identify Ly49 receptors in other strains also identified Ly49 receptors in the NOD strain. These positive signals came from antibodies specific for Ly49A/D, AP, A, D, C/F/H/I, H, G2W and EF but not Ly49I in a binary expression analysis. By combining some of these antibodies into a single panel, the combinatorial expression pattern of Ly49 receptors was confirmed for NOD mice, especially in the pancreas where all 8 combinations of Ly49D, H and G2W were confirmed, to varying degrees. Interestingly, the largest fraction of NK cells was a triple negative population for all 3 Ly49 receptors, but it was unclear if this specific subset expressed any other Ly49 receptor. Given that Ly49A often stained up to 80% of NK cells, much of this ~50% triple-negative population logically had to have expressed at least some Ly49A. However, Ly49A was never included in the same antibody panel as Ly49H and Ly49D, the other predominantly expressed Ly49 receptors. Future high dimensionality analysis of these receptors will make it more feasible to

test the full combinatorial expression of Ly49 receptors on NK cells and whether or not a Ly49 receptor negative NK cell subset exists in NOD mice as it does in B6 mice.

The diverse array of Ly49 receptors on NK cells mediates NK cell responsiveness and tolerance. However, Ly49 receptor transcription had never been profiled comprehensively in a comparison between organs of disease relevance. Here, we report the transcriptional repertoire of Ly49 receptors in BDC2.5/NOD mice for pancreas, lung and spleen. Intriguingly, all Ly49 receptors were expressed, to some degree, in all organs, with no major exon-level differences between organs. This expression pattern represented a dramatic increase in the number of Ly49 receptors previously known to be expressed in NOD mice (especially in the pancreas). We confirmed a subtle difference in surface phenotype on pancreatic NK cells, compared with NK cells from the spleen, for Ly49E and Ly49H. However, the degree of change between organs was quantitatively small or on a very small fraction of NK cells. It remains unclear whether or not these minute differences could explain a functional role for *idd6* in the pathogenesis of T1D.

Previous work indicated that Ly49Q was uniquely expressed by DCs (especially pDCs), macrophages and neutrophils rather than by NK cells in diverse strains, including NOD mice (Belanger et al., 2008; Rahim et al., 2014). Here we report quantitatively low levels of transcripts mapping to the Ly49Q gene in all three organs. These transcripts could indicate that the expression of Ly49Q is controlled post-transcriptionaly or that true protein-level expression exists below the detection level of existing monoclonal antibodies.

A confounding factor for the interpretation of our data is the population-level strategy employed, given the documented stochastic expression of certain Ly49 receptors (Held and Kunz, 1998). Shalek and colleagues recently demonstrated vast differences in the heterogeneity of certain immune cells when analyzed at a population level compared with single-cell resolution

(Shalek et al., 2013). In their study, LPS-stimulated, bone-marrow-derived DCs (BMDCs) showed heterogenous expression of functional genes such as *cxcl10, cxcl1* and *il6* when analyzed at the single-cell level compared with no identifiable heterogeneity when the same set of cells was analyzed in a pool of 10,000 cells. In many ways, their single-cell data for the 10,000-cell pooled condition genes are reminiscent of our population-level expression repertoire. In both cases, the shape of the histograms surrounding exons showed some small variations, but no major exon-level differences were apparent. Given their findings, and the well-documented variegated expression pattern of the Ly49 receptors, single-cell RNAseq analysis of NK cells in different tissues might be a particularly useful strategy, for two reasons. The first benefit would be to define true single-cell transcriptional identity. The second benefit would be to correlate specific Ly49 receptors (representative of specific NK cell "arming" or "licensing") to other genes and transcriptional programs indicative of NK cell function (such as IFN-γ or markers of maturity). This type of analysis could create a vastly different picture as to how certain Ly49s regulate NK cell functions in a given tissue.

The underrepresentation of activating Ly49H was notable because the B6 form of Ly49H does not bind to MHC class I. Rather, Ly49H was shown to play a role in the recognition and clearance of viral proteins, specifically m157, and virally infected cells (Brown et al., 2001). Despite possessing a Ly49H gene, NOD mice were susceptible to MCMV infection unlike their B6 counterparts, presumably because the $Ly49H^{NOD}$ allele was incapable of binding viral protein m157 (Orr et al., 2010). Whether or not Ly49H engaged a ligand in the pancreas, and why that potential interaction decreased Ly49H expression, or limited the frequency of Ly49H+ NK cells remains unclear. Engagement of Ly49H normally causes NK cell activation and degranulation, so the decreased representation of Ly49H may be a tolerogenic mechanism. If decreased Ly49H

causes hyporesponsiveness, it might potentially help explain some of the hyporesponsiveness that was previously described for pancreatic NK cells by Brauner and colleagues (Brauner et al., 2010).

Ly49E is a notable inhibitory receptor due to its unique expression on fetal NK cells, earlier than other Ly49 receptors (Fraser et al., 2002), as well as its expression on epithelial T cells, and NKT cells, but supposedly not on resting adult NK cells (Aust et al., 2011). An exception to this observation is the cytokine-induced expression of Ly49E on mature NK cells (including cytokines such as IL-2 and Il-15) (Gays et al., 2005). Because of these seemingly contradictory findings, the reported increase of Ly49E might indicate either an increased representation of immature NK cells (from the fetal stage) or cytokine-activated mature NK cells. Both scenarios could have functional implications for pancreatic NK cells, but the latter scenario seems more likely given the inflammatory environment in the pancreas and the activated transcriptional phenotype of pancreatic NK cells compared with spleen (unpublished observations).

Two alternative, but non-obvious, hypotheses can be generated regarding the pathogenicity of the NOD *idd6* region, both of which may not involve NK cells. The first possibility is the expression of the Ly49 receptors in non-NK cells, which tends to be an overlooked feature of this family. Second, the *idd6* region also contains the *Nkrp1* family of genes as well as *cd69, cd94, Rmp1, Chok* and a set of resistance genes against the cytomegalovirus family collectively denoted *cmv* (Carnaud et al., 2001).

In regards to non-NK and non-conventional NK cells expressing Ly49 receptors: uterine NK cells, NKT cells, γδT cells, neutrophils, DCs, and macrophages have all been described as expressing some of the Ly49 receptors under certain conditions – each with some functional

consequences. These functions have included pathogen recognition, toll-like receptor (TLR)-signaling, IFN-production, tissue invasion, cell survival and proliferation, as described by Rahim and colleagues (Rahim et al., 2014). Many of these cell types have potential roles in the diabetogenic process. However, nearly all of this work was done in B6 animals and it remains to be seen how much is similarly true for NOD mice. The notable exception to this knowledge-gap is Ly49Q, which has confirmed expression on pDCs in NOD mice, as previously discussed.

High dimensionality analysis by the CyTOF platform was recently used to uncover significantly more heterogeneity in human NK cells than had ever previously been described. Here, we utilized the same technology to assess the heterogeneity of pancreatic NK cells with lymphoid- and myeloid-"flavored" panels of antibodies. Metal-coupled antibodies readily detected NKp46-expressing cells in the pancreas and high-dimensional clustering analysis segregated those NKp46-expressing cells into 33 and 25 unique nodes from the L and MGD panel analyses, respectively. To our knowledge, these figures represent the largest degree of phenotypic heterogeneity ever described for murine NK cells (excluding studies assessing variegated Ly49 expression). Many of these nodes represented a mature and activated phenotype for NK cells, including expression of CD44, CD69, CD27, CCR7, CD25 and CD11b. Some of these receptors were previously reported on pancreatic NK cells by Brauner and colleagues (Brauner et al., 2010), but not in a high-dimensional analysis. Agreeing with our findings, they also reported pancreatic NK cells that expressed low levels of CD127, CD62L and PD1.

The clustering analysis reported here (listing NK cells as between 6.5-7.4% of total pancreatic node diversity) must be an over-representation given the co-clustering of T cell markers, specifically γδT cells. While limiting the accurate analysis of NK cell diversity, our data support γδT cells, another innate-like lymphocyte, as the most phenotypically similar cell

type to NK cells, given the markers assessed. It is also possible that some γδT cells express NKp46, but the relatively low resolution of any individual marker stained with metal-coupled antibodies does not make it particularly suitable to make this determination as compared to conventional flow cytometry (unpublished observations).

Notably, some rare NKp46-containing nodes also had RORγT expression, consistent with an innate lymphoid type 3 (ILC3) cell. However, many of those nodes also expressed γδTCR, implicating γδT cells as a potential source for RORγT, as has been previously described (Martin et al., 2009).

Although we discovered less diversity in murine NK cells than was recently reported for humans – we can explain the differences from a number of perspectives. First, the human study utilized a much larger pool of cells. Second, their cells came from the spleen and were purified from other immune cells as opposed to the "whole-organ" approach that we employed. Third, the human study incorporated a larger number of test subjects. Fourth, most of their subjects were genetically diverse, but even the identical twin analysis would imply unique environmental influences differentiating genetically identical subjects. In our study, we analyzed a genetically identical and environmentally similar set of mice. Finally, their panels were solely dedicated to NK cell markers whereas ours contained markers dedicated to identifying the entire immune system. Therefore, the studies were too different to make any strong claims about the relative diversity between murine and human NK cells.

Ultimately, our data support the new model for NK cell heterogeneity. NK cells express a wide variety of Ly49 and non-Ly49 receptors and activation markers. In the future, it will be crucial to develop a more thorough understanding of murine NK cell diversity and the biological cues that inform it, as well as to determine if this phenotypic diversity is also diverse in function.

Chapter 4: Perspectives on the Biology and Future Research of T_{reg} and NK cells

4.1 Perspectives on emerging NK cell biology

Our understanding of the immune system has gone through a revolution over the last few decades- especially in our understanding of the biology of NK cells. In 1989, a review summarized that NK cells lysed other cells but lacked any form of MHC-restriction, adaptive function or clonal expansion. The authors also concluded that NK cells were not assigned to a formal lineage and had no documented anatomical residency outside of the blood (Trinchieri, 1989). After 25 years of research, we now appreciate that most of those statements were incorrect or incomplete. For example, NK cells recognize MHC class I molecules, have functional memory, can expand clonally, are of lymphocyte lineage and reside in many anatomical locations outside of the blood. Furthermore, emerging research has implicated functional roles for NK cells during pregnancy, hematopoietic/organ transplant rejection, parasitic infection, autoimmunity, HIV infection and asthmatic diseases (Vivier et al., 2008). Finally, we now also appreciate that NK cells are a part of a set of innate lymphoid-like cells, many of which impact immune and non-immune physiology in diverse fashions.

In this dissertation, we provided evidence to support many of these emerging concepts in NK cell biology. First, we discovered how NK cells cross-talk with the adaptive immune compartment and uncovered the conditions permissive to drive NK cell-amplified autoimmunity. From this finding we conclude that IL-2 acts as a bridge between innate and adaptive immunity. Our model implies a more complex network of immune cell cross-talk, responsible for T1D

prevention, than had previously been appreciated. These data add to the developing idea that adaptive immunity instructs innate immunity during autoimmune disease settings (Ji et al., 2002).

Second, we comprehensively profiled the Ly49 receptor family in multiple organs and demonstrated new Ly49 receptors expressed in NOD mice. This study revealed a subtle difference in the expression of Ly49 receptors on pancreatic NK cells compared to spleen. Finally, we demonstrated significant heterogeneity for organ-infiltrating NK cells using high dimensional cytometry profiling. Our second set of studies supported the notion that NK cell phenotypes are dependent upon their local environment and that tissue-infiltrating murine NK cells are more diverse than was previously understood.

4.2 Adaptive features of NK cells and the parallels shared between infection and autoimmunity

The assignment of NK cells to the innate immune system, many decades ago, recently came under fire following multiple lines of evidence that NK cells actually share many "adaptive" features of their B and T lymphocyte counterparts. The "innateness" of NK cells is as true today as it was decades ago when they were first coined "natural" killer cells given their propensity to spontaneously kill target cells without preconditioning. However, it is now clear that NK cells can be long-lived and generate a robust recall response to cytokines (Cooper et al., 2009), haptens (O'Leary et al., 2006) or viruses (Sun et al., 2009; Paust et al., 2010). We add to this growing list of adaptive features of NK cells with supporting data that NK cells participate in

autoimmunity and cross-talk with classical adaptive immune cells. Furthermore, our data implies a greater diversity in NK populations, previously underappreciated for "innate-like" cells.

Given their function and unique phenotype, it is possible that pancreatic NK cells possess disease-relevant adaptive memory, but experimental evidence is lacking in this area. Pancreatic NK cells experience local inflammatory cytokine signaling (which Cooper and colleagues indicated was sufficient to prime a recall response), but also might simultaneously engage β cells through activating receptors such as NKp46, as previously discussed by Gur *et. al.*. These conditions, especially together, seem ripe for NK cell-driven memory formation. In support of this concept, in chapter 2, we uncovered surprising similarities between the NK cell transcriptional response to T_{reg} cell ablation and the NK cell transcriptional response to viral infection. About 50% of induced and repressed genes were shared between these conditions. Thus, although the transcriptional machinery behind NK cell memory is not well understood, given the overlapping transcriptional networks, it is possible that the memory machinery is active in the case of the loss of T_{reg} cells. This overlap also implies that NK cells experience the inflamed pancreas in a manner similar to how they experience viral infections, providing parallels between autoimmunity and infection. One major difference, however, is that most viral infections tend to come in bursts followed by a resolution phase. Progressive autoimmune inflammatory diseases such as T1D present with a long-standing inflammation (different, however, for relapse-remitting diseases such as multiple sclerosis or chronic viral infections). Also hinting towards a "memory-like" NK cell phenotype in the pancreas during T1D is the observation that CXCR6, reported to be required for memory NK cell persistence in Paust *et. al.*, is uniquely expressed in pancreatic NK cells compared with lung and especially spleen (unpublished observations). Whether or not memory NK cells are formed in the pancreas during

T1D and whether or not that (potential) memory is antigen-driven or disease-relevant will need to be explored further.

4.3 Innate and adaptive immune system crosstalk

A simple model for innate and adaptive immune crosstalk offers that innate immunity instructs the adaptive immune response by providing inflammatory signals following early pattern recognition receptor engagement. In this commonly cited scenario, the cytokines produced by innate immunity (acting on antigen presentation machinery) are the bridge between innate and adaptive immune responses. In chapter 2, we provide evidence supporting the notion that T_{conv} and T_{reg} cells instruct and regulate NK cells functions through the expression and sequestration of IL-2, respectively, representing an adaptive control over innate immunity. In turn, NK cells produce diabetogenic IFN-γ, amplifying T cell activation (innate immunity impacting adaptive immunity). This scenario represents a dynamic adaptive-innate inter-dependence in the pancreas via complex cytokine cross-talk. As outlined by Kerdiles and colleagues, NK cell receptor education (intrinsic) and APC-provided cytokine production (extrinsic) were previously characterized as the two primary forms of NK functional control thus far (Kerdiles et al., 2013). Our data, in addition to that of Gasteiger and colleagues, supports a third arm of NK cell functional control: the antigen-driven local adaptive response producing and regulating available IL-2 levels to permit or prevent NK cell responsiveness (Kerdiles et al., 2013).

A number of previous reports experimentally support this complex inter-dependent regulation of innate and adaptive immunity, mediated by IL-2, which we discovered in the pancreas. These reports all described a dependence on T_{reg} cells or T_{conv} cells for the activation or suppression of NK cell functions, as previously discussed throughout this manuscript (including NK cell functions in tumors, during bone marrow rejection and during viral infection). However, from the other side, innate cell-derived cytokines, such as IL-12 and IL-18 enhanced NK cell activation and increased NK cell competitive fitness for IL-2 by increasing NK cell expression of CD25 ((Lee et al., 2012), unpublished observations). Therefore, adding to the complex regulatory feedback loop discussed in chapter 2, NK and T_{reg} cells also differentially impact APCs, and, in turn, the APC-derived IL-12 or IL-18 impacts the NK cell's ability to compete for IL-2. Collectively, T_{reg} cells suppress NK cells, T_{conv} cells, and APCs; T_{conv} cells activate T_{reg} cells and NK cells; NK cells activate APCs and T_{conv} cells; and APCs activate T_{reg} cells, NK cells, and T_{conv} cells.

Surprisingly little is known about the dynamic adaptive-innate inter-dependence discussed here and significantly more research will be needed to determine exactly where and when this cross-talk is relevant. A less reductionist approach will be necessary in order achieve this result, as the common "cell X" impacts "cell Y" by "molecule Z" strategy often utilized in immunology research would miss these multi-cell interdependencies. New approaches that attempt to characterize the changes in the entire immune system under different sets of conditions, such as the CyTOF approach discussed in chapter 3, will be important moving forward to achieve these goals.

4.4 Perspectives on emerging T_{reg} cell biology: context-dependent T_{reg} cell mechanisms

A decade of research has supported the idea that T_{reg} cells are critical mediators of systemic and organ-specific peripheral immunological tolerance. The next stage of T_{reg} cell research will likely focus on how specific populations of T_{reg} cells mediate tolerance (and other immune and non-immune functions) and what mechanisms they use to do so. The efforts to understand how T_{reg} cells protect from T1D, as we explored in chapter 2, serve as an instructive example. We showed that one specific mechanism (IL-2 control via CD25 expression) was relevant to disease compared to another (TGF-β). The emerging picture from our study, and others, is that T_{reg} cells, like many other immune cells, are highly context-dependent. The transcriptional programs co-opted by T_{reg} cells are one mechanism to direct specific functions to specific environmental cues. However, context-dependency is also an emerging feature of T_{reg} cells in non-classical immune contexts such as the adipose tissue (Feuerer et al., 2009a; Cipolletta et al., 2011) and muscle (Burzyn et al., 2013). In the former case, the PPAR-γ transcription factor enables T_{reg} cell control of immune cells in the adipose tissue and the regulation of metabolic indices (Cipolletta et al., 2012). In the latter case, T_{reg} cells distinctly expressed amphiregulin that enhanced muscle regeneration. Efforts like these, and our study of T1D, are unraveling the specific context-dependent mechanisms used by T_{reg} cells in various organs and disease settings. However, more effort will be needed to uncover, specifically and mechanistically, where and how T_{reg} cells function and why they fail in certain settings. Further efforts will also be needed to uncover how else and where else T_{reg} cells might influence NK cell functions.

4.5 Targeting NK cells for immunotherapy

In chapter 2, we presented a model for CD25-expressing T_{reg} cells as buffers to keep tissue IL-2 levels in check. In brief, adding IL-2 to a tissue will preferentially activate T_{reg} cells, due to their expression of the high affinity IL-2R. Excess IL-2 will then be dampened due to the suppressive functions of T_{reg} cells. However, an extreme excess of IL-2, perhaps at the point of CD25 saturation on T_{reg} cells, will be free to impact lower affinity IL-2R-expressing cells such as NK and/or T cells, and cause disease (Tang et al., 2008; Sitrin et al., 2013). This scenario might be the source of the recent failed clinical trial in new onset T1D patients (Long et al., 2012) and why new efforts are being put into place to find the appropriate dosing window for T_{reg} cell-specific activation (Waldron-Lynch et al., 2014). The bright side is that failures to treat diseases due to over-activation of the immune system might be beneficial for a cancer immunotherapy. IL-2 might be especially beneficial given that IL-2 increases both proliferation and IFN-γ production by NK cells, as we show in chapter 2, but also supports the recognition of weaker target cells for killing, as explored by Gasteiger and colleagues (Gasteiger et al., 2013a; Gasteiger et al., 2013b). Notably, chemotherapies induce NK cell ligands (Fine et al., 2010), further increasing the chance of a successful immunotherapeutic effect with a four-pronged attack (more NK cells by proliferation, higher inflammation by IFN-γ production, more stress ligands for NK cells to target and a better chance of killing relatively weaker targets). Clinical and pre-clinical research efforts are being explored for combination therapies in this area (Gutbrodt et al., 2014). Given these findings, next-generation cancer immunotherapies may consider using specific targeted therapies in combination with chemotherapy to enhance tumor clearance. The "super-2" reagent, discussed in chapter 2, or similar derivatives that target NK

cells preferentially compared with T_{reg} cells, could be the key to future successful immunotherapies that avoid off-target effects. While this agent has yet to make it to the clinic, structurally optimized antagonists to the IL-1R have been explored for ophthalmic use (Hou et al., 2013), offering proof of principle support for "designed" immune system agonists/antagonists.

4.6 Diversity of the innate lymphoid cells (ILCs)

NK cells were recently reclassified into the ILC family. ILCs are defined by three major features: the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors, a lack of myeloid and dendritic cell phenotypic markers, and a lymphoid origin and morphology (Spits et al., 2013). A large of body of work identified numerous novel ILC subsets with a range of immune and non-immune functions, many of which were developmentally related as indicated by IL-2R-γ- and Id2-dependency. ILCs can be subdivided into 3 major subfamilies. NK cells (defined by NKp46-expression, production of IFN-γ and dependency on IL-15 and Tbet) are the prototypical member of the ILC1 subset; however, a rare and controversial non-NKp46-expressing cell exists with significant functional and phenotypic overlap with classical NK cells (Vonarbourg et al., 2010). The ILC2 family requires IL-7 for development (Moro et al., 2010), secretes T_H2-associated cytokines such as IL-5, IL-13 and IL-9 (Wilhelm et al., 2011), and expresses T_H2-associated transcription factors (Hoyler et al., 2012). Meanwhile, the ILC3 subset expresses T_H17-associated cytokines (IL-17A and IL-22) and the RORγT transcription factor. One subclass of the ILC3s expresses NKp46 and produces IL-22 at

mucoscal surfaces. As reviewed by Spits and colleagues, the collective ILC family expands often, with each new subset expressing a new combination of surface receptors. Altogether, ILCs are known to express CD4, CD25, Thy1, cKit, IL-7Rα, Sca1, ICOS, NKp46, IL-1R, IL-23R, IL-12R, ST2 and IL-17Rβ in highly diverse and combinatorial fashions. The emerging picture for innate lymphocytes is that they contain a heterogeneity previously only thought to exist in the adaptive immune system. In chapter 3, we explored $CD3^{-}CD19^{-}NKp46^{+}$ cell diversity and reported impressive combinatorial expression of surface receptors typically found on both innate and adaptive immune cells. These receptors included many of the receptors listed for other ILCs. Notably, this gating scheme allowed for the inclusion of other ILC members, including the NKp46-expressing ILC3 population. We also reported the expression of RORγT in some of the nodes co-expressing NKp46. The possibility that an ILC3 population resides in the pancreas, albeit a rare one, should be explored further. Arguing against the inclusion of non-conventional NK cell ILCs in this population, these $CD3^{-}CD19^{-}NKp46^{+}$ pancreatic-origin cells expressed no $T_{H}2$ or $T_{H}17$ cytokines as measured during transcriptional profiling (unpublished observations). Due to their similarity with conventional NK cells, including expression of other NKCRs, it would be interesting to determine whether or not non-NK cell ILCs express any of the Ly49 family receptors and, if so, whether or not those ILCs undergo any form of education as conventional NK cells do.

4.7 The future of "deep profiling" the immune system

Recent technological advances, including the CyTOF technology discussed and utilized in chapter 3, have opened the possibility for extensive profiling of immune system heterogeneity. Already, this technology has uncovered extensive and unexpected diversity in numerous immunological contexts, including drug responses during hematopoeisis (Bendall et al., 2011), CD8+ T cell anti-viral responses (Newell et al., 2012), leukemia (Amir et al., 2013), human peripheral blood NK cells (Horowitz et al., 2013) and, most recently, B cell differentiation (Bendall et al., 2014). The emerging picture from these studies is that the immune system is incredibly diverse and that diversity between and within cellular populations has dramatic effects on how immune cells respond to perturbations (Bendall et al., 2011). No study yet has explored the diversity of the immune system during an autoimmune disease setting. This is perhaps surprising given that autoimmune diseases, especially T1D, are highly complex, with many cell types and mechanisms involved. To that end, in chapter 3, we presented data illustrating the diversity of the NK cell compartment found in the pancreas of prediabetic NOD mice. NK cells, comprising less than 5% of the pancreatic infiltrate, contained over 25 different phenotypic patterns. The pancreas-infiltrating immune system, on the whole, contained at least 385 total nodes depending on the antibody panel used in the assessment. Considering that only roughly 30 parameter panels are utilized in these studies, compared with the thousands or tens of thousands of genes actually expressed by immune cells, the true single-cell diversity will likely be many logs higher than what we reported here. In full, our data illustrates both the impressive diversity of total cellular infiltrate in the pancreas as well as that of the NK cell populations included. How

this diversity develops, is maintained, and whether or not it is functionally consequential will need to be explored further.

These high-dimensional approaches are more than just an exercise in finding as many surface markers as possible. Rather, they may be the key to finding specific rare subsets of cells and phenotypes responsible for complex diseases such as autoimmunity or cancer. The data presented in chapter 3 are just the beginning of a larger effort to comprehensively identify total immune system infiltration in the pancreas, how that immune compartment evolves over the course of disease and to determine what populations or activation patterns are most correlated with disease parameters. By combining this strategy with disease-predictive technologies such as non-invasive magnetic resonance imaging of the pancreas (Denis et al., 2004), we will stratify predicted disease-progressing and non-progressing animals to determine how the insulitis maturation differs between these two conditions and to discover further predictive disease-relevant biomarkers.

4.8 Conclusions

The terms "innate" and "adaptive" immunity were initially helpful descriptors to differentiate what seemed like, at the time, two entirely different physiological systems. Today, the separation of innate and adaptive immunity might be hindering our appreciation for how intertwined and diverse these systems actually are. In this thesis, evidence was presented to support a highly context-dependent cross-talk of T_{reg} cells and NK cells, responsible for control of T1D, with IL-2 at the heart of this cross-talk. We further showed a high-dimensional diversity

in NK cells, not-too-long-ago considered a relatively homogenous lineage but with a growing body of evidence to the contrary. In the future, experimental efforts should further focus on the individual and counteracting context-dependent roles of T_{reg} cells and NK cells, especially in the context of cancer and autoimmunity. Also, it will be important to increase our understanding of cellular cross-talk within and between innate and adaptive immunity. Finally, significantly more work will be needed to assess the true diversity of immune cells and the contribution of specific cellular subsets to normal immune function and disease states.

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Appendix

A. Publication:

Jonathan Sitrin, Aaron Ring, K. Christopher Garcia, Christophe Benoist and Diane Mathis (2013). Tregs Control NK Cells in an Insulitic Lesion by Depriving them of IL-2. The Journal of Experimental Medicine 93:2260. doi:10.1084/jem.20122248

Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2

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Regulatory T (T reg) cells control progression to autoimmune diabetes in the BDC2.5/NOD mouse model by reining in natural killer (NK) cells that infiltrate the pancreatic islets, inhibiting both their proliferation and production of diabetogenic interferon-γ. In this study, we have explored the molecular mechanisms underlying this NK–T reg cell axis, following leads from a kinetic exploration of gene expression changes early after punctual perturbation of T reg cells in BDC2.5/NOD mice. Results from gene signature analyses, quantification of STAT5 phosphorylation levels, cytokine neutralization experiments, cytokine supplementation studies, and evaluations of intracellular cytokine levels collectively argue for a scenario in which T reg cells regulate NK cell functions by controlling the bioavailability of limiting amounts of IL-2 in the islets, generated mainly by infiltrating CD4+ T cells. This scenario represents a previously unappreciated intertwining of the innate and adaptive immune systems: CD4+ T cells priming NK cells to provoke a destructive T effector cell response. Our findings highlight the need to consider potential effects on NK cells when designing therapeutic strategies based on manipulation of IL-2 levels or targets.

The Journal of Experimental Medicine

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Abbreviations used: DTR, diphtheria toxin receptor; NOD, nonobese diabetic; MCMV, murine cytomegalovirus; MFI, mean fluorescence intensity; STAT, signal transducer and activator of transcription; T1D, type 1 diabetes; T reg cell, regulatory T cell.

Regulatory T (T reg) cells , in particular those expressing the forkhead box transcription factor Foxp3, are primary controllers of immune responsiveness and peripheral immunological tolerance (Rudensky, 2011). These critical immunoregulatory cells have been implicated in the control of an assortment of immunological processes, ranging from autoimmunity to infection. In humans, loss-of-function mutations of Foxp3 lead to a severe multi-organ autoimmune and inflammatory disorder called IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked inheritance). *Scurfy* mice, carrying a frameshift mutation in Foxp3, show a similar fatal systemic disease. Moreover, conditional ablation of the T reg cell lineage demonstrated a lifelong requirement for Foxp3-expressing cells to contain highly aggressive, multi-organ autoimmunity, even after normal development of the immune system.

T reg cells also regulate several organ-specific autoimmune diseases, notably type-1 diabetes (T1D), characterized by autoimmune attack specifically on β cells in the pancreatic islets of Langerhans (Bluestone et al., 2008). Supplementation with T reg cells or enhancement of their function protected from T1D, whereas genetic deficiencies in or experimental reductions of T reg cells exacerbated disease in the nonobese diabetic (NOD) mouse model or its T cell receptor (TCR) transgenic derivatives.

Exactly how T reg cells exert their impact on immune responsiveness has been the subject of extensive exploration. To date, numerous protective mechanisms have been ascribed to them, reflecting their expression of several regulatory molecules, either displayed at the cell surface or secreted (Vignali et al., 2008; Josefowicz et al., 2012). It has become clear that the context in which T reg cells perform their regulatory function can shape the mechanisms of immune suppression they use, i.e., the tissular location or inflammatory "flavor" of the response they are participating in (Sojka et al., 2008; Josefowicz et al., 2012).

The behavior of T reg cells in the insulitic lesion of BDC2.5/NOD TCR transgenic mice

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(Katz et al., 1993) serves as an instructive example. This line carries the rearranged TCR genes of a diabetogenic T cell clone isolated from a NOD mouse and has been instrumental in the identification of a spectrum of immunoregulatory genes, molecules, and cells that control the frequency and aggressivity of diabetogenic T cells (André et al., 1996). When the BDC2.5 TCR transgenes are propagated on the NOD genetic background, T cells stereotypically invade the islets at 15–18 d of age and seed a massive infiltration therein; however, progression to diabetes occurs rarely (10–20%) and only months later, reflecting strong immunoregulation (Gonzalez et al., 1997). When a transgene expressing the diphtheria toxin receptor (DTR) under the dictates of the Foxp3 promoter/enhancer elements was crossed into this system (BDC2.5/NOD.Foxp3^{DTR} mice), conditional T reg lineage ablation provoked nearly 100% penetrance of diabetes within days (Feuerer et al., 2009), highlighting the requirement for T reg cells to guard against T1D. Analysis of the insulitic lesion revealed, surprisingly, that the earliest detectable responders to the loss of T reg cells were NK cells, which accumulated to a higher fraction of the infiltrating cells and began to produce IFN-γ within hours. Subsequently, there was increased activation of diabetogenic CD4$^+$ T cells, including their production of IFN-γ. Neutralizing IFN-γ or depleting NK cells dampened pancreatic CD4$^+$ T cell activation and substantially delayed the onset of diabetes. Thus, there seemed to be a direct and continual requirement for T reg cells to keep NK cells, and ultimately diabetes, in check.

Much of the T reg cell–centered research over the last decade has focused on their control of populations typically considered to be participants in adaptive immune responses, especially other T cells and antigen-presenting cells. Less emphasis has been placed on their impact on cells involved in innate immune responses, notably NK cells. This neglect is a bit surprising given that NK cells were long ago found to be hyperproliferative and functionally enhanced in *scurfy* mice (Ghiringhelli et al., 2005). Furthermore, the original report describing T reg ablation also documented a large increase in NK cell numbers (Kim et al., 2007). An exception is the growing body of work on mouse cancer models and human cancer patients that demonstrates a negative correlation between NK and T reg cells, as concerns both presence and function (Shimizu et al., 1999; Ghiringhelli et al., 2005, 2006, 2007; Smyth et al., 2006). The mechanism most commonly highlighted in these studies was T reg mobilization of TGF-β, often in surface-bound form, to directly inhibit NK cell function. In support of this scenario, blockade of TGF-β signaling in NK cells in a mutant TGF-β receptor transgenic model caused a dramatic increase in cell numbers and enhanced secretion of IFN-γ (Laouar et al., 2005).

Given that this axis is still relatively unexplored, particularly in the context of autoimmune disease, we sought to identify the molecular underpinnings of T reg cell/NK cell cross-talk in control of diabetes in the BDC2.5/NOD model. Our explorations were greatly facilitated by the rapidity and synchrony of the diabetogenic changes unleashed by punctual ablation of T reg cells in BDC2.5/NOD.Foxp3^{DTR} mice. This feature permitted us to perform an accurate, detailed inventory of molecular changes over time, and to test mechanistic hypotheses with short courses of inhibitory or enhancing treatments. Our findings need to be considered in future strategies to prevent or dampen T1D.

RESULTS

Acute T reg cell perturbation in BDC2.5/NOD mice rapidly induced signs of activation in pancreas-infiltrating NK cells

To identify molecular pathways underlying the response of pancreatic NK cells to a loss of T reg control, we performed microarray-based gene-expression profiling, as a comprehensive and unbiased approach. NK cells from pancreata of insulitic BDC2.5/NOD.Foxp3^{DTR} mice and control DTR-negative littermates were analyzed 24 h after DT treatment (Fig. 1 A). Even at this relatively early time point, there were clear transcriptional changes in the T reg cell–depleted mice: induction of 89 genes >2-fold (highlighted in red) and repression of 123 genes >2-fold (in blue) compared with 1 and 0 loci, respectively, in an analogous comparison of randomized datasets. The transcripts up-regulated in the absence of T reg cells included indicators of the three canonical activities of NK cells: proliferation (*zbtb32*, 5.9-fold [J.C. Sun, personal communication]), cytokine production (*ifng*, 2.5-fold), and cytotoxicity (*gzmb*, 2.6-fold). Such changes fit well with our previous demonstration that ablation of T reg cells in this model induced cytotoxic activity from, proliferation of, and IFN-γ production by islet-infiltrating NK cells (Feuerer et al., 2009).

The full list of genes whose expression was at least doubled or halved in response to T reg cell removal is presented in Table S1. A quick glance at the induced loci revealed many of them to be characteristic of activated NK cells. More precisely, we compared the response of pancreatic NK cells to T reg depletion with that of splenic NK cells challenged by general (cytokine) or more specific [murine cytomegalovirus (MCMV)] stimuli. The diagonal nature of the red cloud in the fold-change/fold-change (FC/FC) plot of Fig. 1 B denotes substantial overlap between the genes induced in pancreatic NK cells by removal of T reg cells and in splenic NK cells stimulated in culture with IL-12 + IL-18. The tilt toward the horizontal axis signifies that the cytokine stimulus was more potent under these particular experimental conditions. An analogous result was found for the blue-colored repressed transcripts, with an even more pronounced skewing. Overall, 79% of the transcripts that increased or decreased by >2-fold after T reg cell removal were similarly augmented or diminished, respectively, subsequent to cytokine stimulation in culture. Similar, although less striking, observations came from the comparison of pancreatic NK cells responding to a loss of T reg cells and splenic NK cells mobilized by MCMV infection (Fig. 1 C). In this case, 51% of the transcripts induced or repressed >2-fold by T reg cell ablation were enhanced or dampened, respectively. Notably, *ifng, zbtb32*, and *gzmb* were induced in both the cytokine-stimulated and MCMV-induced responses.

We also addressed how rapid and localized the transcriptome alterations were after punctual T reg cell ablation. Fig. 1 D

shows FC/FC plots comparing transcriptional changes in the pancreas and spleen with or without T reg cell perturbation at 8-, 15-, and 24-h time points. The bull's eye nature of the black cloud of expression values at 8 h indicates that the bulk of transcripts were only minimally changed in the two organs. Yet, values for transcripts destined to be up-regulated (red) or down-regulated (blue) in pancreas-infiltrating NK cells had already diverged in the pancreas, which is impressive given that there was no evident loss of T reg cells at this early time point (unpublished data). This divergence was further amplified at 15 h. At 24 h, slight tilts of both the induced and repressed transcript values away from the horizontal axis, toward the diagonal, suggest that the same set of genes was modulated in the spleen, but with delayed kinetics.

T reg control of pancreas-infiltrating NK cells did not operate acutely through TGF-β

Considering that TGF-β has repeatedly been implicated in the control of NK cell functions in other contexts (Uhl et al., 2004; Friese et al., 2004; Lee et al., 2004; Ghiringhelli et al., 2005,

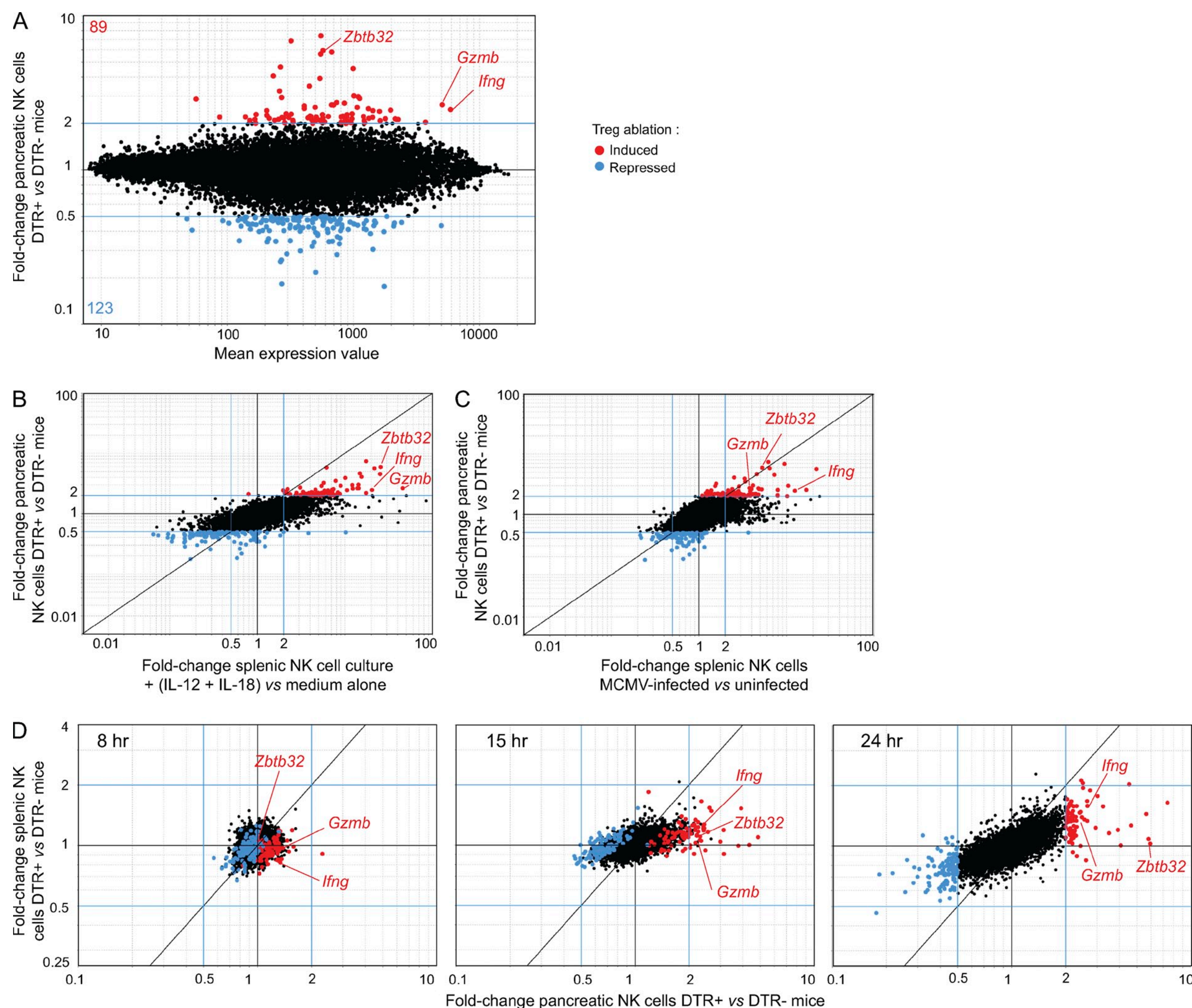

Figure 1. Gene expression changes in pancreatic NK cells soon after T reg cell perturbation. NK cells were sorted from the pancreatic infiltrate 24 h after DT injection into BDC2.5/NOD mice with or without DTR expressed in T reg cells, and were profiled by microarray. (A) Transcript changes. Differential gene-expression (FC) values for DTR$^+$ (n = 3) and DTR$^-$ mice (n = 6) (y axis) versus their two-class mean (x axis). Induced (>2-fold) genes are highlighted in red and repressed (>2-fold) genes are highlighted in blue. (B and C) Activation features. FC/FC plots of the same NK cell data as in A versus NK cells responding to different activation stimuli. Y axis, FC values for DTR$^+$ versus DTR$^-$ mice; x axis, FC values for splenic NK cells cultured with (n = 3) or without (n = 2) IL-12 + IL-18 (see Materials and methods for details; B) or splenic NK cells from C57/BL6 mice 24 h after being infected with MCMV or not (n = 3; C). (D) Time course of transcript changes. Carried out as in A, except additional time points at 8 and 15 h were examined. FC/FC plots comparing gene expression differentials for pancreatic NK cells from DTR$^+$ and DTR$^-$ BDC2.5/NOD mice (y axis) versus splenic NK cells from the same animals (x axis). Multiple replicates of cellular populations were collected (usually n = 3–6) and averaged. Highlighting in A–D represents the same set of genes.

2006; Laouar et al., 2005; Smyth et al., 2006), we wondered whether this cytokine is involved in the ability of T reg cells in the pancreas of BDC2.5/NOD mice to keep the insulitic lesion in check. The slight underrepresentation of TGF-β–dependent genes in the pancreatic CD4+ T cell transcriptome after T reg ablation fit this hypothesis (Feuerer et al., 2009). First, we confirmed that TGF-β could regulate IFN-γ production by NOD-genotype NK cells in an in vitro culture system. Indeed, splenic NK cells stimulated in culture with IL-12+IL-18 produced less IFN-γ in the presence of TGF-β (Fig. 2 A; mean fluorescence intensity [MFI] reduced 77 ± 11%). Moreover, microarray analysis revealed a clear negative correlation between transcript changes provoked by addition of TGF-β to cultures of activated splenic NK cells and by interference with in vivo T reg control of pancreatic NK cells; i.e., the majority of genes up-regulated in the absence of T reg

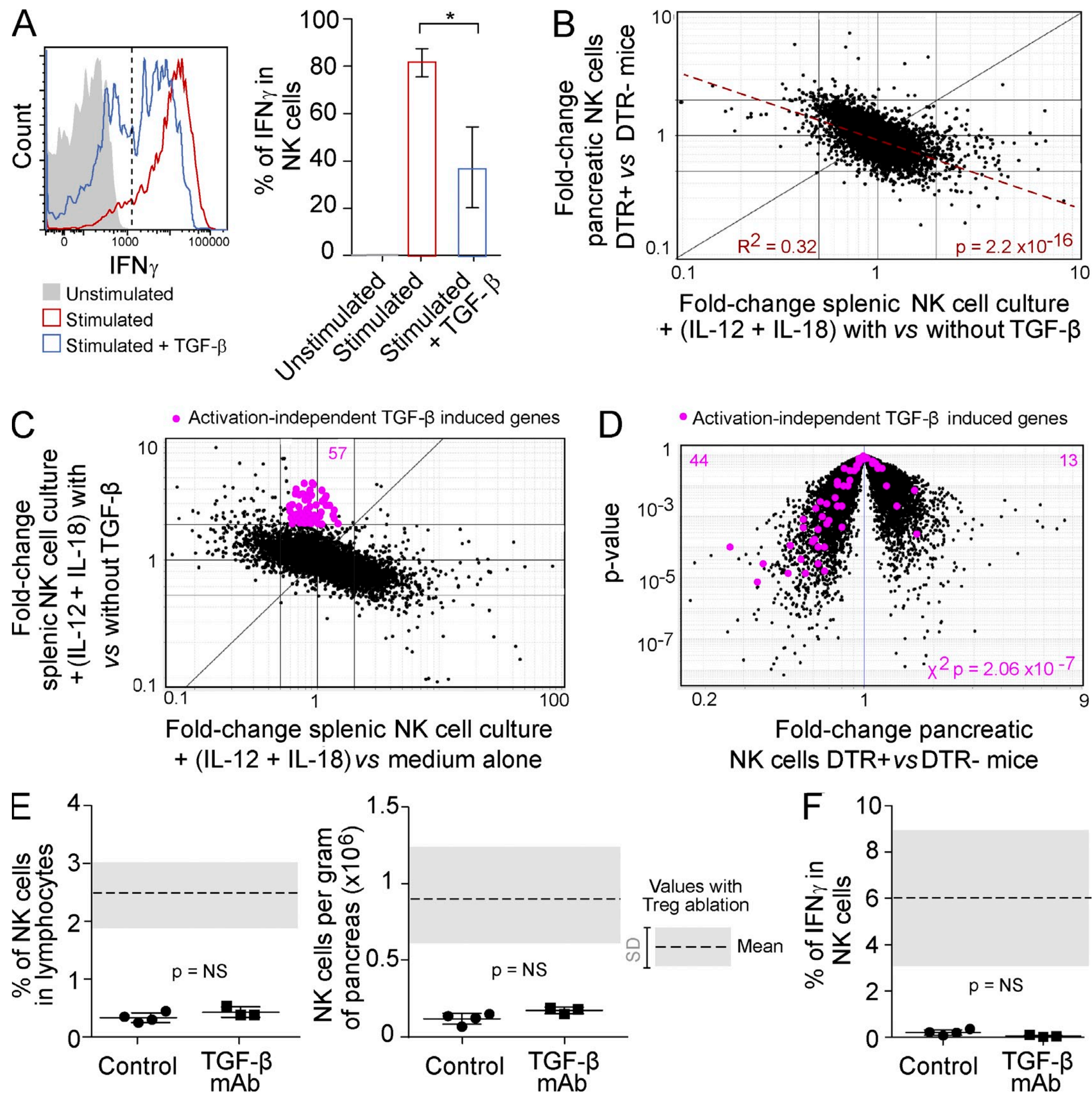

Figure 2. The role of TGF-β signaling. (A, left) Representative flow cytometry profiles of IFN-γ expression in NOD-derived splenic NK cells cultured with (red) or without (gray) IL-12 + IL-18 in the absence of TGF-β, or with both cytokines and TGF-β (blue). (right) Summary data from three independent experiments. Mean ± SD. The p-value was calculated using the two-tailed unpaired Students' *t* test. (B) Reciprocal transcript changes promoted by the loss of TGF-β and T reg cells. FC/FC plot comparing cytokine-activated, cultured, splenic NK cells ± TGF-β (x axis, *n* = 3) versus pancreatic NK cells from BDC2.5/NOD mice ± T reg ablation (y axis, same data as in Fig. 1 A). Dashed red line, linear regression. (C) FC/FC plot comparing transcriptional profiles of cytokine-activated, cultured, splenic NK cells ± TGF-β (y axis, as in Fig. 2 B) versus NK cells ± cytokine-activation (x axis, as in Fig. 1 B). Activation-independent TGF-β–induced genes were highlighted in pink and were superimposed (D in pink) on a volcano plot (p-value vs. fold change) of NK cell transcripts from BDC2.5/NOD mice depleted or not of T reg cells (same data as in Fig. 1). The number of signature genes up-regulated (right) or down-regulated (left) 24 h after T reg cell perturbation are indicated. P-value from the χ^2 test. (E and F) Summary flow cytometry data for BDC2.5/NOD mice treated for 24 h with anti–TGF-β versus either an isotype-control mAb or PBS (combined). Percentage of NK cells of lymphocytes (E) and percentage of IFN-γ+ NK cells (F) for two or three independent experiments. Mean ± SD. Dotted line (mean) and gray shading (SD) represents values achieved after T reg cell ablation (a composite of multiple independent experiments).

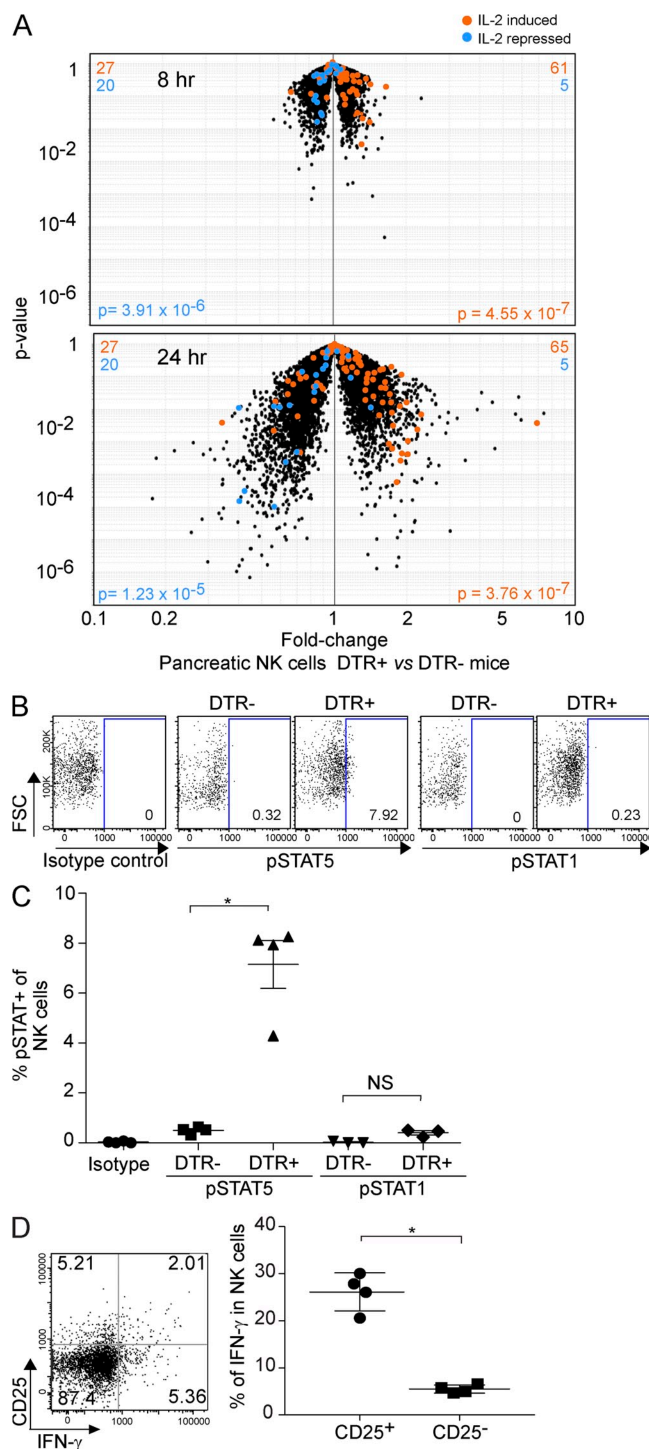

Figure 3. An IL-2 footprint is induced by T reg ablation. (A) IL-2–induced (orange) and –repressed (blue) gene transcripts (Marzec et al., 2008) are superimposed on volcano plots (p-value versus FC) of pancreatic NK cell transcripts up-regulated (to the right) or down-regulated (to the left) by perturbation of T reg cells (data from Fig. 1) at 8 h (top) and 24 h (bottom). P-values calculated using the χ^2 test. (B) Representative flow cytometry plots for NK cells isolated from the pancreas of BDC2.5/NOD mice 24 h after T reg ablation or not. (C) Summary data for three to four independent experiments with mean ± SD. (D, left) Representative flow cytometry plots for pancreas-infiltrating NK cells after T reg cell ablation; (right) summary for the percentage of CD25-expressing versus CD25-negative NK cells simultaneously expressing IFN-γ from at least three independent experiments.

cells were down-regulated by TGF-β, and vice versa (Fig. 2 B). Lastly, we generated a signature for TGF-β's impact on NK cells independent of general activation by comparing transcriptional profiles of NK cells that were cytokine-activated or not and stimulated with TGF-β or not (Fig. 2 C; genes listed in Table S2), and followed its distribution in pancreas-infiltrating NK cells unleashed in the absence of T reg cells (Fig. 2 D). The TGF-β–induced genes were repressed upon removal of T reg cells (i.e., they fall to the left of unity in the FC versus p-value volcano plot of Fig. 2 D). These data were all consistent with the notion that T reg cells might operate through TGF-β signaling to control NK cells in the insulitic lesion.

To directly test this hypothesis, we attempted to mimic punctual T reg cell ablation by injecting a mAb recognizing TGF-β into BDC2.5/NOD mice. This intervention was unable to recapitulate the effects of T reg depletion as neither the fraction/number of pancreatic NK cells (Fig. 2 E) nor their production of IFN-γ (Fig. 2 F) was induced anywhere near the levels observed with T reg ablation (shaded gray). The mAb was bioactive, however, as it substantially reduced the fraction of $CD103^+$ T reg cells, in particular in the mesenteric lymph nodes (not shown), a population known to be TGF-β dependent (Feuerer et al., 2010; Reynolds and Maizels, 2012). Thus, TGF-β may not play an important role in T reg control of NK cell activation in this context, at least not acutely. It is also possible that the insulitic lesion provides an environment that is unusually resistant to neutralization of TGF-β.

Punctual ablation of T reg cells elicited an IL-2 response signature in pancreas-infiltrating NK cells

For several reasons, we wondered whether IL-2 might be a driver of the pancreatic NK cell transcriptome changes provoked by loss of T reg cells in BDC2.5/NOD mice: first, a rapid loss of cells, like T reg cells, that express the high-affinity IL-2R component IL-2Rα (CD25) stands to substantially increase IL-2 bioavailability; second, sequestration of IL-2 by T reg cells is known to be one of their mechanisms of controlling T cells (Barthlott et al., 2005; Scheffold et al., 2005; Pandiyan et al., 2007); third, IL-2 can prime NK cells, both in vitro and in vivo, for proliferation and IFN-γ production (Fehniger et al., 2003; Granucci et al., 2004; Lee et al., 2012); and, finally, previous studies on the NOD mouse model of T1D suggested a limited IL-2 availability in the islet infiltrate (Tang et al., 2008b). To address this possibility, we overlaid an IL-2–responsive gene signature derived from a human $CD4^+$ T cell lymphoma (Marzec et al., 2008) onto a volcano plot depicting the transcriptional response of pancreatic NK cells to punctual T reg ablation (Fig. 3 A). As early as 8 h and continuing through 24 h after DT treatment, there was a significant skewing of IL-2–induced genes within the set of loci up-regulated in pancreatic NK cells and an analogous enrichment of signature IL-2–repressed genes among the down-regulated loci,

suggesting an increased signaling by the cytokine. Such enrichment was also seen with two other IL-2 responsive gene signatures: one derived from murine CD8$^+$ T cells (Verdeil et al., 2006) and the other from human NK cells (Dybkaer et al., 2007; not depicted).

In addition, flow cytometric analysis revealed a substantial increase in the phosphorylation of STAT5, an event downstream of IL-2R engagement and required for signal transduction, in pancreatic NK cells from T reg cell–depleted versus control mice. STAT1, not a member of the signaling cascade downstream of IL-2R, showed minimal additional phosphorylation, which was not statistically significant (Fig. 3, B and C). When we gated on IFN-γ^+ NK cells, the MFI of pSTAT5 was 2.2-fold (±0.7) higher. Furthermore, a higher fraction of CD25-expressing, as opposed to CD25-nonexpressing, NK cells in the pancreatic infiltrate made IFN-γ (Fig. 3 D), suggesting that the former might have a competitive advantage. However, the fact that a clear CD25$^-$ IFN-γ^+ population was detectable indicated that expression of CD25 was not required for IFN-γ production. Collectively, these data indicate that, upon removal of T reg cells, pancreatic NK cells experienced heightened signaling through the IL-2R, which could potentially be responsible for their activation.

IL-2 was required for the activation of pancreas-infiltrating NK cells provoked by loss of T reg cells

To evaluate the importance of IL-2 in the pancreatic NK cell response to acute T reg depletion in BDC2.5/NOD mice, we tested the effect of neutralizing this cytokine with a mAb. Complicating such an experiment was the fact that the mAb could potentially stabilize IL-2 and direct its binding to the high- and/or low-affinity receptor (Boyman et al., 2006). Therefore, we used the mAb JES6-1, as the complexes it forms with IL-2 bind highly preferentially to the α chain of the high-affinity IL-2R (expressed mostly on T reg cells) rather than to the low-affinity IL-2Rβ chain (expressed on NK cells; Boyman et al., 2006). In the context of T reg cell ablation, then, any such in vivo–generated IL-2–JES6-1 complexes should be fairly innocuous.

Treatment of BDC2.5/NOD mice with JES6-1 in conjunction with T reg cell ablation blocked NK cell accumulation, whereas analogous administration of an isotype-control mAb had no detectable effect (Fig. 4 A). Importantly, no significant drop in NK cell number or fraction occurred when JES6-1 was administered to DTR-negative littermate controls, indicating that simple IL-2 starvation did not provoke a wave of NK cell death. T reg cells remained at low levels after DT plus anti–IL-2 injection, demonstrating that IL-2–JES6-1 complexes did not trigger "bounce-back" of the T reg cell population in the pancreas (Fig. 4 B).

IL-2 neutralization also blocked the NK cell production of IFN-γ elicited by punctual T reg depletion, as measured by the percentage of IFN-γ–expressing NK cells (Fig. 4 C) and a reduction in the MFI of IFN-γ in NK cells (55 ± 19%). This is a critical point because we have previously documented that experimental blockade of IFN-γ signaling in this model sufficed in and of itself to halt the characteristic extremely rapid induction of T1D (Feuerer et al., 2009). Thus, IL-2 appears to be a crucial element in the unleashing of pancreatic NK cells when relieved of T reg cell control.

A potential caveat to the use of a mAb that forms IL-2 complexes preferentially capable of engaging CD25 is that NK cells often increase CD25 expression in response to activating stimuli. Therefore, we examined to what extent T reg cell ablation induced expression of this receptor and if mAb blockade altered that effect. Levels of CD25 did not increase on NK cells in the absence of T reg cells; however, T reg ablation combined with mAb treatment did enhance expression of CD25 (Fig. 4 D).

IL-2 supplementation overcame T reg cell control of pancreas-infiltrating NK cells, inducing their activation

We explored to what extent the addition of IL-2 to BDC2.5/NOD mice could surmount T reg cell control of NK cell activities within the insulitic lesion. Given the relatively short in vivo half-life of IL-2 (Donohue and Rosenberg, 1983), we chose to administer IL-2–anti–IL-2 mAb complexes, which stabilize the cytokine and target it preferentially to one or the other of the IL-2 receptors. In this case, S4B6 was the most appropriate mAb, as IL-2–S4B6 complexes bind well to IL-2Rβ–expressing cells, notably NK cells, and poorly to cells displaying IL-2Rα, such as T reg cells (Létourneau et al., 2010). Although treatment of BDC2.5/NOD mice with IL-2–S4B6 complexes did not result in the anticipated expansion of the pancreatic NK cell population (Fig. 5 A), it did elicit IFN-γ synthesis (Fig. 5 B; also true for splenic NK cells; not depicted). The induction of IFN-γ, measured as an increase in IFN-γ^+ cells, was similar to what was seen after ablation of T reg cells (Fig. 2 E and Fig. 4 C). However, we realized that this experiment had an unexpected complication that might explain the lack of NK cell proliferation: although previous studies claimed no effect on T reg cells after treatment of mice with IL-2–S4B6 complexes, we observed an approximate doubling of T reg cells in terms of both fraction of T cells and total cell numbers (unpublished data).

To circumvent this issue, we treated BDC2.5/NOD mice with a mutant form of IL-2 (Super 2), which structurally relieves the dependence of IL-2 on engaging the IL-2Rα chain, thereby shifting the competitive advantage for binding of IL-2 from T reg cells to IL-2Rβ chain–expressing NK cells (Levin et al., 2012). This intervention also induced IFN-γ synthesis by pancreatic NK cells in the absence of population expansion (Fig. 5, A and B), with a less pronounced influence on T reg cells than found in IL-2–S4B6 complex treatment (not depicted).

Although disjunctions between readouts of NK cell proliferation and production of IFN-γ have been described (Cooper et al., 2009), we were surprised to find a divergence in this context because past studies have documented both activities after treatment of mice with IL-2–S4B6 complexes (Jin et al., 2008). However, we noticed that this study and others (Boyman et al., 2006; Mostböck et al., 2008; Létourneau et al., 2010), focused on expansion of NK cells quantified at later time

points. Indeed, when we administered IL-2–S4B6 complexes to BDC2.5/NOD mice and assayed at day 4 (rather than the usual 24 h), there was a clear induction of both proliferation of and IFN-γ synthesis by pancreatic NK cells (Fig. 5 A and B). Thus, supplementation with IL-2 was able to overcome the restraint on NK cells imposed by T reg cells in the insulitic lesion of BDC2.5/NOD mice. Most important was the augmentation of IFN-γ synthesis 24 h after IL-2 administration, as this cytokine is an important and required element in the rapid induction of T1D after T reg ablation. NK cell proliferation was also evident, but was delayed in comaparison with the T reg ablation model, perhaps reflecting a requirement to surpass a higher signaling threshold or the need for an additional cofactor.

To determine whether clinical diabetes could be rescued by supplementing BDC2.5/NOD mice with IL-2, we injected them for 3 d with IL-2–S4B6 complexes. Most complex-injected mice rapidly succumbed to diabetes, starting 6 d after the beginning of treatment. In contrast, none of the control mice developed diabetes during this time frame (Fig. 5 C).

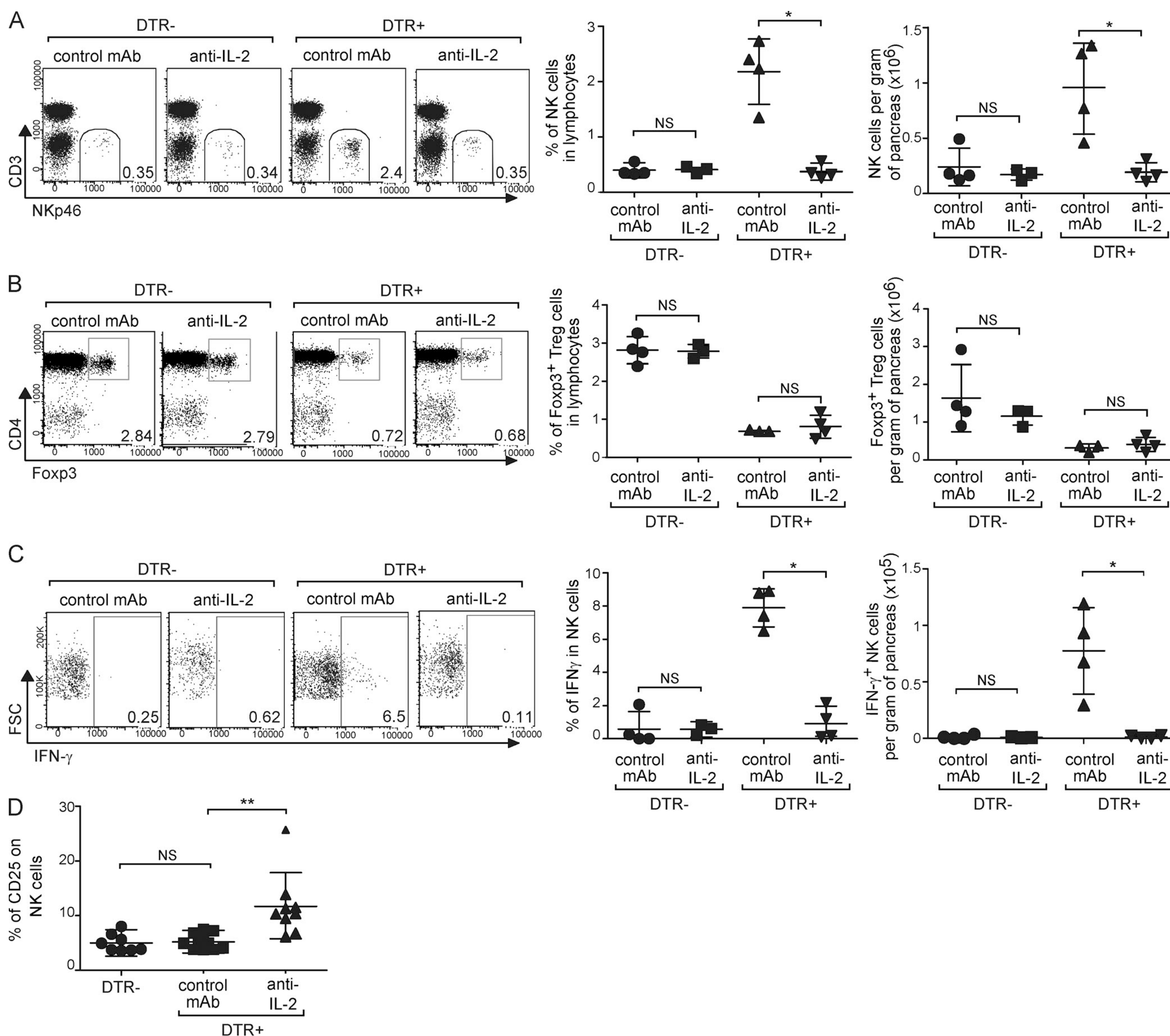

Figure 4. Neutralization of IL-2 prevents the activation of pancreatic NK cells in response to T reg cell ablation. (A–D) The pancreatic infiltrate from BDC2.5/NOD mice (DTR+ or DTR− control littermates) was analyzed 24 h after DT injection ± anti–IL-2 mAb JES6-1 (or isotype control) co-injection. (left) Representative flow cytometry plots. (right) Summary data for fraction and numbers with mean ± SD from three to four independent experiments. (A) NK cells (NKp46+CD3−CD19−) and (B) Foxp3+ T reg cells (Foxp3+CD4+CD3+CD19−) in the pancreatic infiltrate (FSC/SSC lymphocyte gated). (C) IFN-γ–producing NK cells among total NK cells in the pancreatic infiltrate. (D) CD25 expression on pancreatic NK cells.

CD4+ T cells were by far the major producers of IL-2 in the pancreatic lesion in the presence or absence of T reg cells

These findings raised a pair of important questions: what cells produced IL-2 in the pancreatic infiltrate of BDC2.5/NOD mice? And how did IL-2 levels change with T reg ablation? To address the first issue, we performed intracellular staining of IL-2 in pancreatic cells from unmanipulated BDC2.5/NOD mice. $CD45^+$ cells showed a clear IL-2 signal, easily distinguishable from the isotype control background staining. The vast majority of IL-2–expressing cells (>95%) were $CD4^+$ T cells (Fig. 6 A). The remaining signal (~3%) came from $CD3^+CD4^-$ cells, which further analysis revealed to be $CD8^+$ (unpublished data), and double-negative cells (<1%). Next, we compared levels of IL-2–expressing cells in the BDC2.5/NOD pancreatic infiltrate, with or without T reg cell ablation, at 8 h after DT treatment (when the increased transcriptional activity of IL-2 response genes is clearly detectable; Fig. 1 D). This analysis included the entire $CD45^+$ population to ensure a broad coverage. No difference was found in the fraction or number of IL-2–expressing cells, or in the MFI of IL-2–expressing cells (Fig. 6 B). Although it remains possible that at later time points an expansion of $CD4^+$ T cells entails higher IL-2 levels, this scenario is unlikely within the first few hours, as the fraction and total number of IL-2–producing $CD4^+$ T cells remained constant (Fig. 6 C). Thus, T reg cells seem to acutely regulate NK cells by sequestering local IL-2 rather than dampening its synthesis.

DISCUSSION

$Foxp3^+CD4^+$ T reg cells are known to regulate the progression of T1D in several mouse models, and are thought to exert an analogous influence in human T1D patients (Bluestone et al., 2008). Nonetheless, our understanding of the role of T reg cells during the human disease remains very limiting owing to a dearth of preclinical samples, when T reg cells likely exert the most important influences. Mouse models such as the BDC2.5/NOD line, which have facilitated the study of

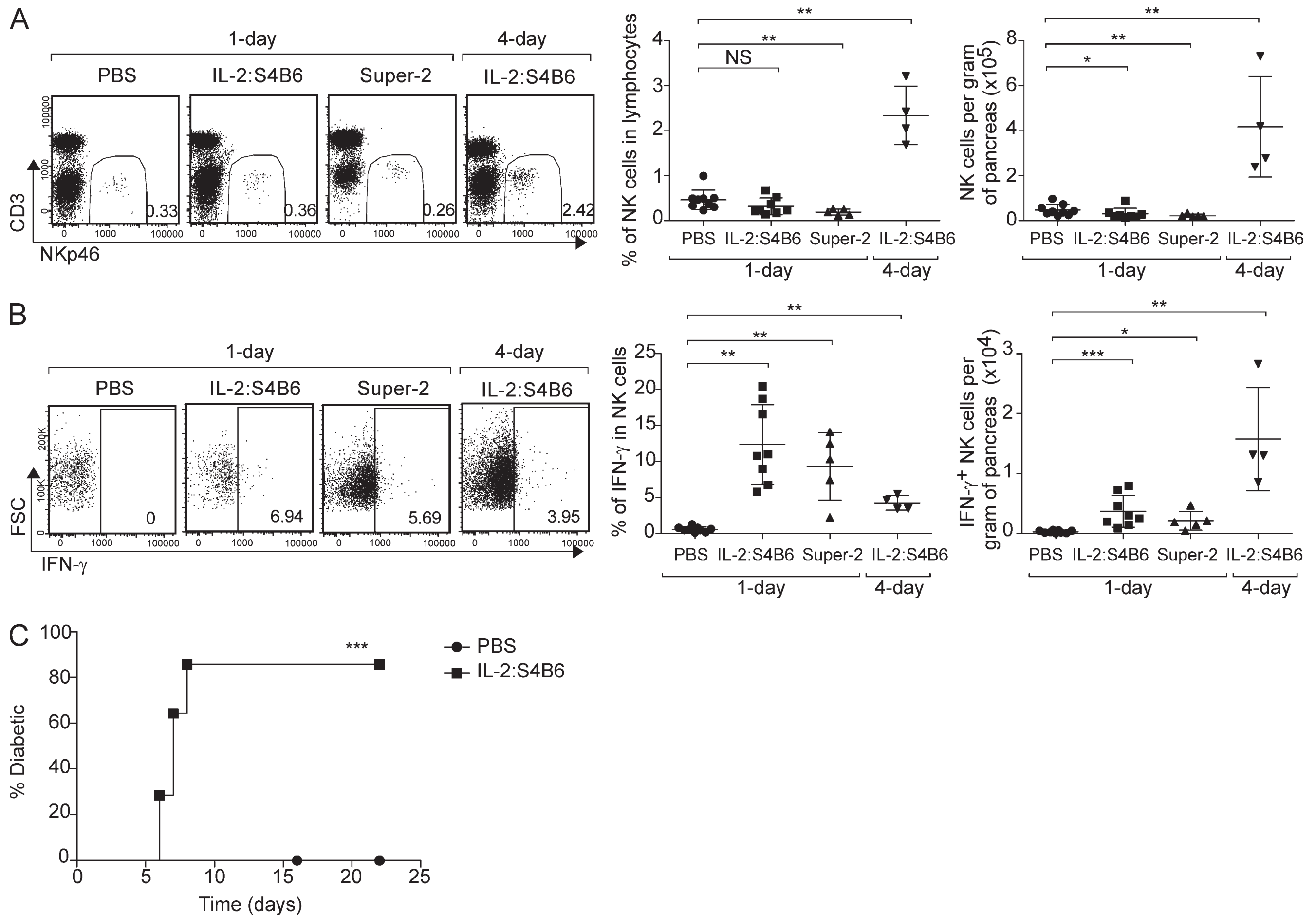

Figure 5. Supplementation with IL-2 induces early IFN-γ production and eventual accumulation of NK cells in the pancreatic lesion, as well as clinical diabetes. Pancreatic infiltrate from BDC2.5/NOD mice was analyzed 24 h after treatment with control PBS, IL-2–S4B6 complexes, or mutant IL-2 analogue Super-2, or 4 d after injection of IL-2–S4B6 complexes. (A, left) Representative flow cytometry data. (middle) Summary data for fraction of NK cells in the FSC/SSC lymphocyte gate. (right) Cell number summary data. (B) Analogous data for IFN-γ–producing NK cells. Mean ± SD, at least three independent experiments for both panels. (C) Summary diabetes data for 2 cohorts after 3 consecutive treatments with IL-2–S4B6 complexes (n = 14 IL-2–S4B6 complex; n = 9 PBS).

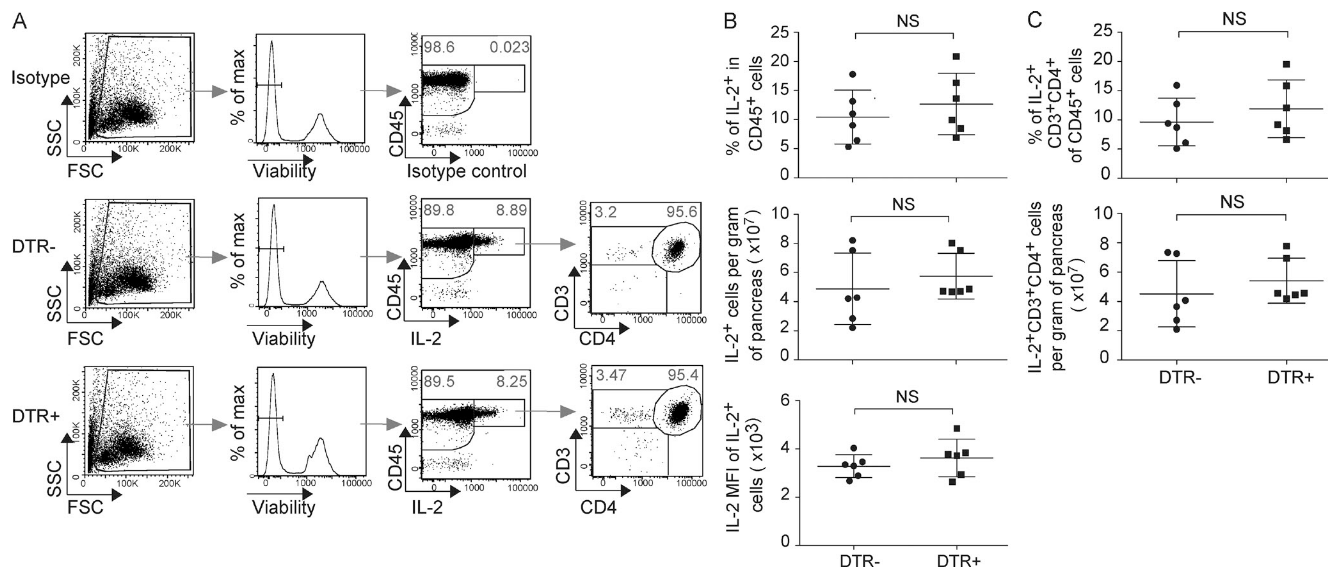

Figure 6. IL-2 production in the pancreas before and after T reg ablation. (A) Representative flow cytometry plots for pancreatic infiltrate from BDC2.5/NOD mice stained and analyzed for IL-2 by intracellular flow cytometry. An extended FSC/SSC gate was taken to include both lymphocytes and leukocytes along with a live/dead stain to exclude dead cells. CD45$^+$IL-2$^+$ cells were analyzed for CD3 and CD4 expression. (B) Summary data for pancreatic infiltrate from mice depleted of T reg cells (8 h with DT, DTR$^+$) or not (8 h with DT, DTR$^-$). Mean ± SD, at least three independent experiments. (C) Summary data, as in B, gated on CD4$^+$ T cells.

prediabetic pathogenic processes, particularly those that play out within the insulitic lesion, are an important resource. We recently reported that T reg cells control the conversion of insulitis to diabetes in this model primarily by reining in the activities of islet-infiltrating NK cells, notably their production of IFN-γ (Feuerer et al., 2009). Neutralization of this cytokine during ablation of T reg cells inhibited the mobilization of T effector (T eff) cells within the islets and drastically reduced the incidence of hyper-acute T1D that typically develops in this model in the absence of T reg cells. Here, we have explored the molecular underpinnings of the NK cell−T reg cell axis, exploiting the robust, rapid, and synchronous phenotypic changes characteristic of the model. Our studies uncovered a previously undocumented scenario whereby T reg cells control NK cell activation in the islet infiltrate by limiting their exposure to IL-2. This mechanism was indicated by the induction of an IL-2–dependent gene signature in NK cells upon T reg cell ablation, a parallel increase in NK cell pSTAT5 levels, reduction in NK cell accumulation and IFN-γ production after treatment of T reg cell–depleted BDC2.5/NOD mice with anti–IL-2 mAb, and a corresponding enhancement of these parameters in BDC2.5/NOD mice supplemented with IL-2.

More precisely, the scenario we propose is that in the insulitic lesion of prediabetic BDC2.5/NOD mice: (a) CD4$^+$ T eff cells (primarily) produce limiting amounts of IL-2; (b) T reg cells in the vicinity efficiently consume most of this cytokine, reflecting their elevated expression of the high-affinity IL-2R, composed of the γ (CD132), β (CD122), and α (CD25) chains; (c) expressing mostly the low-affinity IL-2Rβγ complex, NK cells are deprived of IL-2, dampening their activation; (d) punctual ablation of T reg cells liberates IL-2 at a sufficient concentration to permit NK cell (and eventual T cell) activation, unleashing their proliferation and production of IFN-γ; and (e) IFN-γ, likely in concert with the liberated IL-2, drives the activity of T eff cells, provoking conversion of an innocuous to a pathogenic insulitic lesion and the development of T1D. This sequence of events is consistent with the belief that IL-2 can prime NK cells, both in vitro and in vivo, for proliferation and IFN-γ production (Fehniger et al., 2003; Granucci et al., 2004; Lee et al., 2012). This represents an interesting intertwining of innate and adaptive immunity, wherein the adaptive (CD4$^+$ T cells) primes the innate (NK cells) to promote the adaptive (CD4$^+$ and, eventually, CD8$^+$ T eff cells) immune response.

The primary means reported for T reg control of NK cells thus far is production and cell-surface display of inhibitory TGF-β (Ghiringhelli et al., 2005), a mechanism that seems less critical in the present context. However, a scenario conceptually similar to ours has been proposed for the impact of T reg cells on T cell responses in several experimental models; i.e., T reg and T eff cell competition for limiting IL-2 (Barthlott et al., 2005; Scheffold et al., 2005; Pandiyan et al., 2007). Other studies (see Gasteiger et al. and Gasteiger et al. in this issue) that were carried out on the basis of punctual T reg cell ablation experiments have recently found that T reg control of IL-2 availability is an important systemic control on NK cell homeostasis and activation (A. Rudensky, personal communication).

Our findings should be viewed in the context of an extensive body of work weighing the role of IL-2 in human and mouse T1D (Hulme et al., 2012; Shevach, 2012). For example, *Il2* and *IL2RA* have shown a genetic association with disease in NOD mice and human diabetes patients, respectively. The effects of IL-2–IL-2R signaling on T reg cell homeostasis and function were routinely cited in interpretation of these associations. Indeed, mice devoid of IL-2, IL-2Rα, IL-2Rβ, or STAT5 all succumb to lymphoproliferative disease caused by T reg cell reduction or dysfunction, which can be reversed by administration of exogenous IL-2 or wild-type T reg cells. Nonetheless, given the data presented herein, it seems plausible that allelic variation in IL-2–IL-2R signaling could, instead or in addition, result in aberrant NK cell function and thereby exacerbate disease in a manner not currently appreciated. Consistent with this possibility, the mouse *Klr* and human *killer immunoglobulin-like receptor* (*KIR*) families that modulate NK cell activation have also been associated with diabetes in numerous human studies (van der Slik et al., 2003; Nikitina-Zake et al., 2004; Rodacki et al., 2007; Ramos-Lopez et al., 2009), as well as in the NOD mouse (Rogner et al., 2001). More information on the effects of mutant alleles of elements of the IL-2–IL-2R signaling pathway on NK cells is imperative.

In humans, modulation of the IL-2–IL-2R axis has been achieved through treatment with daclizumab, a mAb targeting IL-2Rα. In multiple sclerosis, where autoreactive T cells recognizing antigens from the central nervous system promote inflammation and demyelination, daclizumab induced a population of $CD56^{bright}$ NK cells that can target and kill $CD4^+$ T cells. Expansion of this NK cell population was associated with enhanced disease outcomes (Rose, 2012). Although multiple mechanisms of action for this mAb have been proposed, increased bioavailability of IL-2 as a result of IL-2Rα blockade would mirror the findings and interpretation reported here.

The role of IL-2–IL-2R signaling in diabetes progression has been experimentally dissected in NOD mice (Hulme et al., 2012; Shevach, 2012). Alone, or in combination with agents such as rapamycin, IL-2 supplementation had disease-modulating effects, in both preventive and curative protocols. Interestingly, one set of studies reported that although low-dose IL-2 treatment suppressed diabetes, high-dose administration actually triggered disease (Tang et al., 2008b). The diabetes-suppressive effect of low-dose IL-2 was interpreted as correction of an imbalance between T reg and T eff cells downstream of a genetic deficiency in IL-2 signaling. The relatively low expression of CD25 on islet-infiltrating T reg cells was taken as evidence of this notion, although it later became apparent that dampened CD25 expression is a characteristic of T reg cells at inflammatory sites in general (Lazarski et al., 2008; Tang et al., 2008a). The diabetogenic effect of high-dose IL-2 was explained as an enhancement of the activities of pathogenic T eff cells (although there was also a striking systemic expansion of NK cells). The results presented here emphasize that the response of islet-infiltrating NK cells to manipulation of IL-2–IL-2R signaling is not to be ignored in interpreting outcomes.

Given a strong rationale from studies on both mice and humans, there has been substantial interest in developing protocols for treating T1D patients with IL-2, likely in combination with other agents (Hulme et al., 2012; Shevach, 2012). The results from a phase I clinical trial wherein IL-2 and rapamycin were administered to 9 T1D patients within 4 yr of diagnosis were recently published, and proved disappointing (Long et al., 2012). There was, as hoped for, a transient augmentation in T reg cell fraction and numbers, accompanied by a more persistent enhancement of their STAT5 phosphorylation levels. However, these changes were accompanied by an unanticipated transient impairment in islet β-cell function, in concert with increases in the NK and eosinophil populations, ultimately resulting in trial closure. As cogently argued by Bonifacio (2012), the detrimental impact on β cells could reflect rapamycin effects and/or influences of the expanded populations of innate immune system cells. Certainly, rapamycin has been reported to inhibit β-cell regeneration and normalization of blood-glucose levels in mice (Nir et al., 2007). However, our findings, especially the rapid induction of diabetes after IL-2 supplementation, highlight the potentially destructive effects of IL-2–mediated unleashing of NK cells. Activation of islet-infiltrating NK cells could be directly cytotoxic or, through production of IFN-γ, could promote the activities of pathogenic T eff cells. This represents another example of the predictive value of murine models of T1D, though only if the available mouse data are reviewed and translation to humans is performed in a precise and critical manner (Shoda et al., 2005).

As recognized early on (Tang et al., 2008b; Grinberg-Bleyer et al., 2010; Hulme et al., 2012; Shevach, 2012), harnessing the tremendous potential of IL-2–IL-2R-based therapies while avoiding the detrimental side-effects is a great challenge. Novel approaches, such as the engineering of designer IL-2 derivatives (Levin et al., 2012), are promising in this regard. Even so, we need to understand much better how the various regulatory and effector populations in the insulitic lesions are orchestrated, and perspicaciously apply this knowledge to the development of therapeutic protocols.

MATERIALS AND METHODS

Mice. NOD/ShiltJ (NOD), BDC2.5/NOD TCR transgenic (Katz et al., 1993), and BDC2.5/NOD.Foxp3^{DTR} double-transgenic (Feuerer et al., 2009) mice were bred in our colony at The Jackson Laboratory, and were genotyped and maintained at Harvard Medical School (under specific pathogen–free conditions). Females between 7 and 10 wk of age were generally used. Protocols were approved by Harvard Medical School's Institutional Animal Care and Use Committee.

In vivo treatments. All in vivo treatments were via intraperitoneal injection. For T reg cell ablation, BDC2.5/NOD.Foxp3^{DTR} mice were administered 1 µg DT (Sigma-Aldrich) in sterile PBS, and samples were taken at the indicated time points. Control mice were littermates lacking the DTR transgene. For blockade of TGF-β, anti–TGF-β mAb (clone 1D11.11) was produced in the laboratory, and 1 mg was injected in sterile PBS 24 h before experimentation or at day 0, 3, and 5 for the long-term experiments. Control mice received an equal amount of isotype-control mAb (MOPC-21;

BioLegend). For neutralization of IL-2, 100 μg anti–IL-2 mAb (JES6-1A12; BioLegend) was injected along with DT, and analysis was done at 24 h. Control mice were treated with 100 μg isotype-control mAb (RTK2758; BioLegend). For IL-2 treatments, IL-2–anti–IL-2 complexes were prepared by adding 5 μg rIL-2 (PeproTech) to 50 μg of an IL-2 mAb (S4B6; BD) in 200 μl sterile PBS before injection, as previously described (Tang et al., 2008b), and analysis was done at 24 h. For treatments longer than 24 h, IL-2 complexes were injected daily, including 2 h before organ harvest on the final day. Mutant cytokine "Super-2" was prepared as previously described (Levin et al., 2012). 100 μg was injected at the outset and at 12 h, and analysis was at 24 h. Control mice were treated with an equal volume of PBS.

Disease assays. For diabetes incidence studies, BDC2.5/NOD mice were injected with IL-2–S4B6 complexes or PBS for 3 consecutive days, and diabetes was assayed by measuring blood glucose levels for up to 3 wk. Obtaining two consecutive draws of >250 mg/dl was considered diabetic.

Cell sorting and flow cytometry. For the initial NK cell microarray experiments, cells were isolated from the pancreas and spleen by mechanical separation with scissors. The pancreas was bathed in a shaking water bath at 37°C in digestion buffer (1 mg/ml collagenase IV [Sigma-Aldrich], 10 U/ml DNaseI [Sigma-Aldrich], and 1% [Thermo Fischer Scientific] in DMEM [Invitrogen]). For all other experiments, postmortem intracardial perfusion was performed with 30 ml room temperature PBS (or cold PBS for the intracellular phospho-STAT stains). After surgical removal of organs, cells were isolated from the pancreas and spleen by mechanically separating with scissors before passing through a 40-μm filter into DMEM supplemented with 2% FBS (Omega Scientific). Bloody samples were treated with ACK Lysing Buffer (Lonza) for 5 min on ice. Cells were Fc blocked (2G42, prepared in-laboratory) before surface or intracellular stains were performed using mAbs against CD3 (145-2C11 and 17A2; BioLegend), CD19 (6D5; BioLegend), NKp46 (29A1.4; BioLegend), CD4 (RM4-5; BioLegend), CD25 (PC61; BioLegend), and CD103 (2E7; BioLegend). For Foxp3 (FJK-16s; eBioscience) and IFN-γ (XMG1.2; BioLegend) stains, fixation/permeabilization were performed according to the manufacturer's instructions (eBioscience). For phospho-STAT staining, including pSTAT5 (C71E5; Cell Signaling Technology), pSTAT1 (58D6; Cell Signaling Technology), and isotype-control (DA1E; Cell Signaling Technology) mAbs, fixation/permeabilization/stains were done according to the manufacturer's instructions. For intracellular IL-2 staining of the pancreas (JES6-5H4 and isotype-control RTK4530; both obtained from BioLegend), cells were purified after PBS perfusion and digestion, using a Percoll gradient (GE Healthcare) according to manufacturer's instructions, and were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 nM; EMD Millipore) for 4 h. GolgiStop (BD) was added to the culture during the last 3 h. Dead cells were discriminated using a live/dead fixable near-IR dead cell stain kit (L10119; Invitrogen), and then stained with fluorescent mAb followed by fixing and permeabilization, all according to the manufacturer's instructions (BD). Flow cytometry was performed using an LSRII (BD), and data were analyzed using FlowJo (Tree Star) software.

Microarrays. Cells were double-sorted to high purity (>99%) on a MoFlo Cell Sorter (Beckman Coulter) directly into TRIzol (Invitrogen). RNA was prepared as described by the Immunological Genome Project (www.immgen.org) and underwent the GeneChip Whole Transcript Sense Target Labeling Assay using the Ambion WT Expression kit and Affymetrix GeneChip WT Terminal Labeling and Controls kit (Affymetrix). The resulting ssDNAs were hybridized to the GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Image reads were processed through Affymetrix software to obtain raw .cel files, and were background corrected and normalized using the RMA algorithm via Affymetrix Power Tools. Multiple replicates of cellular populations were collected (usually n = 3–6) and averaged. Data were analyzed with the Multiplot module from GenePattern. Randomized data were generated using the MultiplotPreprocess module from Genepattern. Data outside an acceptable range of replicate variation was filtered out using a coefficient of variation (CV) filter and when the represented gene was not expressed in any condition. Microarray data are available from the National Center for Biotechnology Information/GEO repository under accession no. GSE39197.

In vitro cultures. Before cell sorting, splenocytes were depleted of B and $CD8^+$ T cells using biotin-labeled mAbs to CD8 (2.43; prepared in-laboratory) and CD19 (6D5; BioLegend), followed by depletion using the CELLection Biotin Binder kit (Invitrogen) according to the manufacturer's instructions. NK cells ($CD19^-CD3^-NKp46^+$) were sorted, and then cultured in sterile complete RPMI (10% FBS, 1X PenStrep [Invitrogen], 2 mM L-glutamine, 55 μM 2-mercaptoethanol (Sigma-Aldrich), RPMI [Invitrogen]), and were activated with rIL-12 (0.5 ng/ml; PeproTech) and rIL-18 (0.5 ng/ml; MBL) for 15–20 h with or without rTGF-β (1 ng/ml; PeproTech). After culture, cells were either stained for intracellular IFN-γ or resorted to high purity for microarray analysis as described above.

Statistical analysis. All nonmicroarray statistical analyses for cytometry experiments were performed with GraphPad Prism software. For in vivo mouse readouts, p-values were calculated using the Mann-Whitney test. For diabetes incidence experiments, p-values were calculated using the Log-rank test. P-values were considered significant at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). χ^2 p-value analysis for microarray expression data (volcano plots) was calculated using Microsoft Excel based on the number of genes dropping to the left or right side of the fold-change distribution.

Online supplemental material. Table S1 lists transcripts differentially expressed in pancreatic NK cells in the presence or absence of T reg cells as described in Fig. 1. Table S2 shows the gene list of an activation-independent TGF-β-responsive signature used in Fig. 2. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20122248/DC1.

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J. Sitrin, C. Benoist, and D. Mathis declare no competing financial interests. K.C. Garcia, and A. Ring declare competing financial interests due to submission of a pending patent application describing the IL-2 superkine (Super-2).

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SUPPLEMENTAL MATERIAL

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Table S1, available as an Excel file, lists transcripts differentially expressed in pancreatic NK cells in the presence or absence of T reg cells, as described in Fig. 1.

Table S2, available as an Excel file, shows the gene list of an activation-independent, TGF-β–responsive signature used in Fig. 2

TABLE S1. Transcripts differentially expressed in pancreatic NK cells in the presence or absence of T reg cells (related to Fig. 1)

Over-represented Genes

Probe	Gene Symbol	Panc DTR-	Panc DTR+ 24 hr	Panc DTR + vs DTR-
10585778	Sema7a	131.37	972.47	7.403
10349603	Il10	81.56	558.78	6.851
10562044	Zbtb32	165.64	983.23	5.936
10531724	Plac8	197.56	1147.35	5.808
10482030	Stom	165.58	934.01	5.641
10570434	Ifitm1	93.63	434.24	4.638
10405211	Gadd45g	360.34	1630.88	4.526
10476314	Prnp	91.29	371.45	4.069
10563377	Sult2b1	220.74	864.49	3.916
10558769	Ifitm1	198.99	694.95	3.492
10516064	Mfsd2a	122.43	396.47	3.238
10363498	Ppa1	501.47	1513.86	3.019
10541307	Usp18	549.57	1635.7	2.976
10543067	Asns	136.33	401.81	2.947
10582275	Slc7a5	574.01	1665.96	2.902
10360398	Ifi202b	29.16	83.93	2.878
10472916	Cdca7	393.59	1077.32	2.737
10483679	Gpr155	458.77	1241.57	2.706
10420308	Gzmb	2786	7368.13	2.645
10421774	Dgkh	368.24	972.44	2.641
10493474	Muc1	381.72	1006.09	2.636
10498350	P2ry14	225.91	587.4	2.600
10502791	Ifi44	280.25	714.15	2.548
10495794	Pde5a	604.36	1538.35	2.545
10366796	Mettl1	232.32	581.47	2.503
10571752	Dctd	754.36	1882.73	2.496
10366586	Ifng	3405.62	8390.93	2.464
10510391	Srm	1147.3	2804.86	2.445
10586591	Car12	120.11	291.66	2.428
10429926	Dgat1	834.91	2025.05	2.425
10584712	Hyou1	946.42	2268.99	2.397
10453887	Cables1	176.1	407.2	2.312
10344713	Ahcy	589.88	1361.78	2.309
10579142	---	592.49	1365.99	2.306
10515943	Ctps	194.82	447.8	2.299
10361760	Timm8a1	102.7	234.94	2.288
10396610	Mthfd1	346.96	791.94	2.283
10439762	Ahcy	542.78	1237.46	2.280
10424349	Sqle	126.81	286.36	2.258
10552311	---	280.78	625.91	2.229
10488816	Ahcy	512.98	1140.89	2.224
10414522	Apex1	285.59	633.18	2.217
10589041	Impdh2	553.34	1226.73	2.217
10393449	Socs3	392.48	870.09	2.217
10408762	Eef1e1	747.93	1656.96	2.215
10407173	Il6st	498.97	1099.79	2.204
10606689	Timm8a1	86.95	191.59	2.203
10384474	Pno1	316.75	696.98	2.200
10351095	Tnfsf4	54.34	119.4	2.197
10426098	Creld2	891.96	1953.82	2.190
10388389	Hic1	170.01	371.51	2.185
10351039	Gas5	193	421.3	2.183
10488785	E2f1	274.09	595.99	2.174
10399636	Mrto4	176.3	383.01	2.172
10392207	Tex2	361.74	784.25	2.168
10527920	Cyp51	136.11	293.73	2.158
10409338	Nop16	842.12	1816.15	2.157
10528143	Ppp1r14b	558.36	1201.92	2.153
10388010	C1qbp	619.92	1330.14	2.146
10473414	Ssrp1	1138.12	2441.71	2.145
10422493	Gpr18	1369.48	2933.74	2.142
10384373	Fignl1	162.65	345.69	2.125
10441436	Snx9	105.87	224.81	2.123
10455588	Hspe1	1453.94	3084.71	2.122
10564960	Furin	1413.12	2997.97	2.122
10394770	Odc1	298.75	632.68	2.118
10461022	Ppp1r14b	554.84	1170.74	2.110
10445767	Treml2	339.47	715.65	2.108
10526120	Tpst1	153.32	321.18	2.095
10375880	Nhp2	214.43	448.9	2.093
10580382	Neto2	97.54	203.59	2.087

Under-represented Genes

Probe	Gene Symbol	Panc DTR-	Panc DTR+ 24 hr	Panc DTR + vs DTR-
10427628	Il7r	2979.32	523.06	0.176
10435907	Cd200r1	456.22	83.68	0.183
10538150	Tmem176a	825.14	178.94	0.217
10455901	Slc27a6	421.17	107.21	0.255
10435948	Ccdc80	425.67	111.17	0.261
10412298	Itga1	1153.11	326.09	0.283
10358421	Rgs18	460.01	131.63	0.286
10480275	Nebl	584.64	174.56	0.299
10544596	Tmem176b	2193.71	670.86	0.306
10493812	S100a4	712.94	228.35	0.320
10434302	Klhl24	1165.05	386.54	0.332
10596303	Acpp	727.89	247.3	0.340
10502890	St6galnac3	309.41	105.89	0.342
10394674	Socs2	952.48	326.45	0.343
10559248	Tspan32	1011	348.72	0.345
10355998	Fam124b	360.77	124.59	0.345
10466779	Pip5k1b	468.04	162.88	0.348
10447056	Qpct	184.25	64.21	0.348
10454856	Psd2	313.03	111.95	0.358
10573082	Inpp4b	1151.63	415.95	0.361
10574246	Gpr114	1087.59	401.8	0.369
10600901	Ar	758.91	286.25	0.377
10365428	Btbd11	753.14	289.94	0.385
10450367	Hspa1a	1972.25	761.8	0.386
10568392	Rgs10	899.19	353.88	0.394
10389190	Gm11435	717.73	283.07	0.394
10416371	Lpar6	2492.42	991.43	0.398
10505911	Dmrta1	255.26	101.78	0.399
10512949	Abca1	539.12	216.02	0.401
10523297	Ccng2	306.74	123.83	0.404
10529375	Mxd4	1127.04	455.29	0.404
10605113	L1cam	399.23	161.4	0.404
10382300	Map2k6	271.76	109.95	0.405
10563706	Mrgpra9	74.76	30.42	0.407
10377927	Rnf167	1792.21	734.11	0.410
10368886	Foxo3	1290.85	530.52	0.411
10430818	Tnfrsf13c	881.06	362.92	0.412
10531952	Abcg3	215.85	89.14	0.413
10363231	Smpdl3a	1714.64	713.85	0.416
10431017	Ttll1	684.29	287.34	0.420
10428698	Sntb1	440.34	185.34	0.421
10427454	Card6	972.58	410.56	0.422
10360040	Fcgr3	557.06	235.57	0.423
10365344	Tcp11l2	488.5	206.64	0.423
10399897	Hbp1	1140.81	483	0.423
10488482	Acss1	616.08	263.3	0.427
10576218	Cpne7	286.64	122.81	0.428
10530503	Cnga1	179.55	77.52	0.432
10601867	Kir3dl1	512.37	221.38	0.432
10497817	Anxa5	2919.25	1264.11	0.433
10358389	Rgs2	2027.06	880.84	0.435
10394054	Cd7	6954.7	3025.97	0.435
10443027	A930001N09Rik	801.16	350.42	0.437
10557481	Ypel3	1502.05	657.54	0.438
10507040	Spata6	438.68	192.48	0.439
10557895	Itgax	1123.68	495.07	0.441
10499160	Cd1d1	233.38	103.26	0.442
10469457	Plxdc2	205.54	91.5	0.445
10356880	St8sia4	1405.15	626.02	0.446
10501046	Gm10673	218.51	97.52	0.446
10429114	Tmem71	1023.48	456.83	0.446
10360684	Ephx1	201.27	90.13	0.448
10378549	Rtn4rl1	1388.79	622.03	0.448
10422598	Sepp1	1676.22	753.42	0.449
10575052	Cdh1	379.28	170.94	0.451
10428376	Angpt1	141.48	63.96	0.452
10606058	Cxcr3	2921.74	1322.36	0.453
10528102	Crot	2060.68	934.54	0.454
10443690	Glp1r	253.58	115.59	0.456
10554094	Igf1r	348.26	158.77	0.456
10371220	Gna15	651.54	297.42	0.456

10592201	Chek1	325.38	678.28	**2.085**
10443836	Rrp1b	360.86	751.55	**2.083**
10445894	Erh	790.23	1644.59	**2.081**
10346303	Hspe1	1057.04	2198.22	**2.080**
10378088	Mybbp1a	585.51	1201.4	**2.052**
10434668	Tmem97	387.29	792.81	**2.047**
10361110	Dtl	367.67	752.63	**2.047**
10473432	Tnks1bp1	611.25	1249.9	**2.045**
10394735	Pdia6	599.15	1225.09	**2.045**
10583732	Ldlr	219.21	447.7	**2.042**
10576034	Irf8	2459.63	5006.46	**2.035**
10346634	Nop58	526.39	1068.95	**2.031**
10347193	Atic	633.66	1283.95	**2.026**
10546163	Mcm2	814	1649.1	**2.026**
10517706	Mrto4	188.68	381.17	**2.020**
10574427	Impdh2	111.52	223.68	**2.006**
10462973	Hells	316.47	634.59	**2.005**
10582295	Odc1	581.58	1165.06	**2.003**

10517287	Man1c1	800.8	365.72	**0.457**
10439744	Cd96	3308.18	1512.27	**0.457**
10459620	Rab27b	351.68	160.99	**0.458**
10350247	Kif21b	1291.15	592.63	**0.459**
10355214	Idh1	189.59	88.08	**0.465**
10350742	Rnasel	1049.22	487.54	**0.465**
10555235	Arrb1	901.46	419.47	**0.465**
10351689	Gm10521	503.23	234.37	**0.466**
10454286	Mapre2	2008.74	936.08	**0.466**
10458581	Gm10008	313.33	146.44	**0.467**
10503259	Trp53inp1	1726.21	807.12	**0.468**
10598750	Gpr34	125.34	58.67	**0.468**
10439239	Dirc2	277.35	130.17	**0.469**
10436304	Abi3bp	165.91	77.94	**0.470**
10479852	Camk1d	798.19	375.79	**0.471**
10598101	Maml2	781.17	368.6	**0.472**
10524310	Ttc28	639.5	301.83	**0.472**
10538802	A930038C07Rik	260.7	123.19	**0.473**
10541114	Rasgef1a	476.27	225.07	**0.473**
10548729	Mansc1	332.34	157.07	**0.473**
10390103	Pdk2	457.33	216.7	**0.474**
10563170	Dkkl1	365.69	173.35	**0.474**
10492815	Tmem154	404.5	191.8	**0.474**
10435930	Cd200r2	583.99	277.08	**0.474**
10485622	Qser1	302.2	143.64	**0.475**
10433057	Calcoco1	952.94	455.41	**0.478**
10404496	Nqo2	1065.12	510.23	**0.479**
10596148	Trf	370.16	177.59	**0.480**
10397346	Fos	872.32	418.69	**0.480**
10476819	Plk1s1	316.68	152.27	**0.481**
10484856	Olfr1259	64.51	31.08	**0.482**
10439634	Gtpbp8	175.26	84.49	**0.482**
10401244	Actn1	333.25	161.22	**0.484**
10549102	Kcnj8	793.57	385.31	**0.486**
10453049	Cdc42ep3	676.57	328.83	**0.486**
10441195	Dscam	338.58	164.95	**0.487**
10475532	Sqrdl	223.55	109.14	**0.488**
10371356	Appl2	646.59	316.37	**0.489**
10415021	Abhd4	657.54	323.22	**0.492**
10387909	Chrne	451.65	222.14	**0.492**
10573319	Podnl1	488.85	240.5	**0.492**
10360315	Fcrl6	763.17	375.93	**0.493**
10595402	Fam46a	452.32	223.1	**0.493**
10602009	Rnf128	190.1	93.98	**0.494**
10502052	Alpk1	223.12	110.33	**0.494**
10576639	Nrp1	186.13	92.12	**0.495**
10479726	Pcmtd2	738.64	365.6	**0.495**
10579744	Large	505.69	251.54	**0.497**
10479159	Zfp831	807.46	402.61	**0.499**
10594774	Ccnb2	763.58	380.93	**0.499**
10390691	Nr1d1	298.37	148.92	**0.499**
10457168	Cd226	2455.26	1225.64	**0.499**

Table S2. Activation-independent TGF-β-responsive signature

Scn3b	Rnf149	2900026A02Rik	Zfp1
Gpr34	Arrdc4	Fam134b	Tiam1
H2-Aa	Epb4.1l2	St8sia1	Ddc8
Tnfsf10	Scn1b	Ccdc80	Arnt2
Adssl1	Osgin1	Anp32-ps	Acsbg1
Mapkapk3	Tmem2	H2-Eb1	B4galnt4
3110043O21Rik	Nrarp	Ece1	Gch1
Spsb1	Anp32a	Nox1	Mobkl2b
Inpp4b	Cd72	H2-Ab1	Fam124b
Cd81	Adam19	1700049G17Rik	Dkkl1
Vipr2	Cpeb4	Ttc39b	Bmf
Gpr68	Dusp2	Ttc39b	Nsun4
Gne	Osbpl1a	Plau	2810408A11Rik
Kcnn4	Ninj1	Klf10	Suox
Cish	Cx3cr1	Skil	Prnd
Pacsin1	Accn3	Myadm	Gpr56
		Gpr68	Dusp4

List of genes derived in Fig. 2 C.