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The role of IL-22 produced by Th17 cells in Type 1 Diabetes

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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(Thesis format: Monograph)

by

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Abstract

Interleukin-22 (IL-22) is produced by T helper 17 (Th17) cells. Th17 cells have been shown to be pathogenic in autoimmune diseases, however their role in type 1 diabetes (T1D) remains controversial. We have shown that Th17-differentiation of naïve T cells can be driven by IL-23 + IL-6 to produce large amounts of IL-22 and induce T1D. Conversely, polarizing T cells using TGF-β + IL-6 led to nonpathogenic Th17 cells that produced lower IL-22 levels. We have shown that neutralizing IFN-γ during polarization leads to a drastic increase in IL-22. We have also found IL-22-producing cells in the pancreas of diabetic NOD mice. The receptor for IL-22 increases in the pancreas of NOD mice during disease providing a target for IL-22. However, neutralization of IL-22 by antibody *in vivo* does not significantly alter disease progression. Therefore, IL-22 may not be the sole factor or play a non-redundant role in T1D pathogenesis.

Keywords

Type 1 Diabetes, Interleukin-22 (IL-22), Non-Obese Diabetic (NOD) mouse, Th17 cells, BDC2.5 NOD mouse, pancreatic islets, beta cells, IL-22Rα1

Dedications

I dedicate this thesis to my parents who have always supported me, especially through my academic pursuits. I would not be where I am today without their constant love, support and generosity.

"Education is the most powerful weapon which you can use to change the world"

-Nelson Mandela

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List of Abbreviations

Antibody	Ab
Antigen Presenting Cell	APC
Aryl Hydrocarbon Receptor	AhR
B Cell Receptor	BCR
Bovine Serum Albumin	BSA
Central Nervous System	CNS
Complete Freund's Adjuvant	CFA
Cytotoxic T Lymphocyte	CTL
Dendritic Cells	DC
Enzyme-Linked Immunosorbant Assay	ELISA
Experimental Autoimmune Encephalomyelitis	EAE
Fluorescence Activated Cell Sorting	FACS
Forkhead box protein 3	FoxP3
Granulocyte Macrophage-Colony Stimulating Factor	GM-CSF
IL-10-related T cell-derived inducible factor	IL-TIF
Immunoglobulin	Ig
Inflammatory Bowel Disease	IBD
Innate Lymphoid Cell	ILC
Intraperitoneal	IP
Intravenous	IV
Lymphoid-Tissue inducer	LTi
Major Histocompatibility Complex	MHC
Multiple Sclerosis	MS
Natural Killer	NK
Natural Killer T	NKT
Non-Obese Diabetic	NOD
Pancreatic Lymph Node	PLN
Pancreatitis-Associated Protein	PAP1

Pathogen Associated Molecular Patterns	PAMP
Pattern Recognition Receptors	PRR
Phorbol Myristate Acetate	PMA
RAR-related orphan receptor gamma splice variant T	RORγt
Real Time Quantitative Polymerase Chain Reaction	RT-qPCR
Regeneration	Reg
Severe Combined Immunodeficient	SCID
Signalling transducer and activator of transcription	STAT
Standard Error of the Mean	SEM
T Helper 1	Th1
T Helper 2	Th2
T Helper 9	Th9
T Helper 17	Th17
T Helper 22	Th22
T Regulatory Cells	Treg
Toll-Like Receptor	TLR
Transcription Factor	TF
Transforming Growth Factor	TGF
Type 1 Diabetes	T1D

Chapter 1

1 INTRODUCTION

1.1 Introduction to the Immune System

The immune system is an intricate network composed of a variety of cells, tissues, and organs that protect a host body from disease. It is highly developed to detect and destroy a large variety of foreign agents—termed pathogens—including viruses, microbes, and parasites while distinguishing these from the host's own healthy tissue. The two main branches of the immune system, the innate and adaptive immune responses, combine to provide a quick, constant level of defence as well as specific and long lasting immunity against foreign invaders. These immune system cells, termed leukocytes, originate in the bone marrow and then migrate to peripheral tissues, the bloodstream, or the lymphatic system.

1.1.1 The Innate Immune Response

The innate immune system is usually described as an organism's first line of defence against foreign pathogens. It is present at all times in all plants and animals and is critical for distinguishing potentially harmful threats. All multicellular organisms have an intricate innate immune response and even single-celled organisms have heritable defense mechanisms (1, 2). Innate immunity is comprised of a number of cells that derive from common myeloid progenitor cells, which can give rise to macrophages, granulocytes (neutrophils, eosinophils, basophils), and dendritic cells (DCs). These three cell types are termed phagocytes as they can engulf and kill pathogens and then induce inflammation, which recruits other immune cells to the site of infection. Another innate immune cell type is the natural killer (NK) cell that chiefly recognizes and kills abnormal cells such as tumour cells or infected cells.

As these cells migrate to sites of infection, it is important that DCs, macrophages and neutrophils can recognize and distinguish foreign particles and to do this they use pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and scavenger receptors. They recognize common patterns of molecular structure expressed by many

pathogens called pathogen-associated molecular patterns (PAMPs) such as mannose-rich oligosaccharides, peptidoglycans or lipopolysaccharides. Once these signals are recognized and the foreign body engulfed, macrophages and dendritic cells migrate to the lymph nodes to become antigen-presenting cells (APCs) whereby they process and display the antigen on their cell surface bound to the major histocompatibility complex (MHC) to activate the adaptive immune system (3). These APCs become activated and produce cytokines and chemokines to recruit more immune cells to the site of infection and present antigens to T lymphocytes for activation (4). DCs are the most potent of APCs as they have the broadest range of antigen presentation and are the only APCs that can activate naïve T cells. They also express large amounts of co-stimulatory molecules such as CD80 and CD86 that are necessary for T cell activation (5).

1.1.2 The Adaptive Immune Response

The adaptive immune system is an organism's more specific response to pathogens. It is highly evolved and only present in certain vertebrates (2). It is responsible for mounting a response against any variation of antigen an organism may encounter and then provide lasting immunity against these antigens to stave off future infection. Lymphocytes, the effector cells of the adaptive immune system, can do this by exhibiting a vast repertoire of antigen receptors on their cell surface. They respond to these antigens by triggering proliferation and differentiation to clonal effector cells (6). The two main types of lymphocytes are bone marrow-derived B cells and thymus-matured T cells, each with their own type of antigen receptor. Their surface receptors are highly specific for one antigen and are generated by somatic hypermutation and recombination to create an extensive range of antigen receptors that collectively respond to a large number of antigens (7).

B cells are critical in the humoral branch of the immune system in which their membrane-bound immunoglobulin (Ig) receptors, termed B cell receptors (BCR), bind to epitopes causing them to terminally differentiate into plasma cells. These plasma cells produce antibodies (Ab) that are the secreted form of their receptors, which travel systemically to tag their specific epitopes for destruction. B cells can also act as APCs since antigens that can cross-link the BCR are internalized and processed to be presented

on MHC class II molecules for recognition by $CD4^+$ T helper (Th) cells (8). In order for B cells to differentiate into clonal plasma cells the Th cells must produce co-stimulatory molecules and cytokines, thus fully activating the cell.

T cells are traditionally more concerned with the cell-mediated branch of the immune system in which activated cytotoxic T lymphocytes (CTLs) can directly destroy unhealthy or abnormal cells such as those infected by viruses. T cell development begins with lymphoid progenitors in the bone marrow that then migrate to the thymus for the remainder of their maturation (9). Here, classical T cells will undergo selection to ensure dysfunctional cells are not being released systemically. First, double positive cells (those that express both CD4 and CD8 receptors) are positively selected for where their T cell receptors (TCRs) must be able interact with MHC molecules with at least weak affinity to ensure reactivity (10). They are also negatively selected for in which most thymocytes with high affinity to self-antigens are destroyed. However, these mechanisms are not perfect, and self-reactive T cells may survive. If this is not controlled by peripheral mechanisms of tolerance it can lead to autoimmunity (11). Matured, naïve T cells then begin to express either CD4 (Th cells) or CD8 (CTLs) co-receptors and leave the thymus to reside in lymph tissues where they can be presented antigens. Unlike B cells, which can recognize and bind antigens directly, the TCR has to bind antigens in the context of MHC on the surface of DCs, macrophages, and B cells. DCs provide co-stimulatory signals to activate a naïve T cell as well as cytokines which determine the type of effector phenotype the T cell will have. Once these T cells are activated, they proliferate and undergo clonal expansion to help clear infection.

The adaptive immune system is also responsible for providing the body with immunological memory that allows the immune system to recognize antigens that have previously been encountered and mount an immediate response to them without having to wait for naïve lymphocyte activation. Rather than become effector lymphocytes, T cells and B cells can also become memory cells that can readily differentiate into effector cells after re-encountering an antigen.

1.1.2.1 $CD8^+$ T cells

$CD8^+$ T cells (CTLs) can induce cell death directly on target cells. They are activated by recognizing antigen displayed in the context of MHC class I molecules and are critical for killing intracellular bacteria-infected cells, virus-infected cells as well as tumour cells. There are two main mechanisms of cell-mediated killing: secreted granules containing perforin and granzyme B, as well as Fas ligand-mediated killing (12). In the first method, perforin inserts itself into the plasma membrane of the target cell where it will form a pore (13). A serine protease called granzyme B may then enter the cell and induce caspase-activated DNase to initiate apoptosis in the target cell. The second mechanism involves Fas-ligand produced by activated T cells which binds the Fas receptor on target cells and induces their apoptosis (12, 14). Activated $CD8^+$ T cells can also produce a large amount of interferon-γ (IFN-γ) which is critical to help clear infection in affected cells (15).

1.1.2.2 $CD4^+$ T cells

The primary job of $CD4^+$ T cells is to activate other cells and influence the immune system. They are MHC class II restricted and have critical roles in activating B cells to make antibody, regulating $CD8^+$ T cell responses, maintaining macrophage function, and supressing immune responses to ensure they are not overly excessive (16). $CD4^+$ T cells, once stimulated by their cognate antigen, can differentiate into a number of effector subsets with specialized phenotypes which allow us to classify them based on cytokine production, surface markers, and/or transcription factor induction. These subsets are induced by their microenvironment, primarily by cytokines that influence their differentiation potential. These cytokines may be a result of nearby helper T cells acting in a paracrine manner to bolster the immune response and deal with the local threat by inducing many naïve T cells to differentiate to the same subset while inhibiting competing subsets. Cytokines induced in one part of the body may also travel larger distances to influence the immune response elsewhere. It is critical that helper T cells drive the immune response in the proper direction and induce the necessary subsets as inappropriate effector induction can lead to inefficient pathogen clearance as well as other concerns in the body. Indeed, it has historically been shown that immune

dominance toward one or another subset can lead to autoimmunity (Th1 mediated) and fetal tolerance during pregnancy (Th2 mediated) (17).

Helper T cells critically rely on master transcription factors and signalling transducer and activator of transcription (STAT) proteins for effector differentiation and cytokine production (16). The STAT family of proteins are induced by cytokines binding to their receptors and will then dimerize and translocate to the nucleus to activate any number of master transcription factors depending on the cell fate (18).

1.1.2.2.1 Th2 Cells

Initially it was thought that there were only two main types of helper T cells: Th1 and Th2. Th2 are $CD4^+$ T cells that have important roles in host defence systems against extracellular bacteria, parasites (widely cited to expel helminth infections), and toxins (19). They also activate naïve B cells to induce class switching, especially towards producing IgE necessary for parasitic clearance and allergic reactions. Th2 cells are induced after activation/stimulation, accomplished by binding of their cognate antigen in the presence of Interleukin (IL)-4. IL-4 may be produced by nearby basophils (20) or by Th2 cells recruiting other naïve T cells to bolster their response. These cells are typically characterized by their IL-4 production as well as up-regulation of the master regulator of transcription factor (TF) GATA3 (16). GATA3 is produced by IL-4-induced activation of STAT-6, and then proceeds to directly induce IL-13 and indirectly up-regulate IL-4 (21, 22). Th2 cells are also known to produce IL-5, IL-9, IL-10, and TNF-α. Activation of Th2 cells also leads to inhibition of competing pathways such as Th1 differentiation by action of IL-4 (23, 24).

1.1.2.2.2 Th1 Cells

Th1 cells are typically considered the more aggressive and inflammatory subset of effector cells. They are responsible for cell-mediated immunity; they activate macrophages to kill intracellular pathogens and stimulate $CD8^+$ CTLs to proliferate and begin killing infected cells. They can also activate B cells to produce antibodies and have a role in the clearance of cancer cells (17). Th1 cells are induced after antigen binding in the presence of IL-12, which can be produced by nearby DCs and macrophages. IL-12

serves to activate STAT-4, which can directly induce Th1 factors such as IL-12 receptor (IL-12Rβ2), and T-box transcription factor T-bet, which can directly induce IFN-γ (16, 25). In the Th1 lineage, STAT-4 can be down-regulated by Th2 factors IL-4 and GATA3 (26), and up-regulated by IFN-γ in a positive feed-back loop (26). Activation of T-bet during Th1 differentiation can also be accomplished by STAT-1 to induce IFN-γ production (27). IFN-γ increases IL-12 production and inhibits production of Th2-type cytokines such as IL-4. Interestingly, it has also been shown that IFN-γ may play a critical role in Th1 induction, as neutralizing this cytokine *in vitro* often reduces Th1 development (28). Other cytokines that Th1 cells produce are IL-2, lymphotoxin, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α.

1.1.2.2.3 Regulatory T cells

The immune system has developed many mechanisms of ensuring that its processes do not stray from necessary natural immune responses. Negative and positive selection, as already discussed, are one part of the regulatory process, however it is not always completely effective: some T cells that can respond to self-antigens abnormally may still leave the thymus. The immune system then has other mechanisms of regulating these potentially harmful responses. Regulatory T cells (Treg) are important components of this protection as seen by Sakaguchi et al. when they transferred a T cell population depleted of Tregs into recipient mice and were able to induce systemic autoimmunity (29). This subset of $CD4^+CD25^+$ T cells can either be generated naturally in the thymus during negative selection by their higher-affinity binding of self-molecules (natural or nTregs) or in the periphery when mature T cells are stimulated under certain conditions in the presence of immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β (inducible or iTregs) (30–32). Tregs make up about 5-10% of circulating $CD4^+$ T cells in mice and humans and have a greater diversity of TCRs than effector cells as they have a bias towards self-peptides (33, 34). Their immunosuppression abilities were shown by Asano et al. after neonatal thymectomy in mice induced systemic autoimmune disease (35).

iTregs are usually under the regulation of a master transcription factor called forkhead box protein 3 (FoxP3) and can express immunosuppressive cytokines such as

TGF-β and IL-10 that will further promote Treg differentiation and effector population inhibition (36). However, recent studies have shown that iTregs may become more specialized to better address the regulation of different $CD4^+$ effector subsets. One study found that IFN-γ induced iTregs to up-regulate the Th1-factor T-bet and that these T-bet-producing Tregs migrated to Th1-mediated inflammatory sites (37). These cells were also more effective at regulating Th1 cells than non-IFN-γ-induced Tregs. Another study found that Tregs that express STAT3 are responsible for mediating responses of Th17 cells whose differentiation also depends on STAT3 (38). Therefore, it has been suggested that iTregs may become more specialized and up-regulate different effector factors in order to better regulate specific effector populations (16). Indeed, perhaps a specific microenvironment that is able to up-regulate TFs and differentiate effector subsets may also induce specific iTregs producing similar factors to restrain their nearby counterparts.

iTreg-mediated immunosuppression may occur through a mix of contact-dependent and contact-independent mechanisms. The cell-contact-dependent approach also includes inhibiting IL-2 and other accessory molecules such as CTLA-4 and co-stimulatory molecules expressed by APCs such as CD80 and CD86 (36). Once these cells become activated, their suppressive targets become non-specific (39). Other Tregs appear to exert their affects independent of TCR specificity, usually by production of IL-10 (40, 41) and TGF-β (42) or even causing cell death via Granzyme B and Perforin (43).

1.1.2.2.4 The Th1/Th2 Paradigm

It was initially suggested in 1986 by Mosmann and Coffman and colleagues (44) that $CD4^+$ T cells might differentiate into phenotypically different subsets depending on local conditions at the time. They suggested two distinct groups of effector T cells and coined the terms Th1 and Th2 cells, recognizable by their cytokine profiles. It was proposed that a balance between Th1 and Th2 was necessary for competent immune function and that predominance of one or the other would have a major impact on disease (45). However, certain inconsistencies were identified, especially pertaining to $CD4^+$ cells and autoimmunity. As the more aggressive, inflammatory responders, Th1 cells were originally thought to be critical for the development of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and Type 1 Diabetes (T1D) (46, 47).

However, seemingly contradictory results were reported concerning Th1-factors when one group neutralized IL-12 *in vivo* and prevented disease in the EAE mouse model (48), while another group reported that knocking out the key Th1 cytokine, IFN-γ, did not prevent disease (49). Indeed, it was concluded that knocking out IFN-γ or its receptor actually exacerbated disease and therefore this suggests IFN-γ plays a role in the down-regulation of pathogenic effector cells in EAE (50, 51). This ambiguity was settled when it was shown that IL-12 is composed of two subunits, p35 and p40, and while the IL-12p35 subunit is not necessary for EAE induction, the subunit common to other cytokines, p40, is essential for disease (52). It was demonstrated that IL-23, one such cytokine that shares the IL-12p40 subunit, is the critical factor responsible for the disease prevention when one of its subunits was neutralized (53). As IL-23 does not fit in with the Th1/Th2 paradigm with regard to autoimmune diseases, another subset of $CD4^+$ T cells was identified as IL-17-producing T helper (Th17) cells (54, 55).

1.1.2.2.5 Th17 cells

Once it was demonstrated that IL-23, rather than IL-12, was responsible for EAE pathogenesis, researchers began focusing on how this cytokine exerts its method of action. It was then shown that IL-23 will generate IL-17-producing T helper cells that can induce EAE when adoptively transferred into naïve mice (56). These cells were then termed Th17 cells and characterized through extensive study (54–56). It was shown that these Th17 cells can be induced from naïve T cells by a combination of IL-6 + TGF-β (57–59). It was also discovered that in the absence of IL-6, a similar cytokine IL-21 in combination with TGF-β could induce Th17 cells (60).

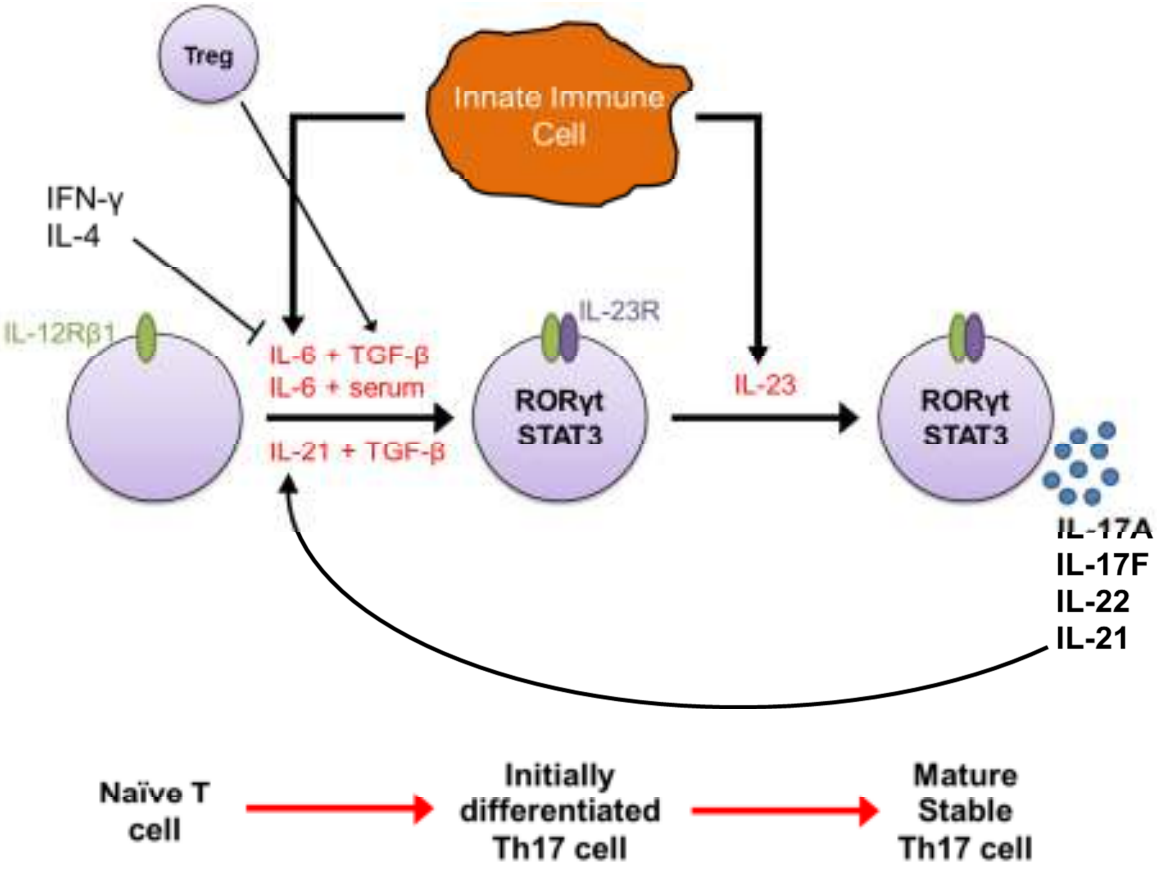
After antigen binding to the TCR, IL-6—which is produced by B cells, activated subsets of T cells, or innate immune cells such as APCs—or IL-21 can directly activate STAT3 to then induce the Th17 master regulator: RAR-related orphan receptor gamma splice variant T (RORγt) (61). This must be executed in the presence of TGF-β, however, as it is absolutely necessary for induction of the Th17 master regulator and of the receptor to IL-23 (62). It is incompletely understood exactly how TGF-β acts to induce Th17 cells, only that in its absence, IL-6 alone, via activation of STAT3, is necessary but not sufficient to induce RORγt (63, 64). The source of this TGF-β is likely nearby activated T

cells or Tregs acting in an autocrine or paracrine manner (59, 65). RORγt can directly up-regulate Th17-cytokines, especially IL-17A and IL-17F, and it has been shown that over fifty per cent of RORγt-expressing cells induce IL-17 (16). RORαt, a related transcription factor, can also somewhat compensate for IL-17 production in the event of RORγt deficiency (64). Other cytokines induced by Th17 cells include IL-21 (which works in an autoamplification loop for increased induction of Th17 cells) and IL-22. Secondary cytokines and chemokines such as IL-22 are not initially expressed by Th17 cells, and therefore need a terminal differentiation or stabilization signal in the form of IL-23. IL-23, produced by DCs, cannot act directly on naïve T cells and IL-6 and TGF-β together are necessary for induction of the IL-23R on the developed T cell surface (66). IL-23 is important in the priming process and maintenance of the Th17 phenotype as it can directly activate STAT3 and further induce effector molecules in mature Th17 cells. The process of Th17 differentiation is depicted in **Fig 1.1**.

The effector function of Th17 cells appears to be the clearance of specific pathogens requiring an extensive inflammatory response that Th1 and Th2 cells cannot sufficiently produce. Specifically, it has been shown that organisms in the bacterial genera *Propionibacterium*, *Citrobacter*, *Klebsiella*, *Borrelia*, and fungal genera *Pneumocysts* and *Candida* all elicit strong Th17 responses (58, 67–70). In general, as Th17 cells are largely reactive to fungal antigens, this may suggest a preferential role in host defence against fungi (71), although Th17-cytokines also show specific clearance of a variety of extracellular pathogens (72, 73). Th17 cells usually exert their functions through secreted effector cytokines; production of these cytokines can have various effects on other immune cells such as the production of proinflammatory cytokines and chemokines that attract neutrophils and other host defence cells to the site of infection (62). This inflammation is related to the role Th17 cells predominantly play in human autoimmune diseases such as psoriasis (74), rheumatoid arthritis (75), multiple sclerosis (76), inflammatory bowel disease (77) and type 1 diabetes (78).

FIGURE 1.1. *Differentiation of a naïve T cell to a fully matured Th17 cell*. Initially, IL-6 produced by innate cells and TGF-β produced by Tregs up-regulate the RORγt and STAT3 transcription factors. This induces expression of the receptor for IL-23. Binding of IL-23 fully differentiates the Th17 cell to stably produce IL-17A, IL-17F, IL-22 and IL-21 which functions in an autofeedback loop to promote differentiation of other naïve T cells.

Adapted from Korn et al., 2009 (62)


Treg
Innate Immune Cell
IFN-γ
IL-4
IL-12Rβ1
IL-6 + TGF-β
IL-6 + serum
IL-21 + TGF-β
IL-23R
RORγt
STAT3
IL-23
RORγt
STAT3
IL-17A
IL-17F
IL-22
IL-21
Naïve T cell
Initially differentiated Th17 cell
Mature Stable Th17 cell

1.1.2.2.6 Th22 cells and Th9 cells

The existence of T helper subsets other than those identified here is a matter of debate in the scientific community. Researchers have characterized other related Th cells that do not necessarily fit into one of the phenotypes previously described, and therefore were classified as novel, distinct subsets. First to be characterized was a novel subset of Th cells that produce an abundance of IL-9 and also IL-10, and these cells were termed Th9 cells (79, 80). They are induced by TGF-β + IL-4 and they do not express cytokines that are characteristic of other subsets such as IFN-γ, IL-4, IL-5, IL-13, or IL-17. It was proposed that these cells could function in helminth infection clearance or the promotion of inflammatory diseases (79, 80). Th9 cells have since been induced from naïve human T cells (81). Another recently identified subset are Th22 cells, which express IL-22, IL-26, and IL-13, and not IFN-γ or IL-17 (82). These cells can be efficiently induced by plasmacytoid DCs in the presence of IL-6 and TNF-α (83) and it is said they have important functions in mucosal antimicrobial host defence, perhaps more superiorly than that of Th17 cells (84). However, these cells are primarily found in the human skin and other barrier sites, (they express skin-homing chemokine receptors CCR4 and CCR10) (83), and have not yet been characterized as exerting their functions in other parts of the body. Both Th9 and Th22 cells are slowly gaining acceptance across the field, but are not as of yet widely recognized as distinct subsets by all researchers. The emergence of new denominations of cells emphasizes the complexity of the immune system and the difficulty in assigning definitive classifications to plastic, variable cells. A summary of the classifications to date is shown in **Table 1.1**.

1.2 Autoimmunity

It is important that both the innate and the adaptive immune system have the ability to distinguish between self and non-self molecules. An excessive immune reaction to innocuous, harmless substances occurs in allergies, while autoimmunity occurs when there is a disproportionate reaction to self-molecules (autoantigens) and the immune system will begin to harm the host's own tissues. This is usually carried out by

Table 1.1 Differentiating factors of $CD4^+$ T cell subsets

T cell subset	Induced by	Cytokine Production	Transcription Factor	Function	Ref.
Th1	IL-12, IFN-γ	IFN-γ, TNF-α, GM-CSF	T-bet, STAT1, STAT4	Inflammation, macrophage activation, CTL activation, clearing cancer cells	(17)
Th2	IL-4	IL-4, IL-5, IL-9, IL-10, IL-13	GATA3, STAT6	Allergic reaction, host defence against extracellular pathogens, activate B cells to secrete Ab	(19)
Treg	TGF-β, IL-10, thymic differentiation	IL-10, TGF-β	FoxP3	Suppress/down-regulate other T cell subsets	(36)
Th17	IL-6 + TGF-β, IL-6 + IL-23, (+ IL-1β) IL-21 + TGF-β	IL-6, IL-17A, IL-17F, IL-21, IL-22	RORγt, STAT3, AhR	Granulocyte activation, inflammation, clearance of pathogens not handled by Th1/Th2, autoimmune diseases	(54–56)
Th22	IL-6 + TNF-α	IL-22, IL-26, IL-13	STAT3, AhR	Mucosal antimicrobial host defence, autoimmune disease in the skin	(82–84)
Th9	TGF-β + IL-4	IL-9, IL-10	PU.1 GATA3	Helminth infection clearance?	(79, 80)

autoreactive effector cells of the adaptive immune system that can either attack self-tissues directly or via autoantibodies. Autoimmune responses are usually the result of a defect in the negative selection process in the thymus, improper regulation of inflammatory responses in the periphery, or because of molecular mimicry, where harmful pathogens try to evade detection by exhibiting antigens that are closely related to those expressed in the host (85). Low level self-reactive responses are necessary for the immune system to clear abnormal cells such as cancer cells, however if strong reactions are not quickly down-regulated they become chronic and can lead to autoimmune diseases. These can be caused by a mix of genetic factors as well as infection and environmental exposures (86). Autoimmune responses can act systemically and affect the entire host body, as in lupus, or target specific organs or tissues, as in T1D.

The accumulation of data from multiple region-specific studies suggests the prevalence of autoimmune diseases affects between 7-10% of the population (87). Some of the more common human autoimmune diseases include psoriasis, multiple sclerosis, and type 1 diabetes mellitus. Psoriasis is an inflammatory skin condition characterised by scaly, thick plaques in the skin. It is thought to be caused by T cell and DC infiltration into the skin producing inflammatory cytokines and chemokines, creating a permissive environment for abnormal proliferation of keratinocytes (88). Multiple sclerosis (MS) is a debilitating, chronic autoimmune neurological disease of the central nervous system (CNS) where immune cells attack and demyelinate axons in the CNS leading to lesions and axon destruction (89). The mouse model experimental autoimmune encephalomyelitis (EAE) is related to the pathology of this disease. Lastly, type 1 diabetes is characterized by immune destruction of pancreatic beta islets which normally produce insulin. This leaves the host unable to regulate insulin production, which is ultimately fatal without medical intervention. We will examine the role that Th cells, specifically Th17 cells, play in autoimmune diseases.

1.2.1 Th17 cells and autoimmune diseases

Although the natural function of Th17 cells is related to host defence, it seems that their main role involves autoimmunity. Beginning with the discovery of IL-17-

producing Th cells in the EAE model, Th17 cells are implicated in numerous other inflammatory autoimmune diseases.

In psoriatic patients, T cells extracted from skin lesions chiefly show a Th17 phenotype (90, 91) which is also seen in peripheral blood of affected patients along with other related cell types when compared to healthy controls (92). An increase in IL-17A mRNA expression as well as other Th17-cytokines is also seen in psoriatic lesions (93, 94). Also a phase II clinical trial showed that use of a monoclonal antibody that targets the p40 subunit of IL-12 and IL-23 demonstrated substantial improvement in psoriatic skin lesions, though this could implicate both Th1 and Th17 cells (74, 95, 96).

The role of Th17 cells in the EAE model of MS is apparent after studies show that IL-23 is critical for induction of disease and that neutralizing this cytokine abrogates disease (53). IL-23 is a necessary factor to induce pathogenic responses in EAE; in its absence, Th17 cells are actually not pathogenic, but rather produce IL-10 (97). There is some debate as to the validity of using EAE as a model for MS (98), however, in human studies it has still been shown that IL-17 and IL-6 are some of the most highly expressed genes in MS lesions (99). Elevated IL-17 mRNA levels have also been shown in MS patients' serum and spinal fluid (76).

1.3 Interleukin 22

IL-22 was first discovered in 2000 while screening for unidentified cytokine transcripts in a stimulated murine thymic T cell line (100, 101). It was then known as IL-10-related T cell-derived inducible factor (IL-TIF) as it shares 22% sequence homology with murine IL-10. Mouse and human IL-22 share 79% homology and the *Il22* gene is located on human chromosome 12 in a similar region to the *Ifng* gene (102). IL-22 is classified as a class 2 α-helical glycosylated cytokine in the IL-10 superfamily of cytokines and both the human and mouse cytokine is 147 amino acids long or 25 kDa (103). Furthermore, IL-22 signals through a receptor complex consisting of a unique IL-22Rα1 subunit, and a common IL-10Rβ2 subunit to initiate downstream signalling (101, 103, 104). First, IL-22

must bind to the IL-22Rα1 subunit, which will cause conformational changes that allow the complex to bind to the IL-10Rβ2 subunit. Binding of the IL-10R is absolutely necessary for IL-22-mediated signal transduction as lack of this subunit leaves cells non-responsive to IL-22 *in vitro* (104). Similar to other class 2 cytokines, it is thought that IL-22 forms dimers and then binds to two IL-22Rα1 subunits (105). Once binding occurs, IL-22 induces phosphorylation of the tyrosine kinases Jak1 and Tyk2 leading to the activation of STAT3 and in some cases STAT1 and STAT5 by phosphorylation of tyrosine residues (106). It was further shown that phosphorylation of a serine residue of STAT3 was necessary for maximum transcriptional activation. This sets IL-22 signalling apart from IL-10 as IL-10 induces phosphorylation of a tyrosine on STAT3, but not a serine residue. Similar to IL-17 signalling, IL-22 has been shown to induce three major MAP kinases MEK/ERK, JNK, and p38 (106).

In the adaptive immune system, IL-22 has been shown to be produced in general by activated T cells including γδ T cells, Natural Killer T (NKT) cells, Th1 cells and especially Th17 cells and Th22 cells (107). It has been reported that Th17 cells produce 100 times more IL-22 than Th1 cells, and 1000 times more than Th2 cells (108). In the innate immune system IL-22 producers include mast cells (100), and a heterogeneous population of innate lymphoid cells (ILCs) which includes NK cells (109), and the RORγt-dependent lymphoid tissue-inducer (LTi) cells (110). These IL-22-producing ILCs express the receptor for IL-23 (111) and are responsible for reinforcing the intestinal barrier against pathogenic bacteria (112). Similarly, LTi cells are usually characterized by their expression of CD117 and CD127 and are critically involved in the development of secondary lymphoid tissues including lymph nodes, Peyer's patches and cryptopatches (113). LTi cells also highly express the genes encoding the MHC class II proteins and have been observed to be critical for controlling $CD4^+$ T cell activation, possibly in an antigen presenting role or by competing with DCs for $CD4^+$ interactions (114). Constitutive expression of IL-22 has also been reported in the thymus and brain, though by which cells specifically has not yet been determined (100).

Though IL-22 is mainly expressed by immune system cells, the targets of this cytokine are mostly non-hematopoietic cells including hepatocytes, keratinocytes, lung

and intestinal epithelial cells, as well as cells in the kidney (115–118). A study by Aggarwal et al. screened many human tissues and found that while the IL-10Rβ2 subunit was expressed across many tissues, the IL-22Rα1 subunit was expressed the highest amount in the pancreas (119). Further study by Shioya et al. specifically found that IL-22Rα1 co-expressed with insulin and glucagon using immunohistochemistry suggesting its expression by beta cells and alpha cells in the pancreas (120). This target expression has led to the belief that IL-22 may mediate cross-talk between immune cells and epithelial cells in specific regions of the body, especially barrier surfaces (107). The IL-22Rα1 subunit is not the only protein that can bind IL-22. Local and systemic regulation of IL-22 can be achieved by binding to the IL-10Rβ2 chain as was observed in heterologous cell lines (101, 104) as well as a soluble IL-22 binding protein. This soluble receptor is denoted IL-22BP or IL-22Rα2 as it shares sequence similarities (approx. 33%) to the IL-22Rα1 extracellular subunit (121). Both chains have been shown to regulate the bioavailability of IL-22 and act as an endogenous antagonist by neutralizing its effects *in vivo* (122). It is uncommon for members of the class II cytokine receptor family to use decoy or soluble receptors to regulate signalling as this has only been seen for type 1 interferons which is not as efficient as IL-22Rα2 in binding its ligand (103). IL-22Rα2 exhibits a higher binding affinity to IL-22 than even the IL-22Rα1 receptor and it has been shown to be expressed widely across many cells and tissues including but not limited to cells in the lungs, intestine, skin, pancreas, thymus and spleen (104, 121, 122).

IL-22 is generally induced in Th17 cells by IL-6 + TGF-β and expanded by IL-23 though it has been shown that IL-6 and IL-21 can each do this alone to activated cells (123). After induction by this polarizing cocktail, 80-90% of IL-22-producing cells also express IL-17 (108, 123, 124). IL-23 is the major inducer of IL-22 and can also induce its production under APC-free conditions in correlation with the amount of IL-23 present. IL-23 signalling depends on the expression of its receptor by induction of STAT3 after cells become activated. IL-23 can also activate STAT3 in an autofeedback loop and therefore IL-22 is also under the regulation of STAT3 as initially shown in response to IL-9 (100). Another critical transcription factor for the induction of IL-22 is the aryl hydrocarbon receptor (AhR) which responds to aromatic hydrocarbons such as those present in environmental pollution. AhR is specifically expressed in Th17 cells and is

directly responsible for their production of IL-22 as AhR-deficient mice could develop Th17 cells, but they lacked IL-22 production (125). However, overexpression of AhR is not sufficient to drive IL-22 production (126) and the mechanism of this induction is not currently known but is thought to be concerned with overcoming the suppressive effects of TGF-β. Though TGF-β is a critical inducer of Th17 cells and therefore IL-22, it is also a potent suppressor as it activates c-Maf to functionally interfere with IL-22 positive TFs RORγt and BATF (127). For optimal IL-22 expression, minimal amounts of TGF-β are necessary.

The function of IL-22 across many tissues has been debated since its discovery. It has been said to have both inflammatory and protective effects in tissues under different circumstances. However, the role of IL-22 in antimicrobial host defence is generally agreed upon. IL-22 induces keratinocytes to produce anti-microbial peptides S100A7, S100A8, S100A9 and β-defensins (108, 128, 129). These effects are notably found at barrier surfaces such as the skin, airway, and intestine and are especially critical against extracellular bacteria *Klebsiella pneumoniae* and *Citrobacter rodentium* (73, 130). IL-22 also has a potent effect on inflammation, recruiting cells and contributing to the expression of IL-6, G-CSF, IL-1α, and LPS-binding protein (73, 82, 129). This cytokine can specifically induce acute-phase reactants in the liver such as serum amyloid A and pancreatitis-associated protein (PAP1) in pancreatic acinar cells (100, 119, 131). Using both local and systemic delivery methods it has also been observed that injection of exogenous IL-22 in mice can induce local and systemic inflammation, respectively (123, 131). In contrast to its inflammatory functions, IL-22 has been shown to play important roles in proliferation, survival and repair in the skin (82). To do this IL-22 up-regulates pro-survival genes Bcl-2, Bcl-X_L and Mcl-1, proliferative factors c-Myc, cyclin D1, Rb2, and CDK4, as well as molecules implicated in the production of protective mucous (132–135). Examples of additional protective effects of IL-22 include amelioration of tissue destruction in mouse models of hepatitis (133, 134) as well as exacerbation of tissue destruction in IL-22-deficient mice using models of colitis (135, 136).

Another protective role IL-22 may play is in the up-regulation of members of the Regeneration (*Reg*) gene family specifically in the pancreas. Reg proteins have been

shown by several groups to induce islet growth and differentiation as well as prevent beta cell apoptosis (137–139). Specifically *Reg1*, *Reg2* and *Reg3δ* genes are up-regulated in pancreatic beta cells by streptozotocin-treated diabetic mice and are correlated with an increase in insulin production, reversal of insulitis and ultimately beta cell regeneration (140–143). Early work in our lab has previously shown that immunization of NOD mice with mycobacterium-containing solutions such as complete Freund's adjuvant (CFA) prevents onset and recurrence of T1D (144, 145) and this was found to be the result of the induced up-regulation of the *Reg2* gene (143). Subsequently, CFA was shown to induce $CD4^+$ Th17 cells to produce their prototypic cytokines IL-17, IL-22 and IL-10 (146). Recently, our lab has shown that IL-22 is exceedingly effective in inducing up-regulation of *Reg1* and *Reg2* genes and may play a role in the protective effects of CFA in T1D (147).

The differential functions of IL-22 in many tissues are paradoxical at best. IL-22 promotes pathological inflammation (as discussed below), yet induces tissue regeneration and prevents its destruction. Therefore, it is also necessary to consider that though IL-22 exhibits protective effects and induces regeneration, such as in the pancreas, this does not signify that it does not play a role in tissue pathogenesis. IL-22 has been shown to act synergistically with other cytokines which could explain these contrasting results. The context in which it is expressed is important as the cytokines IL-17A, IL-17F, and TNF have been shown to act with IL-22 and potentially affect its role (82, 108, 132, 148). Therefore, when considering the presence and function of IL-22, it is imperative to evaluate the local tissue microenvironment and the cytokine milieu.

1.3.1 The IL-22/IL-23 pathogenic axis

In 2000, a new cytokine chain was discovered called p19 and it was shown to form heterodimers with part of the Th1-promoting cytokine, the IL-12p40 chain (149). Combined, these chains form the biologically active cytokine IL-23 found to be produced mainly by activated dendritic cells and macrophages. Target cells of IL-23 seem to mainly be activated T cells, as naïve cells do not begin to express the receptor until after antigen-binding and polarization. As IL-23 is not needed for cell differentiation, it is thought that its major function is to expand/mature Th17 cells after their initial

differentiation, to increase proliferation and to induce prototypic cytokines IL-17 and IL-22 (56, 149). It has also been suggested that IL-23 stabilizes the Th17 phenotype through STAT3-dependent mechanisms and acts as a survival factor (63, 66). What is clear however, is that IL-23 is eventually necessary for prolonged culture of Th17 cells as IL-23p19-deficient mice have limited numbers of Th17 cells (53).

IL-23 initially received a lot of attention as the missing factor in EAE disease progression when IL-12p40 abrogated disease, yet IL-12p35 could not (52). After this finding, researchers began to examine connections between IL-23, the newly discovered Th17 cells and other chronic inflammatory autoimmune diseases. Studies of human autoimmunity have demonstrated genetic links between polymorphisms in the *Il23R* gene and autoimmune diseases: Crohn's disease, spondyloarthropathy, autoimmune thyroid disease, multiple sclerosis, psoriasis, psoriatic arthritis, acquired aplastic anemia, and Behcet's disease (99, 150–155). It is therefore also important to consider products induced downstream of IL-23 signalling, such as IL-22, and the role it may play in IL-23-induced pathogenesis. It has been shown that IL-23 plays a major role in the Th17 inflammatory response in the synovial fluid and tissues in patients with rheumatoid arthritis (156, 157). IL-17 and IL-23 were present in sera of patients but absent in healthy controls, and it was suggested that IL-23 may promote bone erosion by up-regulating cytokines IL-17, IL-6 and IL-8. IL-23p19-deficient mice did not develop clinical symptoms of disease and did not demonstrate joint and bone pathology compared to wild-type mice (158). Further, IL-22 mRNA and its receptor have been detected in synovial tissues of patients and have been shown to up-regulate MCP-1 chemokine to induce pro-inflammatory responses (159). IL-22-deficient mice were also less susceptible to collagen-induced arthritis and point to a pro-inflammatory role (160).

Autoimmune inflammatory bowel diseases (IBD) include Crohn's disease and ulcerative colitis, both in which IL-23 has shown to play a role. Antibodies against IL-23p19 were able to cure established colitis in mice (161) and IL-23 was highly increased in colonic tissue of patients with Crohn's disease (162) though its ultimate role in these diseases is debated. IL-22 expression is also increased in colonic lesions of patients with IBD and high serum concentrations have been correlated with disease (163–165).

The most pronounced relationship between IL-23 and autoimmune disease is shown in the EAE mouse model of MS. IL-23p19-deficient mice are clearly resistant to disease (53), and anti-IL-23 therapy improves EAE symptoms (166). It was further shown that IL-23 is increased in human multiple sclerosis lesions (167). Similarly, IL-22 and IL-17 were detected in human MS lesions while not in healthy controls and both IL-17R and IL-22R were highly expressed on brain vessels (168). IL-22 promotes increased permeability of the blood brain barrier and because of its link to IL-23, it was thought IL-22 may be a downstream factor in MS pathogenesis. This hypothesis proved false as IL-22-deficient mice are not resistant to EAE and therefore suggests IL-22 does not play a non-redundant role in this model (124).

Lastly, the roles of IL-23 and IL-22 in autoimmune psoriasis have been well documented in both mice and humans. IL-23 is overproduced by keratinocytes in human psoriatic skin lesions (169) and an antibody against the p40 subunit of IL-12/IL-23 has demonstrated to be as efficacious as the current standard therapy (96, 170). It was then shown that IL-22 was also present in high levels in skin lesions of patients with psoriasis who also exhibited elevated IL-22 plasma levels correlating with the severity of their disease (128, 129, 171). IL-22 has also been demonstrated to be a crucial factor in two different mouse models of psoriasis; neutralizing IL-22 by use of antibodies prevents the development of disease usually induced by transfer of $CD4^{+}CD45RB^{hi}CD25^{-}$ cells to *scid/scid* mice (172), and neutralization or knock out of IL-22 demonstrated dramatic decreases in disease in mice treated with imiquimod to induce psoriasis-like lesions (173).

Altogether, these reports demonstrate a convincing function of IL-23 in autoimmune pathogenesis, and thus, as a key downstream factor, potentially IL-22 as well. This is interesting because IL-22 may provide the link between autoimmune diseases and environmental pollutants that has been alluded to across the field. AhR expression is restricted to $CD4^{+}$ Th17 cells in mice and binding of its ligand (both by endogenous factors and exogenous pollutants) increases expression of IL-22 and increases the proportions of local Th17 cells (125). Autoimmune pathology is accelerated in mice with AhR activation during EAE onset, however it is not clear if this is due to

increased cytokine production of IL-17 and IL-22 or the pronounced development of Th17 cells. Further research is needed to elucidate the mechanism of environmental effects on autoimmune diseases.

1.4 Th17 subsets

Treatment of naïve $CD4^+$ T cells with TGF-β *in vitro* induces Tregs, while the addition of IL-6 is necessary for the development of Th17 cells. This suggests a reciprocal relationship between Tregs and Th17 cells that depends on the local cytokine milieu. TGF-β generally induces the Treg prototypic TF FoxP3 which mediates Treg differentiation and function (174, 175). It has been proposed that in the absence of inflammatory stimuli, TGF-β induces up-regulation of FoxP3 and Tregs, whereas the presence of pro-inflammatory IL-6 inhibits this increase and instead induces inflammatory Th17 effector cells. IL-6 has been shown to inhibit TGF-β-driven FoxP3 expression, thus explains why it is regarded as key switch factor for a balance of Th17 cells and Tregs (60, 63).

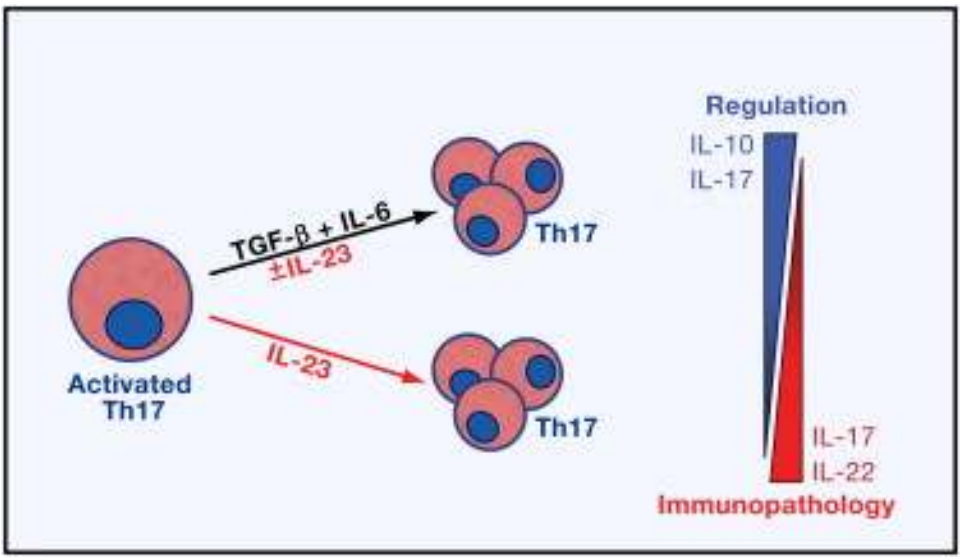
To add to the complexity of T helper subsets, it has been hypothesized that distinct Treg populations may become specialized to mainly control the function of one effector subset. Koch et al. have shown that IFN-γ can induce $FoxP3^+$ Tregs to up-regulate the Th1 prototypic TF, T-bet, and that these cells were found in the proximity of Th-1 mediated inflammation (37). Furthermore, T-bet-producing Tregs were more effective in regulating Th1 effector functions than conventional Tregs, yet controlled Th2 and Th17 responses similarly. It was additionally reported that deletion of a key Th2 differentiation factor (IRF4) in Tregs gave rise to uncontrolled expansion of Th2 cells suggesting IRF4 is necessary for differentiation of a population of Th2-controlling Tregs (176). And lastly, Treg expression of STAT3 has shown to be crucial for Th17-mediated immune pathology suppression (38). Therefore, these results indicate that for each Th effector subset, there may a regulatory counterpart that exhibits a similar phenotype, with up-regulated regulatory factors such as IL-10.

The functions of Th17 cells in autoimmune pathogenesis are not always as explicit as discussed above. This subset of cells was originally determined to be solely pathogenic (89, 177–179), however multiple reports dictate a non-pathogenic effect of Th17 cells (180, 181). Our lab has recently demonstrated that CFA induces Th17 cells after injection, however adoptive transfer of splenocytes from CFA-injected mice polarized with IL-6 + TGF-β delayed diabetes in recipient mice (146). This phenomenon was ultimately resolved in a landmark study by Ghoreschi et al. who demonstrated that differentiation of two distinct Th17-like subsets was possible under different induction conditions (182). They found more than 2000 genes differentially expressed in Th17 cells polarized by IL-1β + IL-6 + TGF-β compared to those with IL-1β + IL-6 + IL-23. Next, they examined the pathogenic potential of each subset and found the cells polarized with IL-23 induced significantly higher incidence and severity of disease in an EAE adoptive transfer model than those polarized with TGF-β, which were poorly pathogenic. Both subsets produced similar Th17 prototypic cytokines such as high amounts of IL-17, and IL-22, and showed high RORγt expression confirming a Th17 phenotype. However, cells polarized with IL-23 induced higher T-bet expression while cells induced with TGF-β produced more IL-9 and IL-10 (182). Importantly, the non-pathogenic population polarized by TGF-β also co-expressed RORγt and FoxP3, further demonstrating that Th17-specific regulatory T cells are induced similarly as the effector cells.

Therefore, in Th17-pathogenic applications, it is important to distinguish which sub-population is exerting the observed functions. The study from our lab observing that Th17 cell induction by CFA results in protective functions against T1D shows a large increase in IL-10 production and demonstrates the role of these regulatory type Th17 cells *in vivo* (146). As shown by Ghoreschi et al. the balance of these subsets relies on the minimal presence of TGF-β—such as the amount in serum-supplemented media—which is necessary for Th17 differentiation, as well as the addition of IL-23. IL-23 seems to be completely necessary for pathogenic functions, though can be nullified in the presence of high concentrations of TGF-β as shown in **Fig. 1.2**.

FIGURE 1.2. *Different induction techniques of Th17 cells can induce either regulatory or pathogenic phenotypes*. During Th17 induction high concentrations of TGF-β can induce developing Th17 cells to produce increased IL-10 and IL-17 and exhibit a regulatory function. High concentrations of IL-23, with minimal amounts of TGF-β induce a pathogenic subset of Th17 cells that produce high amounts of IL-17 and IL-22. The balance of these inducing cytokines determines the functional outcome of Th17 cells.

Adapted from McGeachy and Cua, 2008 (183)


Regulation
IL-10
IL-17
TGF-β + IL-6
±IL-23
Th17
IL-23
Activated
Th17
Th17
IL-17
IL-22
Immunopathology

1.5 Type 1 Diabetes

Type 1 diabetes is an autoimmune disease characterized by the destruction of insulin-producing beta cells in the pancreatic Islets of Langerhans (184). This leaves the affected individual unable to control their blood glucose levels as they are unable to produce insulin. Historically, without medical intervention, T1D can lead to ketoacidosis and death, yet even with treatment this disease has a high risk of potentially life-threatening complications (185). Patients with T1D are usually dependent on synthetic insulin for the remainder of their lives, however this therapy only treats the symptoms and not the underlying autoimmune process. T1D is one of the most prevalent autoimmune diseases in the world and incidence is still increasing 2-5 % (186). Disease onset often occurs in children and prevalence in the United States has been determined to be approximately 1 in 300 children by 18 years of age suggesting this disease is one of the most common chronic childhood diseases (186). Future projections for this disease have stated that incidence will increase by approximately 70 % in children in Europe, and that the age of onset is decreasing thus predicting twice as many diagnoses in children of less than 5 years of age between 2005 and 2020 (187).

Both genetic and environmental factors are thought to play a role in the induction of T1D as a 50 % concordance of disease has been seen in identical twins, yet only 5 % concordance between first degree relatives (188). The genes encoding the MHC have been shown to be the main susceptibility region associated with T1D with more than 90% of diabetic patients expressing a DR3-DQ2 or DR4-DQ8 haplotype (189). Similarly, in the mouse model-counterpart MHC the I-A^{g7} molecule has a unique beta chain with a polymorphism similar to the diabetes-prone human HLA-DQ chain that is necessary for diabetes development (190, 191). Other factors have also been hypothesized to interact with genetic factors and cause T1D; viral infection (192–194), weak or unstable binding of peptide to the MHC (195), or other types of environmental stress (196) have all been implicated in disease progression.

Although T1D is considered a T cell-mediated disease, T cells, B cells, macrophages, and DCs have all been implicated in different roles of disease induction (197). T1D is thought to initiate with an environmental insult to the pancreatic islet cells

which releases autoantigens. APCs present these autoantigens to autoreactive T cells in the local draining lymph nodes which will then infiltrate the pancreatic islets themselves, resulting in inflammation and beta cell destruction. In the NOD mouse model both $CD4^+$ and $CD8^+$ T cells are important for disease onset as depletion of either subtype renders the mice resistant (198–201). The role of cytotoxic $CD8^+$ T cells in diabetes development has been well documented (202). In patients with T1D, $CD8^+$ T cells prevail in affected islets (203–205). MHC class I proteins are highly expressed and associated with beta cells during insulitis and $CD8^+$ T cells were found to predominate islets during beta cell destruction (206). Autoreactive $CD4^+$ T cells have also been identified in infiltrate isolates from the pancreas during insulitis, though the exact role they play in diabetes pathogenesis has yet to be determined (207). Evidence of $CD4^+$ T cell specific mechanisms of disease induction include activating and recruiting cytolytic macrophages (208), activating and recruiting CTLs (209), or apoptosis by FasL coupled with priming by inflammatory cytokines (210).

After evidence that implicates $CD4^+$ T cells in diabetogenesis came to light, it was determined that Th1 cells were the likely culprit as it is a pro-inflammatory subset and subsequently shown to have direct cytotoxic effects on disease. It was discovered that Th1 cytokines such as IFN-γ mediated activation of macrophages and CTLs and promoted infiltration of these cells to ultimately lead to beta cell destruction (211). It was therefore surprising to discover that knocking out IFN-γ delayed disease onset, yet did not prevent diabetes (212) though IFN-γ is highly expressed by both Th1 and CTLs which have both been shown to be intimately associated with disease. Furthermore, splenocytes from these KO mice were fully capable of transferring disease to recipient mice. It was therefore necessary to explore other avenues of $CD4^+$ T cell pathogenesis in T1D.

1.5.1 Th17 cells and T1D

Th17 cells have been getting increased attention for their role in autoimmune diseases such as MS, rheumatoid arthritis and psoriasis. It was then suggested that these pathogenic cells may play a role in the development of T1D as well, assisted by the finding that IL-17 is highly expressed in the pancreas of NOD mice (213). This is further

supported by the co-injection of Th17-expanding cytokine IL-23 with low doses of streptozotocin resulting in disease onset (214). Increased Th17 cells have also been reported in the peripheral blood and pancreatic-draining lymph nodes of patients with diabetes (78, 215).

The mechanisms of Th17 cell differentiation and pathogenesis *in vivo* are incompletely understood though many have been proposed. Monocytes isolated from peripheral blood of patients have been shown to produce IL-6 and IL-1β spontaneously (216). Combination of IL-6 and IL-1β are known to induce IL-17 production from memory T cells, and therefore this study also demonstrated that these monocytes activated *in vivo* induced more Th17 cells in diabetic patients than monocytes from healthy controls. It is thought that diabetes-prone individuals may harbour a subset of monocytes that are predisposed to give rise to pathogenic Th17 cells. Ankathatti Munegowda et al. also demonstrated that OVA-specific Th17 cells could stimulate OVA-specific cytotoxic responses of $CD8^+$ CTLs in C57BL/6 mice (217). It is therefore possible that Th17 cells mainly serve to stimulate CTLs to induce beta cell destruction.

Lastly, two different studies have discovered that transfer of Th17 cells to immunocompromised mice could induce diabetes, but only after cells convert to IFN-γ-producing cells (218, 219). Diabetogenic transgenic BDC2.5 T cells can be polarized into Th17 cells and then injected in an adoptive transfer into NOD.SCID mice, however after disease onset an increased number of cells were producing IFN-γ in the pancreatic lymph nodes. To further complicate the story, neutralization of IL-17 by antibody has been shown to reduce diabetes frequency (215), though genetic knock down of IL-17 was unable to abrogate disease in NOD mice (220). Therefore, Th17 cells may yet be shown to play a role in diabetes pathogenesis, however IL-17 alone is not the critical factor for disease development.

1.5.2 Mouse models of T1D

Mouse models of human diseases are important key tools for researchers to discover the effects of manipulations of potential disease factors. One of the most widely known is the non-obese diabetic (NOD) mouse model of T1D discovered over three

decades ago by researchers at the Shinogi Company (221). NOD mice spontaneously develop disease between 15-22 weeks of age and with similar pathology to human T1D. Progression of the disease is shown by infiltration of $CD4^+$ and $CD8^+$ T cells into the pancreatic islets and subsequent destruction of beta cells. In this mouse strain, infiltration initiates at around three weeks of age followed by progression through a pre-diabetic stage characterized by increasing infiltration and inflammation as well as increased beta cell destruction. Finally, mice become diagnosed with disease when blood glucose levels can no longer be controlled as insulin production is not sufficient from the very low numbers of residual beta cells. Female NOD mice have shown to develop more severe disease and at a higher incidence of 80-90 % whereas only 10-40 % of male NOD mice develop disease (222).

The use of a control, diabetes-free strain of mice is beneficial for testing the effects of particular cells or factors, without the concern of the immune system of a recipient mouse affecting the functions of the cells of interest. Therefore, the severe combined immunodeficient (SCID) mouse was developed in the NOD strain. This strain is characterized by a homozygous recessive mutation in an enzyme involved in DNA repair called protein kinase, DNA activated, catalytic polypeptide (223). Deficiency in this protein precludes the rearrangement of genes coding for the BCR and TCR and therefore leads to an absence of mature B and T cells. NOD.SCID mice are resistant to diabetes as they do not possess T cells and therefore are commonly used in adoptive transfer experiments where injection of stimulated T cells can rapidly cause disease (224–226). The effects of injected cells are usually magnified as NOD.SCID mice cannot employ Tregs to down-regulate autoimmune responses as wild type NOD mice could, and therefore this must be considered in adoptive transfer experiments.

Lastly, to study the effects of a particular antigen and also to facilitate stimulation of naïve T cells, transgenic mice have been utilized in many mouse models. In particular it is valuable to use lymphocytes that are all specific for the same antigen, especially when studying specific diabetogenic autoantigens (207). An islet-specific T cell clone named BDC2.5 was isolated from NOD mice and found to induce disease in recipient NOD mice after adoptive transfer (227). Transgenic NOD mice were then bred to express

the same TCRs as the BDC2.5 clone which is specific for a pancreatic islet autoantigen named Chromogranin A (228). Specifically, Chromogranin A gives rise to several functional peptides such as vasostatin and our lab has demonstrated that the peptide fragment 29-42 of the whole Chromogranin molecule is the antigenic epitope of these BDC2.5 T cell clones (229). For *in vitro* studies, mimotope peptides are often used to increase stimulation and proliferation of T cells. In this study we use PS3, a mimotope peptide discovered by Stadinski et al. to strongly stimulate BDC2.5 cells (228, 229).

1.6 Project Rationale and Foundational Work

1.6.1 Previous Study

Th17 cells have been implicated in many autoimmune diseases though their effects on T1D are still incompletely understood. Previous work in our lab by Dr. Nikoopour has shown the presence of two different subsets of Th17 cells with phenotypically different profiles. Polarization of BDC2.5 splenocytes with IL-6 + TGF-β induces a regulatory population of Th17 cells termed Treg17 cells while induction of Th17 cells with IL-6 + IL-23 gives rise to pathogenic effector Th17 cells termed Teff17 cells. Stimulated Treg17 cells demonstrate higher IL-17 production while Teff17 cells produce more IFN-γ (**Appendix I**). The Treg17 cell population also exhibits less proliferation than both the effector population and non-polarized stimulated cells (**Appendix II**). These cell subsets were then tested for their pathogenic potential in an adoptive transfer of stimulated cells. Incidence of diabetes was 100 % in the Teff17 polarized cells while the Treg17 cells and non-polarized cells were unable to induce disease (**Appendix III**). Interestingly, combination of both Teff17 cells and Treg17 cells decreases proliferation of Teff17 cells in correlation with the ratio of Treg17 cells present (**Appendix IV-A**). Indeed, Treg17 cells are able to down-regulate the pathogenic processes of Teff17 cells as demonstrated by the drastically reduced diabetes incidence after co-injection of both subsets (**Appendix IV-B**). It was observed that one of the major differences between the two Th17 effector subsets was their differential expression of IL-22; the Teff17 cell population produced much higher amounts of IL-22 than either the

Treg17 cell population or the non-polarized cells (**Appendix V**). Differential cytokine production was also observed for IL-17, IL-21, IL-10, AhR and IL-9 between the two Th17 subsets (**Appendix VI**). These results are indicative of the presence of two functionally different subsets of Th17 cells and their corresponding phenotypes. A proposed working model summarizing the major differences between Teff17 cells and Treg17 cells is depicted in **Fig 1.3**.

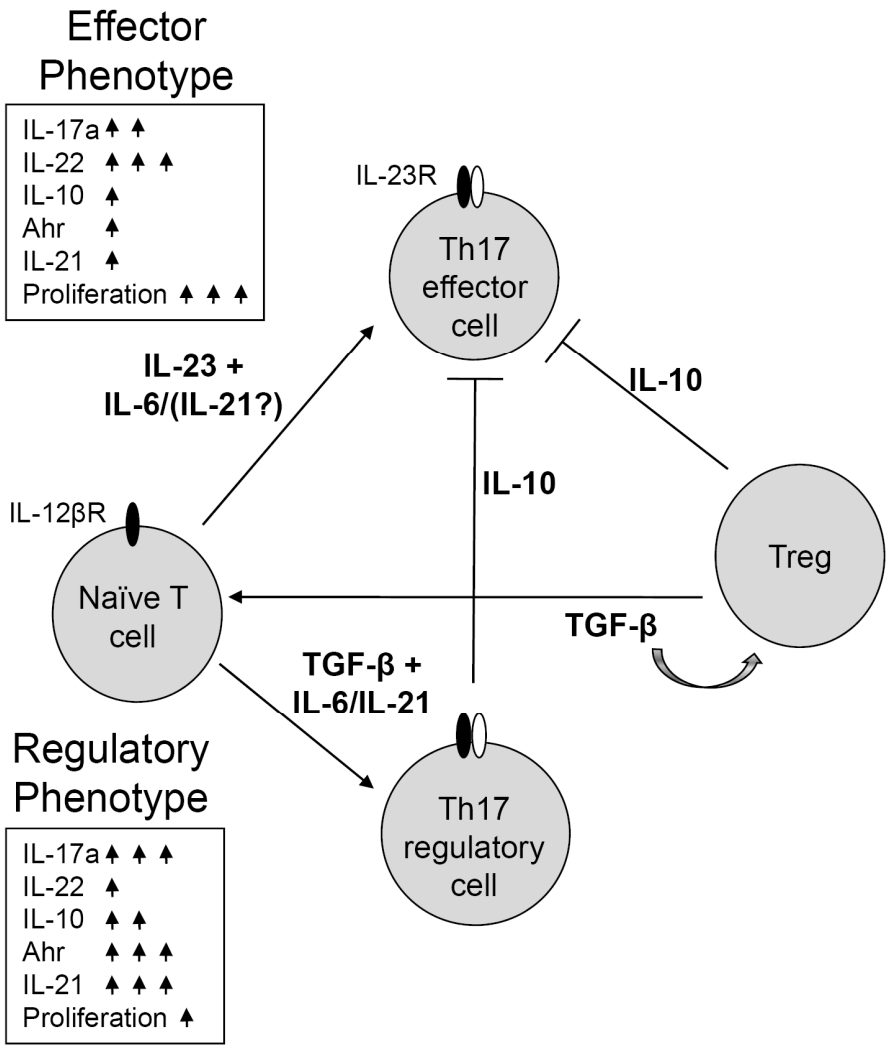
1.6.2 Thesis Rationale

As both IL-17 and IFN-γ were discovered not to be critical for diabetes development we sought to determine which factors were expressed differently enough in these subsets to determine the functional change from pathogenic to protective Th17 cells. From these experiments we have come to the realization that IL-22 was expressed very differently between the functional subsets, with the pathogenic Teff17 cells expressing vastly more. Kuchroo and colleagues were also studying the differential expression of cytokines between pathogenic and protective Th17 populations, though with emphasis on TGF-β3 in the EAE mouse model (178). By microarray analysis of over 400 genes, they discovered a range of cytokines that were up-regulated in slightly different populations of pathogenic cells compared to non-polarized cells and determined this to be the "pathogenic signature". IL-22 was the second-highest gene up-regulated and demonstrated the highest cytokine difference compared to Th0 cells (178). Conversely, IL-10 was the most increased gene in the non-pathogenic signature. Combining these data together with the observation of the highest IL-22R production in the pancreas to provide target cells, as well as the implications of IL-22 in other autoimmune diseases such as psoriasis, we concluded that it was necessary to characterize the role of IL-22 in the pancreas.

We therefore hypothesized that IL-22 produced by Th17 cells has a pathogenic effect on pancreatic islet beta cells and may contribute to type 1 diabetes development. As the previous work in our lab was performed using splenocytes, the first objective of this project was to determine whether similar results could be achieved by polarizing isolated naïve $CD4^+$ T cells and to fully maximize IL-22 production in these cells. We

FIGURE 1.3. *Our proposed model for the induction of functionally different Th17 cell subsets*. Polarization of naïve T cells with IL-6 + TGF-β induces Th17 cells with high IL-17 production with low proliferation and minimal expression of IL-22. Polarization of naïve T cells with IL-6 + IL-23 in serum-supplemented media induces a highly proliferative population of Th17 cells that produces very high amounts of IL-22. The population induced by IL-6 + TGF-β exhibit a regulatory phenotype and are termed Treg17 cells while the population induced by IL-6 + IL-23 is pathogenic and therefore termed Teff17 cells.

Adapted from Nikoopour et al. (manuscript in progress)


Effector Phenotype
IL-17a ↑ ↑
IL-22 ↑ ↑ ↑
IL-10 ↑
Ahr ↑
IL-21 ↑
Proliferation ↑ ↑ ↑
IL-23R
Th17 effector cell
IL-23 + IL-6/(IL-21?)
IL-10
IL-10
IL-12βR
Naïve T cell
Treg
TGF-β
TGF-β + IL-6/IL-21
Th17 regulatory cell
Regulatory Phenotype
IL-17a ↑ ↑ ↑
IL-22 ↑
IL-10 ↑ ↑
Ahr ↑ ↑ ↑
IL-21 ↑ ↑ ↑
Proliferation ↑

next wanted to characterize IL-22 and its receptor in the NOD mouse pancreas as previous studies in the literature were mostly performed on human tissues. Lastly, we wanted to determine the functional effect of IL-22 in diabetes progression by the use of a neutralizing antibody combined with a disease-inducing adoptive transfer model. The implications of this project are towards discovering a key therapeutic target in neutralizing IL-22 or suppressing the pathogenic effect of Th17 cells in T1D.

Chapter 2

2 MATERIALS AND METHODS

2.1 Mice

NOD/Ltj, NOD.SCID and BDC2.5 TCR transgenic NOD mice were bred and housed in a pathogen-free environment at West Valley Animal Care facility at Western University (London, Canada). C57BL/6 (B6) mice were generously provided by Dr. Mansour Haeryfar (Western University). All experiments were performed according to institutional guidelines and those of the Canadian Council for Animal Care. Mice were monitored for disease every 3 days by measuring urine glucose output with Diastix strips (Bayer, Elkhart, IN). Mice were considered diabetic after two consecutive positive (>11.5 mmol/L urine glucose tests. Most mice were used at 4-6 weeks of age unless otherwise stated. In some experiments, diabetic NOD mice were used between 14-26 weeks of age.

2.2 Cytokines and Antibodies

Murine cytokines IL-6, IL-23, and TGF-β were purchased from BioLegend (San Diego, CA). All cytokines were reconstituted and used according to manufacturer's instructions. The following anti-mouse antibodies were purchased from BioLegend (San Diego, CA): anti-CD3ε (clone 145-2C11) was used to coat 24-well plates overnight in 1 ml sterile 1X PBS at 4°C; anti-CD28 (clone 37.51) was added to cultures on anti-CD3 coated plates; anti-IFN-γ (clone XMG1.2) and anti-IL-4 (clone 11B11) were added to splenic or T cell cultures. Anti-TGF-β (clone ID11) was purchased from R&D Systems (Minneapolis, MN). The following anti-mouse, fluorophore-conjugated antibodies were purchased from eBioscience (San Diego, CA): anti-CD4-FITC and -APC, anti-CD8-FITC, -PE-Cy7 or -APC, anti-IFN-γ-FITC, anti-IL-22-PE, anti-IL-17A-APC, anti-Granzyme B-FITC and biotin-conjugated anti-Perforin. Anti-CD4-PE, anti-CD8-PE, PE-conjugated rat IgG1 Isotype control and PerCP-conjugated streptavidin were purchased from Becton-Dickenson and Company (BD, Franklin Lakes, NJ). Anti-CD4-PE/Cy7 was

purchased from BioLegend (San Diego, CA). For western blotting, the primary antibody monoclonal rat anti-mouse IL-22Rα1 was purchased from R&D systems (Minneapolis, MN) and polyclonal goat anti-mouse actin was purchased from Santa Cruz Biotechnology (Dallas, TX). Secondary antibodies used were HRP-conjugated goat anti-rat IgG and HRP-conjugated donkey anti-goat IgG both purchased from R&D Systems (Minneapolis, MN).

2.3 Tissue Preparation

Mice were sacrificed in a CO_2 chamber before spleen, pancreas, and pancreatic lymph nodes (PLN) were harvested. To extract lymphocytes from spleens and PLN, tissues were passed through a 40-µm nylon cell strainer (BD, Franklin Lakes, NJ) to obtain single-cell suspensions. Erythrocytes were lysed in 4 ml ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$ 1 mM Na_2EDTA) for 2 minutes and cells were counted with a haemocytometer. For lymphocyte extraction from the pancreas, the tissue was extracted, cut into 1 mm pieces and incubated in 1 mg/ml collagenase V from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO) in 10% RPMI at 37°C for 30 minutes while shaking. Digested pieces were then forced through a cell strainer and erythrocytes lysed as stated above.

2.4 *In vitro* Stimulation of Splenocytes

Splenocytes from BDC2.5 mice were extracted and seeded into a 96-well plate at $2x10^5$ cells per well with 5 µg/ml PS3 mimotope peptide, SRLGLWVRME (228), which was synthesized, purified and characterized by mass spectrometry in our laboratory as previously described (229). Cytokines were added at the following concentrations: IL-6 (20 ng/ml), IL-23 (20 ng/ml), and TGF-β (5 ng/ml) similar to the Th17 induction concentrations used by Sugita et al. (230). Where stated, the following antibodies were added to cultures: 5 µg/ml anti-IFN-γ (230), 5 µg/ml anti-IL-4 (230), 5 µg/ml anti-TGF-β (127). Cells were cultured for 4 or 5 days as stated.

2.5 Naïve T Cell Isolation

Splenocytes from BDC2.5 mice were extracted as above and naïve T cells isolated using magnetic activated cell sorting (MACS). Cell sorting was performed using Miltenyi Biotec (Auburn, CA) kits to isolate $CD4^+CD62L^+$ cells according to the manufacturer's guidelines. Briefly, magnetic labelling of non-$CD4^+$ T cells and separation using an LS column led to the depletion of non-$CD4^+$ cells. Then positive selection of $CD62L^+$ cells from this fraction was performed using an MS column to achieve a highly enriched (>90 %) sample of $CD4^+CD62L^+$ cells. These cells were then washed, counted and plated at $3x10^6$ cells per well in a 24-well plate that had been coated overnight with anti-CD3 and anti-CD28. Cells were cultured for 4 or 5 days as stated in 10% RPMI (RPMI 1640 medium supplemented with 2mM L-glutamine, 0.5% HEPES, 5 µg/ml penicillin, 100 U/ml streptomycin and 10% (v/v) fetal calf serum (HyClone Laboratories, Logan, UT)).

2.6 Restimulation of Tissue-Extracted Lymphocytes

Lymphocytes were extracted from tissues as described above and restimulated for FACS and quantitative real time polymerase chain reaction (RT-qPCR) analysis. Cells were plated at $3x10^6$ cells per well on a 24-well plate that was coated overnight with 1 µg/ml anti-CD3 and cultured with 1 µg/ml anti-CD28 in 10% RPMI. After 48-72 hours, supernatants were collected and either phorbol myristate acetate (PMA)/Ionomycin was added (see below) or RNA extracted for RT-qPCR analysis.

2.7 Preparation for Cytofluorimetric Examinations

Fluorescence-activated cell sorting (FACS) was used to determine cytokine profiles and surface marker expression of cells after *in vitro* culture. Cells in culture were stimulated with 50 ng/ml (PMA) and 500 ng/ml ionomycin for 4-6 hours. Brefeldin A (5 µg/ml) was also added for the last 4 hours. Cells were then harvested and supernatants collected and stored at -20°C for future applications. Cells were washed with 1X PBS

followed by surface staining for 30 mins at 4°C in 2% bovine serum albumin (BSA) in PBS with the FITC-, PE-, PE-Cy7, and APC-labelled antibodies stated above. Cells were then washed twice with 1X PBS and fixed in 2% formaldehyde with 1% BSA in PBS for 20 mins at room temperature. Cells were again washed and permeabilized with 0.5% Saponin (Sigma) in 2% BSA in PBS with antibodies such as those against IFN-γ, IL-22, IL-17, granzyme B, perforin or appropriate isotype controls either overnight or for 1 hour. Cells were then washed with 1X PBS and data for 100,000 events (unless otherwise stated) were collected using a FACSCalibur (BD Biosciences) at the London Regional Flow Cytometry Facility at the Robarts Research Institute (London, Canada). Analysis of flow cytometry data was performed using FloJo software (Tree Star).

2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

Duoset ELISA kits from R&D Systems (Minneapolis, MN) were used to analyze supernatants from *in vitro* cultures for cytokines IL-10, IL-22, IL-17 and IFN-γ. Manufacturer's protocols were followed directly. Standard curves were generated for each plate to determine sample concentration. Absorbance was determined using a Benchmark Microplate reader (BioRad, Hercules, CA) and data was analysed using Microplate Manager version 4.0 software (BioRad).

2.9 Proliferation Assay

To determine cell proliferation, a tritiated thymidine uptake assay was performed. Splenocytes were plated in a U-bottom 96-well plate at a density of $2x10^5$ cells per well in culture medium containing various cytokines as stated. After 4 days of culture, 1 μCi of tritiated thymidine (or [^{3}H] T) was added to each well for 18 hours. Cells were then harvested using a Tomtec cell harvester onto a Wallac filter (PerkinElmer, Waltham, MA) which was dried for 20 mins at 40 °C. The filter was then placed in a bag with 5 ml of Betaplate scintillation fluid (PerkinElmer, Waltham, MA) and sealed. Radioactivity was measured using a 1450 Microbeta liquid scintillation counter (Wallac).

2.10 RNA Extraction

For RNA extraction from whole pancreatic tissue, mice were sacrificed and approximately 50 mg of pancreatic tissue was placed in Buffer RLT (Qiagen, Mississauga, ON) containing β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Tissues were homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). Total RNA was then extracted using an RNeasy Midi Kit (Qiagen, Mississauga, ON). For RNA extraction from splenocytes, PLNs, or cultured lymphocytes, cells were disrupted in Buffer RLT and β-mercaptoethanol and then homogenized by adding lysate to a QIAshredder spin column (Qiagen, Mississauga, ON). Total RNA was then extracted using an RNeasy Mini Kit (Qiagen). Contaminating DNA was removed from all RNA samples using the DNase Treatment and Removal kit purchased from Ambion (Austin, TX). The concentration of RNA in each sample was then determined by measuring absorbance at 260 nm using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE).

2.11 Quantitative Real Time PCR (qRT-PCR)

For quantification of specific genes using qRT-PCR, 1-2 µg RNA from each sample was reverse-transcribed into first-strand cDNA using oligo dT_{12-18} primers from Superscript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) and using a GeneAmp PCR System 2400 from Applied Biosystems (Foster City, CA). Resultant cDNA was diluted in DEPC water to a consistent concentration for each experiment, usually 225 ng/µl for RNA extracted from *in vitro* cultures, and 500 ng/µl for whole tissues. cDNA was then amplified using Quantifast SYBR Green PCR Kit (Qiagen, Mississauga, ON) according to manufacturer's protocols. Gene-specific primers were purchased from Sigma-Aldrich (St. Louis, MO) and used at a concentration of 1.25 µM. Primers were designed by members in our lab and a complete list of the primers used can be found in **Table 2.1**. Amplification was performed using a Corbett Rotor-Gene 6000 thermocycler (Corbett Life Sciences, San Francisco, CA) and analyzed using the manufacturer-provided Rotor-Gene software. The assay uses a two-step

Table 2.1 Primers used for qRT-PCR in this study

Gene Target	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Product Size (bp)
IL-10	ggttgccaagccttatcgga	acctgctccactgccttgct	191
IL-17	gctccagaaggccctcaga	agctttccctccgcattga	142
IL-22	tcagctcagctcctgtcacat	tccccaatcgccttgatctct	117
IL-23p19	tgctagcctggaacgcacatg	gctgactagaactcaggctgg	342
IFN-γ	tcaagtggcatagatgtggaa	tggctctgcaggattttcatg	92
GM-CSF	gccatcaaagaagccctgaa	gcgggtctgcacacatgtta	114
IL-22Rα1	gctcgctgcagcacactacca	tctgtgtcgggagtcaggcca	247
IL-17Rα	ataccacagttcccaagccag	ggtctgctacgggcaagatg	192
RORγt	ccgctgagagggcttcac	tgcaggagtaggccacattac	230
AhR	cgcaagccggtgcagaaaaca	aggctggccaggcggtctaa	113
β-actin	gcccagagcaagagaggtat	cacacgcagctcattgtaga	117

melting/annealing program over 40 cycles of amplification. The Pfaffl method (231) was used to quantify CT data values, and all primers were determined to be 95-100% efficient.

2.12 Western Blot

Mice were sacrificed and approximately 10 mg of whole pancreatic tissue was immediately snap-frozen in liquid nitrogen. Tissue was then homogenized in lysis buffer containing 1% Triton X-100 and protease inhibitors using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged for 1 min at 5000 rpm and then supernatants were centrifuged at 12 000 rpm for 20 mins at 4 °C. Supernatants were extracted and protein concentration was determined using the Bradford assay using Bio-Rad Protein Assay Dye Reagent Concentrate (Hercules, CA) and a Benchmark Microplate reader (BioRad, Hercules, CA). Data was analysed using Microplate Manager version 4.0 software (BioRad) using BSA as a protein standard (Roche, Laval, QC).

Equal amounts of 15-20 µg total protein from each sample were separated by SDS-PAGE using a 12 % separating gel and then transferred to a nitrocellulose membrane using a TransBlot western blotting apparatus (Bio-Rad, Hercules, CA). The nitrocellulose membranes were then blocked with 5 % skim milk powder (Carnation, Don Mills, ON) overnight on a shaker at room temperature. Primary antibody was applied at a concentration of 1 µg/ml overnight on a shaker at 4°C. Secondary antibody was applied at a dilution of 1:2000 for the IL-22R antibody and 1:8000 for the actin antibody. Blots were developed in TMB solution.

2.13 Adoptive Transfers

In the transgenic adoptive transfer model, splenocytes from BDC2.5 mice were cultured with the indicated cytokines and antibodies as well as PS3 mimotope for 4 days. Cells were then washed with sterile PBS twice to remove excess cytokines and resuspended in PBS. Five-week old female NOD mice were then injected with $5x10^6$ cells each in 200 µl intravenously (i.v.) through the tail vein.

In the NOD.SCID adoptive transfer model, splenocytes from two diabetic NOD mice were pooled, erythrocytes lysed and cells were washed 3 times in PBS. Male and female NOD.SCID mice (8-9 weeks old) were each injected with 10^7 cells in 200 µl i.v. through the tail vein. Mice were monitored for diabetes development every 3 days by urine glucose tests.

2.14 Neutralizing Antibody Treatment

Mice were injected with anti-IL-22 Ab in an adoptive transfer model. Pathogenic cells were injected i.v. in 5 week old mice as described above on Day 0. These mice were also either injected intraperitoneally (i.p.) with 300 µg in 100 µl of rat anti-mouse IL22-01 monoclonal neutralizing antibody (Pfizer)(172) or sterile PBS on Days -1, 2 and 6 for the transgenic adoptive transfer model, or days 0, 3, 6 and 14 in the NOD.SCID adoptive transfer model. The amount of neutralizing antibody injected was chosen due to previous studies by Ma et al. with the same clone (172).

2.15 Statistical Analyses

Statistical analysis was performed using GraphPad Prism 5.01 (La Jolla, CA). Error bars represent standard error of the mean (SEM) among samples. Significant differences between samples were determined using the ANOVA and student's t test with $p<0.05$ considered significant for all experiments. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$.

Chapter 3

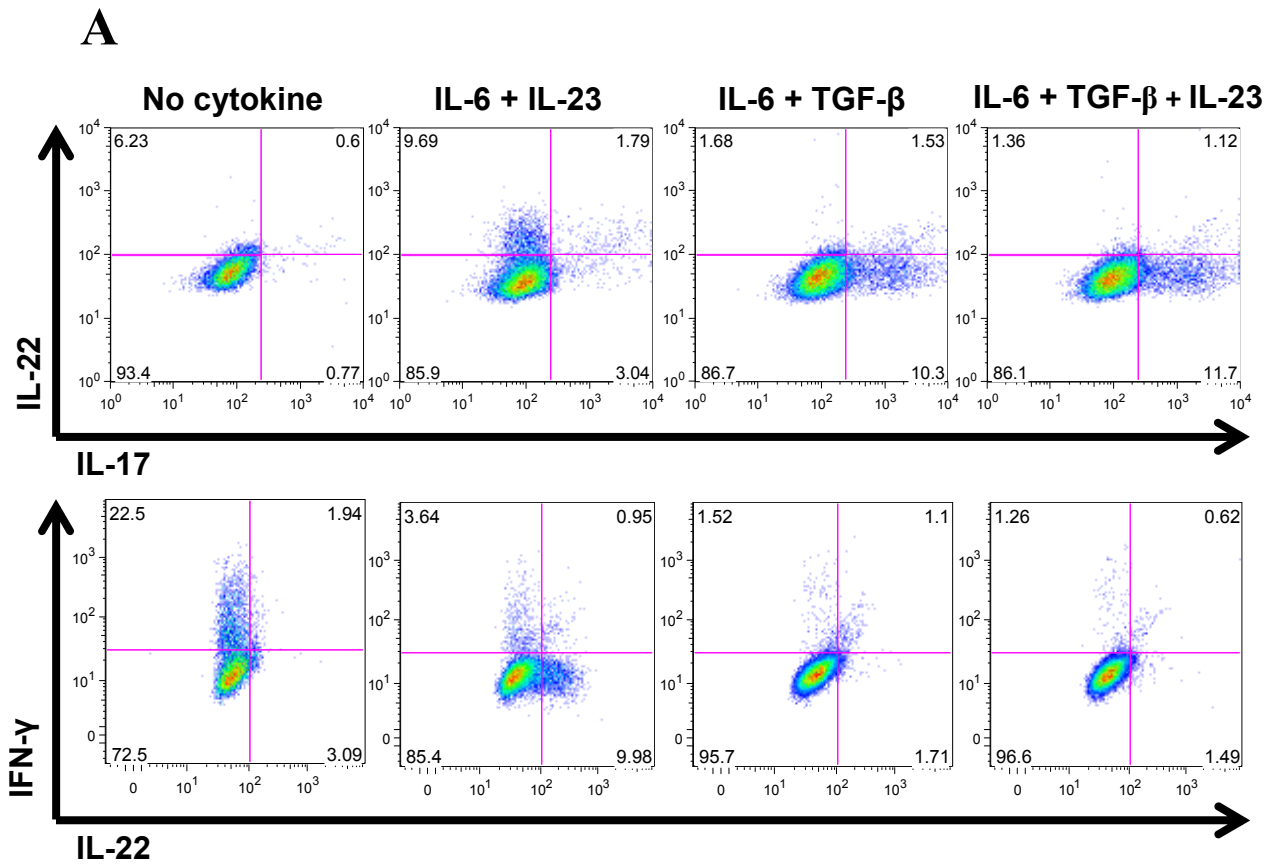
3 RESULTS

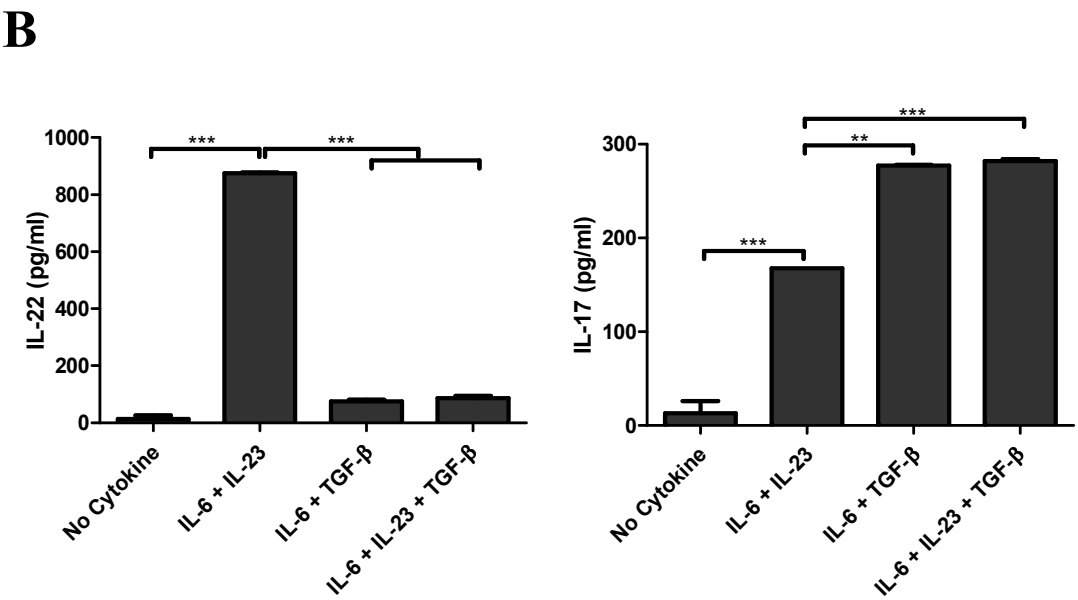
3.1 Characterizing the polarization of naïve T cells into different Th17 cell subsets

T cells can be induced to differentiate into a number of different effector subsets, each characterized by the type of cytokines they produce and transcription factors they express. A relatively new subset of $CD4^+$ T cells called Th17 cells can be polarized from splenocytes in the presence of IL-6 + TGF-β or IL-23 (54, 55). Two functionally different subsets can be induced: an effector subset polarized by IL-6 + IL-23 called Teff17 cells and a regulatory subset polarized by IL-6 + TGF-β called Treg17 cells (182). We wanted to characterize these subsets using isolated naïve T cells rather than bulk splenocytes to specifically examine cytokine production by these T cells. Naïve ($CD4^+CD62L^+$) T cells from splenocytes of BDC2.5 mice were isolated and stimulated by α-CD3 and α-CD28 with polarizing cytokines and neutralizing antibodies to induce Th17 cells. When naïve T cells are polarized with IL-6 + IL-23, a significant increase is seen in the production of IL-22 (**Fig. 3.1A, B**, $p < 0.001$). Such an increase is not seen, however, in either non-polarized cells, or in cells that are Th17-polarized in the presence of TGF-β that was added to the culture. Therefore, this demonstrates that trace amounts of TGF-β present in the media is sufficient for Th17 induction, yet not enough to inhibit IL-22 production. IL-17 production is significantly higher ($p < 0.001$) in Th17-polarized cells compared to non-polarized cells, however maximal IL-17 is produced by the Th17 cell subset induced by the addition of TGF-β, rather than IL-6 + IL-23 (**Fig. 3.1A, B**, $p < 0.01$). The non-polarized group produces much higher amounts of IFN-γ consistent with a Th1 phenotype, though IL-6 + IL-23 polarized cells also express some IFN-γ (**Fig. 3.1A**). This demonstrates an explicit phenotype of Th17-polarized cells and also highlights the distinctions that different methods of induction may have on cytokine production.

For characterization and optimization of Th17 cell differentiation, the Teff17 cell subset, Treg17 cell subset and a non-polarized subset were quantitatively compared in their ability to produce cytokines between 2 days and 5 days after stimulation using qRT-

FIGURE 3.1. *Polarization of isolated naïve BDC2.5 T cells into different subsets shows pronounced differences in cytokine expression.* Spleens and pancreatic lymph nodes were extracted from BDC2.5 mice and naïve cells were isolated by selecting for $CD4^+CD62L^+$ cells. These cells were then cultured in 24-well plates ($3x10^6$/well) that were coated with 5 µg/ml α-CD3 and 5 µg/ml α-CD28 overnight. Th17 effector cells were polarized with 20 ng/ml IL-6 + 20 ng/ml IL-23, Th17 regulatory cells were polarized with 20 ng/ml IL-6 and 5 ng/ml TGF-β and a mix population was cultured with 20 ng/ml IL-6, 5 ng/ml TGF-β, and 20 ng/ml IL-23 for 4 days. Anti-IFN-γ (5 µg/ml) and anti-IL-4 (5 µg/ml) were added to all wells except the No Cytokine sample. Cells were collected and stained for FACS analysis (A); 50 000 events were collected and gated on live cells, then $CD25^+$ cells. (B) Supernatants from cultures were collected and analyzed for IL-22 and IL-17 expression using ELISA. Results are shown as mean concentration ± SEM. The ANOVA test was used to analyze all values with $p < 0.05$ considered significant.

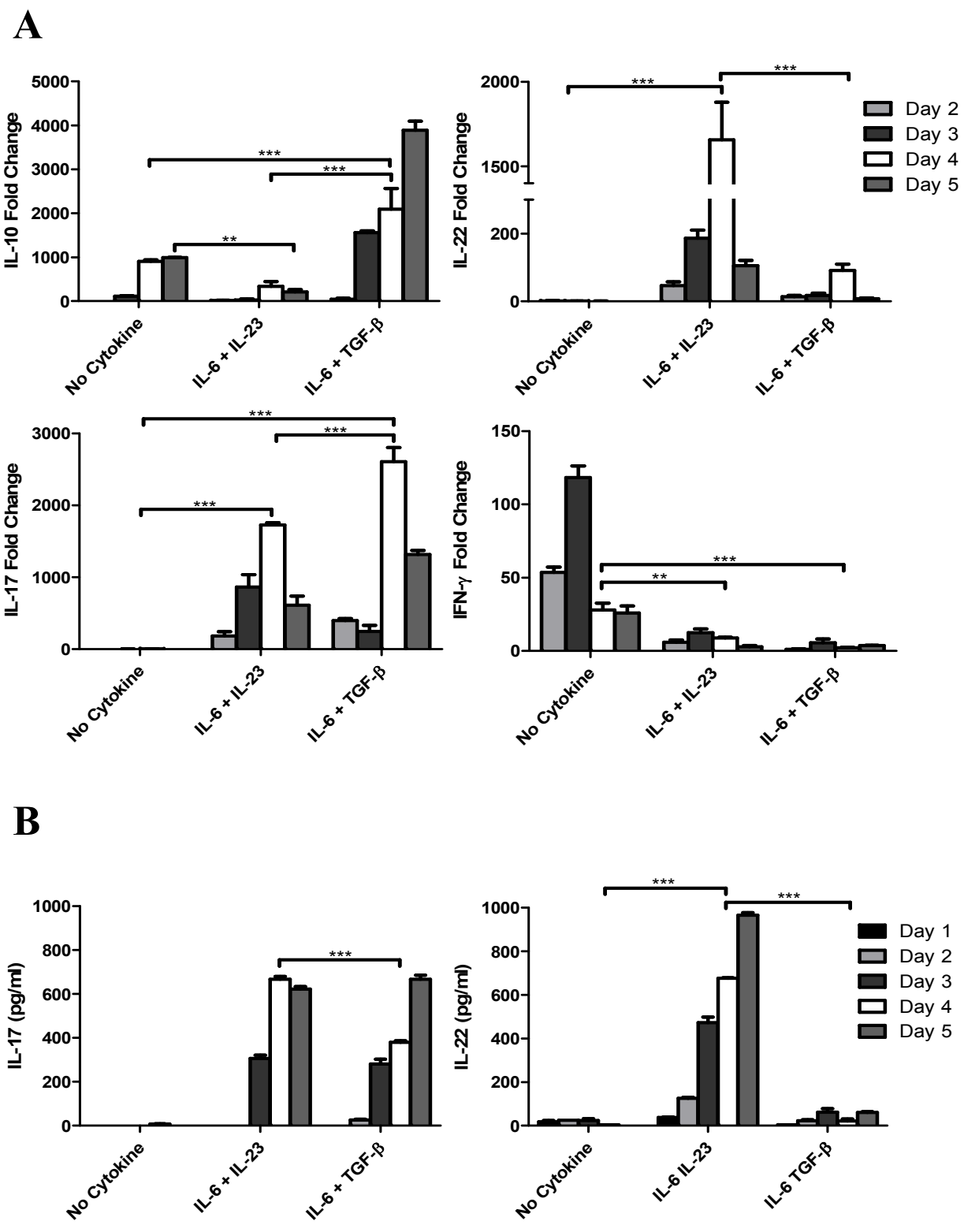

A
No cytokine
IL-6 + IL-23
IL-6 + TGF-β
IL-6 + TGF-β + IL-23
IL-22
IL-17
6.23
0.6
93.4
0.77
9.69
1.79
85.9
3.04
1.68
1.53
86.7
10.3
1.36
1.12
86.1
11.7
IFN-γ
IL-22
22.5
1.94
72.5
3.09
3.64
0.95
85.4
9.98
1.52
1.1
95.7
1.71
1.26
0.62
96.6
1.49


B
IL-22 (pg/ml)
IL-17 (pg/ml)
No Cytokine
IL-6 + IL-23
IL-6 + TGF-β
IL-6 + IL-23 + TGF-β
***
**

PCR. In both Teff17 cells and Treg17 cells, the major Th17 cytokines IL-17 and IL-22 were significantly produced in the highest amounts on day 4 compared to all other days (**Fig. 3.2A**, $p < 0.001$). It seems that after the initial surge of high activation, by day 5 the cells return to a baseline activated state and therefore do not produce these cytokines as quickly afterwards. As day 4 was determined to be the most relevant time point, at which the cytokine potential of these cells is best manifested, statistical comparisons were made based on cytokine production on this day. IL-10 is produced in the highest amounts by Treg17 cells (IL-6 + TGF-β) compared to non-polarized cells and IL-6 + IL-23 polarized cells, which produced significantly lower levels than all other conditions by Day 5 ($p < 0.001$, $p < 0.01$, respectively) (**Fig 3.2A**). Similarly, IL-17 was shown to have the highest production on day 4 by cells polarized by IL-6 + TGF-β, while the IL-6 + IL-23 induced cells can also produce copious amounts of IL-17 compared to the non-polarized group ($p < 0.001$). As expected, IL-22 production was considerably higher in IL-6 + IL-23 polarized cells than all other groups, and showed greater than a 1000-fold increase in IL-22 mRNA production on day 4 compared to the previous day (**Fig. 3.2A**, $p < 0.001$). Lastly, IFN-γ production was measured to determine a Th1-like presence in each of the subsets. On day 4 IFN-γ production was significantly higher in the non-polarized group compared with both Th17-polarized subsets. Interestingly, optimal IFN-γ production occurs by day 3 post-stimulation, which suggests that cells can differentiate to a Th1 phenotype and produce effector cytokines more quickly than cells differentiating into a Th17 phenotype.

IL-17 and IL-22 protein production was confirmed in the supernatants by ELISA (**Fig. 3.2B**). IL-17 production shows somewhat inconsistent results in that using the qRT-PCR assay, Treg17 cells exhibit higher production, whereas analysis of the supernatant points to significantly higher production in Teff17 cells. This is due to the nature of the assays as qRT-PCR analysis is fixed for equal amounts of cDNA whereas the supernatant is affected by the number of cells. Therefore, as Teff17 cells demonstrate much higher proliferation and expansion than Treg17 cells, this is reflected in the assay.

FIGURE 3.2. *IL-22 production is optimal after 4 days of culture in IL-6 + IL-23 polarized cells*. Spleens, pancreatic, and mesenteric lymph nodes were extracted from BDC2.5 mice and naïve cells were isolated by selecting for $CD4^+CD62L^+$ cells. These cells were then cultured in 24-well plates ($3x10^6$/well) that were coated with 10 µg/ml α-CD3 and 2 µg/ml α-CD28 overnight. Th17 effector cells were polarized with 20 ng/ml IL-6 + 20 ng/ml IL-23, and Th17 regulatory cells were polarized with 20 ng/ml IL-6 and 5 ng/ml TGF-β for 4 days. Anti-IFN-γ (5 µg/ml) and anti-IL-4 (5 µg/ml) were also added to all wells except to the No Cytokine sample. Media and cytokines were refreshed after Day 3. RNA was extracted from cells on each of days 2 to 5 and analyzed by RT-qPCR. Relative results using beta actin as a housekeeping gene are shown as mean expression ± SEM. (B) Supernatants from cultures were collected and analyzed for IL-22, and IL-17 expression using ELISA. Results are shown as mean concentration ± SEM. The ANOVA test was used to analyze all values with $p < 0.05$ termed as significant.


A
IL-10 Fold Change
5000
4000
3000
2000
1000
0
***
***
**
No Cytokine
IL-6 + IL-23
IL-6 + TGF-β
IL-22 Fold Change
2000
1500
200
0
***
***
Day 2
Day 3
Day 4
Day 5
IL-17 Fold Change
3000
2000
1000
0
***
***
***
IFN-γ Fold Change
150
100
50
0
***
**
B
IL-17 (pg/ml)
1000
800
600
400
200
0
***
IL-22 (pg/ml)
1000
800
600
400
200
0
***
***
Day 1
Day 2
Day 3
Day 4
Day 5
IL-6 IL-23
IL-6 TGF-β

3.1.1 Titration of subset-inducing cytokines

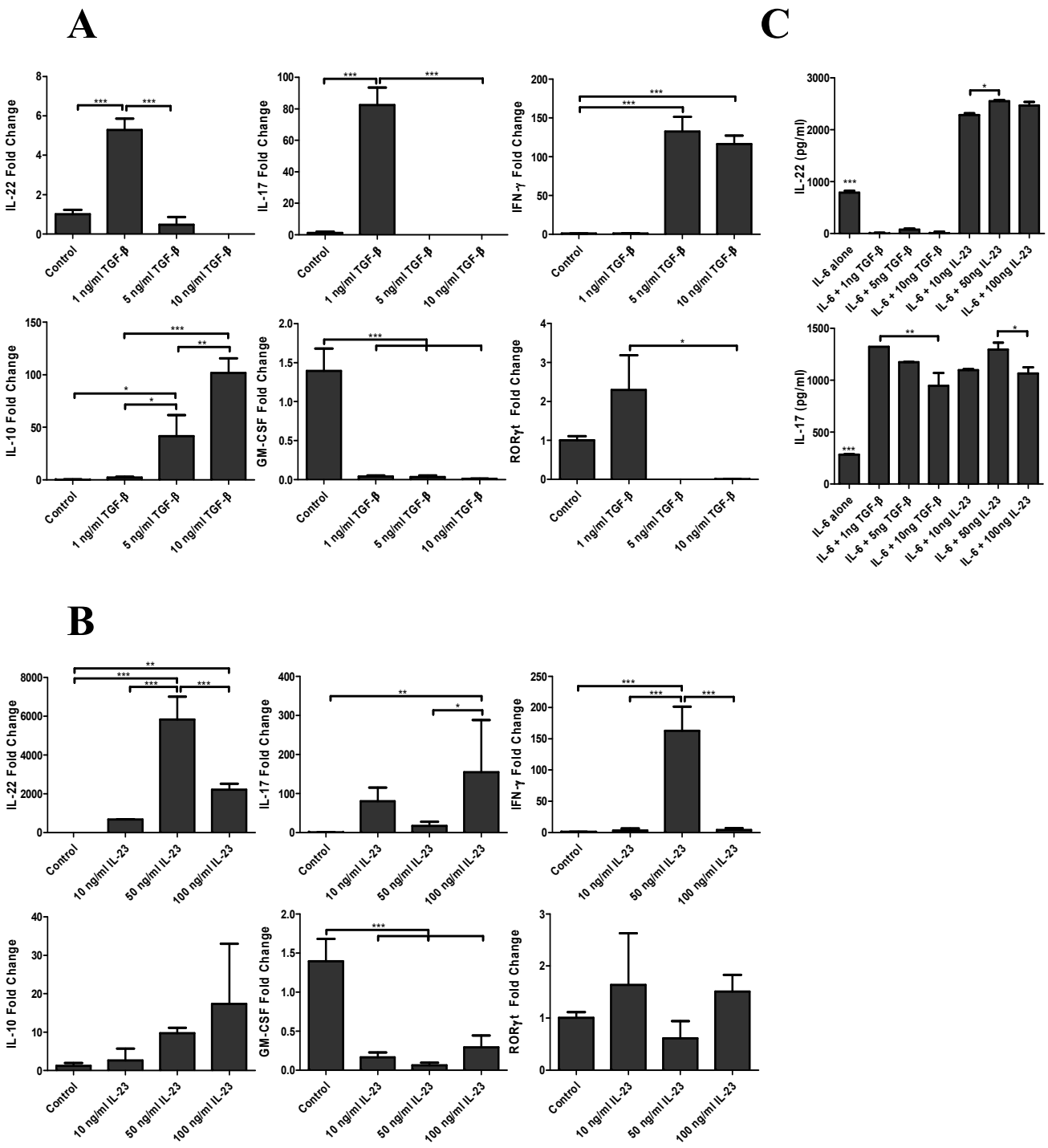
To demonstrate the effects of Th17 cells differentiating in the presence of varying amounts of IL-23 or TGF-β, both cytokines were titrated in BDC2.5 naïve T cell cultures. For Treg17 polarization by IL-6 + TGF-β, titration of TGF-β indicates that lower levels of this cytokine are optimal for Th17 differentiation as 1 ng/ml induces the highest IL-17 and IL-22 fold change when compared to the control without TGF-β or higher concentrations (**Fig. 3.3A**, $p < 0.001$). Indeed, with exceeding amounts of TGF-β, cells may be polarized more towards a Th1-phenotype as shown by their increased production of IFN-γ ($p < 0.001$) or towards a Treg phenotype shown by increased IL-10 production. This is also shown by a decrease in RORγt expression by cells in the presence of increasing amounts of TGF-β ($p < 0.05$).

When titrating IL-23, the optimal concentration for IL-22 production is approximately 50 ng/ml, however, extensive IL-22 production is induced by both 10 ng/ml and 100 ng/ml concentrations as well when compared to the control without IL-23 (**Fig 3.3B**). There are no significant differences in IL-17 production with varying amounts of IL-23, and RORγt is produced evenly without the need for IL-23 to be present. It was also seen that a concentration of 50 ng/ml of IL-23 induced the highest level of IFN-γ production as well ($p < 0.001$).

The above results were confirmed by ELISA, which showed that IL-6 without the inducing factors IL-23 or TGF-β has a significantly different expression of Th17 cytokines IL-22 and IL-17 (**Fig 3.3C**, $p < 0.001$). Few differences were seen between the concentrations of titrated cytokines indicating that IL-23 or TGF-β can elicit Th17 responses that are phenotypically different from each other at lower amounts.

Collectively, these results confirm using isolated naïve T cells that which was previously observed in our lab with bulk splenocytes (see Appendix). Teff17 cells demonstrate the highest IL-22 production, while both Teff17 cells and Treg17 cells exhibit a Th17 phenotype. Treg17 cells produce more IL-10, while non-polarized cells have an affinity for IFN-γ production. Lastly, Th17 cells fully mature 4 days after stimulation.

FIGURE 3.3. *Cytokine expression after titration of TGF-β and IL-23*. Spleens and pancreatic lymph nodes were extracted from BDC2.5 mice and naïve cells were isolated selecting for $CD4^+CD62L^+$ cells. These cells were then cultured in 24-well plates ($3x10^6$/well) that were coated with 10 µg/ml α-CD3 and 2 µg/ml α-CD28 overnight. (A) IL-6 was added to each well (20 ng/ml) and TGF-β was titrated using concentrations between 1 ng/ml and 10 ng/ml as well as a control well with no TGF-β. Anti-IFN-γ (5 µg/ml) and anti-IL-4 (5 µg/ml) were also added to each well. (B) IL-6 was added to each well (20 ng/ml) and IL-23 was titrated using concentrations between 10 ng/ml and 100 ng/ml except for a control well without IL-23. Anti-IFN-γ (5 µg/ml) and anti-IL-4 (5 µg/ml) were also added to each well. RNA was extracted and analyzed by RT-qPCR. Relative results using beta actin as a housekeeping gene are shown as mean expression ± SEM. (C) Supernatants from cultures were collected and analyzed for IL-22, and IL-17 expression using ELISA. Results are shown as mean concentration ± SEM. The ANOVA test was used to analyze all values with $p < 0.05$ considered significant.


A
IL-22 Fold Change
IL-17 Fold Change
IFN-γ Fold Change
IL-10 Fold Change
GM-CSF Fold Change
RORγt Fold Change
Control
1 ng/ml TGF-β
5 ng/ml TGF-β
10 ng/ml TGF-β
C
IL-22 (pg/ml)
IL-17 (pg/ml)
IL-6 alone
IL-6 + 1ng TGF-β
IL-6 + 5ng TGF-β
IL-6 + 10ng TGF-β
IL-6 + 10ng IL-23
IL-6 + 50ng IL-23
IL-6 + 100ng IL-23
B
IL-22 Fold Change
IL-17 Fold Change
IFN-γ Fold Change
IL-10 Fold Change
GM-CSF Fold Change
RORγt Fold Change
Control
10 ng/ml IL-23
50 ng/ml IL-23
100 ng/ml IL-23

3.2 Maximizing IL-22 production by Th17 cells

To better study IL-22 and its effects on diabetes, we first attempted to maximize IL-22 production in Th17-polarized BDC2.5 cells. To do so, we used IL-6 + IL-23 to induce effector cells that can produce high amounts of IL-22. Splenocytes were also used to include APCs and adding PS3, a mimotope antigen specific to the BDC2.5 TCR (228), to fully stimulate the subsets and determine the maximum potential of these cells. We first determined the effect of inhibiting IFN-γ on differentiating cells. We compared non-polarized cells that were antigen-stimulated and treated with α-IFN-γ to those that had not been treated with neutralizing antibody. This yielded a drastic increase in IL-22 (about 5 times more) and some suppression of IFN-γ with the addition of the antibody (**Fig. 3.4**). Interestingly, when cells are polarized with IL-6 + IL-23, neutralizing IFN-γ magnifies this effect by showing an almost 3-fold increase in IL-22-producing cells compared to treatment without the antibody. This effect is also seen with IL-17 as α-IFN-γ induces ~3.5 times more IL-17-producing cells than regular Th17 differentiation alone. Together this shows an increase in IL-22^{+}IL-17^{+} double positive cells by simply neutralizing IFN-γ.

These effects were also observed quantitatively using qRT-PCR. IL-22 production is highest in IL-6 + IL-23 polarized cells in the presence of α-IFN-γ (**Fig. 3.5A**). IL-17 production is similar between IFN-γ-neutralized cells and those that were not, with perhaps less IL-17 in the IFN-γ-neutralized group (**Fig. 3.5A,B**). Interestingly, Th1 cytokines such as IFN-γ and GM-CSF are decreased after *either* polarization using IL-6 + IL-23 *or* α-IFN-γ. This confirms that neutralizing IFN-γ has a similar ultimate effect as polarizing cytokines IL-6 and IL-23: neutralizing IFN-γ influences naïve T cells away from a Th1 phenotype (and thus towards Th17 induction) while polarizing cytokines *direct* cells towards a Th17 phenotype. These results were confirmed using ELISA that IL-6 + IL-23 + α-IFN-γ polarized cells show the highest IL-22 production (**Fig. 3.5B**, $p < 0.01$).

FIGURE 3.4. *Addition of anti-IFN-γ to IL-6 plus IL-23 in culture induces maximal IL-17 and IL-22 production in BDC2.5 cells activated by antigen.* BDC2.5 splenocytes were cultured in a 96 well plate ($2x10^5$/well) with 5 μg/ml PS3 mimotope and with or without 5 μg/ml α-IFN-γ, and 20 ng/ml IL-6 + 10 ng/ml IL-23 for 4 days. Cells were harvested and stained for FACS analysis (A); 200 000 events were collected and gated on live cells, then $CD4^+$ cells.

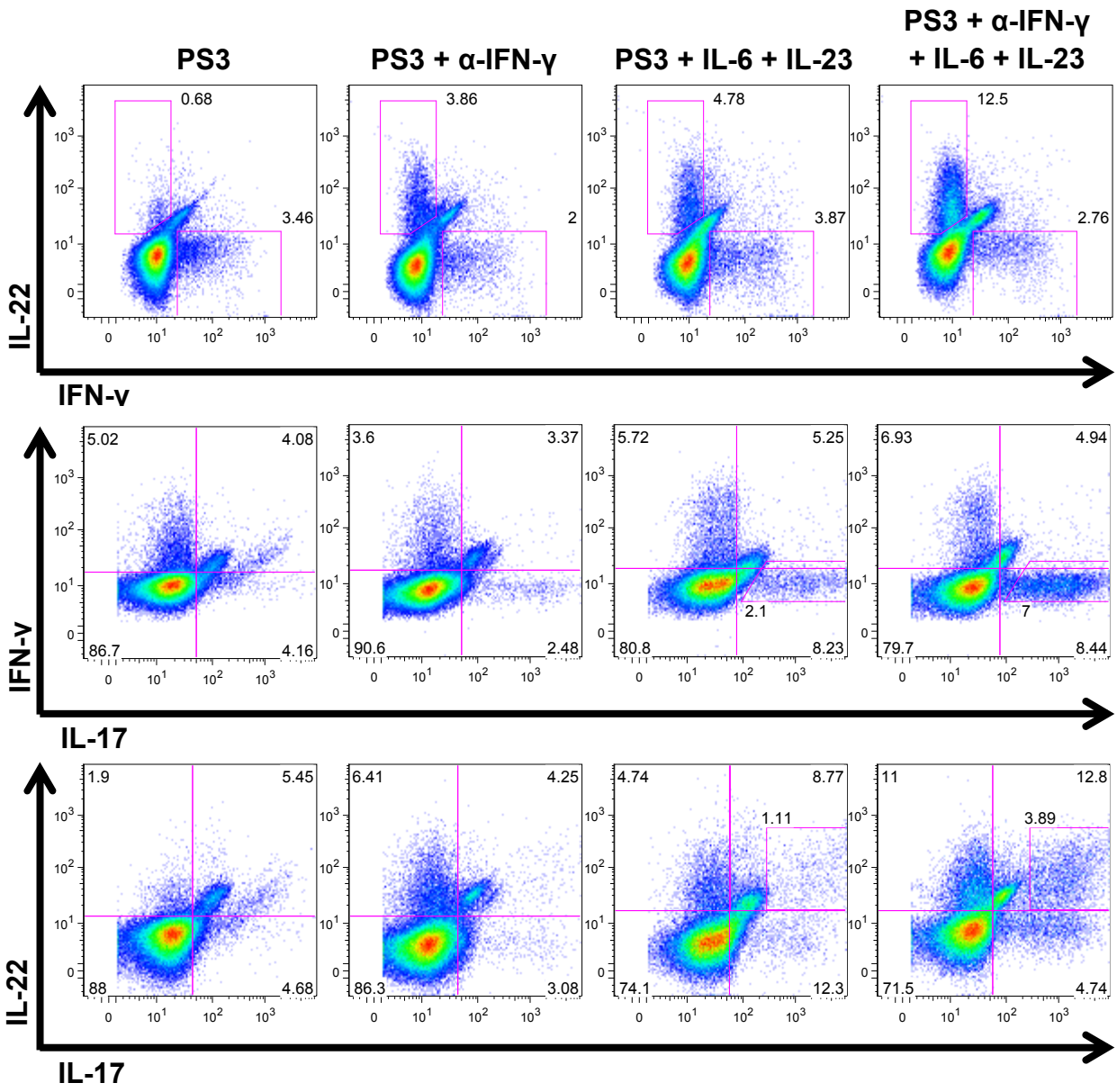
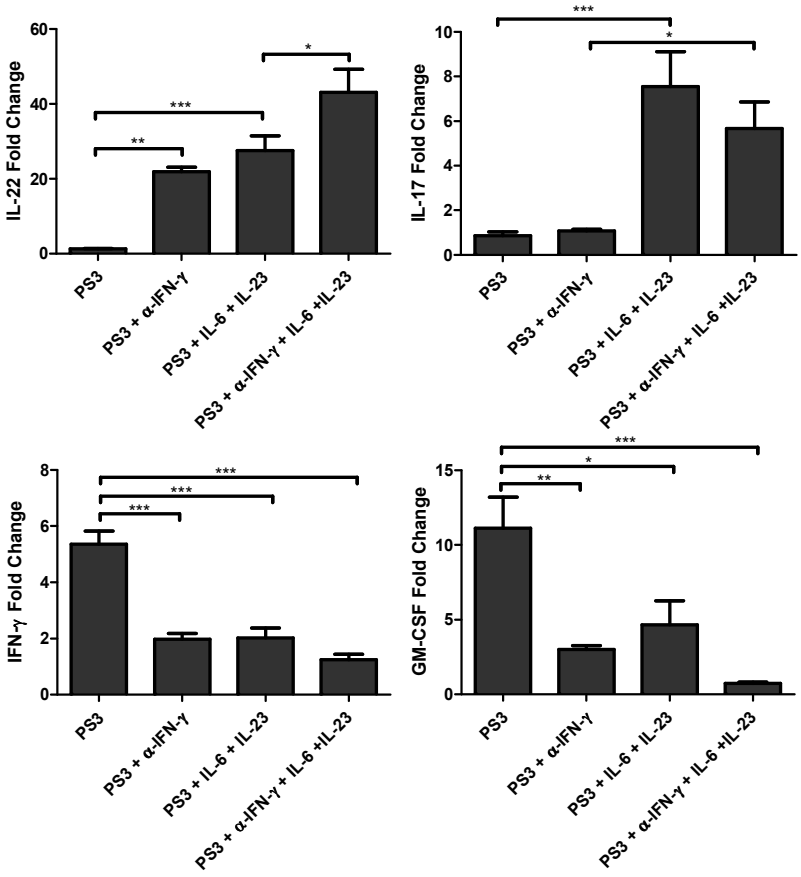
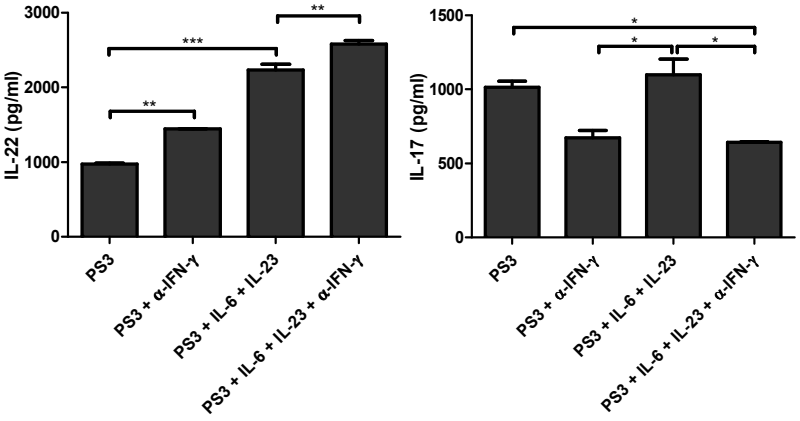

PS3
PS3 + α-IFN-γ
PS3 + IL-6 + IL-23
PS3 + α-IFN-γ + IL-6 + IL-23
IL-22
IFN-v
0.68
3.46
3.86
2
4.78
3.87
12.5
2.76
IFN-γ
IL-17
5.02
4.08
86.7
4.16
3.6
3.37
90.6
2.48
5.72
5.25
2.1
80.8
8.23
6.93
4.94
7
79.7
8.44
IL-22
IL-17
1.9
5.45
88
4.68
6.41
4.25
86.3
3.08
4.74
8.77
1.11
74.1
12.3
11
12.8
3.89
71.5
4.74

FIGURE 3.5. *Addition of anti-IFN-γ to IL-6 plus IL-23 in culture induces maximal IL-22 production and IFN-γ suppression in BDC2.5 cells activated by antigen.* BDC2.5 splenocytes were cultured in a 96 well plate ($2x10^5$/well) with 5 μg/ml PS3 mimotope and with or without 5 μg/ml α-IFN-γ, and 20 ng/ml IL-6 + 10 ng/ml IL-23 for 4 days. (A) RNA was then extracted and cDNA produced from these cells were analyzed by RT-qPCR using gene specific primers. Relative results using beta actin as a housekeeping gene are shown as mean expression ± SEM from 2 different experiments. (B) Supernatants from cultures were collected and analyzed for IL-22 and IL-17 expression using ELISA. Results are shown as mean concentration ± SEM. The ANOVA test was used to analyze all values with $p < 0.05$ termed as significant.

A


IL-22 Fold Change
IL-17 Fold Change
IFN-γ Fold Change
GM-CSF Fold Change
PS3
PS3 + α-IFN-γ
PS3 + IL-6 + IL-23
PS3 + α-IFN-γ + IL-6 +IL-23

B


IL-22 (pg/ml)
IL-17 (pg/ml)
PS3
PS3 + α-IFN-γ
PS3 + IL-6 + IL-23
PS3 + IL-6 + IL-23 + α-IFN-γ

3.2.1 The effect of neutralizing IFN-γ on pathogenicity

To determine whether neutralizing IFN-γ has an effect on the pathogenicity of Th17 effector cells, BDC2.5 splenocytes were polarized with IL-6 + IL-23 and stimulated with PS3 mimotope with or without neutralizing α-IFN-γ Ab. These cells were then adoptively transferred into 5-week-old female NOD mice and diabetes incidence was monitored. According to the Kaplan-Meier survival estimate, no significant difference was seen in disease incidence between these two groups (**Fig. 3.6A**). Analysis of supernatants revealed that the cells polarized with α-IFN-γ had significantly higher IL-22 ($p < 0.001$) and IL-17 ($p < 0.05$) production than the control group (**Fig. 3.6B**).

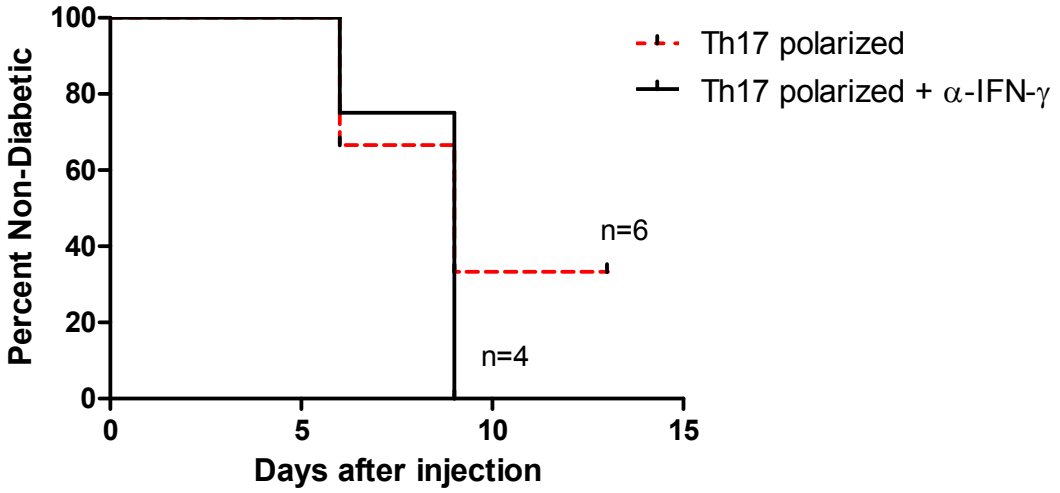
3.2.2 Neutralizing TGF-β to increase IL-22 production

TGF-β is an inhibitor of IL-22 through the action of c-Maf (127). We therefore sought to determine whether neutralizing TGF-β would increase IL-22 production in Th17 cells. BDC2.5 splenocytes were again stimulated with PS3 mimotope and cultured with IL-6 + IL-23 with or without neutralizing antibodies against TGF-β and IFN-γ. Unexpectedly, a combination of α-IFN-γ and α-TGF-β did not induce the highest amount of IL-22 production, which was seen when using α-IFN-γ alone (**Fig. 3.7**). Indeed, polarization in the presence of α-TGF-β actually induced slightly less IL-22 production than polarizing cytokines alone. IL-17 was also decreased in samples containing α-TGF-β, however this was expected as TGF-β helps to induce IL-17 expression.

These results were quantitatively confirmed using qRT-PCR. IL-22 expression was significantly higher in polarized cells with α-IFN-γ than when α-TGF-β was added (**Fig. 3.8A**, $p < 0.01$). Polarized cells with α-TGF-β produced the least amount of IL-17, while these cells produced the most IL-10. Testing the supernatants of each sample reinforced these findings that neutralizing IFN-γ exhibited the highest IL-22 and IL-17 production, while neutralizing TGF-β showed the lowest (**Fig. 3.8B**, $p < 0.05$). Differences in proliferation were evident while observing cultures on the plate so a tritiated thymidine proliferation assay was done. This demonstrated that neutralizing both IFN-γ and TGF-β induces the highest proliferation, while neutralizing only IFN-γ induces more proliferation than just using polarizing cytokines alone (**Fig. 3.8C**).

FIGURE 3.6. *Adoptive transfer of Th17 polarized BDC2.5 splenocytes shows neutralizing anti-IFN-γ antibody does not significantly change the pathogenicity of these cells.* (A) Splenocytes from BDC2.5 mice were cultured with 5 μg/ml PS3, 20 ng/ml IL-6, and 10 ng/ml IL-23 with or without 5 μg/ml α-IFN-γ for 5 days. Cells were collected and $5x10^6$ cells were intravenously injected into four week old female NOD mice in each group. Mice were checked every 3 days for diabetes onset. The Kaplan-Meier survival estimate was used to determine differences in the two groups, however no significant difference was found. (B) Supernatants from the above cell cultures were collected and IL-22 and IL-17 concentrations were measured using ELISA. The Student's t test was used to determine significant differences between the groups ($p < 0.05$).

A


Percent Non-Diabetic
100
80
60
40
20
0
0
5
10
15
Days after injection
Th17 polarized
Th17 polarized + α-IFN-γ
n=6
n=4

B

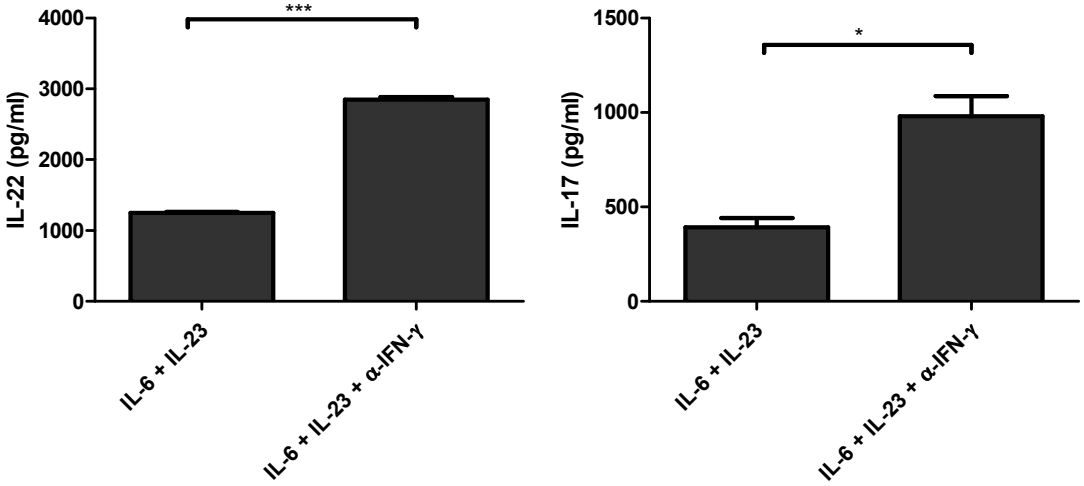

IL-22 (pg/ml)
4000
3000
2000
1000
0
***
IL-6 + IL-23
IL-6 + IL-23 + α-IFN-γ
IL-17 (pg/ml)
1500
1000
500
0
*
IL-6 + IL-23
IL-6 + IL-23 + α-IFN-γ

FIGURE 3.7. *The effects of neutralizing anti-TGF-β and anti-IFN-γ antibodies on IL-22 production from BDC2.5 splenocytes.* BDC2.5 splenocytes were cultured in 96-well plates ($2x10^5$/well) with 5 μg/ml PS3, 20 ng/ml IL-6 and 10 ng/ml IL-23 with or without neutralizing α-IFN-γ (5 μg/ml) and α-TGF-β (5 μg/ml) antibodies for 4 days. Cells were collected and stained for FACS analysis; 100 000 events were collected and gated on live cells, then $CD4^+$ cells.

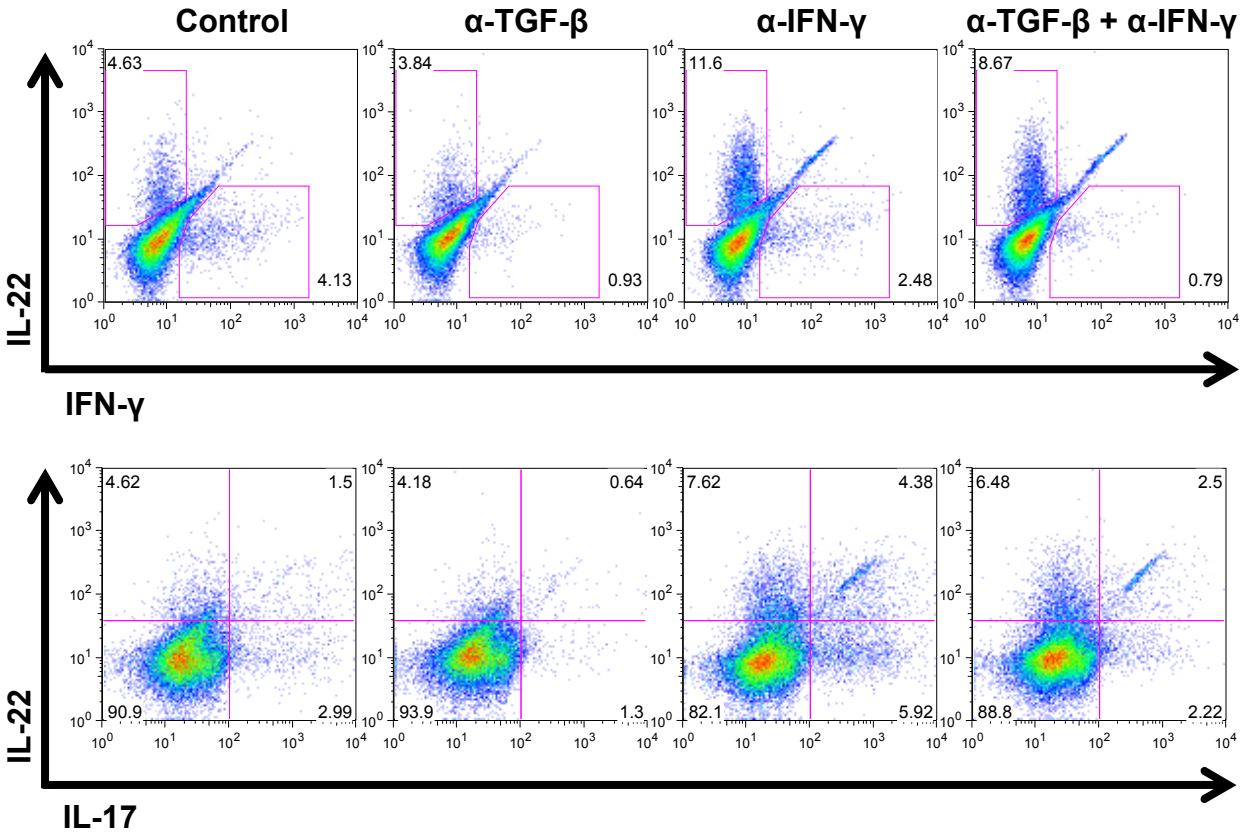
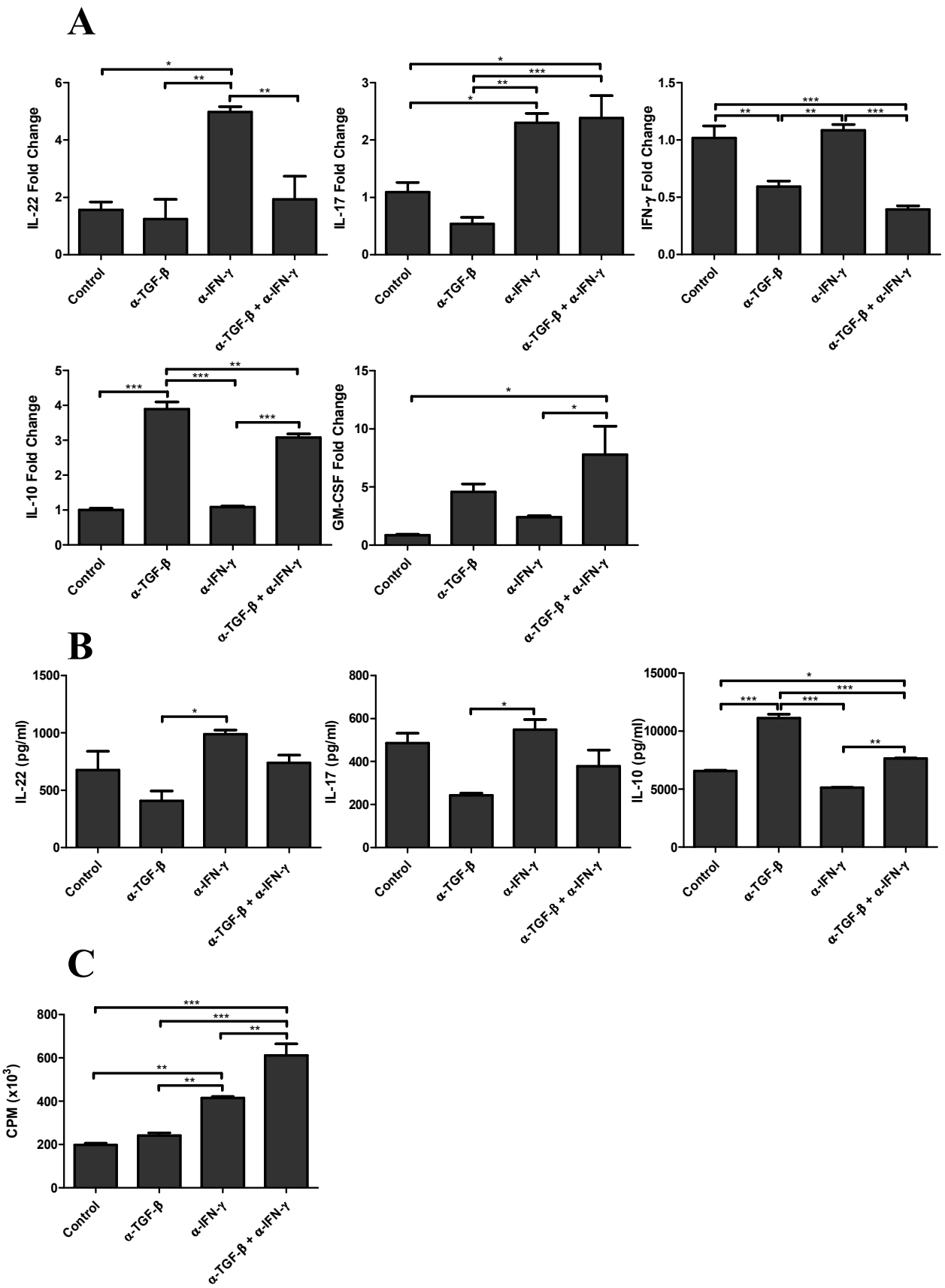

Control
α-TGF-β
α-IFN-γ
α-TGF-β + α-IFN-γ
4.63
4.13
3.84
0.93
11.6
2.48
8.67
0.79
IL-22
IFN-γ
4.62
1.5
90.9
2.99
4.18
0.64
93.9
1.3
7.62
4.38
82.1
5.92
6.48
2.5
88.8
2.22
IL-22
IL-17

FIGURE 3.8. *The effects of neutralizing anti-TGF-β and anti-IFN-γ antibodies on IL-22 production from BDC2.5 splenocytes.* BDC2.5 splenocytes were cultured in 96-well plates ($2x10^5$/well) with 5 μg/ml PS3, 20 ng/ml IL-6 and 10 ng/ml IL-23 with or without neutralizing antibodies α-IFN-γ (5 μg/ml) and α-TGF-β (5 μg/ml) for 4 days. (A) RNA was extracted and made into cDNA which was analyzed by RT-qPCR using gene specific primers. Relative results using beta actin as a housekeeping gene are shown as mean expression ± SEM from 2 different experiments. (B) Supernatants from cultures were collected and analyzed for IL-22, IL-17, and IL-10 expression using ELISA. Results are shown as mean concentration ± SEM. (C) Radioactive thymidine, [^{3}H]T, was added to the culture for 18 h and proliferation was measured using a MicroBeta counter. Results shown are a mean ± SEM. The ANOVA test was used to analyze all values with $p < 0.05$ termed as significant.

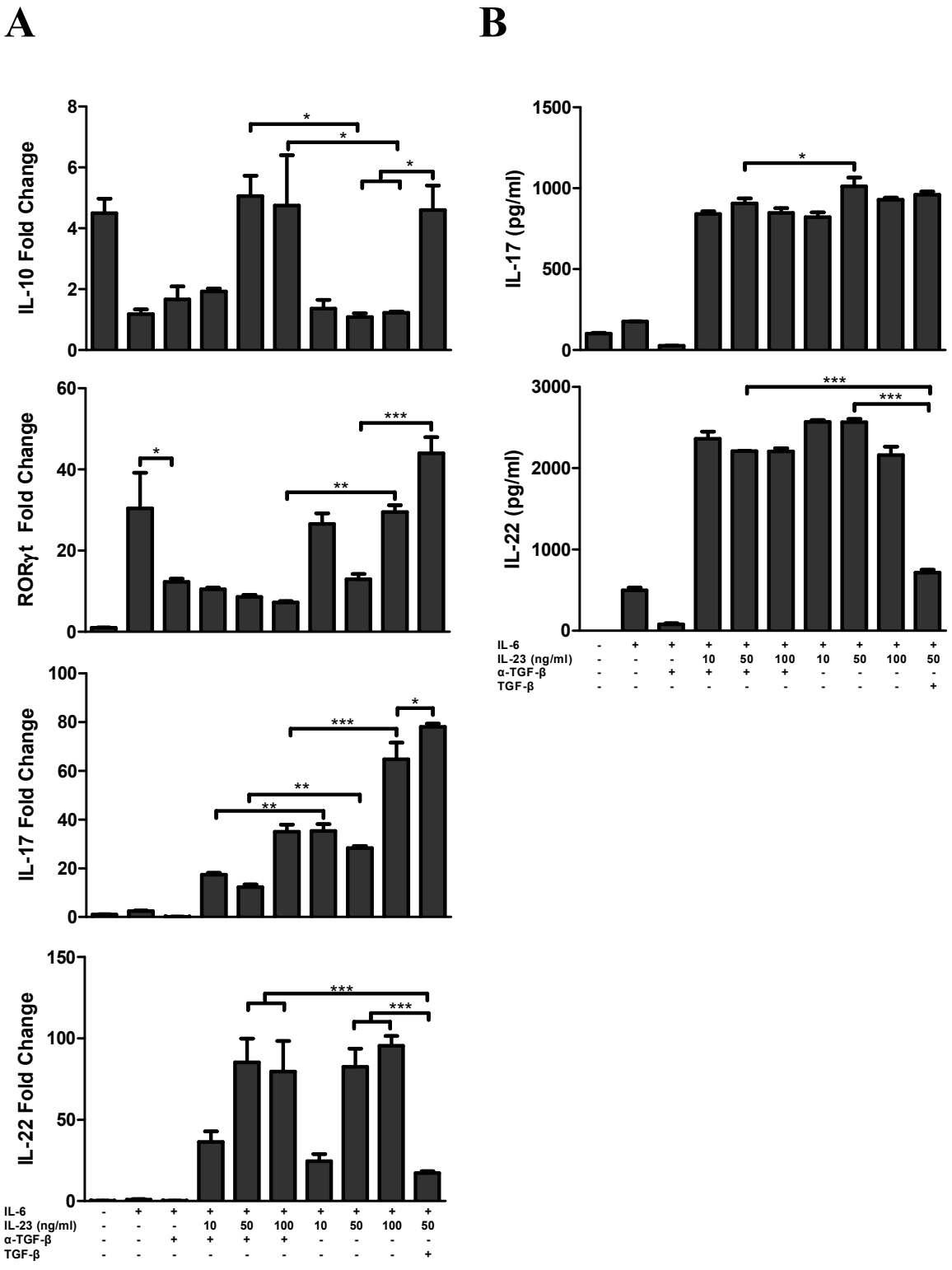

A
IL-22 Fold Change
IL-17 Fold Change
IFN-γ Fold Change
IL-10 Fold Change
GM-CSF Fold Change
Control
α-TGF-β
α-IFN-γ
α-TGF-β + α-IFN-γ
B
IL-22 (pg/ml)
IL-17 (pg/ml)
IL-10 (pg/ml)
C
CPM (x10^3)

We then wanted to confirm the validity of these results using isolated naïve T cells and polarizing them with various cytokine/antibody combinations to rule out contaminating cytokines produced by other cell types. IL-23 was titrated with and without α-TGF-β and compared to control cells polarized without IL-23 and with TGF-β. Cells were compared quantitatively using qRT-PCR to those with the same amount of IL-23 added, and it was found that cells polarized in the presence of α-TGF-β do not produce significantly different amounts of IL-22 (**Fig. 3.9A**). As expected, the addition of TGF-β to the culture, even with a constant amount of IL-23 being present, drastically induces less IL-22 production ($p < 0.001$). To ensure α-TGF-β was present in adequate amounts, the effects on other Th17 factors were measured. Anti-TGF-β has a significant effect on IL-17 expression. When comparing cells polarized with the same amount of IL-23, the samples with α-TGF-β show significantly lower IL-17 production than their counterparts without neutralizing antibody (**Fig. 3.9A**, $p < 0.01, 0.001$). As expected, the highest IL-17 production is seen in cells polarized with both IL-23 and TGF-β. As seen in the previous experiment, α-TGF-β induces significantly higher IL-10 production in polarized cells compared to their non-antibody counterpart (**Fig. 3.9A**, $p < 0.05$). This was unexpected as it was also seen that TGF-β can induce higher amounts of IL-10. Interestingly, while examining RORγt expression in polarized cells, α-TGF-β significantly reduced production in polarized cells, even in the absence of IL-23 (**Fig. 3.9A**, $p < 0.05$). RORγt expression is also the highest in TGF-β-polarized cells ($p < 0.001$). IL-22 and IL-17 production were also confirmed by analyzing supernatants of polarized cells (**Fig. 3.9B**)

3.3 Th17 subset production of Granzyme B and Perforin

To attempt to understand the difference in the ability of one subset to induce disease while a similar subset cannot, we examined Granzyme B and Perforin production by the polarized cell subsets as these proteins can be used as a method of cell-mediated killing. It has previously been shown that islet beta cells are destroyed by these enzymes probably produced by cytotoxic $CD8^+$ T cells, and that a deficiency in perforin in the NOD mouse model results in a reduction of disease (232, 233). We wanted to test

FIGURE 3.9. *Neutralizing anti-TGF-β antibody does not significantly increase IL-22 production from BDC2.5 splenocytes*. (A) Spleens and pancreatic lymph nodes were extracted from BDC2.5 mice and naïve cells were isolated by selecting for $CD4^+CD62L^+$ cells. These cells were then cultured in 24-well plates ($3x10^6$/well) that were coated with 10 μg/ml α-CD3 and 2 μg/ml α-CD28 overnight. Anti-IFN-γ (5 μg/ml), anti-IL-4 (5 μg/ml) and IL-6 (20 ng/ml) were added to each well except the well with no cytokines as indicated. IL-23 was titrated and α-TGF-β (5 μg/ml) or TGF-β (5 ng/ml) were added according to the figure. After 4 days cells were harvested and RNA was extracted and analyzed by RT-qPCR. Relative results using beta actin as a housekeeping gene are shown as mean expression ± SEM of multiple experiments. (B) Supernatants from the above cultures were collected and analyzed for IL-22 and IL-17 expression using ELISA. Results are shown as mean concentration ± SEM. The ANOVA test was used to analyze all values with $p < 0.05$ termed as significant.


A
B
IL-10 Fold Change
RORγt Fold Change
IL-17 Fold Change
IL-22 Fold Change
IL-17 (pg/ml)
IL-22 (pg/ml)
IL-6
IL-23 (ng/ml)
α-TGF-β
TGF-β

whether $CD4^+$ T cells could also use this method in a CD4 transgenic mouse system. Using flow cytometry, we specifically gated on $CD4^+$ cells and observed high production of both Granzyme B and Perforin in these cells. To specifically compare and examine the roles of IL-22-producing cells, IL-22^+Granzyme B^+ double positive cells were gated. IL-6 + IL-23 polarized cells demonstrated increased IL-22-producing Granzyme B positive cells compared to IL-6 + TGF-β or non-polarized cells (**Fig. 3.10A**). However, when looking at Granzyme B production from all $CD4^+$ cells, IL-6 + IL-23 polarized cells had only very slightly more expression than other subsets, and this could be due to a higher proliferation in these cells (**Fig. 3.10B**).

We also explored perforin expression in each of the Th17-polarized subsets and again found a slightly higher frequency of IL-22^+Perforin$^+$ double positive cells in the IL-6 + IL-23 group (**Fig. 3.10C**). However, when gating on all $CD4^+$ cells, no difference is seen in perforin production between IL-6 + IL-23 polarized cells and those induced with IL-6 + TGF-β (**Fig. 3.10D**).

Altogether, we found that neutralizing IFN-γ dramatically increased IL-22 production in polarized Th17 cells. Conversely, neutralizing TGF-β had no effect on expression of IL-22 in these cells. Lastly, polarized Th17 subsets do not differentially express killing-enzymes granzyme B and perforin.

3.4 IL-22-producing cells in diabetic NOD mice

To further delve into the role IL-22 may play in type 1 diabetes (T1D), lymphocytes from aged diabetic NOD mice were characterized from a number of tissues. Infiltrating lymphocytes were extracted from the pancreas using collagenase V and activated for 5 hours with PMA and Ionomycin. Flow cytometry was used to determine IL-22 and IFN-γ production and showed that there are both IFN-γ- and IL-22-producing cells among the infiltrating lymphocytes (**Fig. 3.11B**). A similar percentage of IL-22-producing cells were present in both the general live populations and the $CD4^+$ gated populations suggesting that there may be other infiltrating cells that produce IL-22 than

FIGURE 3.10. *Expression of Granzyme B and Perforin by different subsets of Th17 cells from BDC2.5 splenocytes.* BDC2.5 splenocytes were cultured in a 96 well plate ($2x10^5$/well) and polarized to different subsets of T cells. Th17 effector cells were polarized with 20 ng/ml IL-6 + 20 ng/ml IL-23, Th17 regulatory cells were polarized with 20 ng/ml IL-6 and 5 ng/ml TGF-β and one population was cultured with no added cytokines. PS3 mimotope was used to stimulate all cells for 4 days. Cells were collected and stained for FACS analysis (A-D); 70 000 events were collected and gated on lymphocytes, then $CD4^+$ cells.

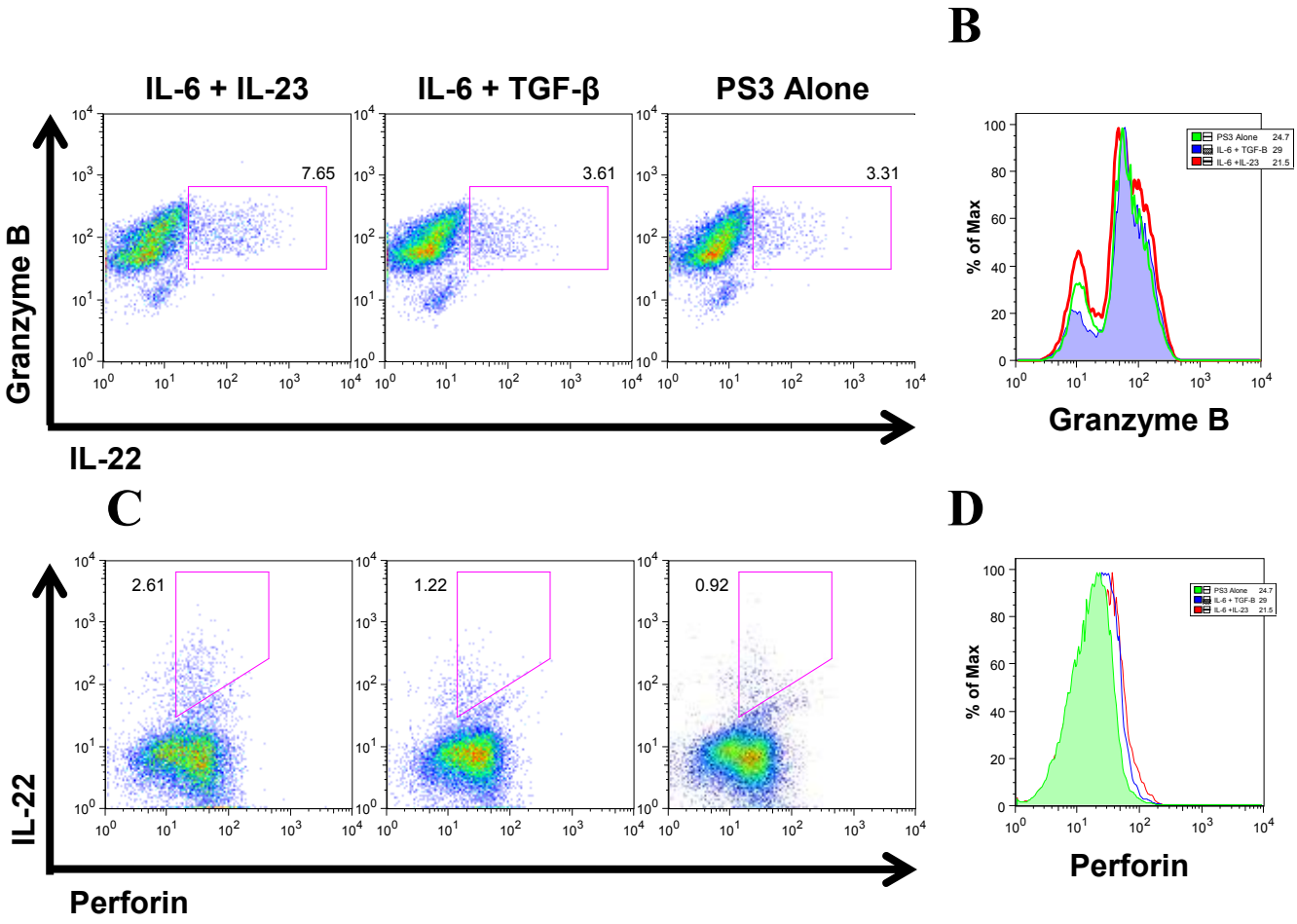
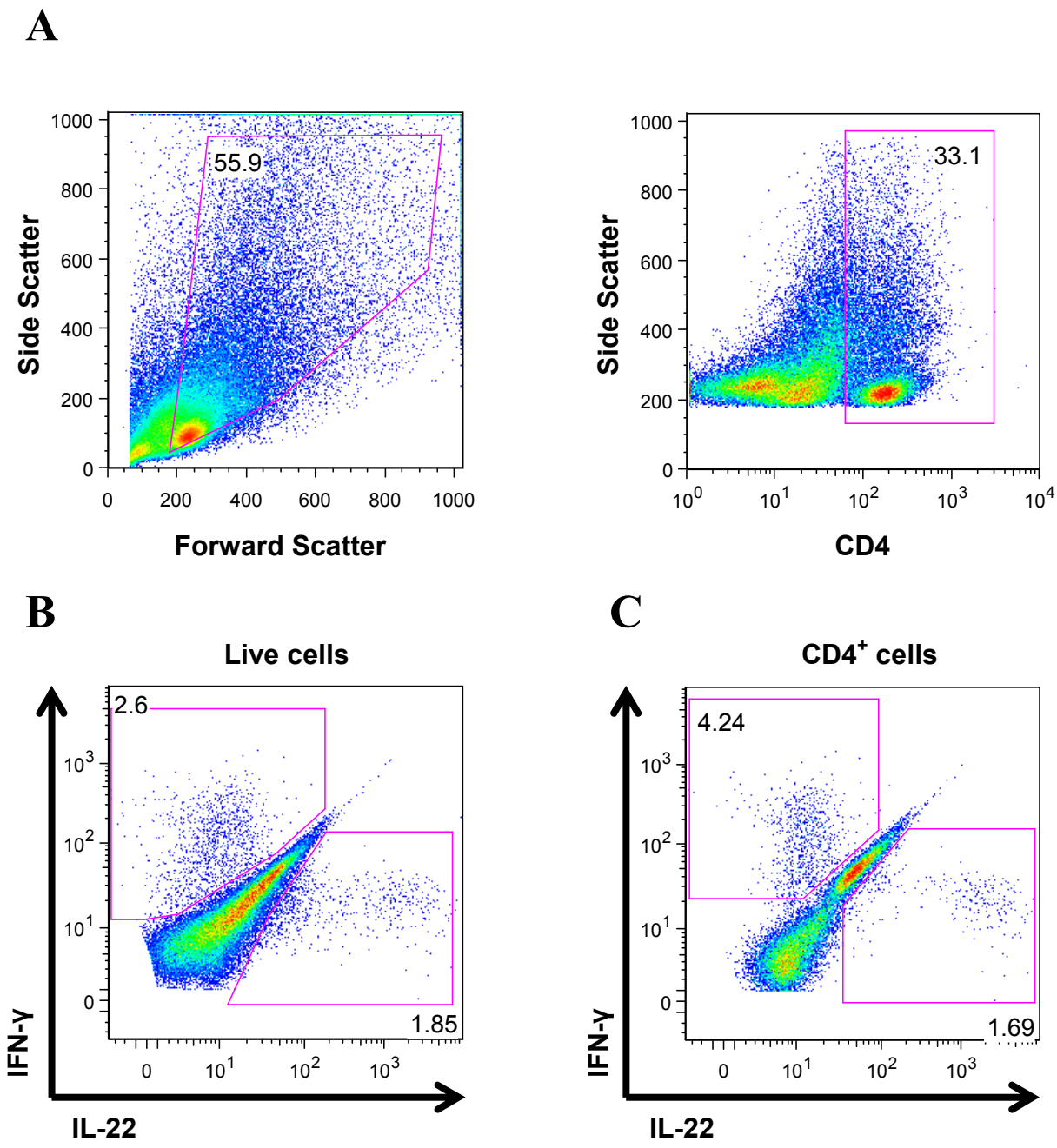

B
IL-6 + IL-23
IL-6 + TGF-β
PS3 Alone
7.65
3.61
3.31
Granzyme B
IL-22
% of Max
Granzyme B
PS3 Alone 24.7
IL-6 + TGF-B 29
IL-6 +IL-23 21.5
C
D
2.61
1.22
0.92
IL-22
Perforin
% of Max
Perforin

FIGURE 3.11. *Unstimulated lymphocytes extracted from the pancreas of diabetic NOD mice show some IL-22 production.* Pancreata were extracted from diabetic NOD mice and infiltrating lymphocytes were isolated by incubating minced pancreatic fragments in 10% RPMI media containing Collagenase Type V for 30 mins to release islets. Cells were then stimulated in 24-well plates with PMA and Ionomycin for 5 hours. Cells were then collected and stained for FACS analysis; Using the gating strategy depicted in (A) 100 000 events were collected and gated on live cells (B), then $CD4^+$ cells (C). Plots are representative of multiple experiments.


A
Side Scatter
Forward Scatter
55.9
33.1
CD4
B
Live cells
2.6
1.85
IFN-γ
IL-22
C
CD4+ cells
4.24
1.69
IFN-γ
IL-22

$CD4^+$ T cells (**Fig. 3.11C**). In both populations the percentage of IL-22-producing cells recovered were very small but somewhat similar to the amount of IFN-γ-producing cells.

To determine the full functional potential of the recovered infiltrating lymphocytes, these cells were stimulated in a 24-well plate coated with α-CD3ε and in the presence of α-CD28 for 48-72 hrs. Flow cytometry was used to compare IL-22 production in infiltrating lymphocytes to isotype or Fluorescence Minus One (FMO) controls. When gating on general live cells, IL-22 production minimally increased compared to both the isotype and FMO controls (**Fig. 3.12A**). However, when gating specifically for $CD4^+$ cells, there seems to be a larger increase in the amount of IL-22-producing cells compared to the FMO control (**Fig. 3.12B,C**). Again, these increases are not drastic but may represent a biological significance.

To determine whether the presence of infiltrating IL-22-producing cells in the pancreas is diabetes-specific, diabetic and non-diabetic NOD mice of the same age were compared. Importantly, when examining $CD4^+$ pancreatic-infiltrating cells, the diabetic NOD mice demonstrated over a 5-fold increase in IFN-γ^+ cells compared to the non-diabetic and an almost 2-fold increase in IL-22-producing cells (**Fig. 3.13B**). The cytokine influence from $CD8^+$ cells was also explored to discover any differences that may be present in diseased mice. As expected, the diabetic NOD mice demonstrated a very high percentage of IFN-γ-producing $CD8^+$ cells, and an increase compared to the non-diabetic mice (**Fig. 3.13C**). A much smaller percentage of these cells express IL-22 as well, however the diabetic NOD did show a greater than 7-fold increase in IL-22-producing $CD8^+$ cells compared to the same aged, non-diabetic control.

3.4.1 IL-22 and IL-22R expression in the pancreas in diabetic and young NOD mice

To explore any differences in IL-22 and other cytokine production in the pancreas compared to younger control mice, lymphocytes infiltrating the pancreas were again isolated using collagenase V and stimulated with α-CD3 and α-CD28. The youngest mice, around 5 weeks old, had few pancreatic infiltrating cells compared to the diabetic but around 1-3 million cells can usually be isolated from these mice. After 48-72 hours of

FIGURE 3.12. *Restimulated lymphocytes extracted from the pancreas of diabetic NOD mice show IL-22 production compared to Isotype and FMO controls.* Pancreata were extracted from diabetic NOD mice and infiltrating lymphocytes were isolated by incubating minced pancreatic fragments in 10% RPMI media containing Collagenase Type V for 30 mins to release pancreatic islets. Cells were stimulated in 24-well plates that had been coated overnight with 1 μg/ml α-CD3. Anti-CD28 (1 μg/ml) was cultured with these cells for 72 hours, with addition of PMA and Ionomycin on the last day. Cells were then collected and stained for FACS analysis including Isotype and Fluorescence Minus One (FMO) controls; 50 000 events were collected and gated on live cells (A), then $CD4^+$ cells (B, C). Plots show two different mice from different experiments and are representative of multiple experiments.

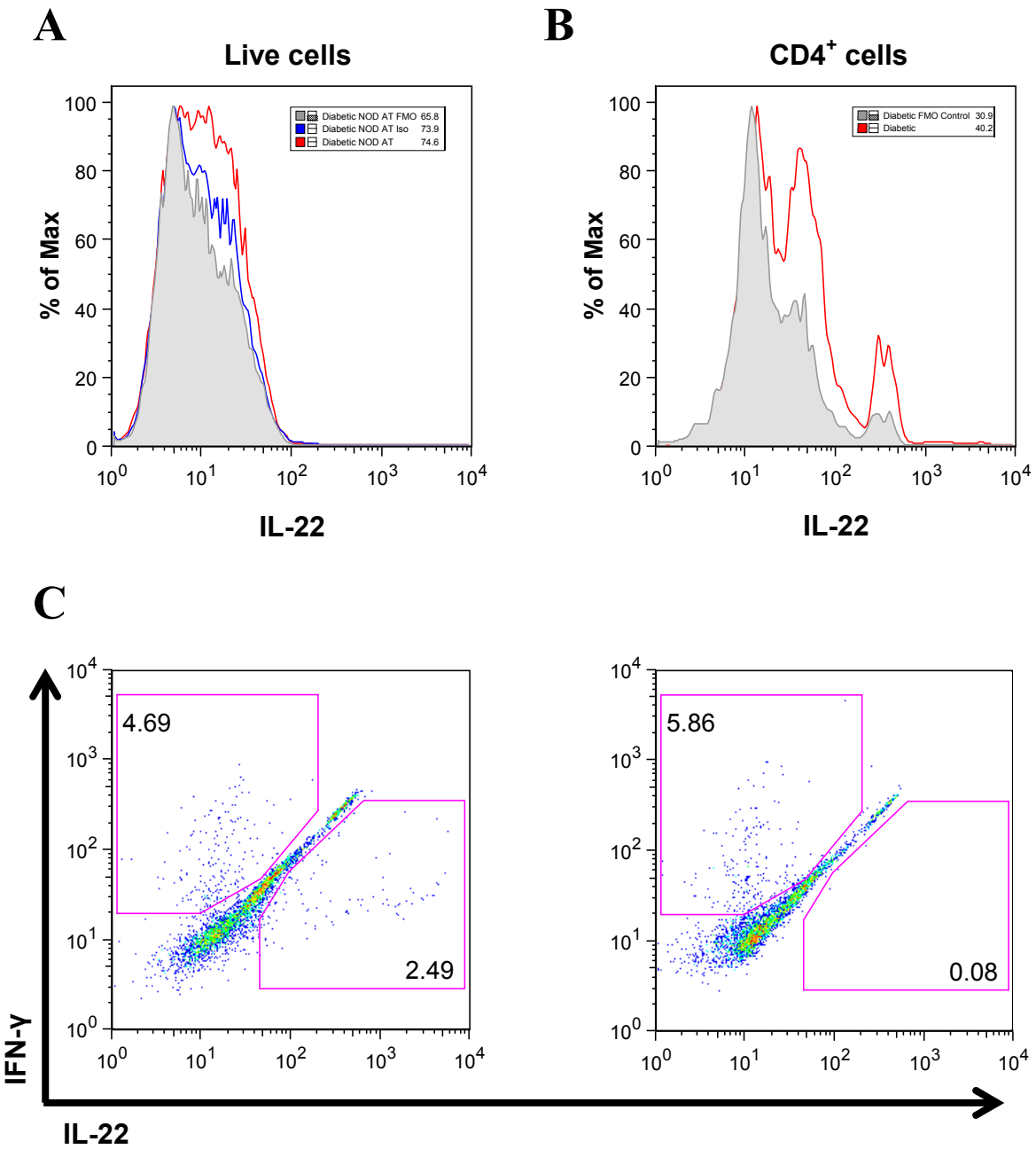
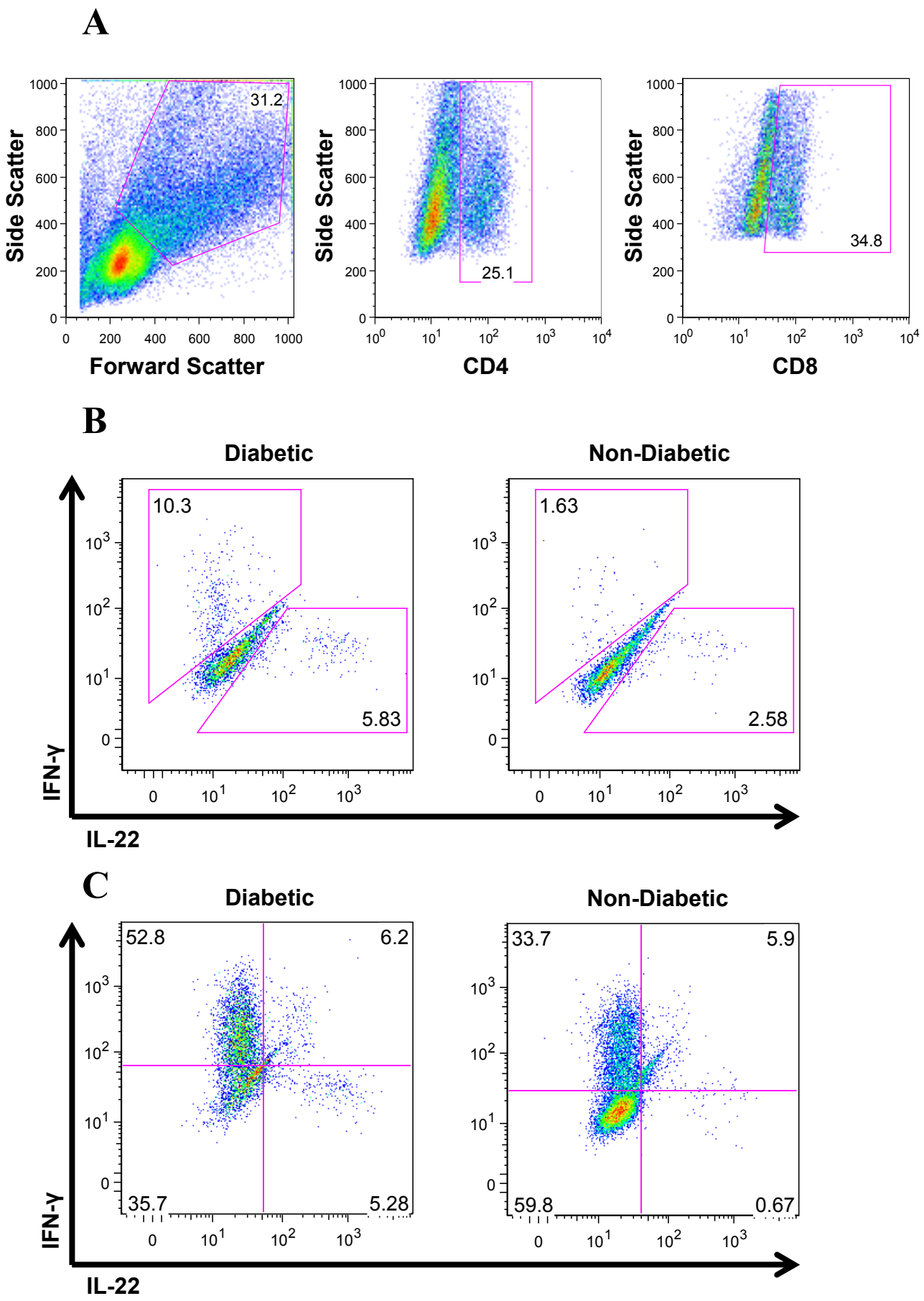

A
Live cells
Diabetic NOD AT FMO 65.8
Diabetic NOD AT Iso 73.9
Diabetic NOD AT 74.6
% of Max
IL-22
B
CD4+ cells
Diabetic FMO Control 30.9
Diabetic 40.2
% of Max
IL-22
C
4.69
2.49
5.86
0.08
IFN-γ
IL-22

FIGURE 3.13. *Restimulated lymphocytes extracted from the pancreas of diabetic NOD mice show more IL-22 production than non-diabetic mice of the same age.* Pancreata were extracted from diabetic NOD mice and infiltrating lymphocytes were isolated by incubating minced pancreatic fragments in 10% RPMI media containing Collagenase Type V for 30 mins to release pancreatic islets. Cells were stimulated in 24-well plates that had been coated overnight with 1 μg/ml α-CD3. Anti-CD28 (1 μg/ml) was cultured with these cells for 72 hours, with addition of PMA and Ionomycin on the last day. Cells were then collected and stained for FACS analysis; Using the gating strategy depicted in (A) 70 000 events were collected and gated on live cells then $CD4^+$ (B) or $CD8^+$ cells (C). Plots are representative of multiple experiments.


A
Side Scatter
Forward Scatter
31.2
CD4
25.1
CD8
34.8
B
Diabetic
Non-Diabetic
10.3
5.83
1.63
2.58
IFN-γ
IL-22
C
Diabetic
Non-Diabetic
52.8
6.2
35.7
5.28
33.7
5.9
59.8
0.67
IFN-γ
IL-22

stimulation, cells were collected and stained for FACS analysis. When gating generally on live cells there appears to be more IL-22-producing cells in the diabetic and 13 week-old pre-diabetic NOD mice compared with the 5 week NOD mouse (**Fig. 3.14A**). After gating specifically on $CD4^+$ cells, this difference is diminished (**Fig. 3.14B**). To determine whether there is a pattern of IL-22 expression among all mice tested, we analyzed the percent positive for IL-22 from each mouse separately and compared to each other group (**Fig. 3.14C**). No significant differences were seen in IL-22 percent positive cells between the three groups of mice, diabetic, pre-diabetic and young mice.

PLNs from these mice were also extracted and cells were restimulated with α-CD3 and α-CD28 for 48-72 hrs. These cells were collected and RNA extracted for quantitative analysis using RT-qPCR. Th17 cytokines IL-22 and IL-17 were significantly higher in the lymph nodes of diabetic mice compared to young controls (**Fig. 3.15**, $p < 0.05$, 0.01 respectively). Other cytokines indicate a strong T cell presence in the diabetic mice as both IL-10 and IFN-γ were increased with disease ($p < 0.05$).

Small pieces of the pancreas from diabetic and young NOD mice as well as B6 control mice were homogenized and RNA extracted for cDNA analysis using RT-qPCR. The diabetic NOD pancreas exhibited significantly higher IL-10 and IFN-γ levels compared to both 4 wk NOD and B6 control mice (**Fig. 3.16A**). Interestingly, the unique IL-22Rα subunit was found to be expressed in significantly higher amounts in the diabetic NOD pancreas compared to young and healthy controls (**Fig. 3.16A**). As this expression is shown at the transcription level, we sought to determine the presence of IL-22Rα in the pancreas at the protein level. Therefore, a western blot of protein extracted from pancreatic tissue was performed and confirmed the protein is expressed in the pancreas of NOD mice at all ages (**Fig. 3.16B**). The pancreas of each group was also assessed for the expression of a number of other factors. The IL-17 receptor, IL-17Rα, was expressed similarly in all groups as well as IL-23. Unexpectedly, transcription factors RORγt and AhR were not expressed differently in the pancreas of diabetic NOD mice compared to controls (**Fig. 3.16A**).

FIGURE 3.14. *IL-22 production in restimulated lymphocytes extracted from the pancreas of diabetic NOD mice and control young, non-diabetic mice.* Pancreata were extracted from diabetic NOD mice and infiltrating lymphocytes were isolated by incubating minced pancreatic fragments in 10% RPMI media containing Collagenase Type V for 30 mins to release pancreatic islets. Cells were then stimulated in 24-well plates that had been coated overnight with 1 μg/ml α-CD3. Anti-CD28 (1 μg/ml) was cultured with these cells for 72 hours, with addition PMA and Ionomycin for 5 hours on the last day (A). Cells were then collected and stained for FACS analysis; 50 000 events were collected and gated on live cells (A), then $CD4^+$ cells (B). Plots are representative one replicate of multiple experiments. (C) Infiltrating lymphocytes from multiple FACS replicates were plotted from 3 groups measuring the IL-22-percent positive $CD4^+$ cells observed from different experiments. The ANOVA test was used to analyze values with $p < 0.05$ termed as significant.

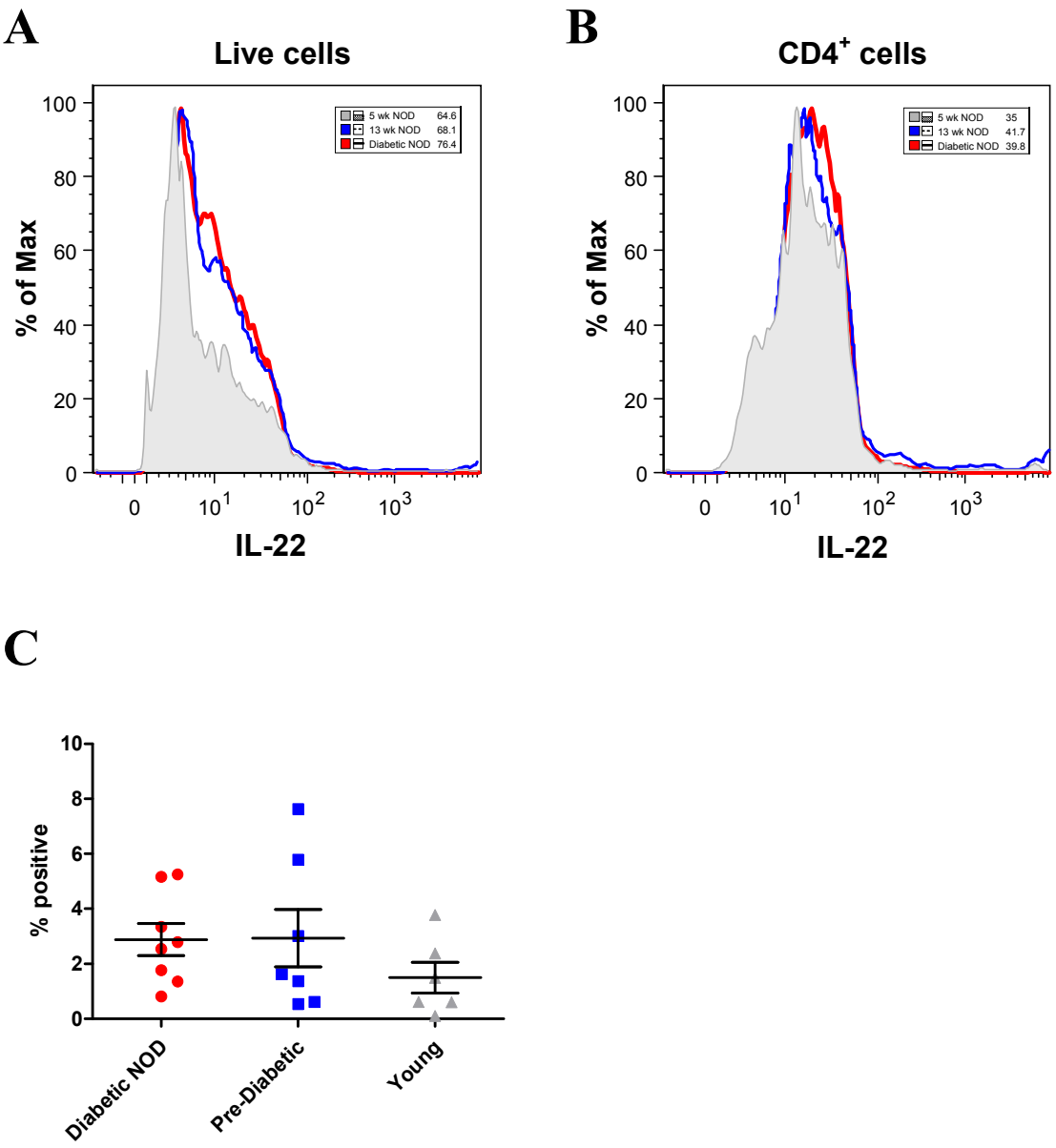

A
Live cells
5 wk NOD 64.6
13 wk NOD 68.1
Diabetic NOD 76.4
% of Max
IL-22
B
CD4+ cells
5 wk NOD 35
13 wk NOD 41.7
Diabetic NOD 39.8
% of Max
IL-22
C
% positive
Diabetic NOD
Pre-Diabetic
Young

FIGURE 3.15. *IL-22, IFN-γ, and IL-10 production in restimulated lymphocytes extracted from PLNs of diabetic NOD mice and control young, non-diabetic mice.* Restimulated PLNs were collected as in the previous figure after 72 hours and RNA was extracted and analyzed by RT-qPCR. Relative results using beta actin as a housekeeping gene are shown as mean expression ± SEM of multiple experiments, n=6 in both groups. The Student's t test was used to determine significance ($p < 0.05$).

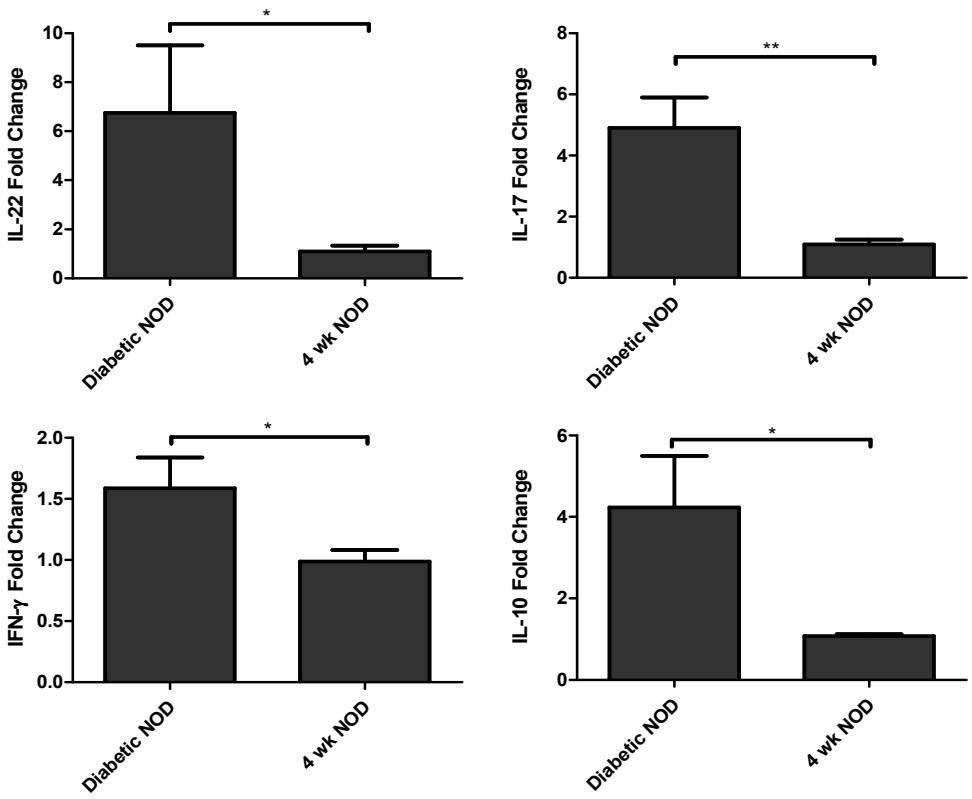
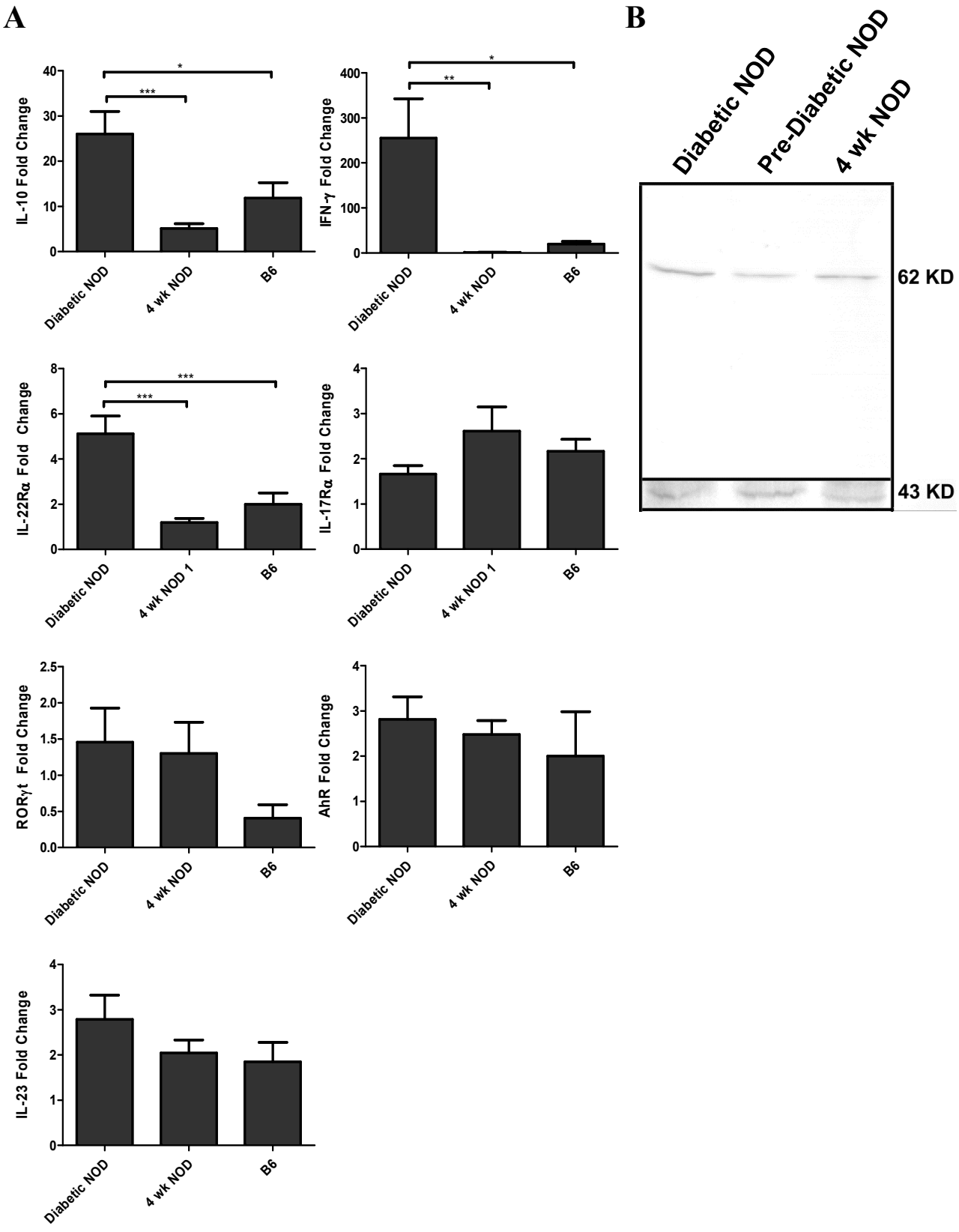

IL-22 Fold Change
10
8
6
4
2
0
*
Diabetic NOD
4 wk NOD
IL-17 Fold Change
8
6
4
2
0
**
Diabetic NOD
4 wk NOD
IFN-γ Fold Change
2.0
1.5
1.0
0.5
0.0
*
Diabetic NOD
4 wk NOD
IL-10 Fold Change
6
4
2
0
*
Diabetic NOD
4 wk NOD

FIGURE 3.16. *Whole pancreatic tissue shows an up-regulation of inflammatory cytokines and factors including IL-10, IFN-γ and IL-22R in diabetic NOD mice compared to young and healthy controls.* (A) RNA was extracted from the pancreas of diabetic NOD mice (n=6), 4-5wk NOD mice (n=5), and 10 wk B6 mice (n=3) and analyzed by RT-qPCR using gene specific primers. Relative results using beta actin as a housekeeping gene are shown as mean expression ± SEM. The ANOVA test was used to analyze all values with $p < 0.05$ termed as significant. (B) Pancreata from diabetic NOD mice, pre-diabetic mice (14 wks old) and 4 wk old NOD mice were excised and snap frozen in liquid nitrogen before homogenization in lysis buffer to collect total protein. Western blots were performed using a monoclonal antibody against IL-22Rα with Actin as a loading control.


A
B
IL-10 Fold Change
IFN-γ Fold Change
IL-22Rα Fold Change
IL-17Rα Fold Change
RORγt Fold Change
AhR Fold Change
IL-23 Fold Change
Diabetic NOD
4 wk NOD
4 wk NOD 1
B6
Pre-Diabetic NOD
62 KD
43 KD

3.5 Neutralizing IL-22 in a Th17 adoptive transfer model

To examine the effect IL-22 has on diabetes pathogenesis, an IL-22 neutralizing antibody was used in adoptive transfer models of NOD mice. BDC2.5 splenocytes were stimulated with PS3 mimotope in the presence of IL-6 + IL-23 for 4 days. On Day 0, 5 week old NOD mice were injected IV with 5 million polarized cells each. These mice were also injected with a neutralizing IL-22 antibody or sterile PBS on days -1, 2 and 6. Diabetes incidence was monitored using urine glucose test strips. The Kaplan-Meier survival estimate was used to determine differences in incidence, however no statistical significant difference was found (**Fig. 3.17**). There *may* be a trend as disease incidence was slightly less in mice given the neutralizing Ab, and in some cases somewhat delayed.

3.5.1 IL-22-producing cells can be recovered from recipient mice after adoptive transfer

To examine IL-22 production by lymphocytes in diabetic recipient mice after adoptive transfer of polarized Th17 cells, tissues such as the pancreas, spleen and PLNs were extracted and lymphocytes isolated. After stimulation by α-CD3 and α-CD28, these lymphocytes were characterized by flow cytometry. IL-22 production was exhibited by lymphocytes in both the spleen and pancreas compared to isotype and FMO controls (**Fig. 3.18A**). IL-22 production by $CD4^+$ cells can be compared between lymphocytes extracted from the pancreas of mice who received IL-22 neutralizing antibody and those that were injected with PBS. Little difference was observed in expression by these lymphocytes, which was expected as the neutralizing antibody works systemically *in vivo* and has no effect on future production of IL-22 (**Fig. 3.18B**). Both IL-22 and IFN-γ production can also be seen in $CD4^+$ cells isolated from the PLNs and spleens of diabetic recipient mice.

FIGURE 3.17. *Neutralizing IL-22 in a Th17 adoptive transfer model.* Splenocytes from BDC2.5 mice were cultured with 5 μg/ml PS3, 20 ng/ml IL-6, and 10 ng/ml IL-23 for 4 days. Cells were collected and $5x10^6$ cells were intravenously injected into female four week old NOD mice in each group. On Days -1, 2 and 6, mice were also injected intraperitoneally with 300 μg of either IL-22 neutralizing Ab or PBS. Mice were checked every 3 days for diabetes onset. The Kaplan-Meier survival estimate was used to determine differences in the two curves, however no statistical significant difference was found.

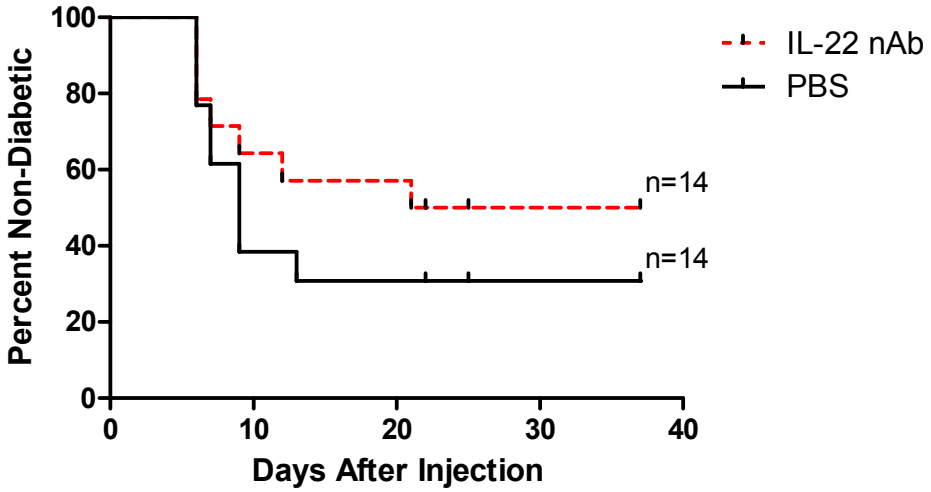
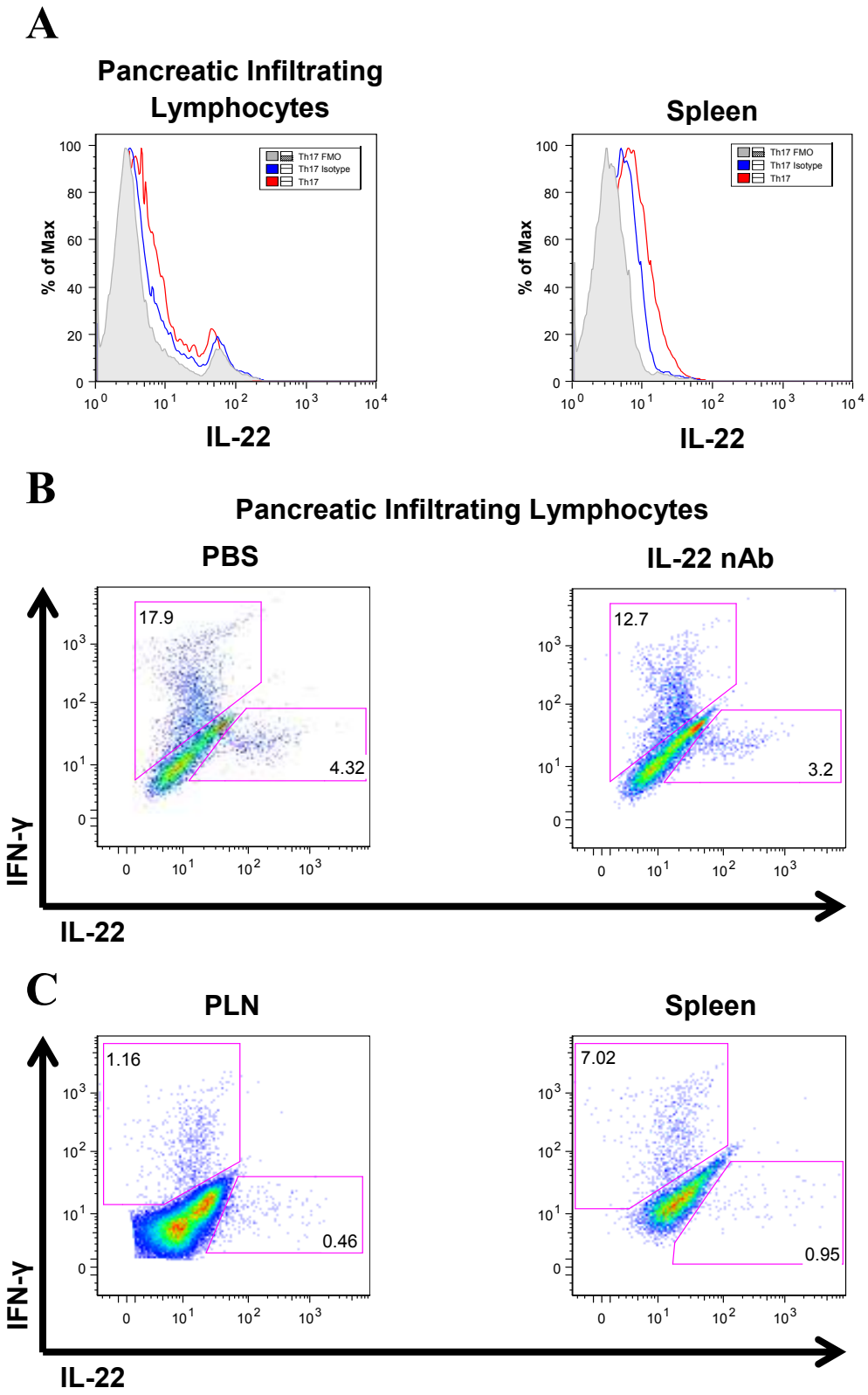

100
80
60
40
20
0
Percent Non-Diabetic
0
10
20
30
40
Days After Injection
IL-22 nAb
PBS
n=14
n=14

FIGURE 3.18. *IL-22 producing cells can be recovered from recipient young NOD diabetic mice.* Pancreata were extracted from diabetic NOD mice and infiltrating lymphocytes were isolated by incubating minced pancreatic fragments in 10% RPMI media containing Collagenase Type V for 30 mins to release pancreatic islets. Spleens and PLNs were also collected from these mice and all tissues were stimulated in 24-well plates that had been coated overnight with 1 μg/ml α-CD3. Anti-CD28 (1 μg/ml) was cultured with these cells for 72 hours, with the addition of PMA and Ionomycin on the last day. Cells were then collected and stained for FACS analysis; 100 000 events were collected and all plots were gated on live cells then $CD4^+$ cells. (A) Infiltrating lymphocytes into the pancreas and spleen in recipient mice show some IL-22 production when compared to Isotype and FMO controls. (B) Little difference is seen in IL-22 production between recipient mice who also receive IL-22 neutralizing Ab and those that receive PBS. However, IL-22- and IFN-γ-producing cells can be recovered in both groups. (C) IL-22- and IFN-γ-producing cells are also recovered from spleens and PLN of recipient mice. Plots are representative of one or two replicates of multiple experiments.


A
Pancreatic Infiltrating Lymphocytes
Spleen
Th17 FMO
Th17 Isotype
Th17
% of Max
IL-22
B
Pancreatic Infiltrating Lymphocytes
PBS
IL-22 nAb
17.9
4.32
12.7
3.2
IFN-γ
IL-22
C
PLN
Spleen
1.16
0.46
7.02
0.95
IFN-γ
IL-22

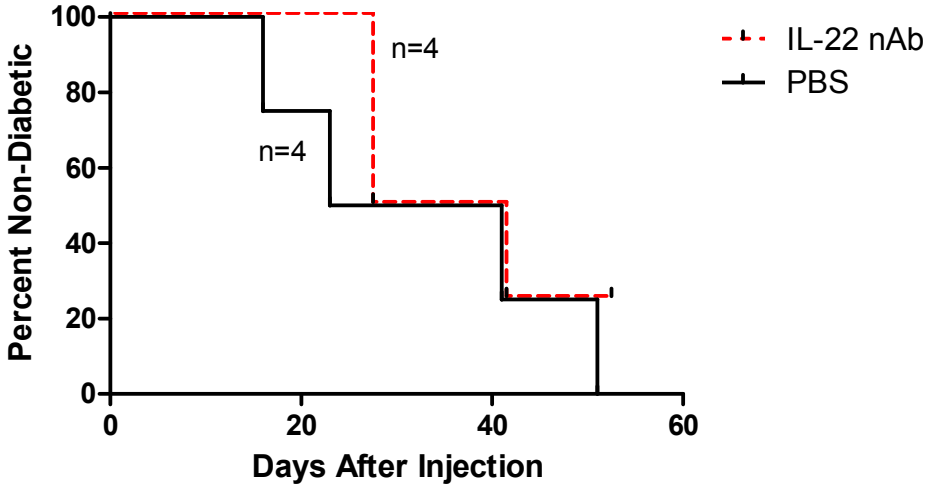
3.6 Neutralizing IL-22 in an adoptive transfer model into NOD.SCID mice

Traditionally, adoptive transfers to cause autoimmune diabetes occur with NOD.SCID mice as recipients to rule out the host's immune system affecting observed results (224–226). Throughout this study we have used NOD mice as injection of polarized Th17 cells into NOD.SCID mice has shown to revert cells to a different phenotype (219). To discover whether IL-22 may play a role in this more traditional adoptive transfer model using NOD.SCID mice, splenocytes from two diabetic NOD mice were pooled and 10 million cells injected IV into 8 wk old recipient NOD.SCID mice. These mice were also injected with either IL-22 neutralizing antibody or sterile PBS on days 0, 3, 6 and 14 after transfer. Diabetes incidence was monitored using urine glucose test strips and the Kaplan-Meier survival estimate was used to determine differences in incidence. No statistical difference was found between IL-22 nAb and PBS groups (**Fig. 3.19A**). It seems at first that in the neutralizing antibody group that disease incidence may be delayed, however as injections were only given for the first two weeks, at 30 days after adoptive transfer both groups show similar incidence.

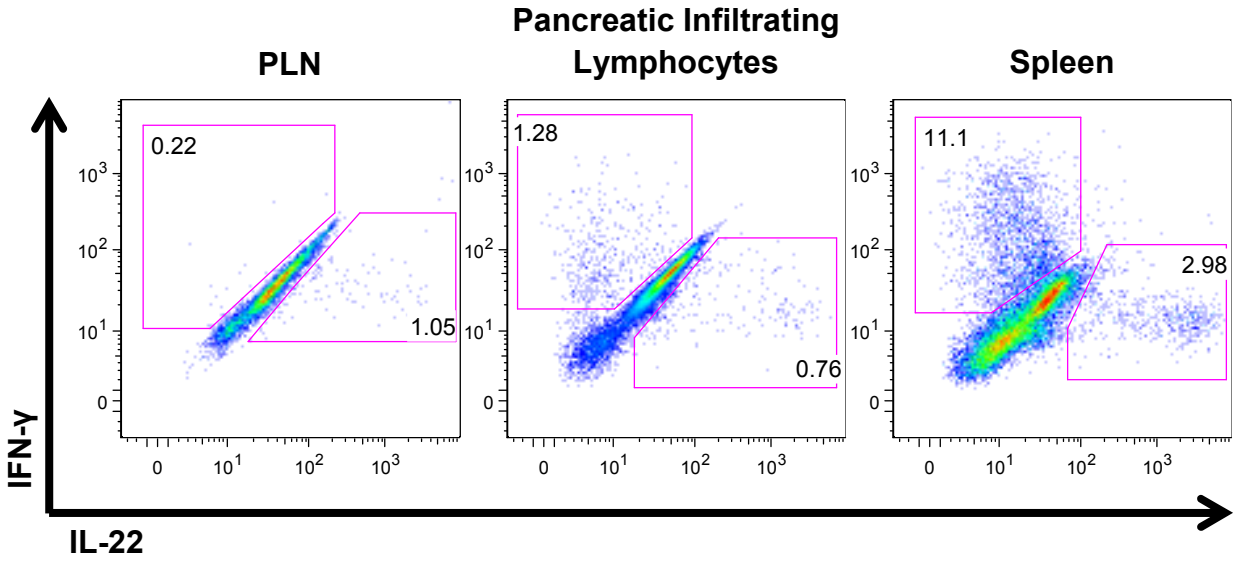
To determine whether IL-22-producing lymphocytes could be recovered from NOD.SCID mice after adoptive transfer, spleens, pancreata, and PLNs were extracted from recipient mice and lymphocytes isolated. After stimulation, IL-22- and IFN-γ-producing cells could be seen by flow cytometry in small amounts in the PLN and pancreas (**Fig. 3.19B**). In the spleen much larger amounts of IFN-γ is demonstrated along with a moderate amount of IL-22.

FIGURE 3.19. *Neutralizing IL-22 in an adoptive transfer of diabetic splenocytes into NOD.SCID mice.* Splenocytes from two 15-25 week old NOD mice were pooled and injected IV into 8 week old NOD.SCID mice at $10x10^6$ cells per mouse. On Days 0, 3, 6, and 14 mice were also injected intraperitoneally with 300 µg of either IL-22 neutralizing Ab or PBS. (A) Mice were checked every 3 days for diabetes onset. The Kaplan-Meier survival estimate was used to determine differences in the two curves, however no statistical significant difference was found. (B) Pancreata were extracted from these diabetic NOD.SCID mice and infiltrating lymphocytes were isolated by incubating minced pancreatic fragments in 10% RPMI media containing Collagenase Type V for 30 mins to release pancreatic islets. Spleens and PLNs were also collected from these mice and single-cell suspensions were made and stimulated in 24-well plates that had been coated overnight with 1 µg/ml α-CD3. Anti-CD28 (1 µg/ml) was cultured with these cells for 72 hours, with addition of PMA and Ionomycin on the last day. Cells were then collected and stained for FACS analysis; 100 000 events were collected and all plots were gated on live cells then $CD4^+$ cells.

A


Percent Non-Diabetic
100
80
60
40
20
0
0
20
40
60
Days After Injection
n=4
n=4
IL-22 nAb
PBS

B


PLN
Pancreatic Infiltrating Lymphocytes
Spleen
IFN-γ
IL-22
0.22
1.05
1.28
0.76
11.1
2.98

Chapter 4

4 DISCUSSION

The purpose of this study was to investigate the role IL-22 may play in the pathogenesis of type 1 diabetes in the NOD mouse model. Characterization of both pathogenic and regulatory subsets of Th17 cells led to the optimization of polarized T cells to maximally produce IL-22 in a transgenic mouse model. In particular, naïve cells were determined to show similar characteristics after Th17 polarization as splenocytes after stimulation. Our focus then shifted to explore the presence of IL-22 and its receptor in the NOD mouse pancreas to confirm that IL-22 *could* be used as a pathogenic or supportive factor in disease induction. Lastly, this study attempted to determine the capacity of IL-22 in type 1 diabetes pathogenesis by elucidating whether or not neutralizing this cytokine had any major effect on disease incidence.

4.1 Characterization of polarized naïve cells into Th17 subsets

Previous work in our lab and others (see appendix and (97, 182, 234–236)) has demonstrated the different phenotypes that can arise from differential induction of Th17 cells. These subsets have been termed Teff17 and Treg17 based upon their ability to induce disease. Induction techniques always include IL-6 and a variety of IL-1β, TGF-β1, IL-23 and even TGF-β3 (64, 71, 178, 237–239). In general, it is observed in all studies that IL-17 production increases; however moderate amounts of IL-23 in the presence of small or even trace amounts of TGF-β is needed for sufficient IL-22 production. In our laboratory, we were most interested in the production of IL-22 and therefore traditionally use only IL-6 + IL-23 to polarize Th17 cells, and allow for the source of TGF-β to come from the media. We have previously shown differential cytokine production from both IL-6 + IL-23 and IL-6 + TGF-β subsets in splenocytes (see Appendix) and wanted to confirm these results using isolated naïve T cells. By characterizing cytokine production in polarized naïve T cells, we can determine the exact effect of these cells on disease,

rather than with splenocytes where other contaminating cells may have an effect. We found that purified naïve T cells from BDC2.5 transgenic mice react similarly to whole splenocytes in that IL-17 production is increased with polarization and that moderate amounts of IL-23 is necessary for IL-22 production. We also observed that TGF-β efficiently inhibits IL-22 and though it is necessary for Th17 induction, the small amount found in the media is optimal and sufficient for production of IL-22. This was first described in human naïve T cells where Th17 cells could be induced without the addition of TGF-β, using only IL-1β and IL-23 (237). Using serum-free media, it was subsequently discovered that without TGF-β, naïve $CD4^+$ T cells do not initially differentiate into Th17 cells, and that in the previous experiments the necessary TGF-β came from serum-containing media (238–240). Our method of excluding TGF-β from our induction cocktail leads to less IL-17 production as TGF-β, along with IL-23, can support the induction of IL-17 via RORγt activation (238). We also see *slightly* more IFN-γ produced by IL-6 + IL-23 polarized cells, even with the addition of the α-IFN-γ antibody, as TGF-β in the Treg17 induction cocktail represses T-bet (241). In agreement with these results, it has been observed by many researchers that a subset of Th17 cells can coproduce IFN-γ, particularly T cells isolated from sites of inflammation such as the brain after experimental autoimmune encephalomyelitis (EAE) induction or the intestine from patients with Crohn's disease (241). Altogether, the up-regulation of inflammatory cytokines IL-22, IL-17 and IFN-γ in IL-6 + IL-23 polarized Teff17 subsets demonstrates an increased disposition towards pathogenesis compared to the IL-6 + TGF-β-polarized Treg17 cells. Alternatively, IL-10 was increased in both the Treg17 subset as well as the non-polarized cells compared to the Teff17 subset, confirming a regulatory bias of Treg17 cells and an increased pathogenic tendency of Teff17 cells. This is due to the nature of the inducing cytokines as continued exposure of cells to TGF-β induces higher IL-10 production and FoxP3 expression, whereas IL-23, while not inhibiting IL-10, will not retain such IL-10 secretion (97).

To observe the temporal induction of Th17 cytokines, naïve cells were stimulated in polarizing conditions and transcription levels of effector cytokines were measured each day. Four days after stimulation, cells demonstrated the highest induction of both IL-17 and IL-22. It seems that by day 5, cells had fully differentiated to a Th17 phenotype, and

without further manipulation maintained a more constant activation level of effector cytokines. Across the field, there are many different methods for inducing Th17 cells with wide variances in the length of time needed to generate these cells. Some use IL-6 + TGF-β for 3 days and then add IL-23 for a remaining 3 or 4 days, while others restimulate memory or resting Th17 cells in the presence of IL-23 and additional cytokines. Future studies will be needed to determine any physiological differences that stem from varying lengths of polarization, however we have shown that Th17 differentiation can be accomplished and vast amounts of IL-22 can be induced after only 4 days if optimal Th17 cytokine production is the objective of the study. This is not to say that all other Th17-related factors are optimal on this day as they were not studied in this report.

To further characterize and optimize the differential role of inducing cytokines IL-23 and TGF-β, both were titrated with IL-6 and neutralizing antibodies in addition to stimulated, isolated naïve T cells. As expected, IL-22 production decreased with increasing TGF-β concentration as TGF-β is a potent inhibitor of IL-22 via the action of transcription factor c-Maf which interferes with IL-22-inducing transcription factors RORγt and BATF (127). Interestingly however, at a low dose of TGF-β (1 ng/ml), IL-22 transcription is higher than the IL-6 alone control group. This demonstrates the balance where TGF-β is needed for Th17 differentiation (and therefore, ultimately IL-22 production) yet at even moderate amounts (above 2 ng/ml) will dramatically inhibit IL-22. However, these differences are minimal with the addition of IL-23. c-Maf also mediates expression of IL-10 through transactivation at its promoter in Th17 cells (242, 243). As TGF-β induces c-Maf expression, IL-10 up-regulation with increased TGF-β is expected.

It has been postulated by some investigators that GM-CSF is a necessary factor for the pathogenesis of Th17 cells (244, 245). Here it is shown that TGF-β, while necessary for Th17 induction, leads to a decrease in GM-CSF compared to a control group polarized with IL-6 alone. GM-CSF expression was also supposedly driven by IL-23 and RORγt (56), however in this study we found that moderate levels of IL-23 also reduced GM-CSF expression compared to the IL-6 alone control. These seemingly

contradictory results can be resolved by the hypothesis that the critical source of GM-CSF in autoimmune diseases is not necessarily the pathogenic Th17 cells themselves during the effector phase of the disease, but the surrounding cells in the initiation phase. GM-CSF was found to be a critical mediator of IL-6 and IL-23 production by DCs and macrophages in the initiation phase of the autoimmune response and is necessary for $CD4^+$ T cell survival, particularly Th17 cells (246). Therefore, in studies of GM-CSF and autoimmune diseases using knockout or neutralizing antibody approaches, Th17 cells cannot differentiate properly and cannot contribute to disease. Furthermore, our data only shows that GM-CSF is down-regulated by addition of TGF-β or IL-23 to culture compared to IL-6 alone and therefore does not determine the absolute amount of GM-CSF produced by these cells. Sufficient amounts may be produced by polarized Th17 cells to induce further IL-6 and IL-23 expression and increase Th17 induction in nearby cells in an autoamplification loop.

Somewhat surprising results were observed in Th17 factors such as IL-17 and RORγt as well as IFN-γ with titration of TGF-β. It has been long understood that TGF-β is a "switch" cytokine with differing roles based on its context and concentration (247). It has also been shown that TGF-β induces RORγt, while at the same time inhibiting its ability to produce IL-17 until related inflammatory cytokines such as IL-1β, IL-6 and IL-21 relieve this inhibition (238). It is possible then that with high concentrations of TGF-β, inflammatory cytokines are unable to relieve this inhibition to produce IL-17 and cells are pushed to a more regulatory phenotype or Th1 phenotype that does not produce IL-17 or RORγt. Indeed, it has been shown that in naïve $CD4^+$ cells cultured in higher than 2.5 ng/ml TGF-β, IL-23R mRNA is decreased and the percentage of IL-17-positive cells is lower (248). The reason behind the initiation of IFN-γ production at these higher concentrations is not understood and may be in line with the hypothesis that excess TGF-β leads to inhibition of Th17 and induction of other subsets. Further TGF-β titration is needed to resolve these conflicting results. Lastly, observing the effect of IL-23 titration confirms that IL-23 has no or little effect on RORγt and IL-17 and supports the notion that IL-23 is majorly involved in Th17 maturation *after* the initial induction (59, 63, 66).

4.1. Maximizing IL-22 production by Th17 cells using neutralizing antibodies

It was previously shown by Lighvani and colleagues that IFN-γ can actually function in an autocrine loop where this cytokine can induce T-bet, a transcription factor responsible for its own production (28). To promote increased cell differentiation to a Th17 phenotype while at the same time inhibiting a Th1 effector cytokine, we used α-IFN-γ in culture with polarizing cytokines and antigen stimulation. Splenocytes from BDC2.5 mice include mostly $CD4^+$ T cells, few $CD8^+$ T cells and DCs and macrophages. By using these splenocytes, we can employ the mimotope antigen PS3 to fully stimulate cells as APCs can efficiently activate naïve T cells. This is ideal as we were aiming to observe the full potential of polarized cells and highlight any differences in cytokine production. BDC2.5 splenocytes were therefore cultured with PS3 with and without α-IFN-γ to determine the effects of neutralizing IFN-γ in the absence of polarization. Noticeable increases in IL-22 and IL-17 were seen, while IFN-γ was partially suppressed. Upon polarization, these effects were magnified as neutralizing IFN-γ induced almost double the amount of IL-22 production compared to using polarizing cytokines IL-6 + IL-23 alone. It is indeed surprising to discover that a population of cells already producing an abundance of IL-22 can further drastically increase this production by neutralization of one opposing factor. An increase in IL-17 was observed as well by flow cytometry signifying an increase in $IL\text{-}22^+IL\text{-}17^+$ double positive cells and indicating that the optimal method for inducing Th17 cells seems to include polarization as well as inhibition of competing differentiation pathways.

We then wanted to determine whether the differential cytokine production by polarized cells with α-IFN-γ had an effect on disease induction. These cells were then transferred into young NOD mice. However, no difference in disease incidence was observed. Though analysis of supernatants confirms a large difference in IL-22 production, with 5 million cells injected into each mouse and the resulting rapid disease induction, they may nevertheless produce excessively high cytokines to mask functional differences. Perhaps the transfer of fewer cells may better highlight the differences in effector cytokine production.

4.1.1. Neutralizing TGF-β does not increase IL-22 production by Th17 cells

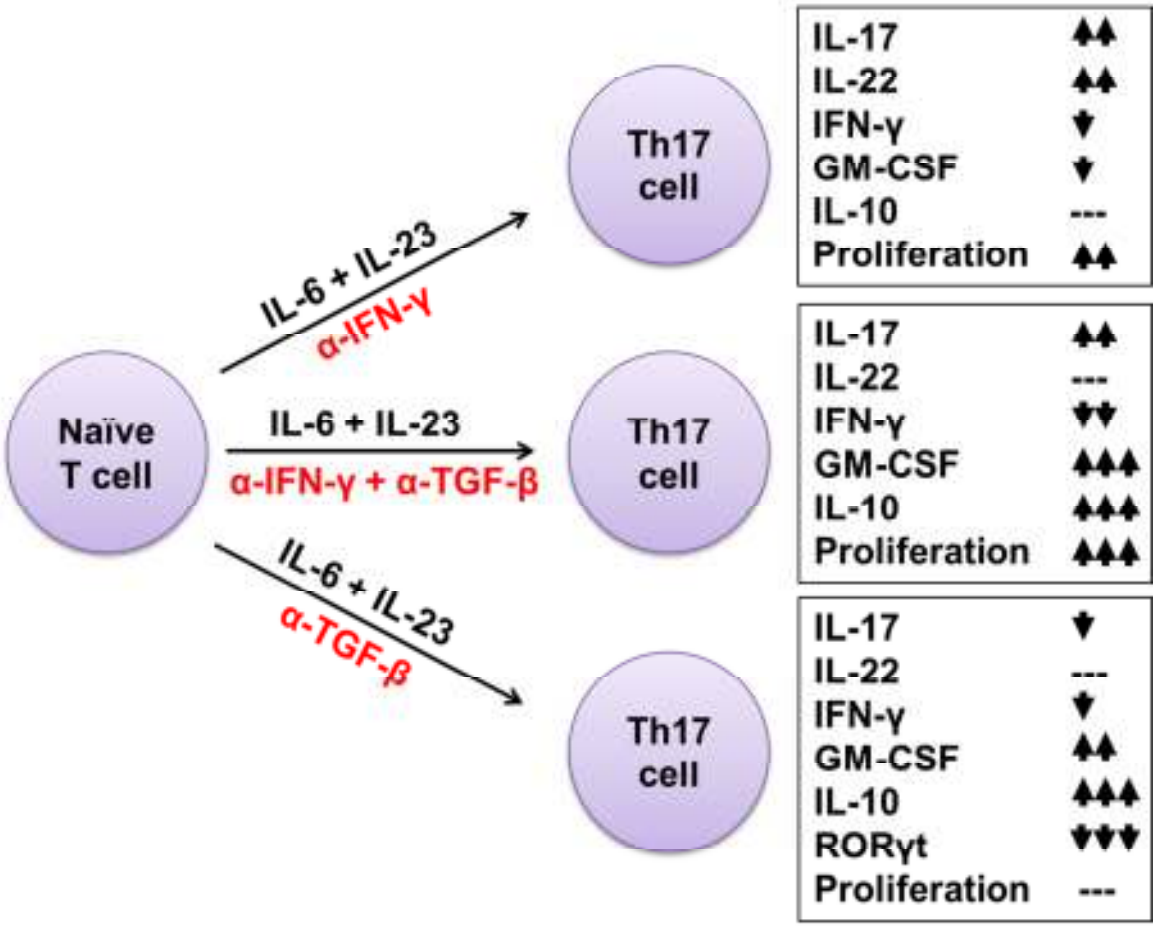
As we had success in establishing the increased production of IL-22 in polarized cells using α-IFN-γ, we sought to test the effects of neutralizing TGF-β, which is a potent suppressor of IL-22. Using a combination of polarized cells with and without α-TGF-β and α-IFN-γ we were able to determine that neutralizing TGF-β did not induce higher IL-22 production beyond the levels observed with anti-IFN-γ alone. These results further supported the finding that α-IFN-γ increases IL-22 and IL-17 production, yet when coupled with α-TGF-β this effect was diminished. Surprisingly, this experiment also shows that neutralizing TGF-β can increase IL-10, which contradicts what was found in the previous experiment—that is increasing TGF-β concentration also increases IL-10. To ensure the validity of these results and to rule out *in vitro* manipulation or cytokine production of innate cells, naïve cells were used in a titration of IL-23 with and without α-TGF-β, while also comparing to cells polarized with TGF-β. Again, it was found that IL-22 production was not different between cells cultured with and without anti-TGF-β. The antibody was at high enough concentrations to decrease IL-17 and RORγt expression, which reinforces the role TGF-β plays in inducing RORγt, which in turn mediates IL-17 production. These results support the findings of a previous study by Rutz et al. where IL-22 production was unchanged, while IL-17 decreased after neutralizing TGF-β Ab was administered at double the concentration used in this investigation (127). Also in agreement with the previous experiment, IL-10 production was increased after TGF-β neutralization, and was expressed in similar amounts as the cells with *added* TGF-β to the culture. These results are in contradiction to data observed by Rutz et al. where neutralizing TGF-β induced lower IL-10 production than the addition of 1 ng/ml TGF-β (127). There is a 5-fold difference in the amount of TGF-β added to culture which could account for this discrepancy as the previous experiment has shown that an increased concentration of TGF-β may result in the down-regulation of Th17 phenotypical cytokines and induce an alternate subset. As for the increase in IL-10 after TGF-β neutralization compared to the same culture without neutralizing antibody, perhaps in the absence of TGF-β, other subsets are polarized and IL-10 is up-regulated by another mechanism.

Altogether, these results have clearly shown that neutralizing IFN-γ leads to an up-regulation of Th17 cells producing IL-22 and IL-17. Conversely, neutralizing TGF-β leads to decreased Th17 differentiation and therefore a decrease in RORγt and IL-17. IL-22 production remains relatively unchanged. We have also shown that for optimal Th17 and IL-22 production, trace amounts—such as that present in serum-supplemented media—are ideal. Some reports show as much as 1.5 ng/ml TGF-β already present in serum-supplemented media, and therefore additional TGF-β may not be needed (249). These data reinforce the role of TGF-β in Th17 differentiation. The differential expression of cytokines and transcription factors by polarized Th17 cells in response to neutralizing antibodies *in vitro* is summarized in **Fig. 4.1**.

4.2. Th17 subsets do not differentially express Granzyme B and Perforin

We attempted to understand functional differences in the polarized Th17 subsets by looking at disparate expression of cytotoxic effector molecules granzyme B and perforin. Most $CD4^+$ T cells from BDC2.5 splenocytes are positive for these enzymes, and as a whole there are very few differences between expression of either granzyme B or perforin between the differently polarized subsets. When looking at cells that also produce IL-22 we see that there are more IL-22^+Granzyme B^+ and IL-22^+Perforin$^+$ in the IL-6 + IL-23 polarized group, however this is to be expected as these cells are predisposed to higher IL-22 production. There is ample evidence that demonstrates that islet beta cells are destroyed by these enzymes produced by cytotoxic $CD8^+$ T cells, and that a deficiency in perforin in the NOD mouse model results in a drastic reduction of disease (232, 233). $CD4^+$ T cells also mediate diabetes either by activating and recruiting cytolytic macrophages (208), activating and recruiting cytotoxic T lymphocytes (CTLs) (209), or apoptosis by FasL coupled with priming by inflammatory cytokines (210). It seems that though Th17 cells may express granzyme B and perforin, there is no difference in expression between pathogenic and regulatory subsets, and therefore either these enzymes do not play a role in $CD4^+$ T cell pathogenesis, or other factors influence

FIGURE 4.1. *The overall effects of neutralizing antibodies on cytokine production in polarized Th17 cells*. Naïve $CD4^+$ T cells were polarized in the presence of IL-6 + IL-23 with and without combinations of neutralizing antibodies α-TGF-β and α-IFN-γ. The effects relative to IL-6 + IL-23 polarized cells without neutralizing antibodies are summarized in this figure. One arrow up/down = increase/decrease, two arrows up/down = 2-fold increase/decrease, three arrows up/down = 3-fold increase/decrease, --- means no change.


Naïve
T cell
IL-6 + IL-23
α-IFN-γ
Th17
cell
IL-17 ▲▲
IL-22 ▲▲
IFN-γ ▼
GM-CSF ▼
IL-10 ---
Proliferation ▲▲
IL-6 + IL-23
α-IFN-γ + α-TGF-β
Th17
cell
IL-17 ▲▲
IL-22 ---
IFN-γ ▼▼
GM-CSF ▲▲▲
IL-10 ▲▲▲
Proliferation ▲▲▲
IL-6 + IL-23
α-TGF-β
Th17
cell
IL-17 ▼
IL-22 ---
IFN-γ ▼
GM-CSF ▲▲
IL-10 ▲▲▲
RORγt ▼▼▼
Proliferation ---

the ability of these cells to destroy beta cells. Further study is needed to conclude the relevance of granzyme B and perforin expression by Th17 cells.

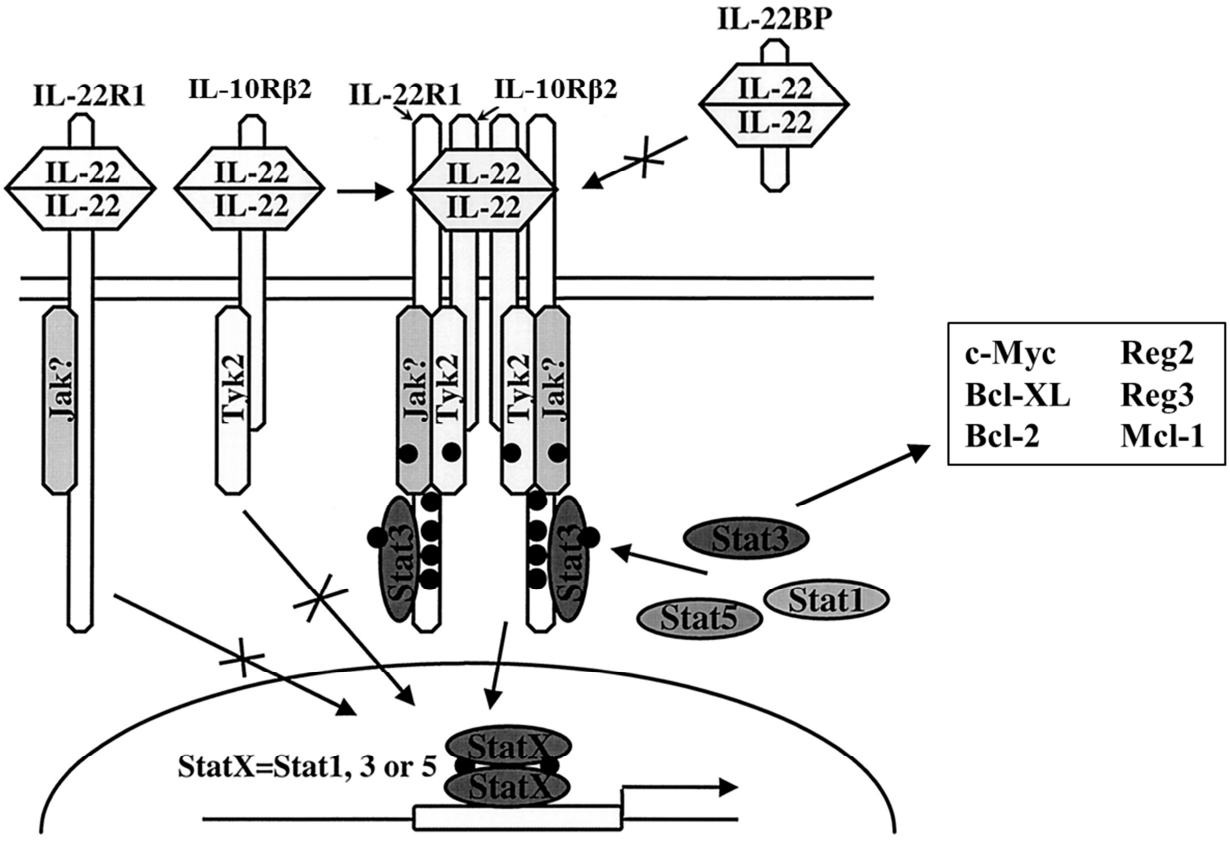
There are a number of other ways IL-22 can have an effect on T1D. As was mentioned above, different studies have shown cytotoxic effects of proinflammatory cytokines on pancreatic beta cells *in vitro* (210, 250, 251). These studies target cytokines such as IL-1α, IL-1β, TNF-α and IFN-γ and show that these cytokines can prime beta cells for Fas-dependent lysis by autoreactive T cells (210). There is currently no evidence suggesting IL-22 specifically up-regulates Fas receptor, however this does not rule out the possibility that IL-22 may affect beta cells by another mechanism, targeting them for destruction.

To better understand downstream processes initiated by IL-22, we looked at the result of receptor signalling. Binding of IL-22 to its receptor activates the STAT3 transcription factor to trigger downstream factors (106) as shown in **Fig. 4.2**. STAT3 mediates transcription of a number of genes such as c-Myc, SOCS3, Mcl-1 and apoptosis-regulators and survival factors Bcl-X_L and Bcl-2 (252–254). It is possible that in diabetes-prone individuals, a polymorphism in IL-22R expressed by beta cells may be dysfunctional leading to improper regulation of the survival/anti-apoptosis pathways. This could also be true for the soluble IL-22 binding protein, IL-22Rα2, which is an *in vivo* antagonist of IL-22 (121, 122). Studies have shown that IL-22Rα2 is implicated in macrophage function (117), and that multiple single nucleotide polymorphisms in the IL-22Rα2 gene are associated with an increased risk of both human multiple sclerosis (MS) and the EAE mouse model (255). Defects in the IL-22 antagonist may lead to dysregulation of IL-22 under disease conditions.

Lastly, IL-22 could play a detrimental role by hindering the regulatory effects of IL-10. IL-10 has been shown to play a protective role in an islet beta cell line by using IL-10 gene transfer vectors (256). This study found that the overexpression of IL-10 was able to block the inhibitory effects of IL-1β on insulin secretion, deter IL-1β production of toxic nitric oxide, prevent the activation of Fas-dependent apoptosis, and inhibit the activity of caspase-3. As IL-10 and IL-22 share a common subunit in their receptors, high

FIGURE 4.2. *The downstream effects of IL-22R signalling*. This pathway illustrates the mechanism of binding of IL-22 to the IL-22Rα1 and IL-10Rβ2 subunits to initiate receptor signalling and activation of STAT3. The transcription factor STAT3 can then mediate expression of downstream activation, proliferation, and survival factors.

Adapted from Kotenko et al., 2001 (104)


IL-22BP
IL-22R1
IL-10Rβ2
IL-22R1
IL-10Rβ2
IL-22
IL-22
IL-22
IL-22
IL-22
IL-22
Jak?
Tyk2
Jak?
Tyk2
Tyk2
Jak?
Stat3
Stat3
Stat3
Stat5
Stat1
c-Myc
Bcl-XL
Bcl-2
Reg2
Reg3
Mcl-1
StatX=Stat1, 3 or 5
StatX
StatX

amounts of IL-22 may compete for binding to the IL-10Rβ2 subunit, and therefore IL-10 may not be able to exert its regulatory functions. At this time, these mechanisms are purely speculative and further study may indicate the type of role IL-22 may play in the pancreas.

Lastly, IL-22 could play a detrimental role by hindering the regulatory effects of IL-10. IL-10 has been shown to play a protective role in an islet beta cell line by using IL-10 gene transfer vectors (256). This study found that the overexpression of IL-10 was able to block the inhibitory effects of IL-1β on insulin secretion, deter IL-1β production of toxic nitric oxide, prevent the activation of Fas-dependent apoptosis, and inhibit the activity of caspase-3. As IL-10 and IL-22 share a common subunit in their receptors, high amounts of IL-22 may compete for binding to the IL-10Rβ2 subunit, and therefore IL-10 may not be able to exert its regulatory functions. At this time, these mechanisms are purely speculative and further study may indicate the type of role IL-22 may play in the pancreas.

4.3. IL-22-producing cells in diabetic NOD mice

It has previously been shown that cytokine production may play a role in $CD4^+$ T cell-mediated induction of T1D (210). Common culprits that have been investigated are IFN-γ and IL-17, the characteristic cytokines produced by Th1 and Th17 cells. Knocking out either cytokine has not fully abrogated disease in the NOD mouse model (212, 220), and therefore the roles of other cytokines have been considered. In this study we aimed to determine the role IL-22 may play in T1D pathogenesis by first examining the presence of IL-22-producing cells infiltrating the pancreas and draining lymph nodes. To directly observe the phenotypes of these cells, we isolated infiltrating lymphocytes from the pancreas of diabetic NOD mice using collagenase and activated them for 5 hours with PMA and ionomycin before staining for FACS analysis. These cells were determined to produce both IFN-γ and IL-22, albeit in low amounts. As a similar percentage of IL-22-producing cells was seen in both live and $CD4^+$ populations, there may be cell types other than T cells expressing IL-22 such as lymphoid tissue inducer cells (LTi) or group 3

innate lymphoid cells (ILCs). Interestingly, even among the $CD4^+$ population, the number of IL-22-producing cells is only around half that of IFN-γ producing cells, with both being relatively low in frequency. Whether the extent of infiltrating cells producing either cytokine is biologically significant to influence disease remains to be seen. These results are supported by a study by Flavell and colleagues, which demonstrates the concomitant presence of IFN-γ-, IL-22-, and IL-17-producing cells in infiltrating lymphocytes isolated from the pancreas of BDC2.5 NOD transgenic mice (257). Their investigation exhibited higher percentages of both IFN-γ and IL-17 positive cells (approximately 15%, and 6%, respectively), however their method of lymphocyte isolation involved the usage of a percoll gradient to specifically identify lymphocytes, rather than other infiltrating cells. Our method of using collagenase to release pancreatic islets and then passing tissues through a screen does not completely rule out contaminating innate cells or connective tissue cells. By using a percoll gradient, the specific lymphocyte population could be purely isolated and therefore have higher percentages of cytokine-producing cells.

To confirm this presence of IL-22 in pancreatic infiltrating cells of NOD mice, IL-22 expression was compared to isotype and fluorescence minus one (FMO) controls. As IL-22 is a cytokine that is not usually expressed in high amounts by cells *in vitro*, it is difficult to differentiate IL-22 staining from non-specific interactions. IL-22 does not stain very brightly, however there seems to be more cells producing IL-22 than those that stain positive for the isotype and FMO controls suggesting a genuine presence of these cells *in vivo*.

Next, we looked at IL-22 production by restimulated infiltrating lymphocytes between diabetic and non-diabetic mice of the same age to determine whether the presence of IL-22 was disease-specific. By restimulating lymphocytes we aimed to achieve near physiological cell phenotypes at the time of disease induction, and also make it more likely that we are looking at T cells, as stimulation may distinguish them from non-stimulated innate or naïve T cells. A higher amount of both IFN-γ- and IL-22-producing $CD4^+$ cells were demonstrated in the diabetic mice compared to the control. A similar observation was made with $CD8^+$ cells. The diabetic mice certainly have more infiltration and therefore more $CD4^+$ and $CD8^+$ T cells present in the pancreas, however

when looking at the characteristics of these cells as a whole, more cells produce inflammatory cytokines IL-22 and IFN-γ than in the non-diabetic mice. Another factor to consider is that the non-diabetic mice are the same age as the diabetic, just not considered diseased according to glucose output levels. That is to say these mice are generally considered pre-diabetic and may have developed disease at any time if they had not been euthanized. Therefore, comparison between mice of the same age may not be fully indicative of disease progression.

4.3.1. IL-22 and IL-22R expression in the pancreas of diabetic and young NOD mice

In addition to observing IL-22 production by pancreatic infiltrating cells in diabetic mice compared with non-diabetic controls, we also wished to determine if there was an age-dependent effect along with a disease effect. We therefore restimulated infiltrating lymphocytes from the pancreas of diabetic, pre-diabetic and young mice to compare using flow cytometry. We found a large difference in IL-22 production in the diabetic compared to the young mice when looking at the general population of cells, however this observation diminishes somewhat while gating specifically on $CD4^+$ T cells. Again, this may suggest the presence of alternate IL-22-producing cells in the pancreas. When comparing IL-22-production in different ages of mice over multiple experiments, it seems there may be a slight increase in cells producing IL-22 as mice age, however this is not statistically significant. Perhaps a higher number of mice studied will further distinguish any differences, as these *in vivo* studies were seen to show a wide range of percentages of IL-22-positive cells.

To further examine differences in cytokine expression between diabetic and young NOD mice, pancreatic lymph nodes were stimulated and quantitated using RT-qPCR. All of IL-22, IL-17 and IFN-γ were discovered to be increased in PLNs of diabetic mice due to increased infiltration of Th1 and Th17 cells in the pancreas. The increase in IL-10 compared to young mice may also be explained by an increase in infiltrating regulatory T cells (Tregs) to the site of inflammation, as in young mice there is little need for large amounts of Treg infiltration in the pancreas. The study by Flavell et al. demonstrate the presence of IL-17, IL-22 and IFN-γ-producing cells infiltrating the

pancreas of BDC2.5 NOD mice and also confirms a lower presence of IFN-γ-producing cells in PLNs compared to the spleen and pancreas (257).

It has been previously shown that the unique alpha subunit of the IL-22R was present in the highest amounts in the human pancreas (119). It was further demonstrated that IL-22R co-localized with insulin and glucagon indicating its presence on beta and alpha cells, respectively, in the pancreas (120). We therefore sought to characterize the IL-22 receptor in the NOD mouse model. In correlation with previous studies examining the receptor protein expression in Balb/c and C57BL6/J mice (258), the IL-22R protein was shown to be present in whole pancreatic samples in all ages of NOD mice, and so we further compared expression in diabetic mice to young controls. Diabetic mice demonstrated increased IL-22R expression compared to control young, healthy mice. This may be a result of higher numbers of infiltrating, IL-22-producing Th17 cells in the vicinity as it has been shown by colleagues in our lab that incubating pancreatic islets with the supernatant of Teff17 cells induces increased expression of IL-22 (147). The same effect was not seen, however on the IL-17R whose expression was similar in all groups. We again noticed an increase in IL-10 and IFN-γ in diabetic mice compared to young mice, but were unable to detect IL-22 and IL-17 consistently in all samples of whole pancreatic tissues (data not shown). However, our lab has previously shown an increase in IL-22 in whole pancreatic tissue of NOD mice as they aged and developed disease (147), and Flavell and colleagues were able to measure the presence of IL-22 in infiltrating lymphocytes extracted from the pancreas, though levels were not compared to 4-week-old controls (257).

4.4. Neutralizing IL-22 in a Th17 adoptive transfer model

Neutralizing antibodies have been used effectively *in vivo* with many models of autoimmune diseases to demonstrate the role a specific factor plays in disease progression. In the diabetes mouse model, anti-IL-17 antibodies were shown to delay incidence of disease when given during the effector phase, yet showed no effect on the

initiation phase (215). Anti-IFN-γ injection along with adoptive transfer of activated BDC2.5 cells decreases diabetes incidence and delays development of disease (259). The IL-22 neutralizing antibody used in this study was shown to drastically reduce psoriasiform skin lesions in a mouse model of psoriasis (173). We sought to determine the effects of IL-22 on diabetes by utilizing an IL-22 neutralizing antibody in the adoptive transfer model of BDC2.5 cells into NOD mice. Though survival differences were not considered statistically different, a trend may be seen where neutralizing IL-22 may *slightly* decrease incidence of disease. As mice were still able to develop disease after injections of neutralizing antibodies it is clear IL-22 may not be the main factor involved in disease. A recent study supports this notion by showing that knocking out IL-22 in NOD mice does not protect against disease (257). To gain a better picture with neutralizing antibody data and to better compare treatment and control groups, it is imperative that the control group have an incidence closer to 100%. A better understanding of the effects of neutralizing IL-22 may have been achieved with a more consistent control group. This is perhaps due to the use of a transgenic mouse model with variances in the ability of the TCR to become stimulated by a mimotope antigen as a difference in proliferation was observed with the same antigen across many different experiments. Therefore, future work may better illustrate the role IL-22 plays in this model of disease.

When lymphocytes are isolated from recipient mice after adoptive transfer, IL-22-producing cells can be recovered along with IFN-γ-producing cells. In the pancreas, there is an increased percentage of IFN-γ-producing cells in adoptive transfer recipient mice compared to aged diabetic NOD mice, while IL-22^{+} cells remain only slightly increased. However, we have shown that IL-6 + IL-23 can induce IFN-γ-producing cells as well as IL-17- and IL-22-producing cells, which could be the major source of IFN-γ, as it is unlikely that 6-week-old mice have such pancreatic infiltration. To rule out their role in disease, adoptive transfer of purified Th17 cells could be performed. This would also be important to study as it has been shown that adoptive transfer of highly purified Th17 cells into NOD.SCID mice induces diabetes, yet these cells show increased plasticity and begin producing IFN-γ in concordance with a Th1 phenotype (219). It was found that this was due to the absence of regulatory T cells in the NOD.SCID system as similar effects

were seen in the NOD mouse when regulatory T cells were depleted (260). Therefore, it is said that Th17 cells are stable in the NOD mouse adoptive transfer model and so it is assumed that injecting purified IL-22-producing Th17 cells into NOD mice would recover fewer IFN-γ-producing cells and more IL-22$^+$ and IL-17$^+$ cells. By specifically using only purified IL-22 producing cells to cause disease, perhaps a neutralizing antibody against IL-22 will have a larger effect.

4.5. Neutralizing IL-22 in an adoptive transfer model into NOD.SCID mice

Because the BDC2.5 transgenic polarized splenocytes into NOD mice adoptive transfer model was not always optimal for diabetes induction, we decided to return to a more traditional model that has been proven throughout the last few decades by injecting diabetic splenocytes into NOD.SCID mice (225). IL-22 neutralizing antibody was injected along with the splenocytes to determine whether IL-22 may play a role in this model, which acts slower than the transgenic model as incidence does not begin until four weeks after injection. At first it seems that there may have been a difference between treatment and control groups as the first two mice in the control group became diabetic between 11 and 4 days prior to the first antibody-treated mouse developing disease. Afterwards however, both groups were nearly identical in diabetes incidence. One reason could have been that more injections of neutralizing antibody could have been given to induce more drastic changes between groups.

4.6. Final Conclusions

In summary, this project has studied the differential cytokine production of polarized naïve T cells in Th17 subsets, the role of neutralizing antibodies on cytokine induction, the presence of IL-22 in the pancreas and pancreatic lymph nodes of diabetic NOD mice and the effect IL-22 may have on disease.

We have observed that polarizing naïve CD4$^+$ BDC2.5 T cells with IL-6 + IL-23 induces much higher IL-22 expression than the addition of TGF-β. We have further

shown that for optimal Th17 cytokine and transcription factor production including IL-17 and RORγt, polarization should occur for 4 days and minimal amounts of TGF-β are optimal, such as that present in serum-supplemented media. We attempted to increase IL-22 production in Th17 cells to better study its effects. It was found that neutralizing IFN-γ demonstrated a profound increase in IL-22 production as the Th1-differentiation pathway was unfavourable. Conversely, neutralization of TGF-β, an IL-22 inhibitor, did not increase IL-22 production as Th17 induction was impeded as well. We therefore conclude that, specifically for maximal IL-22-producing Th17 cells, naïve T cells should be polarized with IL-6 + IL-23 + α-IFN-γ in serum-supplemented media.

We then examined the *in vivo* characteristics of pancreatic-infiltrating lymphocytes from diabetic NOD mice. We confirmed the presence of IL-22-producing cells in both the pancreatic-infiltrated and pancreatic lymph node populations, albeit in small percentages. We also observed an increase in IL-22-producing cells infiltrating the pancreas of diabetic mice compared to non-diabetic mice of the same age. It was determined that IL-22 expression was increased in the pancreatic lymph nodes of diabetic NOD mice compared to young, healthy controls and that IL-22R expression in the pancreas was up-regulated in diabetic mice as well. Lastly, in functional assays to determine the effect IL-22 has on diabetes incidence, the results are inconclusive and may indicate the lack of a major role in disease induction. This is supported by a recent study by Flavell and colleagues which demonstrated that knocking out IL-22 in NOD mice does not protect against diabetes (257). However, there are many avenues to consider on this matter such as redundant roles of inflammatory cytokines in the pancreas. It was originally hypothesized that TID was a result of Th1 pathogenesis, and that its prototypic cytokine, IFN-γ played a major role. However, knocking out the gene coding IFN-γ did not reduce incidence of disease, though the onset was delayed (212). These cells were also able to induce disease in recipient mice after adoptive transfer. There is ample evidence to suggest a role of IFN-γ in T1D pathogenesis, and therefore it is possible that in the absence of this cytokine, other inflammatory cytokines become more abundant and provide a similar effect as IFN-γ. This redundancy may account for the lack of conclusive evidence that one factor is solely responsible for disease induction. One group examined the effect of knocking out the receptors for both IL-17 and IFN-γ in the NOD mouse

model and found that the double deficient mice exhibited decreased incidence of disease and delayed onset compared to even the single KO mice (261). Therefore, the strongest model to elucidate may be the effects of knocking out two inflammatory factors—such as IL-22 and IFN-γ—to determine whether in the absence of one cytokine, another cytokine can cause a redundant effect.

These data illustrate a potential role for IL-22 in the pancreas that has yet to be determined. The high expression of IL-22R in the pancreas coupled with the presence of IL-22-producing cells isolated from the tissues suggests IL-22 may function as a mediator of cross-talk between cells. The finding that both IL-22 and its receptor are increased in diabetic mice points to the role being of a more pathogenic nature, however it is possible that IL-22 responds to pathogenic signals in the pancreas and has a protective effect. Colleagues in our lab have demonstrated that IL-22 can induce regeneration genes in beta cells in the pancreas and that neutralizing IL-22 abolishes this effect (147). Whether this effect is due to an inflammatory signal or because IL-22 plays an active role in the destruction of beta cells, thereby turning on genes to regenerate them, is not fully understood. Another study has shown that increased IL-22 expression can protect mice from acute pancreatitis (258). Further work in this area may uncover an important mechanism of IL-22 action in the pancreas that is not yet known.

4.7. Future Directions

This study has shown a possible role for IL-22 in the pancreas and in pathogenesis of diabetes in the NOD mouse model. To better illustrate the potential for IL-22 in the pancreas, immunohistological staining could confirm the expression of IL-22R on beta cells in NOD mice, as well as display the extent of infiltration of IL-22-producing cells and their proximity to islet beta cells. This was unsuccessfully attempted in this study and further work to optimize this protocol may lead to interesting results.

To better elucidate the capacity of IL-22 in diabetic disease models it is necessary to fully observe the effect of knocking out this cytokine in the NOD mouse. This was

initiated by Flavell and colleagues, however another step could be to examine the effect of double deficiency with either IL-17, to inhibit the two prototypic Th17 cytokines, or IFN-γ, to discover any overlapping effects of these cytokines.

Lastly, as mentioned above, the IL-22Rα2 binding protein has been shown to have an effect in the pathogenesis of MS or EAE. Therefore, it may be prudent to examine the effects of dysregulation of the IL-22 antagonist in a diabetes disease model.

Type 1 diabetes is a complex, multi-faceted disease of the immune system with many unknown factors contributing to pathogenesis. After decades of research, the scientific field has made many advances in identifying key regulators that may mediate progression of disease, however conflicting reports reveal that there is likely not one single, major effector in every case of disease presentation. Further research is necessary to identify predispositions to type 1 diabetes as well as better therapeutic options for those living with this devastating disease.

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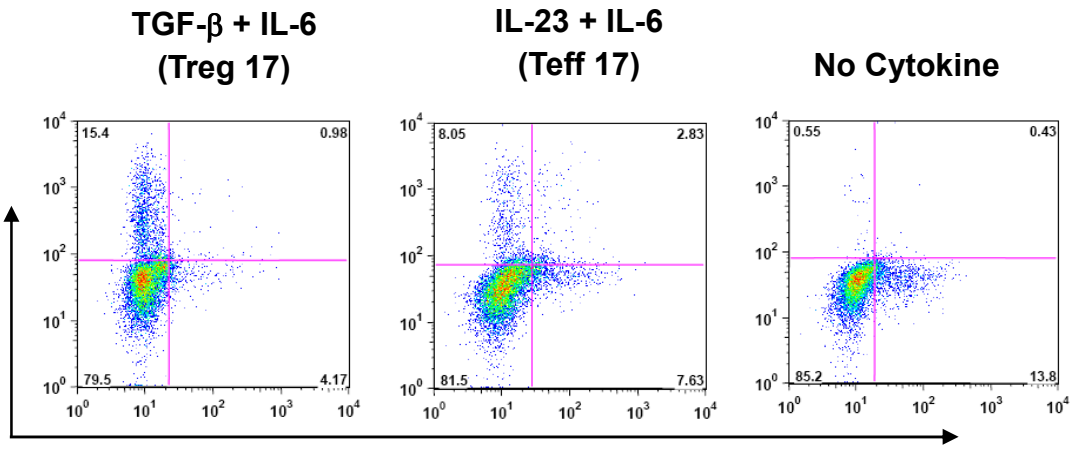
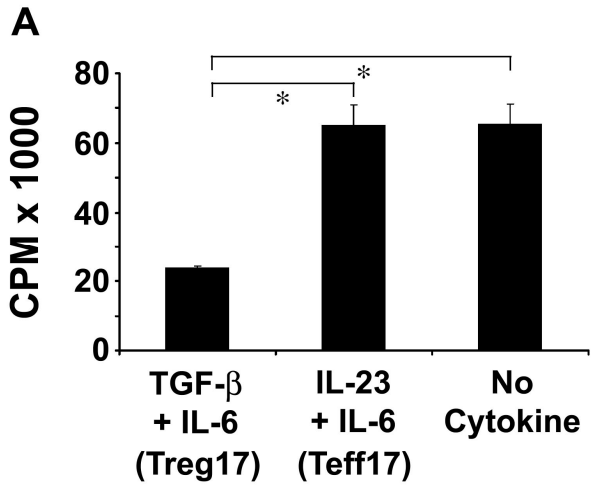
Appendices

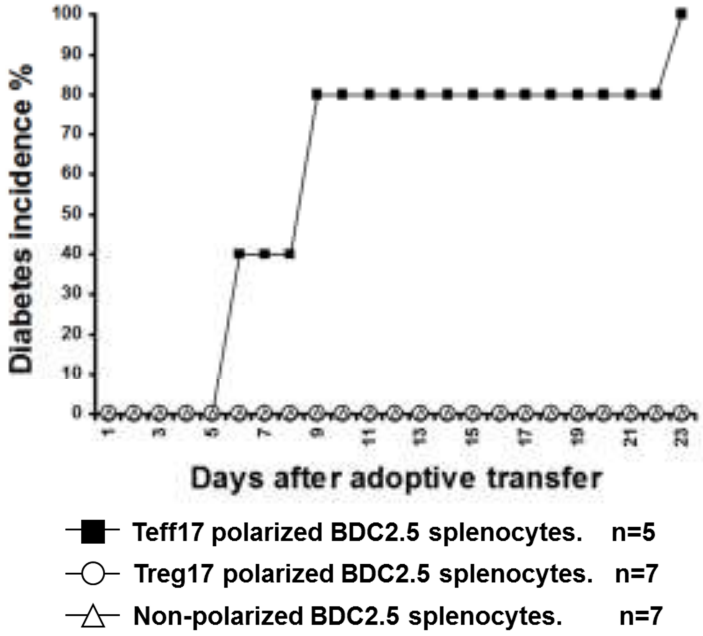
Appendix I. *Differential cytokine production in Th17-polarized splenocytes*. BDC2.5 splenocytes were cultured on 96-well plates and stimulated with PS3 mimotope (5 μg/ml) with polarizing cytokines IL-6 (20 ng/ml), TGF-β (3 ng/ml) or IL-23 (10 ng/ml) as indicated. Cells stimulated with PS3 mimotope without cytokine treatment were used as a control. After 5 days cells were stained with fluorescent antibodies and FACS analysis performed. Cells were gated on live then $CD4^+$ cells.


TGF-β + IL-6
(Treg 17)
IL-23 + IL-6
(Teff 17)
No Cytokine
15.4
0.98
79.5
4.17
8.05
2.83
81.5
7.63
0.55
0.43
85.2
13.8

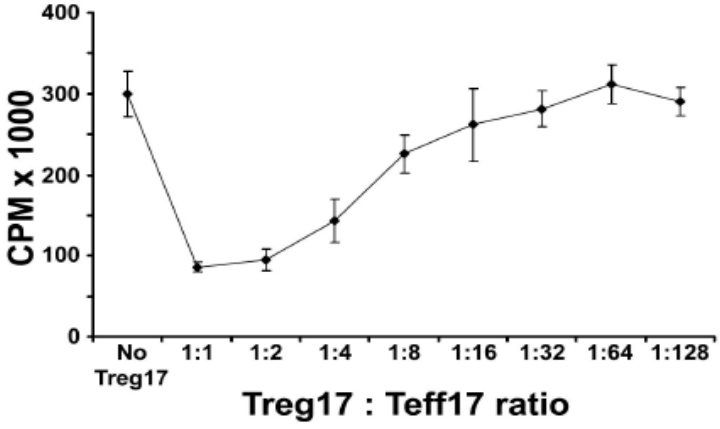
Appendix II. *Differential proliferation exhibited by Th17-polarized splenocytes*. BDC2.5 splenocytes were cultured on 96-well plates and stimulated with PS3 mimotope (5 μg/ml) with polarizing cytokines IL-6 (20 ng/ml), TGF-β (3 ng/ml) or IL-23 (10 ng/ml) as indicated. Cells stimulated with PS3 mimotope without cytokine treatment were used as a control. After 72 hours [^{3}H] thymidine was added and proliferation was measured. * indicates significant difference for 3 experiments ($p < 0.05$).

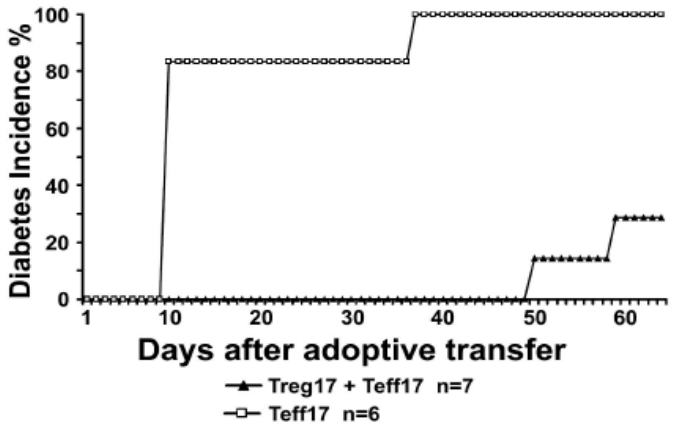

A
CPM x 1000
80
60
40
20
0
*
*
TGF-β
+ IL-6
(Treg17)
IL-23
+ IL-6
(Teff17)
No
Cytokine

Appendix III. *Different polarization techniques of Th17 cells leads to functionally different effector subsets.* BDC2.5 splenocytes were cultured on 96-well plates and stimulated with PS3 mimotope (5 μg/ml) with polarizing cytokines IL-6 (20 ng/ml), TGF-β (3 ng/ml) or IL-23 (10 ng/ml) as indicated. Cells stimulated with PS3 mimotope without cytokine treatment were used as a control. After 5 days cells were injected IV into the tail vein of NOD mice in an adoptive transfer. Diabetes incidence is depicted. All mice injected with the Teff17 polarized cells developed diabetes, $p < 0.001$.

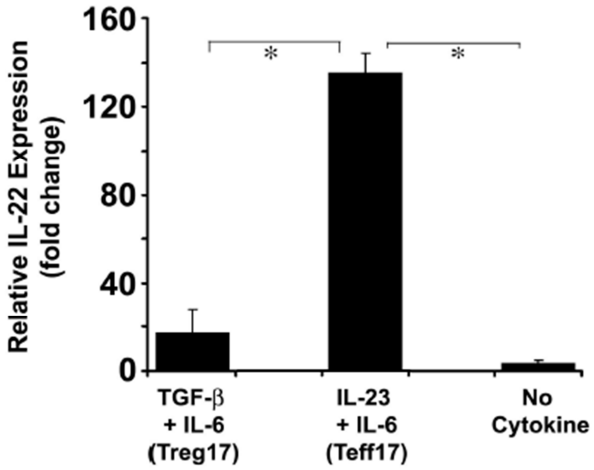

Diabetes incidence %
100
90
80
70
60
50
40
30
20
10
0
1
3
5
7
9
11
13
15
17
19
21
23
Days after adoptive transfer
Teff17 polarized BDC2.5 splenocytes. n=5
Treg17 polarized BDC2.5 splenocytes. n=7
Non-polarized BDC2.5 splenocytes. n=7

Appendix IV. *Treg17 cells can suppress both proliferation and the pathogenic effects of Teff17 cells*. BDC2.5 splenocytes were cultured on 96-well plates and stimulated with PS3 mimotope (5 μg/ml) with polarizing cytokines; Treg17 cells were polarized by IL-6 (20 ng/ml) + TGF-β (3 ng/ml) and Teff17 cells were polarized by IL-6 (20 ng/ml) + IL-23 (10 ng/ml). (A) After 5 days the two subsets were replated together and restimulated with a serial dilution of the ratio of Teff17 cells to Treg17. After 72 hours [^{3}H] thymidine was added and proliferation was measured. (B) Equal numbers (5 x 10^{6}) of Treg17 cells and Teff17 cells were injected IV into the tail vein of NOD mice in an adoptive transfer. Treg17 cells blocked disease development for an extended period of time ($p < 0.03$).

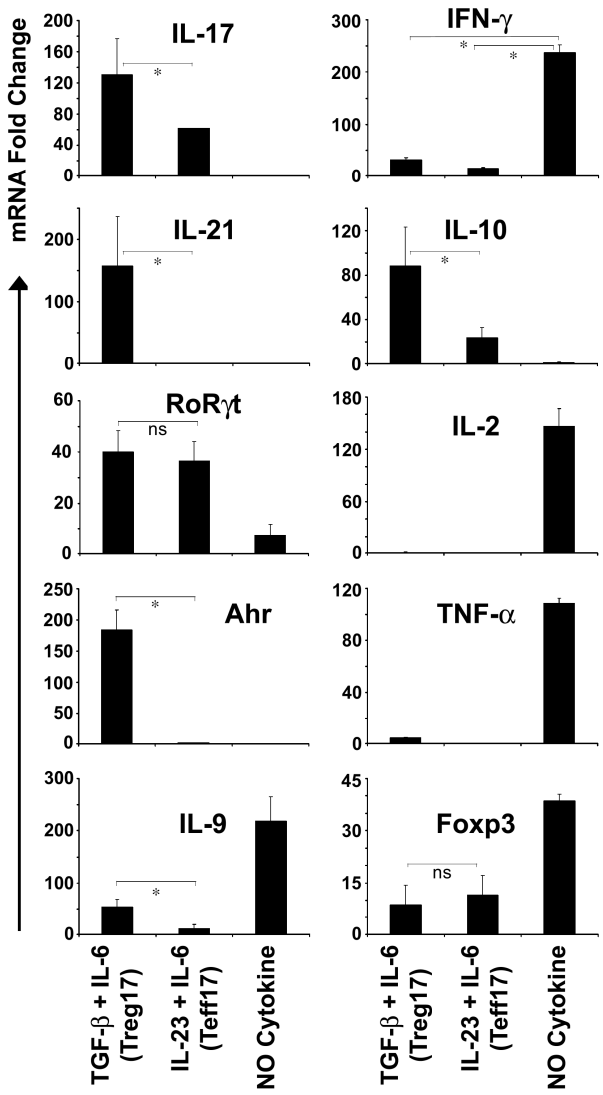

400
300
200
100
0
CPM x 1000
No Treg17
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
Treg17 : Teff17 ratio


100
80
60
40
20
0
Diabetes Incidence %
1
10
20
30
40
50
60
Days after adoptive transfer
Treg17 + Teff17 n=7
Teff17 n=6

Appendix V. *Teff17 cells demonstrate increased IL-22 production compared to Treg17 cells and non-polarized cells.* BDC2.5 splenocytes were cultured on 96-well plates and stimulated with PS3 mimotope (5 μg/ml) with polarizing cytokines IL-6 (20 ng/ml), TGF-β (3 ng/ml) or IL-23 (10 ng/ml) as indicated. Cells stimulated with PS3 mimotope without cytokine treatment were used as a control. After 5 days cells were collected and RNA extracted. mRNA expression was measured using RT-qPCR and IL-22 expression was shown to be much higher in the cells polarized by IL-6 + IL-23. Results are shown as fold change and are relative to beta actin which was used as a housekeeping gene.


Relative IL-22 Expression
(fold change)
160
120
80
40
0
*
*
TGF-β
+ IL-6
(Treg17)
IL-23
+ IL-6
(Teff17)
No
Cytokine

Appendix VI. *Differential cytokine and transcription factor expression is observed between two Th17 subsets.* BDC2.5 splenocytes were cultured on 96-well plates and stimulated with PS3 mimotope (5 μg/ml) with polarizing cytokines IL-6 (20 ng/ml), TGF-β (3 ng/ml) or IL-23 (10 ng/ml) as indicated. Cells stimulated with PS3 mimotope without cytokine treatment were used as a control. After 5 days RNA extracted and mRNA expression was measured using RT-qPCR. Results are shown as fold change and are relative to beta actin which was used as a housekeeping gene.


mRNA Fold Change
IL-17
IFN-γ
IL-21
IL-10
RoRγt
IL-2
Ahr
TNF-α
IL-9
Foxp3
ns
TGF-β + IL-6 (Treg17)
IL-23 + IL-6 (Teff17)
NO Cytokine

Curriculum Vitae

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