LOSS OF CSF2 EPIGENETIC REGULATION THROUGH ABERRANT GM-CSF INDUCED STAT5 SIGNALING CONTRIBUTES TO TYPE 1 DIABETIC MYELOID CELL DYSFUNCTION

By

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To Sally, Mom, Dad and Gran
ACKNOWLEDGMENTS

It is with deepest gratitude that I acknowledge my family, friends and colleagues who contributed profoundly to my professional and personal lives. I wish to sincerely thank my mentors Dr. Sally Litherland and Dr. Mark Atkinson for their continued support and generosity of time, guidance, materials and insight. Without their patience, encouragement of creativity and open exchange of thoughts and ideas this degree would not have been possible. In addition to my mentors, I wish to gratefully acknowledge my advisory committee members, Dr. Ammon Peck and Dr. Peter McGuire. Their advice and support has been invaluable throughout this process.

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By

Erin Lee Garrigan

December 2008

Chair: Mark Atkinson
Cochair: Sally Litherland
Major: Medical Sciences

Defective myeloid differentiation and activation contribute to immunopathogenesis of autoimmune type 1 diabetes (T1D) in humans and non-obese (NOD) mice by retarding their antigen presenting cell function, altering their cytokine production, and promoting a pro-inflammatory microenvironment through aberrant expression of prostaglandin synthase, PGS2 (COX2). Monocytes of at-risk/T1D humans and macrophages of the NOD mouse have elevated autocrine GM-CSF production and persistent signal transduction/activator of transcription (STAT5) phosphorylation. GM-CSF activates STAT5 binding at its own gene promoter within the Idd4.3 region, as well as at the enhancer of the COX2 gene, Ptgs2. Since STAT5 acts as adaptor protein for either deacetylase or acetylase enzymes mediating epigenetic chromatin modification, we hypothesize that the loss of Csf2 gene regulation in autoimmune cells is mediated by changes in epigenetic control perpetuated by GM-CSF activating persistent STAT5 then also genes such as Ptgs2. Our specific aims are to characterize epigenetic modification at regulatory sites upstream of the Csf2 and Ptgs2 genes mediated by STAT5 binding and determine if such changes are related to GM-CSF-induced 1) STAT5 function and 2) Csf2/Ptgs2...
gene expression in autoimmune myeloid cells, and 3) the genetic sequence of regulatory regions
diabetes susceptibility loci.

A small volume flow cytometric analysis was developed to measure STAT5Ptyr levels
in T1D patients and NOD mice for use as a potentially clinically-useful biomarker assay for
autoimmune T1D susceptibility. On average, T1D patient monocytes STAT5Ptyr levels were
two fold higher than healthy controls. However when these data are analyzed by gender, the
results showed a statistically significant female bias for this biomarker in the subject group.

Our phenotypic and genotypic studies of the NOD mouse and subsequent congenic
derivatives of this model narrow diabetes susceptibility attributed to Chr. 11 and GM-CSF
overproduction with persistent STAT5 phosphorylation to a small (200bp) un-transcribed
regulatory region within the $Csf2$ promoter. In the NOD, this region contains a loss of STAT6
binding site (potential anti-inflammatory mediator) and a gain of a STAT5 binding site.
Homologous sites were not found in the T1D patient versus healthy control, suggesting alternate
regulatory mechanisms contributing to the persistent STAT5 phosphorylation in human
autoimmune monocytes.

Our results support the potential of epigenetic control as a mechanism underlying the
chromosomal dysregulation seen in autoimmune diseases and indicate that epigenetic control of
gene expression can provide a new avenue for discovery of potential prevention/therapeutic
intervention targets.
CHAPTER 1
INTRODUCTION

Autoimmune Disease Susceptibility

Autoimmunity is the condition in which the immune system fails to recognize the difference between the body’s own tissues and a foreign matter, and thus can become intolerant of its own components (1). When tolerance is lost, the immune cells respond to the body’s own tissue as it would to a foreign invader, often resulting in a self-directed immune response which can damage the body’s organs and tissues. Autoimmunity arises from a complex mix of environmental, genetic and immunological factors. Some autoimmune responses may arise as a result of infection or exposure to other foreign elements leading to the development of antibodies that have cross reactivity with self tissue.

Type 1 Diabetes (T1D): An Autoimmune Disorder

Diabetes is a disorder generally characterized by the body’s inability to respond to insulin signaling, either through loss of insulin production, resistance in the insulin receptor, or from a failure to adequately use insulin. Insulin is a necessary hormone that facilitates the uptake of glucose contained in food from the blood into the cells around the body. Improperly regulated glucose levels can lead to tissue damage and can become life threatening. Diabetes affects an estimated 20.8 million children and adults in the United States (2).

Onset of T1D results from a complex blend of genetic predisposition and one or many environmental trigger(s). Two twin studies, conducted in Denmark and the United States, observed the presence of islet and beta cell auto-antibodies in monozygotic and dyzygotic twins where one or both siblings was afflicted with insulin dependent diabetes (3, 4) While the studies differed in their conclusions of which was more important in diabetes etiology, genetic or
environmental triggers, they serve to highlight the multi-factorial nature of diabetes pathogenesis.

Prior to the onset of clinical symptoms of T1D, an early signal of autoimmunity is the appearance of circulating immune cells and antibodies directed against islet and beta cell antigens (5, 6).

**T Lymphocyte Tolerance and T1D**

Type 1 Diabetes Mellitus is classified as an autoimmune disorder based on the destruction of insulin producing beta cells in the pancreas by the immune system. The immune mediated beta cell destruction involves cells of both lymphoid and myeloid origin, including CD4+ and CD8+ T lymphocytes and Antigen Presenting Cells (APC), and it may also be influenced by an inflammatory cytokine microenvironment. Random rearrangements of germline sequence contribute to antigenic diversity within the T Cell Receptor (TCR) but may also give rise to auto-reactive T lymphocytes. During central tolerance of healthy individuals, the potentially auto-reactive T cells are negatively selected when they recognize self peptides presented in the context of self MHC (7). Auto-reactive T cells may become anergized by contacting an antigen-MHC complex in the absence of co-stimulatory molecules, thus adding an additional level of regulation of potentially immunopathogenic T cells (8). Errors in T cell tolerance may promote the escape of auto-reactive T cells which can contribute to the onset of autoimmunity. Serreze et al. (1993) asserted that abnormal maturation of bone marrow derived APC in non-obese diabetic (NOD) mice are affected by both MHC and non-MHC linked diabetes susceptibility genes. He found that the NOD has APC that are unable to adequately express β cell auto antigens during tolerance (9). The results of these defects are that NOD APC function at suboptimal levels and are very poor at inducing and maintaining self-tolerance.
Regulatory T Cells and Inflammation in T1D

NOD mice that exhibit homozygous expression of the MHC allotype H-2\(^{g7}\), the MHC region linked to diabetes susceptibility. This MHC molecule has been shown to present diabetogenic antigens, which can activate β cell auto-reactive effector T cells. NOD APC expressing H-2\(^{g7}\) molecules are known to preferentially activate these auto-reactive T cells but poorly activate regulatory T cells. Interaction of NOD derived T cell precursors with APC that present an MHC haplotype other than H-2\(^{g7}\) prevents diabetogenesis (10-12).

Defects in the regulation of auto reactive T cells which destroy insulin producing β pancreatic cells have been implicated in diabetic pathogenesis. In addition, there is growing evidence that defects in the maturation of antigen presenting cells also contribute to the development of diabetes (13). Moreover, errors in cytokine signaling between these immune cells lead to a cellular miscommunication, contributing to both de-regulation of T cell ontogeny and peripheral APC dysfunction, which allows the autoimmune responses of T1D to progress unchecked. Chronic inflammation is a common phenotype in many autoimmune diseases and it is thought to play a role in loss of tolerance induction by APC (14).

Myeloid APC Dysfunction in T1D

Myeloid APC development is advanced by a succession of cytokine signals that push cells down differentiation paths to become different cell types. For example, interleukin 3 (IL-3) activates autocrine GM-CSF (granulocyte macrophage colony stimulating factor), which in turn activates G-CSF (granulocyte colony stimulating factor) to promote granulocyte differentiation. IL-3, in conjunction with GM-CSF also stimulates M-CSF (macrophage colony stimulating factor), to promote monocyte and macrophage development (15).

In this cascade, GM-CSF is a multifunctional cytokine exerting different influences in myeloid cells differentiation and mature monocyte/macrophage/ dendritic cell functionality.
GM-CSF regulates the proliferation and differentiation of hematopoietic stem cells but it also helps to direct mature cell function (16, 17). GM-CSF is thought to be involved in autoimmunity both through its regulation of the stages of maturation of macrophages and granulocytes, but also for its role in activating the inflammatory process in mature macrophages (14, 17). Through its stimulation of Jak2/STAT5 signaling, GM-CSF activates STAT5 (by phosphorylation with Jak2) to up-regulate expression of the dual functional enzyme, prostaglandin synthase/cyclooxygenase 2 (PGS2/COX2), and subsequent production of Prostaglandin E2, a key lipid mediator in the inflammatory process (18, 19). Finally GM-CSF stimulates the gene expression of Interleukin 10 (IL-10), which down regulates the signaling cascade (20).

**Study Background**

Chase et al. (1979) initially observed that T1D patients had high levels of PGE2 in their plasma, indicative of a chronic pro-inflammatory state (21). Monocytes from Type 1 Diabetic human patients exhibited a high production of COX-2 (cyclooxygenase 2), also known as PGS2 (prostaglandin synthase 2), an enzyme involved in the production of pro-inflammatory prostaglandins, specifically Prostaglandin E2 (PGE2). *Ptgs2* is the gene that encodes the COX2 enzyme. No genetic abnormalities were observed in the coding region of the NOD and TID patient *Ptgs2* gene, nor in the five prime promoter regions or the three prime poly A tail. Since the COX2 gene appeared to be normal healthy control humans, it was necessary to investigate an alternate explanation for the aberrant COX2 expression in human T1D monocytes, so regulation of *Ptgs2* was examined. PGE2 production by COX2 is important not only in promoting inflammation but also in blocking toleragenic APC maturation as well as inhibiting the signal for activation induced cell death (AICD) from the APC from being received by the T cell. COX2 activity in T1D patient peripheral blood monocytes was resistant to suppression by IL-10; whereas, COX2 expression was suppressed by the addition of IL-10 to control human monocytes.
cultures (18). In addition GM-CSF, a strong activator of IL-10 and COX2 expression in macrophages, was found to be abnormally high in individuals with or at risk for T1D. These findings suggested that both activation and suppression of COX2 in individuals at-risk or with T1D may be the result of the disruption of these cytokine’s regulation of the \textit{Ptgs2} gene expression.

In non-autoimmune myeloid cells, GM-CSF stimulation alone is not sufficient to induce COX2 expression. Yamaoka et al. (1998) reported that normally, activation of COX2 expression requires LPS and GM-CSF stimulation and it is accomplished through Jak2/STAT5 signal transduction (20). However, in diabetic monocytes and macrophages, GM-CSF stimulation alone is adequate to activate COX2 expression (22). This activation makes COX2 resistant to IL-10 suppression even though GM-CSF expression in these same cells is completely IL-10 sensitive. These findings suggest that some other component in COX2 activation is also abnormally regulated and that the expression of GM-CSF itself is aberrantly regulated in NOD and T1D myeloid cells.

GM-CSF acts on gene expression through the activation of transcription factors such as PI3K, MAPK, and the JAK/STAT pathway (14). Work by Yamaoka et al. indicates that GM-CSF activation of the Jak2/STAT5 signaling pathway is critical to its influence on the \textit{Ptgs2} expression (23). STAT5 is an effector molecule in epigenetic regulation (24, 25). After phosphorylation (by Janus Kinase), two STAT5 molecules dimerize and translocate to the nucleus where it binds DNA at the motif TTCNNNGAA (18). In addition to a DNA binding domain, the STAT5 dimer contains an acetylase and de-acetylase binding site, making it an adaptor molecule to facilitate the opening and closing of chromatin to influence gene expression based on the primary signal transduction. Epigenetic regulation entails the modification of
histones and the DNA itself with small molecules such as acetyl, methyl, ubiquitin and sumo groups (26-28). These modifications alter the topological structure of chromatin, allowing for the opening and closing of gaps in the nucleosome cores and making the DNA sequences in the region accessible or inaccessible to DNA modification, replication, repair, and transcriptional enzymes. These rapid and inheritable changes in chromatin structure are potentially crucial to cytokine induced gene regulatory functions.

To examine whether GM-CSF overproduction was affecting COX2 expression through JAK2-STAT5 signaling, Litherland et al. (2005) looked at Jak2 and STAT5 function in T1D human and NOD mouse myeloid cells. The results of these studies showed that STAT5 was persistently phosphorylated in un-activated T1D patient peripheral blood monocytes and NOD mouse monocytes and macrophages (13, 22). Moreover, GM-CSF was found to stimulate its own production (via the JAK2/STAT5 pathway). These data suggest that overproduction of GM-CSF in autoimmune cells may contribute to abnormal autoimmune monocytes and macrophage development and activation as well as the abnormal COX2 expression in autoimmune T1D patients and NOD mice (29). This aberrant expression of COX2 in T1D patients and its subsequent production of PGE2 may assist in the establishment of an inflammatory microenvironment which may promote loss of beta cell specific auto-reactive T cell tolerance and successive activation, advancing the tissue damage observed in Type 1 Diabetes.

We have observed increased COX2 expression and PGE2 production by COX2 in un-activated autoimmune monocytes, as well as elevated GM-CSF production, increased STAT5 phosphorylation and IL-10 resistant COX2 activity in these cells. Because STAT5 is strongly activated by GM-CSF in T1D patients, we asked how GM-CSF activation of STAT5 is affecting COX2 expression and potentially disease pathology.
Abnormal regulation of STAT5 could indicate that STAT5 (after having been activated by an initial GM-CSF signal) is feeding back to stimulate the continuous production of GM-CSF (through activation its gene, \(Csf2\)) as well as affecting expression of other genes (e.g. \(Ptgs2\)) that are controlled through the same signaling pathway. To test this hypothesis, we analyzed both monocytes in T1D human blood samples and monocytes and macrophages from NOD and congenic mice. Within the human system, we determined whether the observed phenotypes (elevated GM-CSF, persistent STAT5 phosphorylation and increased COX2 expression) were correlative enough to be useful as biomarkers for susceptibility screening among known at risk populations. Through a study of mouse strains derived through congenic breeding, we investigated the genetic input in these three phenotypes independently as well as their potential interactions in T1D immunopathogenesis.
CHAPTER 2
MATERIALS AND METHODS

Human Sample Collection and Preparation

Patient blood samples are obtained from healthy volunteers (age range 4 to 46 years; Table 2-1), T1D patients, and their immediate relatives, through collaboration with Dr. Michael Clare-Salzler M.D and Dr. Mark Atkinson. Samples are collected with informed consent and under IRB (human blood samples) approved protocol number 372-1996.

Human peripheral blood was initially processed on Ficoll gradients and the monocyte layer (PBMC) was separated from whole blood with Ficoll gradient centrifugation. Later, whole blood was used directly in the analyses. The PBMC layer was collected, washed and counted for viable cells. The monocytes were plated on tissue culture dishes and fed with fresh sterile medium alone or with 1000U/ml of GM-CSF (Biosource). Cultures were then processed according to protocol detailed in the Mouse and Human Cell Processing section.

Small Volume Flow Cytometric Analysis of STAT5Ptyr in Peripheral Blood Monocytes

Fluorescence Activated Cell Sorting (FACS), also known as flow cytometry, was used to determine the presence of tyrosine phosphorylated STAT5 (STAT5Ptyr) in the myeloid cells. Intracellular flow cytometry was carried out with phosphate modification specific anti STAT5 Ptyr monoclonal antibodies that were conjugated with APC (chemical name) or PE (chemical name). Surface staining with anti CD11b- FITC/PE was used to identify mouse myeloid cells or with CD14-PE to identify human peripheral blood monocytes.
Antibody Conjugation

Anti Phosphorylated STAT5A/B antibody (STAT5Ptyr, Upstate) and Normal Mouse IgG antibody (Upstate) were conjugated with PhycoLink Activated Allophycocyanin (APC) or PE (Prozyme, Glyko) for use in fluorescent immunohistochemical analyses (e.g. FACS, deconvolution microscopy, etc.). The conjugation was accomplished according to the protocol from the PhycoLink Allophycocyanin (APC) Conjugation Kit (Product code PJ25K). The final conjugated antibody concentrations were measured by Bradford Assay by comparison to a BSA/Bradford dye comparative standard curve measured at 655nm. Dye conjugation was calculated from Absorbance at 655nm relative to an APC/PE dye standard. Stock antibody concentrations were diluted to 1mg/ml (STAT5Ptyr-APC) and 0.01mg/ml (mouse IgG-APC) in PBS based on optimum on optimum fluorescence when calibrated with human peripheral blood monocytes.

Flow Cytometry Analysis of Activated STAT5

Blood samples were collected by venipuncture or by finger prick from Type 1 diabetic patients as well as non autoimmune control subjects under IRB approved protocols. Samples were processed within 45 minutes of collection. In keeping with the goal of developing a small volume biomarker assay, 150µl of whole blood was incubated for 10 minutes with anti human CD14 (a macrophage marker) (BD Pharmingen) and unlabeled anti mouse IgG (Sigma). Cells were then fixed and made permeable with BD cytofix/cytoperm (BD Biosciences) and incubated for 10 minutes. Cells are then split into two equal volumes, washed with saponin buffer (containing BSA, Sodium Azide, saponin and PBS in ddH2O) and incubated for one hour in 3µl of 1mg/ml STAT5Ptyr-APC or 1µl 0.01mg/ml mouse IgG-APC. After incubation cells are again washed with saponin buffer and stored in an isotonic buffer solution until flow cytometric analysis.
Deconvolution Microscopy

Cell samples prepared for Flow Cytometry were also examined by microscopy to confirm activated STAT5 staining. Samples were adhered to a charged slide by centrifugation in a cytospin centrifuge. The specimens were counter stained with 1ng/ml DAPI and washed and held at 4C in the dark until analysis. Deconvolution microscopy (Olympus IMT/DeltaVision Deconvolution) was used to determine the sub-cellular location of STAT5Ptyr in a set of 20-30 - 0.01micron optical slices per image of isolated cells labeled for STAT5 Ptyr.

Animal Models

Four to thirty week old male and female mice (The Jackson Laboratory, Bar Harbor, ME) were used for all studies. NOD, C57BL/6, NOD.LC11, B6.NODC11 and B6.NODC1 mice were maintained in the University of Florida Pathology SPF mouse colony in microisolator cages with food and water ad libium throughout the study. NOD background congenic mice (NOD.LC11) and its sub-congenic derivatives (NOD.LC11e and NOD.LC11b) were originally developed at the University of Virginia specific pathogen free (SPF) colony and were a generous gift from our collaborator, Marcia McDuffie, MD (30) (Figure 2-1). The B6.NODC11b and B6.NODC1tb strains were derived for this study from B6.NODC11 and B6.NODC1 congenic mice which are housed at the University of Florida College of Medicine Pathology Department SPF colony. These B6 background strains were originally derived by Yui and Wakeland (31). Sub-congenic strains of the B6.NODC11 and NOD.LC11 mice and the B6.NODC11bxC1tb bi-congenic strain were bred from these strains. Genotyping was performed from tissue collected by tail or ear biopsy at the time of weaning to confirm genetic intervals using PCR analysis (20, 32). All procedures were conducted according to IACUC approved protocols B083 and D754.
Bone Marrow and Tissue Collection, Cell Culture and Sample Preparation

Control, NOD, sub-congenic and bi-congenic strain mice were analyzed at weaning, 5-12 weeks of age and at 20 weeks of age. Mice were euthanized by over-anesthetizing and cervical dislocation. Blood monocytes were collected either at the time of tail or ear biopsy or by cardiac puncture after euthanasia, then analyzed for percentage of phosphorylated STAT5 by flow cytometry. Peritoneal macrophages were collected by injecting ice cold RPMI medium supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic mix (Cellgro-Mediatech, Herndon, VA) into the peritoneal cavity. The lavage fluid was then withdrawn and washed with cold media by centrifugation. The long bones of the hind limbs were excised marrow cells were flushed out of the bones using a 30-guage needle and syringe filled with cold RPMI medium supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic mix (Cellgro-Mediatech, Herndon, VA). The marrow cells and lavage harvested macrophages were washed with cold media then the red blood cells in samples were lysed by incubation in sterile, cold 0.84% NH4Cl buffer. The remaining bone marrow cells and macrophages were then plated on tissue culture dishes and fed with fresh sterile medium alone or with 1000U/ml of GM-CSF (Biosource) with or without 2µg/ml anti-GM-CSF blocking antibodies (Endogen) for 24 hours at 37°C/5%CO2. After incubation, 1ml of the cultured cell supernatant was collected and frozen at -80°C to measure GM-CSF concentration by Luminex and ELISA as well as PGE2 by ELISA (GE/Amersham). Cells were then processed according to protocol detailed in the Mouse and Human Cell Processing section.

Mouse and Human Cell Processing

Cultures were maintained for 24hr or 48hr at 37°C/5%CO2, then washed and re-supplemented for an additional 24hr or 48hr in culture at 37°C/5%CO2. An aliquot of cells was taken to confirm phenotypic identification and phosphotyrosine STAT5 analysis by flow
cytometry as previously described (22, 42). Cells were fixed in situ with 1%(v/v Cf)
formaldehyde (methanol-free, Sigma-Aldrich, St. Louis, MO) added in the remaining media for
10min at 37°C, then washed with 1x PBS. Cells were sonicated for 5 seconds in SDS Lysis
Buffer (Table 2-2) + protease inhibitors (Roche, Indianapolis, IN) to disrupt membranes and
shear chromatin to approximately 1000bp fragments then frozen for later analysis with
Chromatin Immunoprecipitation (ChIP).

**Chromatin Immunoprecipitation (ChIP) Analysis of STAT5 Binding at the Csf2 Promoter**

Frozen cell extracts detailed in the Mouse and Human Cell Processing section were
thawed. The samples consisted of four to five million cells from bone marrow cultures or ex vivo
peritoneal macrophages and were divided into aliquots for each run of the analysis. The aliquots
used for immunoprecipitation (IP) were pre-cleared with salmon sperm DNA Protein A agarose
beads (Upstate), then incubated overnight at 4°C with anti-STAT5Ptyr antibodies (Upstate).
After incubation, the antibody-bound chromatin complexes were precipitated using salmon
sperm DNA Protein A agarose beads, and washed extensively with a series of increasing
stringency buffers (low salt, high salt, LiCl, TE; table 2-2). A non-specific antibody control
(mouse IgG, UpState) and a sham IP containing no extract were run as negative controls. Total
cell and ChIP extract aliquots were dissociated from the beads in 1% SDS, 0.1M Bicarbonate
buffer (Fisher Scientific, Atlanta, GA). Sodium chloride was then added to a final concentration
of 500mM and the samples incubated 4 hours at 65°C to reverse the formaldehyde crosslinks.
DNA was purified from these aliquots for PCR amplification of DNA sequences from Csf2
promoter which have been shown to be epigenetic regulatory sites for inducible Csf2 expression
and from thePtgs2 gene (33, 34).

In Double ChIP (dbChIP) analyses, chromatin complexes first precipitated with anti-
STAT5Ptyr antibodies were dissociated from the antibody-Protein A agarose beads and then re-
precipitated with anti-histone H3 antibodies (UpState USA) prior to DNA de-crosslinking and purification as described above (35).

**Polymerase Chain Reaction**

ChIP DNA was eluted in a buffer containing 20% SDS, 1M NaHCO₃ and water. The DNA/Protein crosslinks were reversed (with four hour incubation in NaCl then addition of 0.5M EDTA, 1M Tris-HCl and Proteinase K) and the remaining DNA was purified by Phenol/Chloroform Extraction. PCR reactions were set up using Eppendorf Master Mix (2.5x), 2% DMSO (Sigma-Aldrich) and primers (IDT) and run on a Mastercycler (Eppendorf) with cycle protocol; 98C 5min, 94C 30 sec, 55-60C (dependent on the primer set used) 30sec, 72C 30sec, for 35 cycles. Amplification products were separated on a 2% agarose gel (SeaKem Fisher Scientific) and visualized by ethidium bromide (Fisher Scientific) intercalation.

**Real Time PCR**

DNA samples were volume matched to 100ng of their Total DNA aliquots (no IP) in all PCR reactions. DNA isolated from ChIP extracts was used as a template in a reaction containing SYBR® Green PCR Master Mix (Applied Biosciences, Foster City, CA or BioRad, Hercules, CA), primers specific for Csf2 promoter region or COX-2 enhancer (IDT), and 2% DMSO (Fisher Scientific). The Real Time PCR reaction was completed in a continuous fluorescence detector (MJ Research) and amplification quantitation was compared on the basis of R value calculated as $R = 2^{(\Delta\Delta C_{t} \text{ (Non specific Ig ChIP c(t) – anti-STAT5Ptyr ChIP c(t))})}$. Statistical data analyses were performed using Prism 4/5 (Graph Pad, San Diego CA).

**Luminex and ELISA**

Supernatant media from myeloid cells cultured at 37C/5%CO2 for 24hr and 48hr in was collected for GM-CSF Luminex analysis. Luminex quantifies the amount of GM-CSF and other cytokines made by the treated cells using fluorescently labeled beads coated with antibodies to a
specific cytokine. The amount of cytokine (i.e., bound specific antibody + fluorescent beads) in a sample is quantified by fluorescence detected on a Luminex flow cytometer versus a standard curve for each cytokine tested. GM-CSF was quantified using Luminex (Upstate Beadlyte) and ELISA (BD biosciences) and PGE2 was detected using ELISA (GE/Amersham).

**Sequence Analysis of Csf2 Gene Promoter and Ptgs2 gene enhancer**

**Mouse Sequence Analysis**

Approximately 50ng of genomic DNA from each mouse strain was prepared from liver and amplified by PCR using Master Mix (Eppendorf or Roche Biosciences) reagents and primers (3’ CTA AAA CAT GTT TCT TGG CTA; 5’ AAA TAA GGT CCA GCC CAA TG) designed to amplify the -3 to -969bp sequence upstream of the Csf2 gene. The amplified DNA was gel purified using Qiagen gel extraction reagents and phenol/chloroform extraction (Qiagen, Valencia CA). The amplified fragment was then used as template in a Big Dye PCR amplification reaction (Applied Biosystems) and sequenced using an AB capillary sequence analyzer (Applied Biosciences). ChromasLite and ClustalW freeware were used for the sequence analysis and alignment.

**Human Sequence Analysis**

Primers specific to the Csf2 promoter and Ptgs2 enhancer regions in the human genome that are homologous to the regions in the mouse genome were designed using Net Primer. Primers for the region upstream of hPTGS2 (5’GGGGCGAGTAAGGTAAAGGAAGGC; 3’ ACATTAGCGTCCCTGCAAATTCTG Sigma-Genosys) select for a region approximately 397 bp in length and include two STAT5 binding sites (33). The primers designed to amplify the human GM-CSF gene (Csf2) (5’ GTGGATTGGAAGACTTGTTGACTG; 3’ TTCACATGCTCCAGGGCT Sigma-Genosys) generate a PCR product of length 1993bp. Human DNA samples were purified using Qiagen Blood and Cell Culture DNA Mini Kit
(Qiagen, Valencia CA) and amplified by PCR using Eppendorf 2.5X Master Mix (Fisher Scientific) and either Csf2 promoter or Ptgs2 enhancer primers. The Eppendorf thermocycler program for the GM-CSF promoter amplification was 95°C 5 minutes, 55.6°C 30 seconds, 72°C 2 minutes, 25°C 30 seconds. The amplification program for the Cox2 enhancer region differs by annealing temperature and elongation time (95°C 5 minutes, 57°C 30 seconds, 72°C 30 seconds, 25°C 30 seconds). PCR amplification products were visualized on 1% agarose gel (SeaKem Fisher Scientific) and purified using DNA Clean and ConcentratorTM-5 (Zymo Research) then amplified again in a Big Dye PCR amplification reaction (Applied Biosystems) and sequenced using an AB capillary sequence analyzer (Applied Biosciences). Human sequence was analyzed and compared to consensus sequences in VectorNTI.

Table 2-1. Characteristics of patient, control and at-risk samples collected for flow cytometric and sequencing analysis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy Control (n = 24)</th>
<th>Type 1 Diabetic** Patient (n = 24)</th>
<th>At risk* Individual (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Age Range</td>
<td>4.97 – 44.89</td>
<td>6.56 – 35.1</td>
<td>7.42 – 46.35</td>
</tr>
</tbody>
</table>

* At risk individual for this study is defined as any individual with a familial history of T1D, HLA susceptibility haplotypes and/or auto-antibodies associated with T1D.
** Mean disease duration = 6.59
Figure 2-1. Chromosomes 11 and 1 on mouse strains derived through congenic breeding to isolate regions of the Idd4.3 diabetes susceptibility locus as well as the Ptgs2 enhancer region, respectively. The cluster of strains to the left is Chromosome 11 and the cluster of strains to the right reflects loci on Chromosome 1.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS lysis buffer</td>
<td>1% SDS, 0.5M EDTA, 10mM Tris; pH 8.1</td>
</tr>
<tr>
<td>Low salt buffer</td>
<td>0.1% SDS, 1%Triton-X100, 2mM EDTA, 2.4mMTris, 0.15mM NaCl; pH 8.1</td>
</tr>
<tr>
<td>High salt buffer</td>
<td>0.1% SDS, Triton –X100, 2mMEDTA, 2,4mM Tris, 0.5mM NaCl; pH 8.1</td>
</tr>
<tr>
<td>LiCl buffer</td>
<td>0.25M Lithium Chloride, 1mL IGEPAL-CA630, 1% Deoxycholic acid, 16.5mMTris, 0.5M EDTA; pH 8.1</td>
</tr>
<tr>
<td>TE buffer</td>
<td>1M Tris HCl, 0.5M EDTA; pH 8.0</td>
</tr>
</tbody>
</table>
CHAPTER 3
ACTIVATED STAT5 LEVELS IN HUMAN T1D PATIENTS, NON-AUTOIMMUNE CONTROLS AND AT-RISK INDIVIDUALS

Human Flow Cytometry

Human peripheral blood mononuclear cells from healthy controls, T1D patients and at-risk individuals were analyzed by flow cytometry to measure the levels of phosphorylated STAT5. At-risk individuals are those with first degree familial relation to a person with T1D or with measured auto-antibodies associated with T1D including those against insulin, beta cell and GAD antigen.

Consistent with 2005 reports by Litherland et al. we found that the percentage of STAT5Ptyr+/CD14+ untreated peripheral blood monocytes was elevated approximately two fold in Type 1 Diabetic patients versus non autoimmune control individuals (*p= 0.0002, Mann Whitney U test) (Figure 3-1).

Because T1D patients showed a wide range of levels of activated STAT5 (STAT5Ptyr) we wanted to determine if there was any influence of gender on levels between the sample groups. To this end we analyzed the flow cytometry data and found a statistically significant gender bias among females with T1D (*p= 0.0015, Mann Whitney U test) (Figure 3-2). This gender bias may influence the usefulness of this assay as a biomarker for T1D. Sample data was also analyzed by age; but no correlation was observed between age and STAT5Ptyr levels (data not shown).

Chromatin Immunoprecipitation (ChIP) Analysis of Human PBMC

T1D and non-autoimmune control PBMC were analyzed by Chromatin Immunoprecipitation (ChIP) to isolate and identify chromatin in the CSF2 and PTGS2 regulatory regions associated with epigenetic modifications, STAT5 binding and active transcription (Figure 3-3). GM-CSF seems to promote site specific STAT5 binding at locations where
epigenetic regulation occurs within the autoimmune T1D patients. When stimulated with GM-CSF in vitro we report enhanced STAT5Ptyr binding in autoimmune cells versus healthy control cells within the CSF2 promoter region and PTGS2 enhancer region (Figure 3-3). Our findings implicate loss of cytokine-induced suppression of epigenetic modification in non-coding regulatory regions as a mechanism for promoting the aberrant expression of genes or genetic regions (e.g., Idd loci) in autoimmune diabetes.
Figure 3-1. Flow cytometric analysis of STAT5 levels in human healthy controls, at-risk individuals and T1D patients.

Figure 3-2. Comparison of STAT5Ptyr expression in controls and T1D patients evaluated by gender.
Figure 3-3. Chromatin Immunoprecipitation (ChIP) analysis of STAT5 binding at various regions upstream of \textit{CSF2} and \textit{PTGS2} in human peripheral blood monocytes (PBMC) with and without GM-CSF stimulation. Autoimmune (AI) and healthy control (C) PBMC were cultured with (G) and without (0) GM-CSF. Real Time PCR analysis was performed on the ChIP extracts with primer sets specific for regions A, B, C and D to assess how STAT5\textsubscript{Ptyr} binding is affected by GM-CSF stimulation. Region A represents an approximately 1kb non-transcribed region upstream of \textit{CSF2}. Region B represents the enhancer region of the COX-2 gene, \textit{PTGS}. Region C encompasses a 191 bp region within the \textit{CSF2} promoter and region D represents a 115 bp region upstream of the gene.
CHAPTER 4
NOD MOUSE MODEL ANALYSIS

Increased GM-CSF Expression and STAT5 Phosphorylation in NOD Bone Marrow Cells and Peritoneal Macrophages

GM-CSF is an important contributing cytokine in both myeloid cell differentiation and in the inflammatory process mediated by mature myeloid cells. We examined GM-CSF and STAT5 levels within immature bone marrow precursor cells and mature peritoneal macrophages of autoimmune NOD mice and non-autoimmune C57BL/6 control mice. As was previously reported by the Litherland lab (13, 22, 36), NOD bone marrow cells have increased GM-CSF expression (*p=0.0450, Mann-Whitney U test) and high STAT5 phosphorylation compared to C57BL/6 mouse bone marrow cells (Figure 4-1). However, the STAT5 phosphorylation in NOD bone marrow cells was significantly lower than in more mature cells(*p= 0.399, ANOVA, Figure 4-1b), despite its comparably high GM-CSF production (Figure 4-1A).

Enhanced STAT5 Binding on the Csf2 Gene Promoter in NOD Macrophages and Bone Marrow Cells

To investigate the potential role of STAT5 in the regulation of Csf2, we performed Chromatin Immunoprecipitation (ChIP) with STAT5Ptyr antibody to examine STAT5 associated chromatin. Extracted ChIP DNA was analyzed by real time PCR using primers designed to amplify the first 1000 bp of the Csf2 promoter region (based on known de-acetylase binding sites)(33, 34). STAT5 proteins in NOD peritoneal macrophages exhibit strong binding on sequences within the Csf2 promoter region without exogenous GM-CSF stimulation. In contrast, STAT5 chromatin binding at the same sequence was decreased by 4.5 logs in C57BL/6 macrophages in real time analysis and undetectable by western blot (Figure 4-2).

STAT5 binding within the entire promoter region (-3 to -969 bp) of Csf2 upstream of the transcriptional start site and within subsets of that region was analyzed by STAT5Ptyr mediated
ChIP and real time PCR for NOD and C57BL/6 bone marrow cells (Figure 4-3A, B). Within the entire promoter region, stimulation with GM-CSF produced an overall decrease in STAT5 binding in both the NOD and C57BL/6 mice (Fig 4-3A-right). However, STAT5 binding within identified epigenetic regulatory regions of the promoter was enhanced in NOD bone marrow cells stimulated with GM-CSF compared to un-stimulated versus the C57BL/6 GM-CSF stimulated bone marrow cells which showed a decrease in STAT5 binding compared to the untreated group (Figure 4A,B). These findings suggest that GM-CSF is enhancing STAT5 binding at specific regulatory regions within the Csf2 promoter of NOD but is reducing STAT5 binding at all sites within the C57BL/6 Csf2 promoter region. These analyses do not define if STAT5 binding at these sites is synergistic or independent at each individual site.

**Sequence Analysis of Csf2 Promoter Region Defines STAT5 Binding Site Polymorphisms**

In order to examine the genetic components involved in the GM-CSF induced STAT5 binding within the Csf2 promoter region we performed a sequence analysis of this region in all congenic strains and compared in a ClustalW sequence alignment analysis (Figure 4-3). The overlapping NOD region of the B6.NODC11b and the NOD.LC11e mouse strains defines the region most likely responsible for the GM-CSF and STAT5 phenotypes seen in the NOD (enhanced GM-CSF expression (Figure 4-5) and persistent STAT5 phosphorylation (Figure 4-6)). This shared NOD.LC11b and NOD.LC11e region contains at least 2 potential STAT5 GAS binding sites (33, 34). It is this region of the Csf2 gene promoter and not the coding sequence that defines the contribution to diabetes resistance conferred on the NOD.LC11 by correction of the Idd4.3 loci on an NOD genetic background. However, insulitis in the B6.NODC11b conferred by this promoter region is minor in comparison with the NOD.L11b strain, suggesting that other components of the Idd4.3 region upstream of the Csf2 promoter may also contribute to this disease phenotype. In the more proximal region of the Csf2 promoter, 2 more STAT5
binding sites and one adjacent half site (GAA) are also altered in the NOD and found in the diabetic congenic NOD.LC11e mouse strain and corrected in NOD.LC11b strain mice which the GM-CSF and STAT5 phenotypes are normal and the diabetes disease incidence is greatly reduced.

**Bicongenic B6.NODC11bxC1tb Mice Have a Macrophage Islet Infiltration**

NOD.LC11 mice develop a strong T cell-dominant peri-islet insulitis which remains non-invasive despite having fully autoimmune phenotypes present in their T cell population [Marcie 2000]. In contrast, Pancreatic tissue was analyzed from B6.NODC11b and B6.NODC1tb mice shows very little infiltration, mainly limited to peri-ductal areas of the pancreas near islets (Figure 4-7A, B). However, an invasive infiltration of was seen in the bicongenic B6.NODC11bxC1tb male (Figure 4-7C) and female mice (Figure 4-7D). This infiltrate was devoid of T cells but showed a modest infiltrate of macrophages within the islet.

**NOD GM-CSF and STAT5 Phenotypes Segregate with the NOD CSF2 Promoter, Not the CSF2 Gene**

GM-CSF production of congenic mouse bone marrow cells (Figure 4-5A) and peritoneal macrophages (Figure 4-5B), was increased in strains that contained Csf2 promoter region from the NOD, including the NOD.LC11e mouse strain which has the NOD promoter with C57L Csf2 gene sequence, but not in strains where this region was derived from the C57BL/6 or C57L non-autoimmune strains. These data indicate that the Csf2 promoter, not its coding sequence, is responsible for GM-CSF overproduction seen in these mice and in the NOD.

The STAT5 phosphorylation phenotype was stronger in un-activated monocytes (Figure 4-6A) than in macrophages (Figure 4-6B) of the NOD.LC11e and B6.NODC11b strains compared to the NOD.LC11b or control strains. STAT5 phosphorylation levels in neither the
B6.NODC11b nor NOD.LC11e myeloid cells recreated the levels observed in the NOD mouse, suggesting some other component in or acting on the Idd4.3 region contributes to this phenotype.

The amount of STAT5 DNA binding at the Csf2 promoter in ChIP analyses was also defined by the presence of NOD sequence in this region (Figure 4-8). Moreover, exogenous GM-CSF treatment of bone marrow cells from the NOD.LC11b restores STAT5 binding within the Csf2 promoter region, but not in bone marrow from other strains containing NOD sequence in this region, and decreased STAT5 binding in bone marrow cells from NOD and strains containing NOD sequence in this region (Figure 4-8A). However, untreated ex vivo macrophages from the B6.NODC11b and the NOD.LC11e mice which containing NOD DNA in the Csf2 promoter upstream of the microsatellite insertion in the region had enhanced STAT5 DNA binding at the site (Figure 3-8B). These findings suggest that bone marrow cells require specific amount of GM-CSF stimulation to activate STAT5 binding on its own gene’s promoter and promotion of its own expression. However, over-stimulation (double the amount expressed in NOD macrophages) can block this effect.

Effects of NOD CSF2 Promoter Polymorphisms on GM-CSF Induced STAT5 Binding at the PTGS2 Enhancer

We previously reported that the high GM-CSF production of NOD and T1D human myeloid cells could enhance the PGS2/Cox2 production of these cells, even when given alone without additional activation stimuli, such as by LPS (22, 37). ChIP analysis of STAT5 binding at the Ptgs2 enhancer region showed that NOD bone marrow myeloid cells treated with GM-CSF in vitro have STAT5 bound to this sequence, while control strain myeloid cells do not (Figure 4-8A). In contrast, GM-CSF treatment of NOD peritoneal macrophages diminished the high level of STAT5 binding at the same site in untreated NOD macrophages. Furthermore, GM-CSF enhances STAT5 binding at the Ptgs2 enhancer site in control strains C57BL/6 and C57L
Figure 4-9B. ChIP analyses of the NOD chromosome 11 B6.NODC11b congenic mice which have NOD genetic sequence at the Csf2 promoter but not at the Ptgs2 region. Even though these cells had strong binding without stimulation; like in the NOD, exogenous GM-CSF treatment of bone marrow and macrophage cells did not enhance their STAT5 binding at the Ptgs2 enhancer (Figure 4-9A, B). Furthermore, macrophages but not bone marrow cells from NOD.LC11e mice that have the NOD Csf2 promoter, C57L Csf2 coding region, and NOD Chromosome 1 in their genetic make up, show enhanced STAT5 binding as seen in the NOD with GM-CSF treatment. Congenic B6.NODC1tb mice that have the Ptgs2 containing chromosome 1 region from the NOD on an otherwise C57BL/6 genetic background, show no binding of STAT5 at this site in bone marrow cells without GM-CSF treatment; but have strong STAT5 binding at the Ptgs2 enhancer in their macrophages comparable to the NOD without exogenous GM-CSF stimulation. NOD.LC11b mouse myeloid cells which have NOD chromosome 1; and therefore, NOD sequence at the Ptgs2 enhancer, do not exhibit STAT5 binding at this site until stimulated with exogenous GM-CSF in culture. These findings suggest that GM-CSF differentially regulates STAT5 interactions with chromosome 1 chromatin while influencing myeloid cell maturation and mature macrophage activation. Also, induction of STAT5 binding on Csf2 regulatory sequences on Chromosome 11 enhances this Chromosome 1 sequence interaction in the NOD.

As a confirmation that STAT5 binding may influence the interaction between these two genetic regions, the same GM-CSF, STAT5, and PGE2 phenotypes were measured in myeloid cells from sub-congenic mice bred for the NOD Ptgs2 containing region (Chromosome 1 telomeric, C1t and C1tb) on a C57BL/6 genetic background (B6.NODC1tb strain). We also looked at the phenotypes of myeloid cells from bi-congenic C57BL/6 genetic background mice that have both the NOD Ptgs2 containing-region on chromosome 1 (C1tb) and the Csf2 promoter
region on chromosome 11(C11b) (B6.NODC11bxC1tb strain) (Figures 4-2 – 4-7). Both of the B6.NODC1tb and the bi-congenic B6.NODC11bxC1tb mice strains show STAT5 binding at the Ptgs2 enhancer when stimulated with GM-CSF (Figure 4-9). However, only the bi-congenic mice had STAT5 binding at both Ptgs2 enhancer and Csf2 promoter sites (Figures 4-8 and 4-9), as well as increased STAT5 phosphorylation and enhanced GM-CSF production without GM-CSF stimulation. These results indicate that GM-CSF induced STAT5 binding at these 2 regulatory sites is associated with the expression of Csf2 and GM-CSF stimulated expression of Ptgs2 in the NOD.

We measured PGE2 production in cultures of congenic mouse bone marrow and macrophage as an indicator of COX-2 expression, and we found that PGE2 production was greatly enhanced in the NOD.LC11b and the B6.NODC11bxC1tb mice (Figure 4-10A, B). These findings are consistent with a mechanistic model where STAT5 activated by GM-CSF is binding to regulatory sequences to promote the expression of both Csf2 and Ptgs2, and suggest that polymorphisms in the Csf2 promoter region affects the abnormal regulation of both genes in the NOD.
Figure 4-1. Granulocyte Macrophage Colony Stimulating Factor Production and STAT5 Phosphorylation are aberrantly high in NOD Mouse Myeloid Cells. A) Four to five million bone marrow cells and adherence-isolated peritoneal macrophages were cultured without supplementation for 24hr at 37C/5%CO2. The p values listed were obtained from Mann-Whitney U test analysis of the data. Patterned bars indicate the mean GM-CSF production from NOD samples and open bars the mean of C57BL/6 samples. Error bars represent SEM. B) Ex vivo myeloid cells from NOD and C57BL/6 mice (peritoneal macrophages, peripheral blood, and bone marrow cells) were collected and fixed within 4hr of collection and then analyzed for phosphorylated STAT5 by intracellular flow cytometry. The p values listed were obtained from Mann-Whitney U test analysis of the data. Patterned bars indicate the mean %STAT5Pyr+/CD11b+ cells detected in NOD samples and open bars the mean of C57BL/6 samples. Error bars represent SEM. The p values listed are from pair wise (Student t or Mann Whitney U) or group wise ANOVA analyses.
Figure 4-2. Macrophage Chromatin Immunoprecipitation (ChIP) Analysis shows STAT5 binding within the promoter region upstream of the gene which encodes for GM-CSF, Csf2. Inset: Western blot analysis of STAT5 proteins isolated from ChIP assay in NOD and C57BL/6 mice. Key: 5P = ChIP anti-STAT5 precipitated DNA, T = total cellular DNA from un-precipitated fixed cell extracts, Ig = ChIP non-specific mouse IgG precipitated DNA, W = DNA-free water control. Patterned bars (log of mean R values) and gel represent data obtained from 3 independent runs of each strain. Error bars represent SEM. Published work by Federica Seydel et al. (2008) (29)
Figure 4-3. Chromatin Immunoprecipitation (ChIP) analysis of GM-CSF induced STAT5 binding upstream at multiple sites within the *Csf2* promoter involves DNA secondary structure. Four million cell cultures of NOD and C56BL/6 mouse bone marrow cells (BM) were grown for 24hr in the presence (GM or G) or absence (0) of 1000U/ml GM-CSF before being fixed and extracted for ChIP analysis. Aliquots of 100ng of total DNA extracted from ChIP protein-chromatin complexes precipitated with anti-STAT5 antibodies were amplified using primers to potential epigenetic modification sites within the *Csf2* promoter region (33, 34). A) Real time PCR analysis using specific primers to amplify and identify *Csf2* promoter regions previously identified as epigenetic control sites for *Csf2* gene expression. Non-patterned bars indicate cells without treatment (0) and hatched bars indicate cells treated with GM-CSF (G). Data
representative of 2-3 sample sets. B) Non-patterned bars indicate cells without treatment (0) and hatched bars indicate cells treated with GM-CSF (G). Data representative of two (combined A-I) and three (Promoter -3 to -969bp) sample sets. The p values indicate one-way ANOVA analysis (above graphs) or Mann-Whitney U tests (on graph) of pairwise comparisons.
Figure 4-4. Sequence Analysis of Csf2 Promoter region and definition of the STAT5 binding site polymorphisms involved in NOD myeloid cell phenotypes and chromosome 11 diabetes susceptibility. Yellow boxes represent STAT binding sites. Pink highlight is a microsatellite insertion.
Figure 4-5. The GM-CSF Production by Congenic Mouse Bone Marrow Cells and Peritoneal Macrophages. A) GM-CSF production in mouse bone marrow cells. B) GM-CSF production in mouse peritoneal macrophages.
Figure 4-6. The STAT5 Phosphorylation by Congenic Mouse Monocytes and Peritoneal Macrophages. A) Phosphorylation of STAT5 at the tyrosine residue (STAT5Ptyr) in mouse monocytes. B) STAT5Ptyr in mouse peritoneal macrophages.
Figure 4-7. Histology of Pancreas Tissues of B6.NOD and NOD.L sub-congenic mice. Congenic mouse pancreas slices were stained with H and E (A, B, C). Immunostaining was also performed against CD3 and F4/80 (D).
Figure 4-7. Continued.
Figure 4-8. Chromatin Immunoprecipitation (ChIP) analysis of STAT5 binding at the Csf2 promoter regions in congenic mice. A) Quantitative real time PCR analysis of mouse ChIP samples with and without GM-CSF stimulation in mouse bone marrow cells. B) Quantitative real time PCR of activated STAT5 binding at the Csf2 promoter in mouse peritoneal macrophages without GM-CSF stimulation.
Figure 4-9. Chromatin Immunoprecipitation (ChIP) analysis of STAT5 binding on the *Ptgs2* enhancer in GM-CSF stimulated congenic mouse bone marrow and macrophages. STAT5 binding at the *Ptgs2* enhancer in congenic mouse bone marrow cells (A) and peritoneal macrophages (B).
Figure 4-10. The PGS2/COX2 expression and PGE2 production in congenic mouse monocytes and macrophages with and without GM-CSF stimulation. A) PGE2 production in mouse monocytes. B) PGE2 production in mouse macrophages.
CHAPTER 5
HUMAN SEQUENCE ANALYSIS

Given the STAT5 binding site changes in the promoter/enhancer regions of GM-CSF and COX2 genes observed in the NOD mouse model, we postulated that the same binding site changes might occur in humans. To that end, we sequenced the promoter regions of homolog GM-CSF and COX2 genes in human type 1 diabetics (T1D) as well as healthy controls to look for STAT5 binding site changes (Figures 5-1; 5-3).

**Gene Sequence Analysis: GM-CSF (CSF2)**

Upon assembly of the sequencing contigs obtained from four T1D patients and four healthy control samples, a STAT5 binding site (TTCN3GAA) was noted at -1015 relative to the transcriptional start site. Another STAT5 binding site was located at -962 in the reverse orientation (AAGN3CTT) 53 bases downstream of the above noted STAT5 site. Considering the traditional orientation independent mechanism of enhancers (3527033), it was not surprising to find a STAT site in this context. In addition, a STAT6 binding site (TTCN4GAA) was located 73 downstream of the STAT5 site at -942 (Figure 5-2). Both the STAT5 and STAT6 binding sites are identical between the T1D, controls and the published genomic sequence.

**Gene Sequence Analysis: COX2 (PTGS2)**

We also evaluated the PTGS2 enhancer region of 10 T1D patients and 12 healthy controls. Primers specifically designed to amplify the non-transcribed region approximately 2kb upstream of the CSF2 were created based on GAS sequences (Interferon Gamma Activated Sequence) reported by Yamaoka et al. (20, 38) (Figure 5-3). Upon examining a 397bp sequence within the enhancer region of PTGS2, we confirmed the location of the GAS motif sequences and found one to be a preferential STAT5 binding site (TTCN3GAA) and the other a preferential STAT6 binding site (TTCN4GAA). The STAT5 binding site, located at -757 relative to the
transcriptional start site, is intact within the sequences of the T1D, controls and the published genomic sequence (Figure 5-4). The STAT6 binding site was observed at -713, approximately 44bp upstream of the STAT5 binding site, and was also intact (Figure 5-4).

Additionally, a C/G heterozygote polymorphism 8 bases downstream of the STAT5 binding site was observed in 3 of the analyzed samples. Of the 22 total samples analyzed, the heterozygosity was found in both T1D and control samples (Figure 5-4).
Figure 5-1. Granulocyte Macrophage Colony Stimulating Factor gene (CSF2) map. The above is a schematic representation of the promoter region upstream of the GM-CSF gene, CSF2. The location of the amplified region is delineated by forward and reverse primers, FP1,2,3 and RP1,2,3, respectively. Also defined are the STAT5 and STAT6 binding sites within the region.

Figure 5-2. Sequence alignment of the GM-CSF promoter region from healthy control and T1D DNA shows intact STAT5 and STAT6 binding sites.
Figure 5-3. Prostaglandin Synthase 2 gene (PTGS2) map. The enhancer region upstream of the COX-2 gene, PTGS2. The location of the amplified region is delineated by forward and reverse primers, FP1 and RP1, respectively. Also defined are the STAT5 and STAT6 binding sites within the region.

Figure 5-4. Sequence alignment of the COX-2 enhancer region from healthy control and T1D DNA shows intact STAT5 and STAT6 binding sites.
The inability of APC to effectively induce tolerance and regulate effector cell function is thought to play a contributing role in the immunopathology of T1D (11). Chronic inflammation and aberrant myeloid differentiation and activation may be factors in this loss of APC tolerance. The pro-inflammatory cytokine GM-CSF is required for two signals in myeloid cells: differentiation into mature monocytes, macrophages and dendritic cells, capable of tolerogenic activity, and as an inflammatory signal in mature monocytes and macrophages to activate production of pro-inflammatory cytokines and prostanoids. Along with M-CSF, GM-CSF is found in abundance at sites of inflammation and localized autoimmunity (such as in rheumatoid arthritis) (16). It has been suggested that during inflammation, GM-CSF, in concert with M-CSF and G-CSF form an important network of communication between myeloid cells and adjacent cells which may activate mature myeloid cell populations to produce pro-inflammatory mediators (37, 38). Our previous data showed that GM-CSF is expressed in abnormally high levels in NOD mice and T1D patient autoimmune myeloid cells and this subsequently activates transcription factors STAT5A and STAT5B which mediate epigenetic regulation (20, 23). Along with our finding that in the NOD mouse, GM-CSF feeds back to regulate its own gene expression, it is possible that in autoimmune cells a positive autocrine/paracrine feedback loop is established which promotes maintenance of the pro-inflammatory microenvironment characteristic of autoimmune disease.

Our ChIP binding studies within the Csf2 promoter region suggest that there is a critical level of GM-CSF stimulus needed to influence STAT5 binding within the region. We suggest that in the NOD mouse, the persistent presence of activated STAT5 and its subsequent abnormal binding at the Csf2 promoter mediate enhanced expression of the gene and increased production
of GM-CSF. B6.NOD C11b and NOD.LC11e congenic mice share an NOD-derived region within the \textit{Csf2} promoter that encompasses approximately -1kb to -250bp away from the TATA box of \textit{Csf2} gene. These mice recreate the three observed autoimmune phenotypes

A proposed mechanism for this aberrant epigenetic regulation lies in the function of STAT5 as an adapter for de-acetylase binding and activity (39). Prolonged de-acetylase activity could promote protracted assembly and stabilization of transcriptional machinery and thus enhanced transcription of \textit{Csf2} leading to over-production of GM-CSF. STAT5 binding data from the bi-congenic mouse (NOD background at both the \textit{Ptgs2} enhancer on Chr. 1 and \textit{Csf2} promoter on Chr 11) suggest that there is interaction between Chromosomes 1 and 11, mediated by GM-CSF. STAT5 binding this site affects not only GM-CSF expression, accompanied by its continued activation of STAT5, but also affects GM-CSF stimulation of \textit{Ptgs2} expression, apparently through stimulating STAT5 binding at the \textit{Ptgs2} gene enhancer. We suggest a model by which abnormal GM-CSF regulation contributes to persistent inflammation via over-production of the inflammatory mediator enzyme COX-2, with enhanced GM-CSF inflammatory cytokine production would perpetuate the pro-inflammatory cycle. In conjunction with preliminary histology data from the bi-congenic mouse, showing macrophage infiltrate into the islet in the absence of T cell infiltrate, we propose that the pro-inflammatory macrophages and monocytes may migrate into the islet tissue and create an inflammatory microenvironment that may facilitate the migration and activation of destructive effector cells. In order to further investigate this claim, comparative histology data is needed to look at the presence of macrophages in pancreatic tissue of non-autoimmune mice.

Within the NOD mouse \textit{Csf2} promoter we observed a loss of STAT6 binding site (and a potential loss of anti-inflammatory mediation through IL-4 suppression) and gains of a STAT5
binding site which may in part explain abnormal STAT5 binding within the promoter region. Homologous sites were not found in the T1D patient versus healthy control, suggesting alternate regulatory mechanisms contributing to the persistent STAT5 phosphorylation in autoimmune monocytes. However, we did observe a binding site pattern within both the Ptgs2 enhancer and Csf2 promoter in humans that consists of two STAT5 binding sites and one STAT6 binding. In order to understand how STAT family molecular interactions impact transcription of these genes and possibly other, it may be valuable to perform a genome wide assessment of this particular binding pattern.

The Ptgs2 enhancer sequence lies between the IL-10 gene and the Ptgs2, it is feasible that GM-CSF regulation of the expression of both Csf2 and Ptgs2 may be affected by the enhanced STAT5 binding at the site. A future direction for this study would be to expand sequence analysis in mouse and human samples to include the IL-10 gene as well as the non-transcribed region between the IL-10 and COX-2 genes in order to look for additional regulatory elements that may contribute to altered transcription of these two genes. An insulator sequence element, which acts to modulate gene expression between two adjacent genes with differential expression patterns, could affect transcriptional regulation when located between an enhancer and a promoter by blocking gene expression stimulated by the enhancer (40, 41). It is also possible that a microsatellite insert within the non-transcribed region could affect bi-directional enhancer binding depending on where it is added, thus changing the expression patterns of the surrounding genes.

Based on research demonstrating three shared phenotypes between human Type 1 Diabetic patients and autoimmune NOD mice; persistent STAT5 phosphorylation, COX-2 over-expression and GM-CSF over-production (20, 23), we developed a small volume “blood drop”
assay of activated STAT5 levels within human peripheral blood monocytes to determine the reproducibility of the phenotypic findings for potential use as a minimally invasive T1D biomarker.

Assay validity for T1D is uncertain for the entire population, although it may be useful in females as a biomarker for T1D or as an early indicator of autoimmunity. STAT5 is activated by estrogen and progesterone in addition to other hormones so that may be a contributing factor in the usefulness of this assay as a biomarker in studies of gender bias seen in autoimmunity.

Our studies in humans were limited to analysis of peripheral blood monocytes. Gaipa et al. reported that bone marrow precursor cells of juvenile myelomonocytic leukemia, patients exhibited elevated levels of STAT5 in response to GM-CSF stimulation (39). In future studies it may be valuable to investigate the functional assays of the project within the context of autoimmune bone marrow precursor cells and autoimmune macrophages. This may also be useful in elucidating the mechanisms driving the observed phenotypes within the NOD mouse and human T1D patient given that the human sequence data did not produce a direct homolog to the mouse binding site changes within the GM-CSF gene promoter region.
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BIOGRAPHICAL SKETCH

Erin Garrigan was born in Tarpon Springs, Florida in 1984 to Patrick and Deborah Garrigan. After attending Palm Harbor University High School in Palm Harbor, Florida she graduated from the International Baccalaureate Program in 2003, and continued on to the University of Florida.

In spring 2005, Erin joined the lab of Dr. Sally Litherland in the Department of Pathology, Immunology and Laboratory Medicine, where she worked as a research assistant until enrolling in graduate school in 2007. Erin Garrigan graduated from the University of Florida in 2007 with a Bachelor of Science degree in interdisciplinary studies with a focus on biochemistry and molecular biology and a minor in nutrition. She conducted her master’s research in Dr. Mark Atkinson’s lab under the co-mentorship of Drs. Sally Litherland and Mark Atkinson. Erin received her master’s degree in Medical Sciences from the University of Florida in December, 2008.