Functional Deficiencies of Granulocyte-Macrophage Colony Stimulating Factor and Interleukin-3 Contribute to Insulitis and Destruction of β-Cells

Short title: GM-CSF, IL-3 and Diabetes

Thomas Enzler, Silke Gillessen, Michael Dougan, James P. Allison, Donna Neuberg, Darryl A. Oble, Martin Mihm, and Glenn Dranoff

1Department of Medical Oncology, Dana-Farber Cancer Institute and Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115

2Howard Hughes Medical Institute and Program in Immunology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

3Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute and Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115

4Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

5Present address: Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, School of Medicine, University of California San Diego, La Jolla, CA 92093

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†To whom correspondence should be addressed at:

Dana-Farber Cancer Institute, Dana 520C, 44 Binney Street, Boston, MA 02115

e-mail glenn_dranoff@dfci.harvard.edu

TE, SG, MD, DAO, and MM performed research, TE, SG, MD, DN, DAO, MM, and GD analyzed data, JPA contributed a vital reagent, and TE and GD wrote the paper.

Abbreviations: type I diabetes (T1D); non-obese diabetic (NOD); granulocyte-macrophage colony stimulating factor (GM-CSF); interleukin-3 (IL-3); interferon-gamma (IFN-γ); cytotoxic T lymphocyte associated antigen-4 (CTLA-4); major histocompatibility (MHC)
Abstract

The pathogenesis of type I diabetes (T1D) involves the immune-mediated destruction of insulin producing β-cells in the pancreatic islets of Langerhans. Genetic analysis of families with a high incidence of T1D and non-obese diabetic (NOD) mice, a prototypical model of the disorder, uncovered multiple susceptibility loci, although most of the underlying immune defects remain to be delineated. Here we report that aged mice doubly deficient in granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) manifest insulitis, destruction of insulin producing β-cells, and compromised glucose homeostasis. Macrophages from mutant mice produce increased levels of p40 after LPS stimulation, whereas concurrent ablation of interferon-gamma (IFN-γ) ameliorates the disease. The administration of antibodies that block cytotoxic T lymphocyte associated antigen-4 (CTLA-4) to young mutant mice precipitates the onset of insulitis and hyperglycemia. These results, together with previous reports of impaired hematopoietic responses to GM-CSF and IL-3 in T1D patients and NOD mice, indicate that functional deficiencies of these cytokines contribute to diabetes.
Introduction

T1D is a chronic autoimmune disease in which a loss of tolerance to insulin producing β-cells in the pancreatic islets results in impaired glucose homeostasis. T1D clusters in families and is frequently associated with other autoimmune disorders, suggesting that an underlying genetic susceptibility compromises tolerance to multiple normal tissues. NOD mice are widely used as a model for T1D, as they display many similar aspects of disease pathogenesis and harbor a general predisposition to autoimmunity, which is modulated by genetic background. In NOD mice, the development of diabetes proceeds from an initial phase of insulitis, characterized by T and B cell infiltrates in the absence of β-cell damage, to an aggressive stage in which β-cells are destroyed and glucose homeostasis is disrupted.

Extensive linkage analysis of families with T1D and NOD mice yielded more than 20 genetic susceptibility loci. Among these, the major histocompatibility (MHC) class II locus exerts the most potent influence on disease development. Several non-MHC related genes have also been implicated, including insulin, CTLA-4, IL-2, CD25, the protein tyrosine phosphatase PTPN22, and the membrane transporter NRAMP-1. Nonetheless, multiple additional loci remain to be identified, although characterization of these gene products has been hampered by the large number of immune defects associated with disease and a limited understanding of the key pathogenic mechanisms.

Antigen presenting cells are thought to play an important role in the development of diabetes. Dendritic cells and macrophages contribute to the maintenance of tolerance through central deletion of auto-reactive thymocytes and the induction of recessive and dominant modes of suppression in the periphery. Among the phenotypic abnormalities
observed in T1D patients and NOD mice are the impaired responses of hematopoietic cells to GM-CSF and IL-3. Alterations in the number and/or function of dendritic cells, macrophages, and granulocytes derived from cultures of hematopoietic precursors in GM-CSF and IL-3 have been described, but the contribution of these cytokine defects to altered antigen presenting cell function in vivo and the pathogenesis of diabetes remains unclear.

We previously established roles for GM-CSF and IL-3 in the maintenance of immune homeostasis through promoting the efficient phagocytosis of apoptotic cells by macrophages. Consistent with other strains of mice that display an impaired uptake of dying cells, aged GM-CSF and, to a greater extent, GM-CSF/IL-3 doubly deficient mice developed a systemic lupus erythematosus (SLE)-like disorder characterized by anti-double stranded DNA antibodies and immune complex-mediated glomerulonephritis. Here we report that aged GM-CSF/IL-3 deficient mice also develop insulitis, destruction of insulin producing β-cells, and compromised glucose homeostasis. Similar to T1D patients and NOD mice, disease pathogenesis in this model involves p40 and CTLA-4, suggesting that functional defects in GM-CSF and IL-3 contribute to autoimmune diabetes.
Methods

Mice. GM-CSF-\textsuperscript{22}, IL-3-\textsuperscript{23}, GM-CSF/IL-3-\textsuperscript{24}, IFN-\gamma-\textsuperscript{25}, and GM-CSF/IL-3/IFN-\gamma-\textsuperscript{20} deficient mice were backcrossed at least nine generations onto the C57Bl/6 strain and housed under specific pathogen-free conditions. Genotypes were confirmed by PCR, as previously described \textsuperscript{20}. All mouse experiments were conducted under a protocol approved by the AAALAC-accredited Dana-Farber Cancer Institute IACUC.

Pathology. Pancreas were fixed in 10% buffered formalin, embedded in paraffin, cut at 5 \textmu m sections, and stained with hematoxylin and eosin. Islets were examined in 7 to 11 fields per specimen at 100X. Inflammation was evaluated as peri-insulitis and insulitis. Peri-insulitis was noted when an aggregate of lymphocytes surrounded the islet. The inflammatory infiltrates were graded as 1-3+; 1+ represented an infiltrate of less than 10 cells, 2+ an infiltrate of 10-50 cells, and 3+ an infiltrate greater than 50 mononuclear cells. Insulitis was noted when lymphocytes were present within the islets. Each islet was evaluated for necrosis as evidenced by: marked nuclear pallor and loss of nuclear content with vacuolization of the cytoplasm and ghost-like remnants of cells; or marked nuclear pyknosis surrounded by shrunken, intensely staining cytoplasmic masses.

Immunohistochemical staining for CD3, CD4, CD8, and B220 proteins was performed by an automated method on the Ventana ES immunohistochemistry instrument (Ventana Medical Systems, Inc., Tucson, AZ) utilizing an indirect biotin avidin diaminobenzidine (DAB) detection system on contiguous formalin-fixed paraffin-embedded (FFPE) 4-micron sections from a representative block in each case. Monoclonal antibodies were from BD Biosciences.
To stain for islet cell hormone production, we treated 5-µm paraffin sections with 3% H₂O₂ overnight to quench endogenous peroxide and then added goat serum for blocking. An anti-insulin mAb (Biogenex, Mu029-UC) was incubated overnight at 4°C followed by a horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (Santa Cruz, sc-2055) for one hour at room temperature. The staining was developed with the DAB detection system as above, followed by hematoxylin counterstaining. Somatostatin and glucagon immunohistochemical stainings were performed similarly using rabbit polyclonal anti-somatostatin (N1551, Dako, Carpinteria, CA), and rabbit polyclonal anti-glucagon (18-0064, Zymed, San Francisco, CA), and using the HRP-labeled goat anti-rabbit secondary Ig (sc-2054, Santa Cruz). For insulin and somatostatin double-stainings, somatostatin was detected using rabbit polyclonal anti-somatostatin Ig (N1551, Dako), HRP-labeled goat anti-rabbit polymer (K1395, DAKO), and the DAB chromagen (SK-4100, Vector), while insulin was detected with the anti-insulin monoclonal antibody (clone HB125, Biogenex), the alkaline phosphatase labeled secondary reagent (K1395, DAKO), and fast red (K0597, DAKO). Appropriate positive and negative control sections were included with each immunohistochemical staining reaction. The histological samples were analyzed using an Olympus BX41 microscope, and microscopic images were taken with a SPOT digital camera and SPOT software (Diagnostic Instruments, Sterling Heights, MI).

Blood sugar measurements. Age and sex matched mice were studied after 6 hours of fasting. Blood sugars were measured with the ExacTech R·S·G·™ testing system (Abott) using samples obtained from tail veins. For glucose tolerance tests, mice were injected intraperitoneally with a 3 g/kg of body weight glucose solution in a volume of 50 µl 0.9% NaCl
solution per 10 g of body weight. Glucose measurements from tail blood were performed at t = 0, 15, 30, 60, and 120 min.

Anti-CTLA4 antibody experiments. Supernatants from the 9H10 hybridoma were collected and purified on a HiTrap™ protein A sepharose column (Amersham Bioscience). The anti-CTLA-4 monoclonal antibodies were eluted with 0.1 M citric acid pH 3.0 and flow through was collected in 1 ml aliquots and rapidly neutralized with 100 µl 1 M Tris-Cl pH 8.0. Protein concentrations were measured by Bradford analysis (Biorad). Aliquots with the highest protein concentrations were pooled and antibody concentrations were determined by coating a MaxiSorp™ ELISA plate (Nunc) with 1:50 diluted pooled aliquots following an incubation at 4° C overnight. Hamster IgG (Pharmingen) was used for standardization. After washing, the plates were incubated with an alkaline phosphatase-conjugated goat anti-hamster Ig (Southern Biotech, Inc.) and developed with p-nitrophenyl-phosphate (BioRad). The absorbances at 405 nm were measured. 100 µg of purified anti-CTLA4 or hamster IgG monoclonal antibodies were injected into the peritoneal cavities of young female mice at the age of 12, 15, and 18 d in a volume of 200 µl 0.9% NaCl. Blood sugar measurements from tail vein blood started at d 10 after the first injection.

Cytokine measurements. 5x10^5 or 1x10^6 peritoneal macrophages were cultured in DMEM supplemented with 2 mM glutamine and 10% fetal calf serum and stimulated with 100 ng of LPS (E. coli serotype B5:055, Sigma) for 24 h. Supernatants were harvested for ELISA and pellets were used for RNA isolation (Qiagen method). For Real-time PCR, reverse transcriptions were performed with 0.5 µg of total RNA using Superscript® First Strand
Synthesis System (Invitrogen). cDNA was amplified with specific primers and output was monitored by SYBR Green (Roche) and the ABI Prism 7700 System (PE Applied Biosystems). The results were normalized to the level of cyclophilin mRNA. The primer sequences were: p40f atccagcgcaagaagaaaa, p40r ggaacgcacctttctgtta, p35f atgaagctctgcacccatt, p35r cagatagcccatcaccctg, p19f ccagcgggacatatgaatct, p19r tggatacggggcattatt, and TNF-α f acagaaagcatgatccgcg, TNF-α r gccccccatctttttggg, cyclophilinf atggtcaaccacgctgt, and cyclophilinr ttcttgctgtttgttaacttggc. Absolute data were normalized to cyclophilin for each reaction according to the formula: 2 (Power) [CT(Cyclophilin)-CT(Sample)]. For ELISAs, culture supernatants were concentrated with Centricon 25 columns (Amicon, Beverly, MA) and then analyzed with the mouse IL-12 ELISA kit (BD Biosciences) according to manufacturer’s instructions.
Results

*GM-CSF/IL-3 deficient mice develop insulitis and β-cell destruction.*

Previous studies characterized the development of pulmonary alveolar proteinosis and a SLE-like disorder in mice deficient in GM-CSF and IL-3\(^{20,22}\). Further analysis of aged mutant mice (8+ months) on the C57Bl/6 background revealed inflammatory pathology in the pancreas (figure 1 and Table 1). While wild type controls demonstrated minimal abnormalities, GM-CSF singly deficient animals manifested large lymphoid aggregates that circumscribed a significant proportion of the islets; these aggregates rarely penetrated the islets and were not associated with necrosis. Approximately 60% of the infiltrates were graded as 3+, with more than 50 mononuclear cells per reaction. IL-3 singly deficient mice\(^23\) also displayed peri-insulitis, albeit of reduced intensity.

Mice doubly deficient in GM-CSF and IL-3 showed more severe disease, with lymphocytic infiltrates extending into the islets, which in some cases evidenced necrosis of scattered single cells. Immunohistochemistry revealed the presence of both CD3\(^+\) T cells and B220\(^+\) B cells in these reactions. To determine whether this insulitis resulted in a loss of β-cells, we performed immunostaining for insulin. Compared to the wild type controls, GM-CSF/IL-3 doubly deficient mice showed a marked reduction in the size of many islets, and the total number of insulin secreting β-cells was greatly reduced. Moreover, immune mediated destruction was evidenced by the lymphocyte satellitosis of β-cells, which was accompanied by islet cell cytoplasmic swelling and nuclear degeneration. Mega-islets were not observed.

To determine whether these pathologic features were associated with abnormalities in glucose homeostasis, we measured fasting blood sugars in age-matched mice (8
months). Analysis of 30 wild type animals yielded a mean fasting glucose of 96 mg/dl, with a standard deviation of 14.3 (figure 2). The fasting sugars for IL-3 deficient mice (n=8) were comparable to wild type controls (mean 91.5 mg/dl, s.d. 5.4; p=0.21), whereas GM-CSF deficient animals (n=10) showed a modest elevation of borderline statistical significance (mean 107 mg/dl, s.d 13.9; p=0.04). In contrast, 22 of 30 GM-CSF/IL-3 doubly deficient mice demonstrated fasting hyperglycemia (mean 147 mg/dl, s.d. 10.4; p <0.0001), whereas the other eight were comparable to controls (mean 99.1 mg/dl, s.d. 11; p=0.26). Young GM-CSF/IL-3 deficient mice (one or two months) did not show fasting hyperglycemia (not shown), although additional studies are required to define the precise time frame of disease onset.

To characterize glucose homeostasis further, we administered a glucose challenge intra-peritoneally into 8 months old mice and followed blood sugar levels serially (figure 3). The maximum glucose levels achieved at 30 minutes post-infusion were comparable among wild type (n=8, mean 213 mg/dl), GM-CSF deficient (n=5, mean 256 mg/dl, p=0.59), and IL-3 deficient (n=6, mean 252 mg/dl, p=0.13) animals. However, nine of twelve GM-CSF/IL-3 deficient mice showed significantly greater peak blood sugars (mean 402 mg/dl, p=0.00008) that remained elevated at the end of the test, whereas the remaining three were comparable to controls. All of the double deficient mice with a diabetic glucose tolerance profile demonstrated an elevated fasting blood sugar. Taken together, these results indicate that a high proportion of aged GM-CSF/IL-3 deficient mice develop autoimmune diabetes. However, further work will be necessary to understand the mechanisms underlying the heterogeneity in disease development in these animals.

GM-CSF/IL-3 deficient mice show altered macrophage cytokine production
Our previous analysis of the mechanisms underlying the development of SLE in GM-CSF/IL-3 deficient mice uncovered a marked impairment in macrophage phagocytosis of apoptotic cells. A similar defect has been reported in NOD mice, raising the possibility that some macrophage abnormalities in diabetes might reflect alterations in GM-CSF and IL-3 function. In this context, several studies have shown increased macrophage production of p40 in response to LPS, and this cytokine has been mapped to the Idd4 (mouse) or IDDM18 (human) susceptibility loci. Persistently elevated NF-κB activity and compromised phagocytosis of apoptotic cells might contribute to the enhanced p40 production.

To examine whether inflammatory cytokine profiles were altered in GM-CSF/IL-3 deficient mice, we harvested peritoneal macrophages and stimulated them in vitro with 100 ng LPS for 24 hours (figure 4). Real-time PCR disclosed a marked increase in p40 transcripts of the cytokine deficient macrophages compared to wild type controls (p=0.001), using cyclophilin as a reference standard. An ELISA performed on culture supernatants confirmed the increased production of p40 protein (p=0.001). Since p40 is a subunit of the heterodimeric cytokines IL-12 and IL-23, we also examined expression levels of the unique p19 and p35 chains. Real-time PCR demonstrated increases in both transcripts compared to wild type controls (p=0.05), whereas TNF-α levels were not altered.

While recent studies have underscored a central role for IL-23 and Th-17 cells in experimental allergic encephalomyelitis and collagen-induced arthritis, previous work showed that IL-12 administration could exacerbate diabetes in NOD mice, in part through the stimulation of IFN-γ secreting Th1 cells that infiltrate the islets. Although p40 and IFN-γ deficient NOD mice display minimal alterations in the frequency or kinetics of
diabetes development\textsuperscript{36-38}, the progression to diabetes in several models is associated with increased expression of IFN-\(\gamma\) induced genes\textsuperscript{39-41}. Thus, IFN-\(\gamma\) might play an important role, although other pathways may contribute to disease development in the absence of this cytokine.

To determine whether IFN-\(\gamma\) is involved in the diabetes in GM-CSF/IL-3 deficient mice, we introgressed an IFN-\(\gamma\) null allele and thereby generated triply cytokine deficient mice on the C57Bl/6 background (figure 5). GM-CSF/IL-3/IFN-\(\gamma\) deficient mice (n=10) showed significantly reduced fasting blood sugars compared to GM-CSF/IL-3 deficient mice (\(p<0.0001\)), and these levels were similar to IFN-\(\gamma\) deficient mice and wild type controls. Moreover, glucose tolerance tests for GM-CSF/IL-3/IFN-\(\gamma\) deficient mice (n=6) were significantly improved compared to GM-CSF/IL-3 deficient animals (n=12). Mean peak glucose values were 190 mg/dl for triple knockouts compared to 402 mg/dl for double knockouts with diabetes (\(p=0.00004\)). Consistent with these metabolic parameters, pathologic examination of the pancreas of aged GM-CSF/IL-3/IFN-\(\gamma\) deficient mice failed to disclose peri-insulitis or insulitis. Interestingly, however, 2 of 16 triply deficient mice analyzed showed islet cell adenomas. Immunostaining revealed that these pancreatic lesions were primarily composed of insulin secreting \(\beta\)-cells, rather than glucagon or somatostatin producing cells, although the insulin staining was more punctate and heterogeneous than in wild type mice. These findings raise the possibility that insulin metabolism and/or islet cell homeostasis might be altered by the compound cytokine deficiency. In this context, we previously demonstrated that GM-CSF/IL-3/IFN-\(\gamma\) deficient animals succumb to a high incidence of hematologic and solid malignancies within a background of chronic infection\textsuperscript{20}. Further studies are required to define the precise impact of chronic infection and cytokine dysfunction on islet cell function.
CTLA-4 antibody blockade stimulates rapid progression to destructive insulitis in GM-CSF/IL-3 deficient mice

Whereas diabetes development in GM-CSF/IL-3 deficient mice manifests a relatively long latency, the addition of other susceptibility loci might accelerate disease pathogenesis. Indeed, recent investigations have delineated a key role for the negative immune regulator CTLA-4 in diabetes. Reduced expression of a soluble isoform of CTLA-4 in T1D patients or of a ligand-independent CTLA-4 product in NOD mice is associated with increased disease risk. Consistent with these findings, the administration of blocking antibodies to CTLA-4 to young NOD or BDC2.5 TCR transgenic mice precipitated destructive disease, although older animals were refractory to this manipulation. Moreover, lentiviral mediated RNAi knockdown of CTLA-4 similarly evoked insulitis.

In view of these data, we wondered whether disrupting CTLA-4 function might increase the kinetics of diabetes development in GM-CSF/IL-3 deficient mice, thereby uncovering a potential important interaction between cytokine deficiency and loss of negative immune regulation. We thus administered a blocking anti-CTLA-4 monoclonal antibody to 12 days old GM-CSF/IL-3 deficient animals, which do not demonstrate islet pathology at this age (figure 6). Administration of anti-CTLA-4 antibody to age matched wild type mice failed to elicit fasting hyperglycemia, in agreement with earlier reports that CTLA-4 blockade alone was insufficient to provoke diabetes. Similarly, the injection of isotype control antibody into 12 days old GM-CSF/IL-3 deficient mice did not evoke disease. In contrast, 22 of 30 young GM-CSF/IL-3-deficient mice treated with anti-CTLA-4 antibody developed fasting hyperglycemia within three weeks. Mean fasting sugars were
153 mg/dl (s.d. 19.5) for diabetic cytokine knockouts versus 99.8 mg/dl (s.d. 13.1) for anti-CTLA-4 antibody treated wild types (p<0.0001); the remaining eight treated GM-CSF/IL-3 deficient animals were comparable to controls (mean fasting glucose 102.5 mg/dl, s.d. 11). Moreover, the administration of anti-CTLA-4 antibody to four weeks old GM-CSF/IL-3 deficient mice failed to provoke disease, consistent with the narrow window of CTLA-4 function in NOD and BDC2.5 transgenic mice 44.

Pathologic examination of anti-CTLA-4 antibody treated GM-CSF/IL-3 deficient mice that developed diabetes revealed lymphocytes infiltrating the islets (figure 7). In contrast, wild type mice that received anti-CTLA-4 antibody and GM-CSF/IL-3 deficient mice that received control antibody did not show islet abnormalities. Together, these results show that defects in CTLA-4 cooperate with deficiencies of GM-CSF and IL-3 in the pathogenesis of diabetes.
Discussion

The ability of GM-CSF to enhance protective immune responses is well established. Vaccination with irradiated tumor cells engineered to secrete GM-CSF stimulates potent, specific, and long-lasting anti-tumor immunity through improved antigen presentation by dendritic cells and macrophages. Similarly, the provision of GM-CSF through pharmacologic strategies activates innate immune effectors and increases antigen-specific T and B cell responses against diverse pathogens.

The role of GM-CSF in immune regulation, however, is less clear. Transgenic mice in which GM-CSF was expressed in the stomach epithelium developed gastritis, whereas GM-CSF expression in the islets either exacerbated or inhibited diabetes, depending upon the genetic background. GM-CSF deficient mice displayed reduced susceptibility to collagen-induced arthritis and experimental allergic encephalomyelitis, which correlated with the induction of weaker auto-reactive T cell responses, albeit preserved antibody reactions. We previously reported that GM-CSF deficient and, to a greater degree, GM-CSF/IL-3 doubly deficient mice developed a SLE-like disorder with anti-double stranded DNA antibodies and immune complex deposition in the glomeruli of the kidney. The studies presented here extend these findings to demonstrate that GM-CSF/IL-3 deficient mice also manifest insulitis and compromised glucose homeostasis. Consistent with NOD mice, the pathogenesis of diabetes in this model involves p40, IFN-γ, and CTLA-4.

Many studies have documented impaired responses of hematopoietic cells from NOD mice and T1D patients to GM-CSF and IL-3. These abnormalities appear to reflect cell-intrinsic defects in myeloid cells, resulting in altered numbers or maturation of...
dendritic cells, macrophages, and granulocytes. However, the contribution of these defects, if any, to diabetes pathogenesis remained unclear. In this context, our characterization of hematopoiesis in GM-CSF/IL-3 doubly deficient mice uncovered only a mild eosinophilia that was likely due to enhanced IL-5 signaling through the βc subunit shared among GM-CSF, IL-3, and IL-5. Contact hypersensitivity reactions were markedly diminished, though, and this involved a reduction in priming hapten-specific responses.

Macrophages from GM-CSF/IL-3 deficient mice are impaired in the phagocytosis of apoptotic cells. This defect has been linked to the development of SLE in several systems, including NOD mice, which manifest a similar abnormality. The importance of efficient clearance of dying cells in diabetes has been highlighted by studies that reveal a developmental window of β-cell death that is linked to dendritic cell infiltration of islets and the transport of islet cell antigens to draining lymph nodes. Efficient capture and presentation of islet cell antigens is critical for the maintenance of anergic and regulatory T cells that protect against diabetes. Furthermore, the cross-talk between dendritic cells and CD1d-restricted NKT cells also contributes to tolerance, and it is thus noteworthy that optimal dendritic cell activation stimulated by GM-CSF secreting tumor cells similarly requires CD1d-restricted NKT cells.

Defects in the uptake of dying cells by macrophages might contribute to autoimmune disease through the decreased production of immunoregulatory cytokines such as TGF-β and the increased production of pro-inflammatory cytokines such as IL-12. Indeed, the production of p40 following stimulation of macrophages from GM-CSF/IL-3 deficient and NOD mice was markedly increased, and these findings are consistent with the identification of p40 as a disease susceptibility locus in NOD mice and
possibly T1D patients \textsuperscript{27,28}. Additional studies are required to determine whether these defects impact thymic selection.

Characterization of cytokine profiles in stimulated GM-CSF/IL-3 deficient macrophages revealed elevated levels of both IL-12 and IL-23. While recent work has shown that IL-23 plays a major role in the inflammatory pathology of collagen-induced arthritis and experimental allergic encephalomyelitis \textsuperscript{32,33}, earlier studies demonstrated that the administration of IL-12 to NOD mice exacerbated the disease \textsuperscript{34,35}. The effects of IL-12 involved the stimulation of IFN-\(\gamma\) producing Th1 cells, although the cytokine also worsened disease in IFN-\(\gamma\) deficient NOD mice through additional cytotoxic mechanisms that included Fas-ligand. This redundancy of function may help explain why IFN-\(\gamma\) deficient NOD mice display only minor differences in disease development compared to wild type mice \textsuperscript{37,38}. Indeed, IFN-\(\gamma\) target genes are highly up-regulated during the progression from benign to destructive insulitis triggered by cyclophosphamide or CTLA-4 antibody blockade \textsuperscript{39-41}. Our finding that IFN-\(\gamma\) is required for insulitis and compromised glucose homeostasis in GM-CSF/IL-3 deficient mice supports the idea that IFN-\(\gamma\) regulated pathways are important in autoimmune pathology \textsuperscript{60}. Moreover, the development of islet cell adenomas, but not insulitis in some GM-CSF/IL-3/IFN-\(\gamma\) deficient mice raises the possibility that IFN-\(\gamma\) might also participate in islet cell growth control. In this context, the distinction between islet cell adenoma and hyperplasia is based upon pathologic criteria of size (greater than 2 mm) and alterations in cell morphology. Additional studies are required to clarify the molecular mechanisms underlying these alterations.

Consistent with the identification of CTLA-4 as a disease susceptibility locus in NOD mice and possibly T1D patients \textsuperscript{42}, we found that administration of anti-CTLA-4
blocking antibodies to young GM-CSF/IL-3 deficient mice precipitated diabetes in a high proportion of animals. As in the BDC2.5 transgenic TCR model, CTLA-4 blockade was only active within a narrow time interval \textsuperscript{43,44}. Studies of the BDC2.5 mice indicated that the CTLA-4 effect was restricted to the initial contact of naïve T cells with antigen loaded dendritic cells in the draining lymph node; once insulitis was established, CTLA-4 blockade was inactive. Further studies are necessary to delineate whether a similar mechanism accounts for the therapeutic window in GM-CSF/IL-3 deficient mice. It will also be of interest to examine the impact of blocking antibodies to PD-1 in this model, particularly since IFN-γ is a potent stimulus for PD-L1, which can be expressed at high levels in the islets \textsuperscript{61-63}.

Taken together, the studies presented here indicate that functional deficiencies of GM-CSF and IL-3 contribute to diabetes. The more severe pathology observed in the doubly deficient mice compared with the single knockouts likely reflects some redundancy in signaling through the shared βc subunit \textsuperscript{24}. Since macrophages from NOD mice and T1D patients show increased GM-CSF production \textsuperscript{18,19}, gene products involved in cytokine receptor signaling or downstream targets are candidates for disease susceptibility loci. Indeed, alterations in STAT5 activity have been reported in T1D patients and NOD mice \textsuperscript{18,19,64}. It is tempting to speculate that gene products involved in the defective phagocytosis of apoptotic cells by GM-CSF/IL-3 deficient macrophages may be critical determinants of autoimmunity.
Acknowledgments

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Table 1. Pathologic analysis of insulitis in GM-CSF and IL-3 deficient mice

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Figure Legends

Figure 1. GM-CSF/IL-3 deficient mice develop insulitis. (A). Wild type mouse with normal islets, original magnification X 100. (B). Insulitis and peri-insulitis in GM-CSF/IL-3 deficient mouse, original magnification X 200. (C). B220⁺ B cells in the islets of GM-CSF/IL-3 deficient mouse, original magnification X 400. (D). CD3⁺ T cells in the islets of GM-CSF/IL-3 deficient mouse, original magnification X 400. (E). Insulin producing β-cells in wild type mouse, original magnification X 200. (F). Decreased insulin producing β-cells in GM-CSF/IL-3 deficient mouse, original magnification X 200. (G). Insulitis with β-cell destruction in GM-CSF/IL-3 deficient mouse, original magnification X 400.

Figure 2. Aged GM-CSF/IL-3 deficient mice develop fasting hyperglycemia. Blood sugars were determined for wild type (n=30), GM-CSF⁻/⁻ (n=10), IL-3⁻/⁻ (n=8), and GM-CSF/IL-3⁻/⁻ (n=30) mice. 22 of 30 GM-CSF/IL-3 doubly deficient mice showed fasting hyperglycemia compared to controls (p <0.0001, Wilcoxon rank sum test).

Figure 3. GM-CSF/IL-3 deficient mice show abnormal glucose tolerance tests. Wild type (n=8), GM-CSF deficient (n=5), IL-3 deficient (n=6), and GM-CSF/IL-3 deficient (n=12) mice were injected intra-peritoneally with a glucose challenge and tail bloods were serially analyzed. Nine GM-CSF/IL-3 deficient mice showed significantly greater peak blood sugars that remained elevated at the end of the test compared to all other groups (p=0.00008, Wilcoxon rank sum test).
Figure 4. GM-CSF/IL-3 deficient macrophages produce increased inflammatory cytokines in response to LPS stimulation. (A). Real-time PCR analysis shows increased levels of p19, p35, and p40 in GM-CSF/IL-3 deficient macrophages compared to wild type controls. (B). GM-CSF/IL-3 deficient macrophages secrete elevated levels of p40 protein compared to wild type macrophages. Shown are three animals per group. Similar results were observed in three independent experiments.

Figure 5. IFN-γ is required for diabetes in GM-CSF/IL-3 deficient mice. (A). Fasting blood sugars were determined for IFN-γ deficient (n=8) and GM-CSF/IL-3/IFN-γ deficient (n=10) mice and compared to GM-CSF/IL-3 deficient mice shown in figure 2. GM-CSF/IL-3/IFN-γ deficient animals showed significantly reduced fasting blood sugars compared to GM-CSF/IL-3 deficient mice (p<0.0001). (B). Glucose tolerance tests were performed on GM-CSF/IL-3/IFN-γ deficient mice (n=6). Peak glucose levels were significantly reduced compared to GM-CSF/IL-3 deficient animals shown in figure 3 (p=0.00004). (C). Absence of insulitis in a GM-CSF/IL-3/IFN-γ deficient mouse, original magnification X 200. (D). Islet cell adenomas in GM-CSF/IL-3/IFN-γ deficient mouse, original magnification X 200. (E). Insulin (red) and somatostatin (brown) staining in adenoma in GM-CSF/IL-3/IFN-γ deficient mouse, original magnification X 400.

Figure 6. CTLA-4 antibody blockade precipitates diabetes in young GM-CSF/IL-3 deficient mice. (A). Time course of disease development. GM-CSF/IL-3 deficient (n=30) or wild type mice (n=25) were injected intra-peritoneally with 100 µg of anti-CTLA-4 antibody (clone 9H10) three times as indicated (arrows). A cohort of GM-CSF/IL-3 deficient mice (n=15) was similarly treated with isotype-matched IgG control antibody.
Fasting blood sugars were determined 50 days after the initiation of antibody injections. 22 of 30 treated GM-CSF/IL-3 deficient mice developed fasting hyperglycemia (p<0.0001 versus all other groups).

**Figure 7.** Anti-CTLA-4 antibody triggers insulitis in young GM-CSF/IL-3 deficient mice. (A). Normal islets in a wild type mouse treated with anti-CTLA-4 mAb, original magnification X 60. (B). Normal islets in a young GM-CSF/IL-3 deficient mouse treated with control Ig, original magnification X 200 (C). Insulitis in a young GM-CSF/IL-3 deficient mouse treated with anti-CTLA-4 mAb, original magnification X 400.
Functional deficiencies of granulocyte-macrophage colony stimulating factor and interleukin-3 contribute to insulitis and destruction of β-cells

Thomas Enzler, Silke Gillessen, Michael Dougan, James P Allison, Donna Neuberg, Darryl A. Oble, Martin Mihm and Glenn Dranoff