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Research In-Press Preview **Metabolism**

Imatinib (Gleevec) reverses type 1 diabetes (T1D) in NOD mice and is currently in clinical trials in individuals with recent-onset disease. While research has demonstrated that imatinib protects islet β cells from the harmful effects of ER stress, the role the immune system plays in its reversal of T1D has been less well understood, and specific cellular immune targets have not been identified. In this study, we demonstrate that B lymphocytes, an immune subset that normally drives diabetes pathology, are unexpectedly required for reversal of hyperglycemia in NOD mice treated with imatinib. In the presence of B lymphocytes, reversal was linked to an increase in serum insulin concentration, but not an increase in islet β cell mass or proliferation. However, improved β cell function was reflected by a partial recovery of MafA transcription factor expression, a sensitive marker of islet β cell stress that is important to adult β cell function. Imatinib treatment was found to increase the antioxidant capacity of B lymphocytes, improving reactive oxygen species (ROS) handling in NOD islets. This study reveals a novel mechanism through which imatinib enables B lymphocytes to orchestrate functional recovery of T1D β cells.

B lymphocytes protect islet beta cells in diabetes prone NOD mice treated with imatinib

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Conflict of Interest
The authors declare no conflict of interest.

Abstract

Imatinib (Gleevec) reverses type 1 diabetes (T1D) in NOD mice and is currently in clinical trials in individuals with recent-onset disease. While research has demonstrated that imatinib protects islet beta cells from the harmful effects of ER stress, the role the immune system plays in its reversal of T1D has been less well understood, and specific cellular immune targets have not been identified. In this study, we demonstrate that B lymphocytes, an immune subset that normally drives diabetes pathology, are unexpectedly required for reversal of hyperglycemia in NOD mice treated with imatinib. In the presence of B lymphocytes, reversal was linked to an increase in serum insulin concentration, but not an increase in islet beta cell mass or proliferation. However, improved beta cell function was reflected by a partial recovery of MafA transcription factor expression, a sensitive marker of islet beta cell stress that is important to adult beta cell function. Imatinib treatment was found to increase the antioxidant capacity of B lymphocytes, improving reactive oxygen species (ROS) handling in NOD islets. This study reveals a novel mechanism through which imatinib enables B lymphocytes to orchestrate functional recovery of T1D beta cells.

Introduction

T1D results from an absolute insufficiency of insulin, the hormone exclusively produced by islet beta cells to promote glucose uptake and storage in peripheral tissues. In the course of disease, adverse interactions between immune cells and islet beta cells lead to almost complete loss of beta cell mass in T1D. Successful strategies to ameliorate autoimmune destruction need to target both the immune system and islet cells, and identifying such strategies remains an important therapeutic goal. Imatinib (Gleevec) improves insulin responsiveness in the NOD animal model of T1D and is presently being evaluated in an ongoing phase 2 clinical trial in early onset T1D patients (NCT01781975)(1). Although the mechanism of action is still unclear, imatinib appears to act by reducing stress levels in islet beta cells, which are proposed to be created in the islet under hyperglycemic and immune-infiltrating conditions (2–6). In the NOD mouse the c-Abl tyrosine kinase promotes beta cell ER stress by enhancing IRE1-a activity, which drives the unfolded protein response leading to ER stress and ultimately beta cell death. Imatinib is thought to impinge on this process by inhibiting c-Abl, thus preventing beta cell death (6). Although inhibiting c-Abl signaling through imatinib therapy leads to reduced beta cell stress, no immune requirement has been identified for successful treatment.

While the preservation of islet beta cell function is a vital component of T1D treatment, it is unlikely to be sustained unless simultaneously targeting the underlying immune dysfunction. Prior studies have focused on the effects of imatinib on innate immune cells and T lymphocytes but it seems that these mechanisms are not at work in diabetes reversal (1, 7–12). B lymphocytes are also a critical immune target of imatinib in some clinical trials reported in oncology (13). This observation led us to hypothesize that B lymphocytes could also be immune targets of imatinib in diabetes treatment. This hypothesis is bolstered by the central antigen-presenting role that B lymphocytes play in T1D pathogenesis (14–21). In fact, the action of B lymphocytes is so pivotal to T1D development that autoantibodies produced by autoreactive B lymphocytes now define the earliest stage of the disease (termed Stage 1), which precedes the occurrence of hyperglycemia (22).

Our investigation demonstrates that imatinib improves NOD islet beta cell activity by elevating the anti-oxidant capacity of B lymphocytes that surround and invade the islets. The amelioration of oxidant stress allows the remaining islet beta cells to functionally recover, as revealed in part by improved expression of MafA, a redox sensitive and functionally important beta cell enriched transcription factor. This study defines a new inducible mechanism of tissue-protection by B lymphocytes, which may lead to novel therapeutic targets and opportunities to promote tissue recovery from autoimmune attack.

Results

Imatinib targets B lymphocytes that are essential for diabetes reversal in NOD mice

To investigate the hypothesis that imatinib targets B lymphocytes in T1D, we analyzed splenocytes isolated from imatinib-treated and untreated prediabetic NOD mice. These mice were treated with imatinib mesylate (1.5mg/mouse) or saline for 7 days via intraperitoneal injection. A significant decrease in total splenocyte numbers was found in imatinib-treated mice, with B lymphocytes accounting for the majority of the lost cell numbers **(Figure 1A and B)**. In contrast, no significant effect on T lymphocytes was observed, as previously reported **(Figure 1B)** (1). Additionally, imatinib treatment led to preferential apoptosis of B lymphocytes in *ex vivo* assays **(Supplemental Figure 1A)**. Because imatinib only partially depleted the B lymphocyte population, we considered that its protective effect resulted from either depleting auto-aggressive B lymphocytes or by enriching those producing a beneficial function among the remaining cells. We first assessed the capacity of imatinib to deplete insulin reactive B lymphocytes in NOD.VH125 mice that possess a small population of B cells that can bind insulin. We observed no significant decrease in insulin-reactive B lymphocytes (**not shown).** Consequently, we evaluated the ability of imatinib to reverse diabetes in an animal devoid of B lymphocytes, to determine whether imatinib induced a protective effect in B lymphocytes to reverse disease.

To examine the role of B lymphocytes in diabetes reversal, we utilized an immune cell transfer model of diabetes induction. In this model splenocytes were removed from a diabetic NOD WT donor mouse and depleted of all B lymphocytes via MACS positive selection (B220+). The B lymphocyte negative fraction was then transferred into NODRag1$^{-/-}$ mice, which lack an adaptive immune system and develop diabetes only when donor NOD immune cells are transferred (**Figure 1C).** Blood glucose was monitored daily and mice were enrolled in one treatment group after two blood glucose readings of >200 mg/dl. Mice were then randomly assigned to imatinib therapy (1.5mg/mouse), imatinib therapy plus transfer $20x10^6$ splenocytes that were B lymphocyte-depleted, or imatinib plus transfer of $20x10^6$ B lymphocytes (purified by B220$^+$ MACS selection) from prediabetic NOD mice. As we found that normalization of blood glucose occurred very early in NOD WT mice reversed by imatinib **(Supplemental Figure 2A and B)**, we assessed diabetes reversal after 4 days on

therapy. Only mice that received B lymphocytes reversed diabetes; none of the imatinib-treated mice without B lymphocytes demonstrated diabetes reversal **(Figure 1D, and Supplemental 2C)**. As imatinib targets the c-Abl kinase, we next assessed the c-Abl signaling system in NOD B lymphocytes to determine whether these cells possessed a signaling profile that sensitizes them to imatinib therapy.

B lymphocytes in NOD mice have altered c-Abl signaling

Imatinib exhibits enhanced preference and inhibitory potency against the inactive form of c-Abl (23). The active conformation of c-Abl is induced by phosphorylation at tyrosine-residue 412 (Y412). Since hyper-phosphorylation of this residue imparts imatinib resistance in many BCR-ABL driven cancers (24, 25), we hypothesized that reduced phosphorylation in B lymphocytes could explain increased sensitivity to apoptosis to imatinib in NOD mice. The B lymphocyte compartment is composed of multiple cell subsets, many of which contribute to T1D pathology and can respond differentially to imatinib therapy; for this reason we assessed c-Abl signaling in a cell subset specific manner utilizing phosphoflow cytometry (13, 14, 16, 21, 26–30). In B lymphocytes c-Abl is phosphorylated downstream of the B-cell receptor (BCR) and CD19 signaling complex (31). Therefore, we assessed c-Abl phosphorylation (Y412) after stimulation of the BCR (anti-IgM Fab_2) in both NOD and C57BL/6J (B6) mice.

Analysis of c-Abl phosphorylation revealed decreased c-Abl signaling in transitional B lymphocytes in NOD mice as compared to B6 mice (**Figure 2A, 2B, and Supplemental Figure 3A)**. Further analysis confirmed c-Abl pathway alterations in transitional B cells, as downstream proteins CrkL and Protein kinase c delta had reduced phosphorylation in comparison to marginal zone B lymphocytes **(Supplemental Figure 3B and not shown)**. In contrast, c-Abl phosphorylation levels were similar in the marginal zone and marginal zone precursor cells. Follicular and immature B lymphocyte subsets demonstrated a lower capacity to signal through c-Abl in both strains, a potential indication that reduced c-Abl activity leaves these subsets sensitized to imatinib (**Figure 2C and Supplemental 3A and B**). These data suggest that imatinib may differentially affect c-Abl activity in B lymphocyte subsets.

Imatinib preferentially depletes B lymphocyte subsets with reduced c-Abl signaling

Having identified that c-Abl signaling was lowest in follicular and transitional subsets, we hypothesized that these subsets would be preferentially depleted by imatinib therapy in NOD mice. We utilized flow cytometry to analyze the B lymphocyte compartment before and after imatinib therapy. Utilizing B220, IgM, CD19, CD21 and CD23, we were able to subset the splenic B lymphocyte compartment similarly to previous reports (32). Analysis of mature B lymphocytes revealed preferential depletion of follicular B lymphocytes while marginal zone B lymphocytes and their precursor population remained intact (**Figures 3A, B, C**). Immature splenic B lymphocytes demonstrated preferential loss of transitional 1 and transitional 2 B lymphocytes while transitional 3 B lymphocytes, largely thought to be an anergic population of B lymphocytes, were not impacted (**Figures 3D, 3E**). In agreement with our signaling data, only those cells with the lowest c-Abl signaling activity were robustly depleted (**compare Figure 2A to Fig 3**). Nonetheless, depletion was incomplete and we hypothesized the remaining B lymphocytes may have resistance to imatinib therapy, a phenomenon characteristic of immune cells treated with imatinib in cancer studies (33–35). These alterations in B lymphocyte function could impact beta cell health in the islet as B lymphocytes represent the most prominent component of the cellular islet infiltrate in NOD mice (36).

Imatinib restores beta cell function only in the presence of B lymphocytes

To understand how the islet cell population was impacted in imatinib-mediated, B lymphocyte-dependent reversal of diabetes, we analyzed beta cells in imatinib-treated NODRag1$^{-/-}$ mice in the presence or absence of B lymphocytes. We found that 4 days following imatinib treatment, neither the beta cell nor alpha cell area appeared changed (**Figure 4A, 4B and 4C**). Moreover, there was no obvious change in Pax6+ endocrine cells, proliferating insulin+ Ki67+ cells, or apoptotic insulin+ TUNEL+ cells (**Supplemental 4A**). Collectively, these results suggested that the rapid and robust improvement in glycemia in imatinib-treated mice with B lymphocytes reflected improved islet beta cell function, as further indicated by the relatively elevated serum insulin levels in fasted mice and relatively unchanged glucagon levels (**Figures 4D and Supplemental 4B**).

Increased levels of ROS have been shown to deleteriously impact islet beta cell function, in part, through reductions in beta cell-enriched transcription factors important in maintaining beta cell function. To understand the role B lymphocytes play in modulating beta cell function in imatinib therapy, we analyzed by immunofluorescence for markers of beta cell identity, function, and maturation in non-diabetic control mice, newly diabetic NOD mice, and NOD mice treated with imatinib with or without B lymphocytes (37–42). Levels of the Pdx1, Nkx6.1, and Nkx2.2 transcription factors (**Supplemental Figure 5A**) appear unchanged in all conditions, whereas MafA was significantly reduced in newly diabetic and B lymphocyte deficient NODRag1$^{-/-}$ islet beta cells. Strikingly, MafA levels were partially recovered in insulin positive cells of NODRag1$^{-/-}$ mice when imatinib therapy included B lymphocytes (**Figure 4E and 4F**). MafA, a potent insulin-driving transcription factor important to adult beta cell function, is extremely sensitive to increased levels of oxidative stress (43). In contrast to MafA, Ucn3 (urocortin 3), a mature beta cell marker and hormone co-packaged within insulin secretory granules, did not recover following imatinib treatment (**Supplemental Figure 5B**) (37, 42, 44–46). The recovery of MafA and improvement in blood glucose homeostasis suggests that B lymphocytes act to relieve oxidant stress on beta cells, as observed in hematologic cells treated with imatinib but previously unrecognized in tissue recovery from autoimmune attack (33).

B lymphocytes acquire enhanced ROS antioxidant capacity after imatinib therapy.

Resistance to the effects of imatinib is a common problem encountered in the treatment of hematologic malignancies and is often associated with increased ROS handling proteins, including SODs and the glutathione system (33–35). We hypothesized that the remaining B lymphocytes enhance ROS handling to promote beta cell recovery of ROS-sensitive transcription factor MafA following imatinib treatment. Although we observed no increase in the uptake of cystine, a precursor to glutathione assembly and a primary mechanism of imatinib-induced ROS regulation (**Supplemental Figure 6A**) (47), we did find a modest increase in SOD2 (i.e. the manganese dependent superoxide dismutase) in both splenic and pancreatic B lymphocytes **(Supplemental 6B-G),** which reflects an activation of the B lymphocyte program to handle ROS at both sites. Interestingly, SOD2

expression was highest in the marginal zone at baseline, a potential second mechanism for their resistance to imatinib-induced depletion, whereas the transitional and follicular B lymphocytes demonstrated an increase in SOD2 after imatinib therapy **(Supplemental 6D**). While this increase in SOD2 was modest and would not likely account for beta cell recovery by itself, we hypothesized that this change may be a sign of an improved overall ROS handling system in B lymphocytes, leading to a change in their antioxidant capacity.

To assess whether a change in overall ROS handling was induced by imatinib treatment, a cellular dye (H2DCFDA) that fluoresces with increasing levels of intracellular ROS was used. A robust decrease in intracellular ROS was found in the B lymphocytes of NOD mice treated with imatinib (**Figure 5A and B).** Previous studies have demonstrated that lymphocytes can release antioxidant proteins to handle increased oxidative stress and is a mode by which B lymphocytes could provide antioxidant capacity to the surrounding beta cell microenvironment (48). Assaying cell supernatants from B lymphocytes incubated in media alone or in the presence of imatinib in the Hydroxyl Radical Antioxidant Capacity (HORAC) activity assay revealed that imatinib-treatment increased ROS-neutralizing capacity in the media (**Figure 5C and D).** These results explain how islet-invading B lymphocytes could extend tissue protection to nearby islet beta cells following imatinib treatment.

Discussion

Imatinib is an attractive therapeutic for T1D that can be delivered orally on an outpatient basis and has the potential to both promote beta cell recovery and establish longer lasting beta cell protection from the immune system. Here we have uncovered a novel, inducible interaction between the immune system and beta cells, which allows the immune system to facilitate beta cell recovery. We have determined that imatinib partially depletes follicular mature and immature B lymphocyte populations and induces the remaining B lymphocytes to enhance their antioxidant capacity including secreted anti-oxidants. Beta cells in the presence of these imatinib-treated B lymphocytes have partial restoration of MafA, a previously unobserved effect of imatinib within islet beta cells. Overall our data suggest a model in which local B lymphocytes under the influence of imatinib create a shift in oxidative stress that allows immediate beta cell recovery and leads to functional islet preservation.

Most studies in NOD mice have demonstrated B lymphocytes are deleterious to beta cell health through priming of T lymphocytes to mediate beta cell death (14–18, 21). While the activity of B lymphocytes is central to the progression of T1D, this study illustrates a treatment that repurposes B lymphocytes to deliver islet protective function, a novel finding of our data. Anti-islet autoantibodies have long been recognized as the signature of new onset disease. More recently, our understanding of these autoantibodies has evolved to reveal that they are present years to decades before the onset of dysglycemia and hence can be used to predict and even diagnose diabetes well in advance of clinical signs (22). These established clinical features are understood mechanistically in the NOD mouse model, where B lymphocytes are required for disease pathogenesis (16). In this model, islet-reactive B cells activate destructive T lymphocytes (14). These B lymphocytes are in fact required as antigen presenting cells and diabetes cannot proceed in their absence. Clinically, targeted B lymphocyte depletion has delayed loss of insulin C-peptide in patients with new onset disease, but unfortunately disease progression returns once B lymphocytes repopulate (18–20, 49). Unlike current B lymphocyte directed therapies, imatinib reprograms B lymphocytes with putative roles in pathology to instead orchestrate beta cell protection.

In our investigation, we found B lymphocytes that were most affected by imatinib possessed a reduction in the capacity to phosphorylate c-Abl at Y412, which favors a conformation amenable to imatinib binding. These cells were potently depleted, especially at the transitional zone stage where autoreactive cells emerge to maturity and become capacitated to participate in the immune response. The cells that remained after imatinib treatment had enhanced redox-handling capacity as revealed by their ability to neutralize oxidative challenge. Further evidence of change in their redox handling system was observed both in the form of expression of MnSOD (SOD2), which was seen in both spleen and pancreas, and in the ability to secrete antioxidants, thereby neutralizing local oxidants. Interestingly, some forms of MnSOD can be secreted, though we did not specifically assess that here as there may be multiple factors contributing to the enhanced redox capacity (50). Further investigation is needed to determine what proteins and signaling pathways are necessary for this ROS response in NOD mice after imatinib treatment. The capacity for B lymphocytes to respond to imatinib by upregulating the redox system has been well established in the cancer literature where imatinib is used for treatment of malignancies including B cell acute lymphoblastic leukemia (51). We did not observe any clear features of enhanced ROS production in B lymphocytes following imatinib though this may have occurred prior to our measurements. The observed decrease in local ROS availability may also limit further T cell activation as ROS can enhance T cell activation in an ongoing immune response(52, 53).

The beta cell is exquisitely sensitive to oxidative stress and has little of its own internal defense mechanisms to prevent oxidative injury (54–56). The MafA transcription factor is a very sensitive target of this oxidative stress, with levels being substantially reduced in T1D and T2D islets (37, 43, 57). Here we observed a significant reduction in MafA+ insulin+ islet cells of hyperglycemic NOD mice, which was partially reversed in animals under imatinib therapy in the presence of B lymphocytes. This finding adds new insight into the previously established effects of imatinib at the level of the beta cell, which have focused more on the alleviation of ER stress and prevention of apoptosis (1, 6). Prior studies performed *in vitro* have established that imatinib can enhance insulin production through increasing levels of Nkx2.2 (5). We did not observe an increase in Nkx2.2

levels *in vivo*; however, we did similarly observe improved insulin levels following recovery. Imatinib has previously been shown to significantly reduced ER stress-mediated beta cell apoptosis in NOD islets prior to diabetes diagnosis (6). Here we did not observe a profound change in beta cell apoptosis, which could be due to the relatively late time-point of assessment, after NOD mice were hyperglycemic. We predict, however, that the residual beta cells remaining, upon initiation of imatinib therapy, recover their functional status due to the enhanced ROS handling capacity of the pancreatic B lymphocytes and improvement in MafA expression.

The translation of Gleevec (imatinib) to human clinical trials represents an exciting opportunity for utilization of an FDA approved drug that could be rapidly approved for use in humans with T1D. Our studies indicate a need to analyze the immune compartment with a focus on B lymphocytes in clinical studies and highlight a novel interaction between immune and beta cells. It is important to point out that our data indicate altered c-Abl signaling in B lymphocytes from NOD mice at baseline that could play a role in the predisposition of these cells to gain ROS regulatory capacity. Our data suggest that NOD B lymphocytes are especially sensitive to imatinib therapy (**Supplemental Figure 1A**), but it is unclear whether the same is true in humans with T1D. However it is likely to be efficacious since clinical data suggest that the ROS system is a universal target of imatinib in hematologic cells (33–35). As mounting evidence indicates T1D is a heterogenous disease, the cellular antioxidant response to imatinib may represent an important biomarker for predicting efficacy of imatinib therapy in patients with T1D.

Methods

Animals
C57BL6/J (B6), NOD/ShiLtJ (NOD), and immunodeficient NODRag1$^{-/-}$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were housed in a specific-pathogen–free facility at Vanderbilt University. B cell deficient NOD.μMT mice were a gift from David Serreze (Jackson Labs, Bar Harbor, ME).

Imatinib Treatment and Diabetes Reversal
Imatinib Mesylate (Eton Biosciences) was dissolved at a concentration of 1.5mgs/100ul of saline. As the original studies in NOD mice utilized gavage to deliver Gleevec (imatinib) suspended in peanut oil, we first determined whether imatinib delivered via i.p. was equally effective at reversing T1D (1). We determined that it was highly effective in reversing diabetes in newly diabetic NOD mice (2 consecutive blood glucose readings >200 mg/dl) **(Supplemental Figure 2A and B)**. In initial studies 100uls of Imatinib was injected into diabetic NOD mice for up to 3 weeks. For studies involving cellular analysis Imatinib was injected for 5 days at a concentration of 1.5mg/100uls. NODRag1$^{-/-}$ mice were given 10x10^6 splenocytes depleted of B220$^+$ cells by MACS (Miltenyi 130-049-501). Once diabetic these mice were given 1.5mg of Imatinib in 100uls of saline alone or concurrently with B lymphocytes (20x10^6), purified via B220$^+$ selection.

Flow Cytometry
Splenocytes or Pancreatic lymphocytes were stained with the following fluorophore-conjugated antibodies: B220 (RA3-6B2), CD43 (S7), CD86 (GL1), CD40 (HM40-3), CD80, CD69 (H1.2F3), CD4 (RM4-5), CD8a (53-6.7), CD25 (7D4), IgM (II/41) purchased from BD Biosciences (San Jose, CA) and CD21 (7G6), CD23 (B3B4) purchased from eBioscience (San Diego,CA). For intracellular staining Sod1 (Abcam Polyclonal), Sod2 (Abcam Polyclonal), and c-Abl Y412 (247C7 Cell Signaling Technologies Danvers, MA) were utilized. In the case on intracellular staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer set (#00-5523-00 eBioscience) according to manufacturer's specifications. Cellular ROS was detected using H2DCFDA (ThermoFisher) according to manufacturer's recommendation. Cystine conjugated to FITC was stained as previously described.

Phosphoflow measurement of c-Abl (Y412)
Total splenocytes were isolated from NOD and C57Bl/6J mice. These splenocytes were rested for 30 mins at 37 degrees C in DMEM culture media (10% FCS, Pen/Strep, and 2ME). Following a rest, the cells were stimulated with an equal volume of media containing 10ug/ml of anti-mouse IgM (Jackson ImmunoResearch). At the end of the time course cells were fixed with 1.5% PFA at room temperature for 10 mins then washed and stained with extracellular subsets markers. Cells were then permeabilized with 0.1% Triton before being stained for phospho c-Abl.

Tissue Preparation and Immunostaining
Pancreata were fixed in 4% (vol./vol.) paraformaldehyde, embedded in OCT, and cut to 6μm. Sections were blocked with 5% (vol./vol.) normal donkey serum in 0.5% BSA/PBS (wt/vol.) and incubated with primary antibodies overnight at 4°C. Cyanine dye (Cy)2-, Cy3-, or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, 1:2,000) were used for fluorescent detection. DAPI (diamidino-2-phenylindole) dye (SouthernBiotech) was used to detect nuclei in immunofluorescent images. The following primary antibodies were used: insulin (DAKO, A056401-2, 1:1,000); glucagon (Cell Signaling, #2760, 1:2,000); Ki67-mouse (BD Pharmingen, 550609, 1:1,000); MafA (Novus Biologicals, NBP1-00121, 1:500); Nkx6.1 (Novus Biologicals, NBP1-49672, 1:500); Urocortin 3 (Phoenix Pharmaceuticals, H-019-29, 1:1000); Pax6 (Covance, PRB-278P, 1:1000); Nkx2.2 (Santa Cruz, sc-15015,1:2000) and Pdx1 (gift from C. Wright, Vanderbilt University, 1:20,000). Images were collected on a Zeiss Axioimager M2 (Jena, Germany) fluorescent microscope.

For beta and alpha cell area measurements, six sections (~240 μm apart) were analyzed for insulin or glucagon positive area with HRP-secondary staining using the DAB substrate kit (Vector Labs, Burlingame, CA) and counterstained with eosin. Images were collected on an Aperio ScanScope (Leica, Buffalo Grove, IL, USA) whole slide scanner and the percentage of beta and alpha cell area relative to whole tissue area (eosin) was calculated. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on sections using the Click-iT TUNEL Assay kit (ThermoFisher, C10617).

Measurement of serum insulin
Serum from fasted NODRag1$^{-/-}$ mice was collected via retroorbital bleed. Serum was separated from platelets by centrifugation of whole blood. Insulin concentration was determined by Luminex assay by the Vanderbilt Hormone and Analytical Core.

HORAC Activity Assay
Analysis of the antioxidant capacity of supernatants from Imatinib treated B lymphocytes was carried out according to manufacturer's recommendation (Eagle Biosciences). Briefly, 10x10^6 B lymphocytes were incubated in 60 uls HBSS+1% fetal calf serum for 5 hours at 37C in the presence of 10uM Imatinib or with media only. As a control wells with only 60uls of HBSS+1% FCS with or without Imatinib were incubated on the same plate for the same time period. At the end of the assay cells were pelleted via centrifugation and 20uls of supernatant was acquired for each well, all samples were assayed in duplicate. Supernatant was transferred to a black-walled optical plate to which was added 120uls of assay media, 20uls of Fenton reagent and 20 uls of hydroxyl radical solution. Wells were thoroughly mixed and fluorescence was measured every minute for 45 minutes on a fluorescent plate reader. As an assay control gallic acid with a known antioxidant capacity was run on the same plate.

Statistics
Statistical analysis was performed with GraphPad Prism V5 (La Jolla, CA), using the Student's t-test for comparison of two normally distributed conditions. One- or two-way analysis of variance followed by Sidak's multiple comparison post-test was used to compare multiple groups. Statistical comparisons with $p \leq 0.05$ values were deemed significant. The error bars represent mean +/- standard error of the mean.

Study Approvals
All procedures were approved by the IACUC at Vanderbilt University prior to initiation of any experiments.

Author Contributions
CSW, JMS, JK, BTS, EMH, RWS and DJM designed, executed and analyzed experiments. CSW, JMS, RWS, and DJM wrote the manuscript, which all authors reviewed.

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Figure 1:

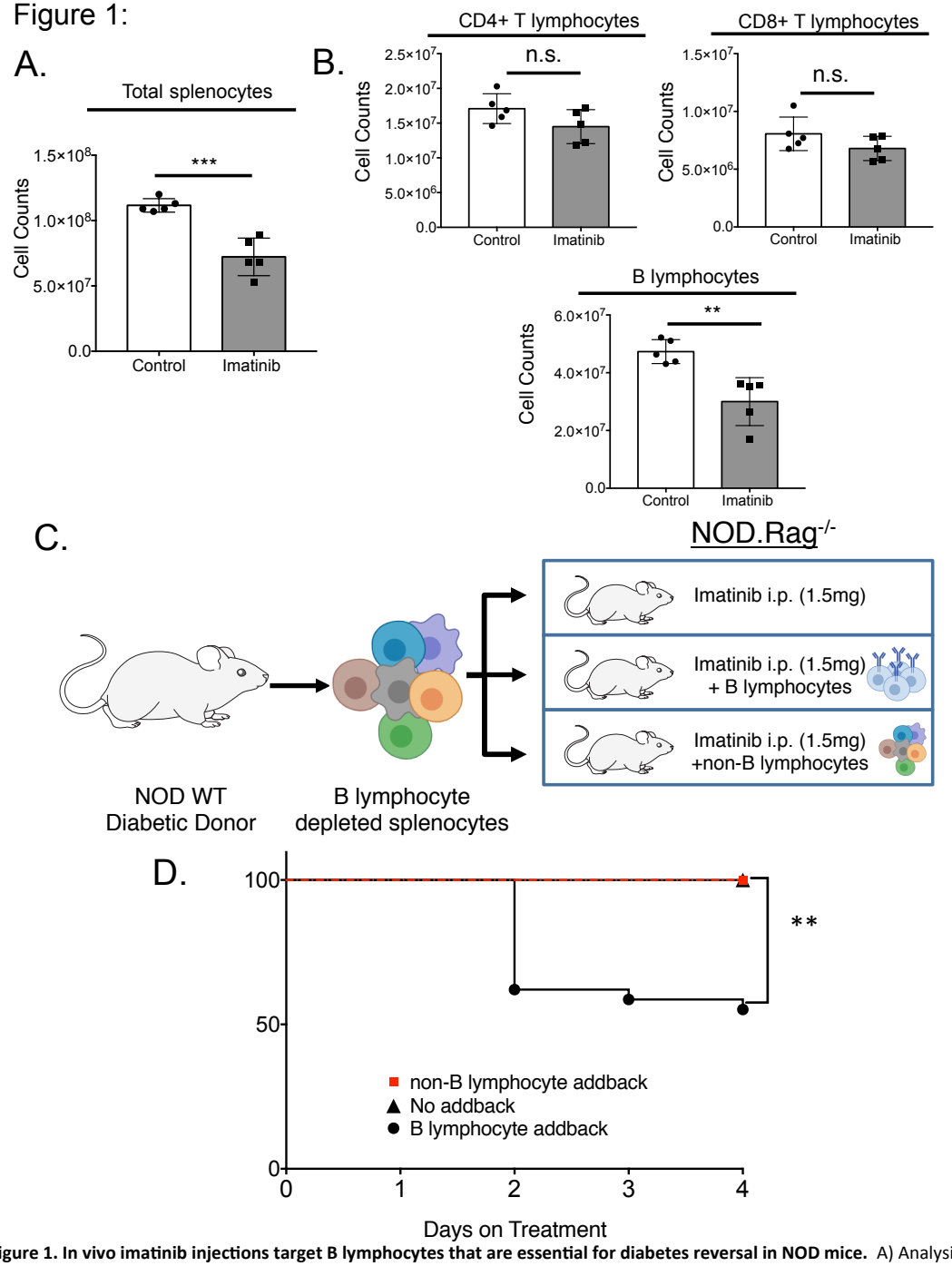

Figure 1. In vivo imatinib injections target B lymphocytes that are essential for diabetes reversal in NOD mice. A) Analysis of splenocytes revealed a reduction in total splenocyte numbers in NOD mice following imatinib (***p = 0.022 Student's t-test). B) B lymphocytes were preferentially depleted in NOD mice (**p = 0.041 Student's t-test) as compared to T lymphocytes (p = 0.071 Student's T-test) (n=5 per group, representative of 5 experimental replicates). C) B220 MACS depleted splenocytes ($10x10^6$) from a diabetic NOD donor mouse were transferred into immunodeficient NODRag1$^{-/-}$ mice. Mice were allowed to become diabetic and at time of diabetes were given imatinib injections alone, with $20x10^6$ B cell depleted splenocytes and imatinib, or $20x10^6$ MACS-purified B lymphocytes and imatinib. D) Blood glucose levels of diabetic mice on imatinib therapy revealed that only mice that received B lymphocytes and imatinib together had normalization of blood glucose. (n=15 in no addback, n=6 in non-B lymphocyte addback, n=29 in B lymphocyte addback group. (Mantel-Cox log-rank test **p=0.002) (Data compiled from 5 separate experiments) Shown in C).

Figure 2:

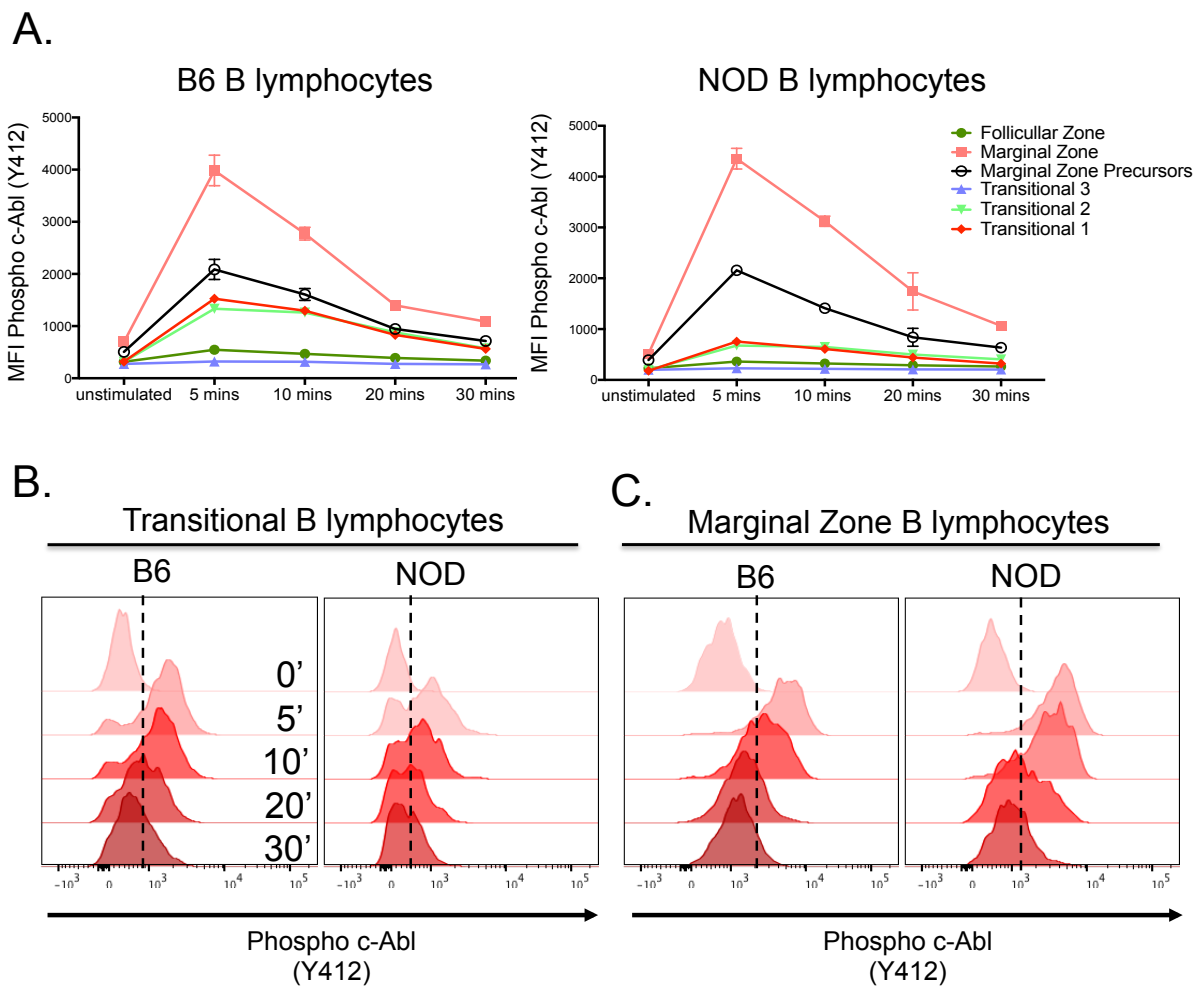

Figure 2. Specific NOD B lymphocyte subsets possess reduced c-Abl phosphorylation in response to BCR stimulation. A) Splenocytes from B6 and NOD mice were stimulated with anti-IgM $F(ab)_2$ (10ug/ml) and subsequently fixed and stained for markers of B lymphocyte subsets and phosphorylated c-Abl(Y412) over a course of 30 minutes. Transitional and follicular B lymphocytes had reduced dynamic phosphorylation in both strains. c-Abl phosphorylation in B) transitional B lymphocytes from NOD mice was further reduced compared to B6 ($p<0.0001$ one-way ANOVA) while C) marginal zone B lymphocytes were relatively similar between strains. (n=3 in each group, representative of 10 repeats)

Figure 3:

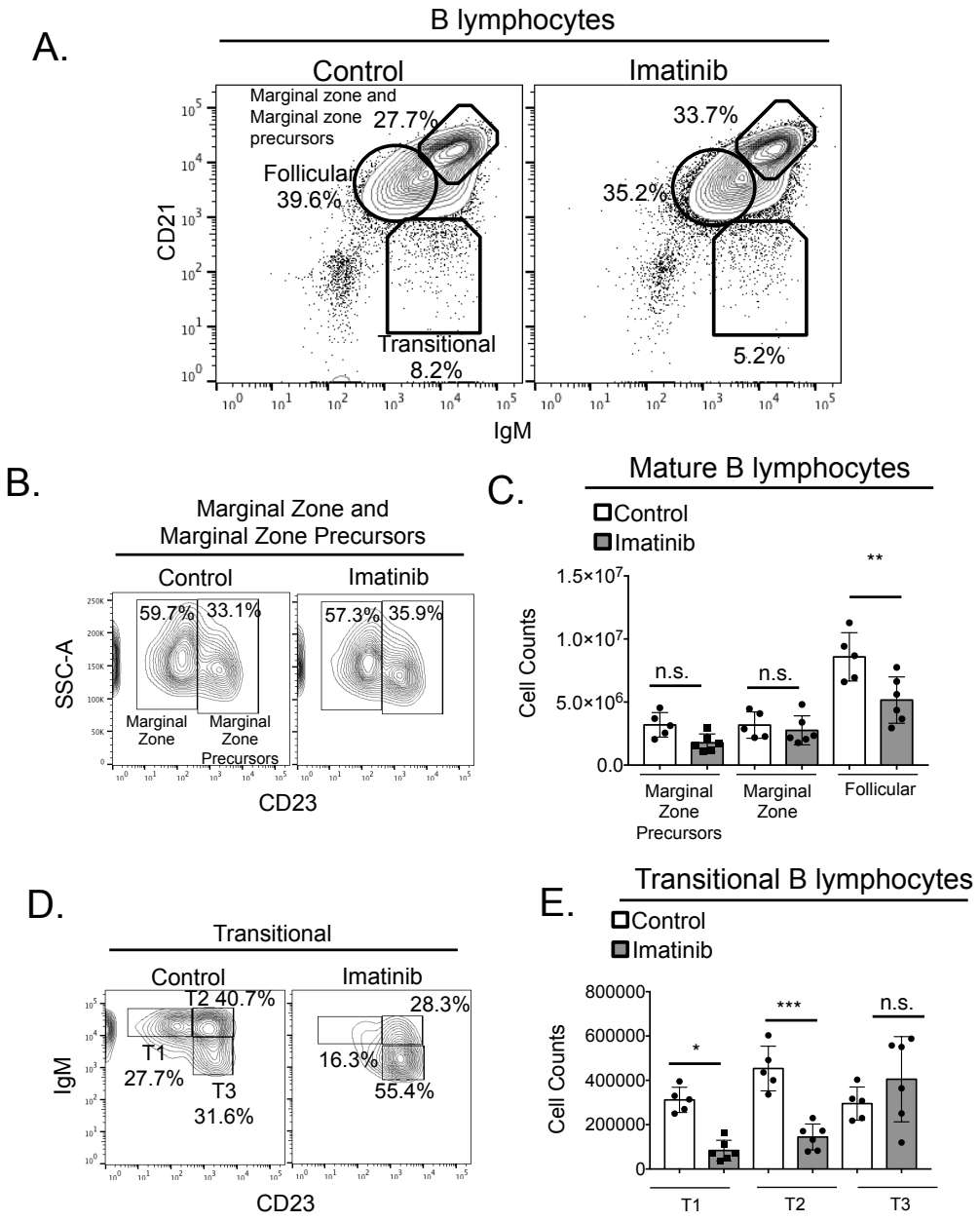

Figure 3. Imatinib preferentially depletes B lymphocyte subsets with reduced c-Abl signaling capacity.
A) Subset analysis of B lymphocytes revealed that among mature B lymphocytes, follicular B lymphocytes (**p = 0.0031 two-way ANOVA followed by Sidak's multiple comparisons test) were depleted. (n=6 in each group) B) Marginal zone (n.s. p = 0.99 two-way ANOVA followed by Sidak's multiple comparisons test) and marginal zone precursors (n.s. p = 0.51 two-way ANOVA followed by Sidak's multiple comparisons test) remained intact. (n=6 in each group) Quantified in C. D) Immature B lymphocytes were also depleted, including preferential depletion of T1 (*p = 0.013 two-way ANOVA followed by Sidak's multiple comparisons test) and T2 (**p = 0.005 two-way ANOVA followed by Sidak's multiple comparisons test) subsets but not anergic T3 (n.s. p = 0.51 two-way ANOVA followed by Sidak's multiple comparisons test) B lymphocytes. (n=5 controls and 6 imatinib treated) This is quantified in E). (Data representative of at least 5 repeats; group means are also shown on contour plots)

Figure 4:

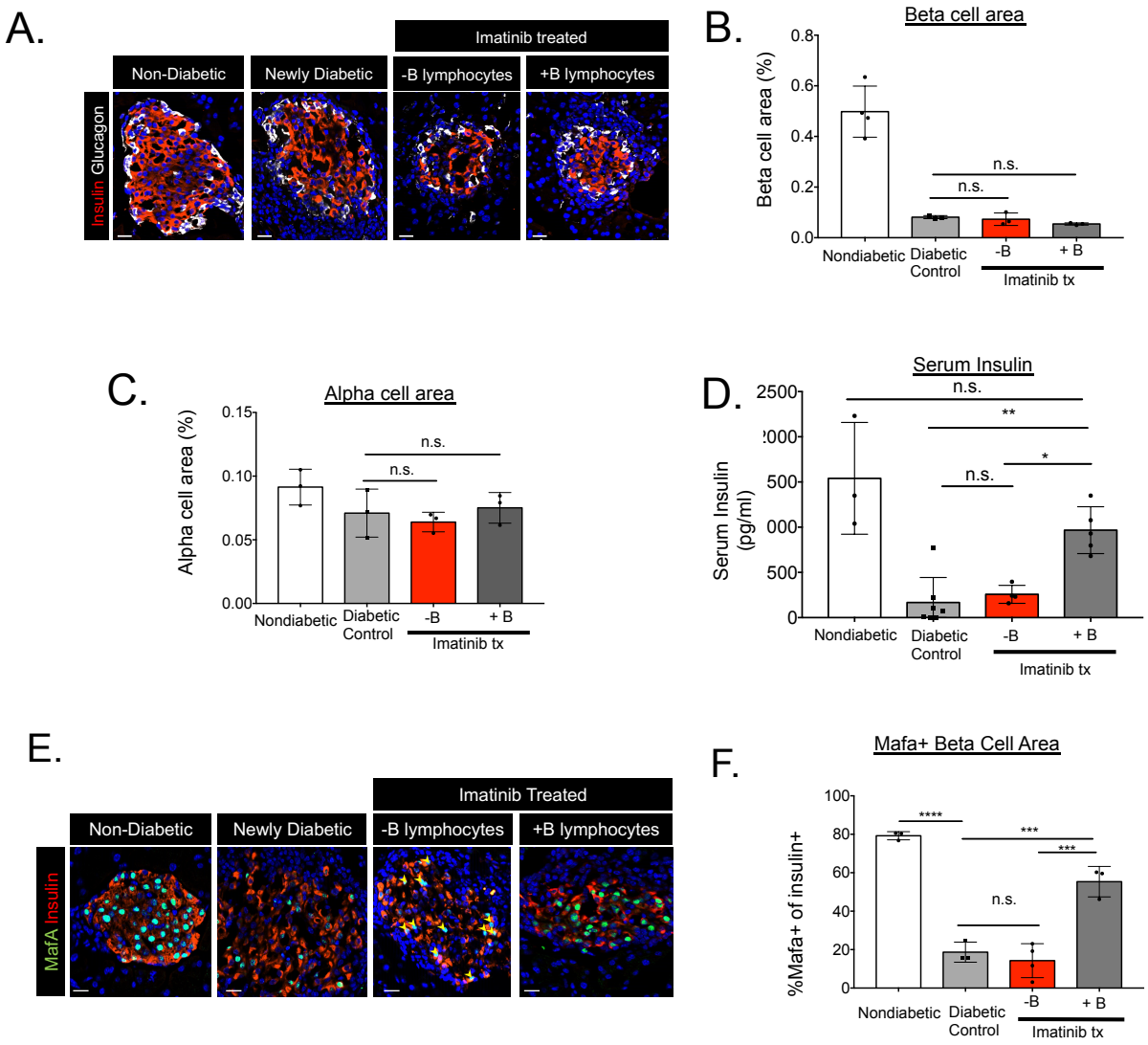

Figure 4. Imatinib therapy restores beta cell function but not beta cell mass in mice with B lymphocytes. A) Pancreatic sections from WT, newly diabetic (two consecutive blood glucose readings > 200mg/dL) and imatinib-treated mice with or without B lymphocytes (4 days after treatment initiated) were evaluated for beta cell area and alpha cell area by staining with insulin or glucagon specific antibodies. B and C) Neither beta cell nor alpha cell area increases following imatinib treatment. (n=3 in each group two-way ANOVA followed by Sidak's multiple comparisons test) D) Serum insulin levels are partially restored in NOD mice following imatinib (**p=0.0032, *p=0.022 two-way ANOVA followed by Sidak's multiple comparisons test). (n=3 in control, n=7 diabetic control, n=5 imatinib+B lymphocytes, and n=4 imatinib-B lymphocytes) E) MafA and insulin staining of pancreatic sections reveals loss of MafA from insulin+ cells in newly diabetic mice. Only imatinib-treated mice with B lymphocytes restored MafA in insulin+ cells. F) Quantification of MafA+insulin+ cells revealed partial recovery in imatinib-treated mice with B lymphocytes as compared to newly diabetic mice or imatinib-treated mice with no B lymphocytes. (****, $p < 0.0001$; ***, $p < 0.0005$; n.s. = not significant two-way ANOVA followed by Sidak's multiple comparisons test. Scale bars = 20μm) (n=3 in each group)

Figure 5:

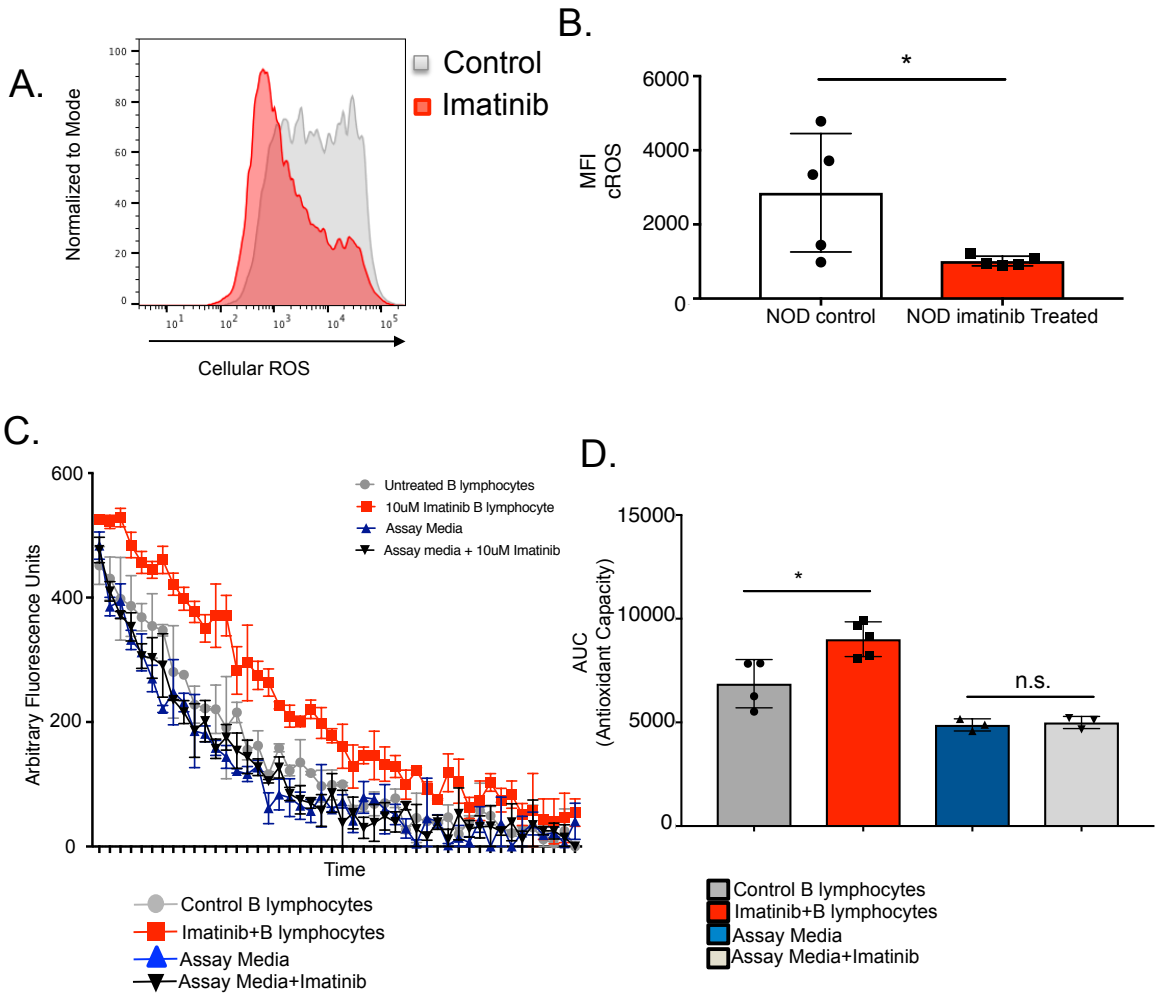

Figure 5. B lymphocytes acquire enhanced ROS handling capacity after imatinib therapy. A) Cellular ROS analysis by flow cytometry of B lymphocytes from imatinib-treated and control NOD mice indicated that B lymphocytes from imatinib-treated mice possessed reduced cellular ROS quantified in B) (*p = 0.03 Student's t-test)(n=5). C) B lymphocytes from NOD mice were purified by MACS and incubated in HBSS+FCS with or without imatinib (10uM). Supernatants were collected and their antioxidant capacity was measured by a fluorescent quenching assay (HORAC). Antioxidant capacity is revealed by prolongation of a ROS-sensitive fluorescent probe. Supernatants from imatinib-treated B lymphocytes prolonged fluorescence (red) as compared to B lymphocytes incubated with HBSS+FCS (gray), HBSS+FCS alone (blue), or HBSS+FCS+10uM imatinib(black). D) The area under the curve was calculated for biologic replicates and plotted demonstrating a significant increase in secreted antioxidant capacity of B lymphocytes (* p = 0.015 two-way ANOVA followed by Sidak's multiple comparisons test) (representative of at least 3 experimental repeats)