# Role of macrophages and B cells in the resolution of T2DM following bariatric sleeve gastrectomy

**by**

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## I. Abstract:

Sleeve gastrectomy (SG) results in significant improvements in glycemic control in obese patients with type 2 diabetes (T2DM). Animal models of diet-induced obesity have demonstrated roles for numerous immune cell subsets in the pathogenesis of insulin resistance, but the effects of SG on immunity are unknown. We hypothesized that SG improves insulin sensitivity by altering intestinal and splenic immune cell milieu. C57Bl/6J mice fed normal (lean) or high fat chow (obese) underwent SG (lean n=4; obese n=10) or sham (lean n=5; obese n=8) surgery. Oral glucose tolerance tests (OGTT) were performed at 2 weeks post-op. Major immune cell compartments in the jejunum, liver, and spleen were then profiled using the novel time-of-flight mass cytometry (CyTOF) platform. SG improved glycemic control by OGTT in lean and obese mice and multivariate viSNE analysis of CyTOF data demonstrated changes in B cell and macrophage populations after SG. SG reduced splenic $CD19^{+}CD21^{+}CD23^{+}$ B cells by 27% ($p=0.0009$) in obese and 45% in lean mice ($p=0.04$) compared to shams. Further, obese-SG mice exhibited decrease in jejunal $CD19^{+}CD21^{+}CD23^{+}$ B cells ($p=0.009$) and increase ($p=0.03$) in $MHCII^{neg}F4/80^{+}CD11b^{+}$ splenic macrophages compared to obese-shams. Splenic and jejunal macrophages in obese-SG mice also exhibited elevated anti-inflammatory M2 polarization. We therefore conclude that SG improves glycemic control in association with decreased splenic follicular B cells in obese and lean mice, suggesting a weight-independent effect of surgery. In obese mice, SG also promotes increased anti-inflammatory M2 macrophage polarization, which may represent a downstream effector phenotype. Reduction of pathologic inflammation may therefore contribute to the metabolic benefits of SG and support a role for immune-targeted therapies for diabetes.

## II. Introduction:

Obesity and type 2 diabetes mellitus (T2DM) are serious public health problems for which novel therapeutic strategies are needed. Metabolic and bariatric surgery (MBS), including sleeve gastrectomy (SG), is an effective and durable treatment for obesity that is often accompanied by rapid improvement in glycemic control in patients with T2DM.[1,2,3,4] Bariatric procedures, which were initially hypothesized to work solely through mechanical restriction of food intake or decreased intestinal absorption, have been shown to induce a diverse array of post-operative physiologic changes in energy homeostasis, gut hormone signaling, bile acid secretion, and microbial communities.[5,6] Little is known, however, about the effects of bariatric surgery on systemic or local immune cell recruitment and signaling, or the contributions of the immune system to alterations in glycemic control after bariatric surgery.

MBS represents a rapidly growing category of therapeutic procedures for patients with severe obesity. An International Federation for the Surgery of Obesity and Metabolic Disorders (IFSO) survey of 62 national bariatric societies and groups projected that 580,000 MBS surgeries were performed worldwide in 2014, rising to 685,874 surgeries in 2016.[7] This is in stark comparison to the roughly 16,000 procedures performed in the early 1990s and the roughly 150,000 procedures performed in 2003.[7,8,9] In 2016, 92.6 percent of procedures were primary interventions and 7.4 percent were revisional in nature.[7] According to a recent IFSO survey, currently the sleeve gastrectomy (SG) is the most commonly performed procedure in the world.[7] It comprises 53.6 percent of all primary bariatric surgical procedures; a trend that has been consistent since 2013 when SG eclipsed Roux-en-Y gastric bypass (RYGB), which now accounts for only 30.1 percent of all MBS procedures world-wide.[7,10] In addition to laparoscopic SG, new endoscopic approaches have been developed to alter gastric anatomy, including endoluminal sleeve gastroplasty (ESG) and primary obesity surgery endoluminal (POSE).[11,12]

Laparoscopic SG was originally developed as a standalone operation in 1999 by Dr. Michel Gagner as a means of staging a more complicated bypass procedure, the biliopancreatic diversion with duodenal switch (BPD-DS), in high-risk patients.[13] In a standard laparoscopic SG, the patient's abdomen is accessed through laparoscopic ports and the greater curvature of the stomach is identified and elevated. Using electrocautery or ultrasonic scalpel, the greater sac is entered, and the greater curvature of the stomach dissected from the omentum and the short gastric vessels. A bougie or gastroscope is then passed through the mouth and into the stomach, where it is aligned with the lesser curvature to serve as a template. An endoscopic linear cutting stapler is then used to serially staple and transect the stomach lateral to the template, extending approximately 5-6 cm proximal to the pylorus to the Angle of His. Once dissected, the greater curvature segment can be removed from the peritoneum through a laparoscopic port. The resulting tubular stomach segment has a volume of 50- to 100-mL.

Outcomes following SG demonstrate durable weight loss. A 2019 meta-analysis by Sharples and Mahawar of five, randomized controlled clinical trials with 5-year follow-up demonstrated an average excess weight loss (EWL) of 57.3 percent and resolution of T2DM (as defined by maintenance of HbA1c $< 6.0\%$ without medication) in 27.5 percent of patients.[14] SG also resulted in resolution of hypertension in 48.4 percent of patients and resolution of dyslipidemia in 55.2 percent of patients.[14] Other comorbidities of obesity, such as gastroesophageal reflux, obstructive sleep apnea, and osteoarthritis have also been shown to improve or resolve in many patients following SG.[14]

SG is often distinguished from other bariatric procedures, such as RYGB, as a "purely restrictive" operation, yet the mechanisms by which SG promotes weight loss and improved glycemic control are complex. Although the volume of the stomach is reduced by approximately 80% following SG, no correlation has been demonstrated between stomach volume and weight loss in patients.[15] In rodent models, SG results in an initial decrease in food intake, however, there is no difference in long term

caloric intake between animals undergoing SG and sham surgery, despite significant weight loss in the former group.[16] Moreover, both female humans and rats who have undergone SG are able to meet the increased energy demands of pregnancy and lactation by increasing their daily caloric intake.[17] Similarly, rats that have undergone SG are capable of defending their post-surgical weight following a period of food deprivation, similar to sham-operated animals, by increasing their caloric intake.[16]

It has also been shown that improved glycemic control following SG occurs prior to and independent of significant weight loss in patients with obesity and T2DM. Our group recently showed that, in a retrospective study of 187 obese patients with T2DM who underwent SG at Brigham and Women's Hospital, 39% of patients no longer required T2DM medication at the time of discharge at a mean of 2.5 days post-operatively.[18] Wallenius et al similarly demonstrated a significant improvement in homeostatic model assessment of insulin resistance (HOMA-IR) scores as early as 2 days post-operatively in patients who underwent SG, long before significant weight loss could occur.[19] Other studies in patients undergoing SG or RYGB show a weight-independent restoration in the acute insulin response to intravenous glucose, the loss of which is characteristic of diabetic beta cell dysfunction.[20, 21, 22] Similarly, mice treated with SG exhibit weight-independent improvement in islet cell function and insulin secretion 2 weeks post-operatively.[23] Together, these data suggest that SG affects improvement in glycemic control, not simply as a result of weight loss, but through complex, and likely multifactorial, alterations in both insulin secretion and peripheral insulin resistance.

A large body of evidence has established roles for many physiologic changes believed to contribute to weight loss and metabolic improvement following SG that extend beyond the simple restriction of caloric intake. SG has been shown to accelerate gastric emptying and decrease intestinal transit time, possibly through altered gastric vagal signaling,[24] speeding the delivery of chyme to the distal intestine and increasing anorexigenic signal production.[25, 26] Circulating ghrelin levels have been shown to decrease

following SG, likely due to removal of the ghrelin-producing cells in the gastric fundus, which may contribute to decreased appetite, increased satiety, and improved insulin sensitivity after surgery.[27, 28] An extensive literature has shown that SG results in increased fasting and postprandial plasma glucagon-like peptide 1 (GLP-1) levels,[29, 30] though Wilson-Perez et al. demonstrated that GLP-1 receptor-deficient mice exhibited similar weight loss and glycemic control after SG, suggesting that GLP-1-dependent signaling is not essential to the beneficial effects of SG.[31] Similarly, increased levels of peptide YY (PYY)[32, 33] and cholecystokinin (CCK),[33, 34, 35] and decreased production of glucose-dependent insulinotropic peptide (GIP),[36, 37] may play roles in increased gastric emptying and enhanced distal nutrient delivery, increased satiety, and improved glucose homeostasis following surgery. Total plasma bile acids, which have been found to exhibit direct and indirect influences on food intake, energy metabolism and glycemic control via interaction with the Takeda G protein-coupled receptor 5 (TGR5) and the farnesoid-X receptor (FXR),[38] have also been found to increase in patients following SG,[39] and FXR-deficient mice that undergo SG do not exhibit improved glycemic control.[40] Increasing evidence also points to alterations in the post-surgical microbiota as a contributor to weight loss and improved glycemic control, with studies correlating SG with induction of lean-associated microbial phenotypes in both animals and humans.[40, 41, 42]

Intriguingly, little is yet known about the effects of SG on the local and systemic immune milieu, despite well-established links between systemic inflammation and insulin resistance.[43] Diet-induced obesity is associated with chronic, low-grade inflammation, including elevated pro-inflammatory cytokine levels, most notably tumor necrosis factor-α (TNF-α), and recruitment of pro-inflammatory immune cells such as Th1 T cells, CD8+ T cells, macrophages, dendritic cells, that have been shown to directly contribute to obesity-related insulin resistance.[44, 45, 46, 47, 48, 49 , 50, 51, 52, 53] A high-fat Western diet, rich in saturated fats and low in fiber, also induces changes in gut microbial communities that have

numerous effects on intestinal homeostasis.[54] Increased local and systemic production of TNF-α, interferon-gamma (IFN-γ), IL-12, and IL-6 in turn contribute to intestinal barrier dysfunction[54] leading to the leakage of bacterial and dietary antigens that contribute to adipose tissue inflammation through a Toll-like receptor (TLR)-dependent mechanism.[55]

Macrophages are contributors to this pro-inflammatory obesity state, with pathogenic macrophage accumulation observed in the subcutaneous and visceral adipose tissue of both mice and humans.[56] Notably, adipose macrophage accumulation in diet-induced obese (DIO) mice is also associated with a switch from an "alternatively activated" M2 phenotype to a pro-inflammatory M1 phenotype.[45]

Similar studies in DIO mice by Winer et al. have demonstrated that B cells accumulate in VAT during obesity pathogenesis and are sufficient to confer insulin resistance.[50] DIO mice had higher levels of IgG2c in visceral adipose tissue (VAT) and serum, and transfer of purified IgG from DIO mice to normal-chow, $B^{null}$ mice was associated with impaired glucose metabolism. By contrast, DIO mice lacking B cells showed impaired M1 macrophage accumulation and TNF-α production. Together, these data indicate that B cells may contribute to obesity and diabetes through antibody production and regulation of macrophage activity.

Little is known, however, about the contributions of these immunologic phenotypes to weight loss and glucose homeostasis after bariatric surgery. In order to assess these changes, our lab has developed a murine model of SG that improves glucose tolerance in C57Bl/6 mice in both obese animals fed a high-fat diet (HFD) and lean animals fed a normal chow diet (NCD) as early as two weeks post-operatively. Mice treated with a sham surgical procedure (ligation of the short gastric artery) are co-fed and serve as negative controls. Consistent with human bariatric patients, obese mice that undergo SG exhibit decreased body weight post-operatively, increased glucose tolerance by oral glucose tolerance testing, and lower fasting blood glucose despite continued exposure to the high-fat chow; sham

operated mice maintain a high weight, reduced glucose tolerance, and elevated fasting blood glucose. By contrast, lean animals fed NCD do not exhibit significant weight loss after undergoing SG, but they nonetheless develop improved glycemic control parameters post-operatively in comparison to sham-operated animals.

Given the evidence supporting a role for inflammatory VAT macrophage and B cell accumulation in the pathogenesis of obesity and insulin resistance, we hypothesized that SG contributes to improved glycemic control in mice by reducing the size and inflammatory polarization of macrophage and B cell pools both locally in the gastrointestinal tract and systemically in the spleen and liver. To test this hypothesis, we utilized our murine model of SG in conjunction the mass cytometry by time-of-flight (CyTOF) platform, which uses heavy metal-tagged antibodies to murine intra- and extra-cellular antigens to characterize and quantify immune cell phenotypes at the single-cell level. Using this modality, we were able to both screen for immunophenotypic changes across innate and adaptive immune cell pools as a function of diet and surgical intervention, while also quantifying these changes at the single cell level.

## III. Materials and methods

*Mouse model of sleeve gastrectomy:*

Male C57Bl/6 mice were maintained under specific pathogen-free conditions on a normal murine chow diet (NCD) or high-fat chow diet (HFD) for 6 weeks. Mice were then randomized to undergo either sleeve gastrectomy (SG) or ligation of the short gastric artery (sham). Under isoflurane anesthesia, SG and sham procedures were performed via a small midline laparotomy. In SG, the stomach was dissected free from its surrounding attachments. The short gastric vessels between the stomach and spleen were divided and a tubular stomach was created by

removing 80% and 100% of the glandular and non-glandular stomach, respectively, using a surgical stapler-divider. Sham operation consisted of a similar gastric dissection, short gastric vessel ligation, and manipulation of the stomach along the staple line equivalent. Mice were then individually housed thereafter to allow for monitoring of food intake, weight, and behavior. After surgery, SG and Sham mice were maintained on Recovery Gel Diet (Clear $H_2O$, Westbrook, ME) for 6 days and then restarted on their respective original chow formulation.

*Oral glucose tolerance tests (OGTT):*

OGTT was performed at 2 weeks and 4 weeks post-operatively. Animals underwent a 4 hour fast (8 am to noon). During OGTT, mice received 2mg/g of oral D-Glucose (Sigma-Aldrich, St. Louis, MO) and serum glucose levels were measured from the tail vein at 15, 30, 60, and 120 min with a OneTouch Glucometer (Life Technologies, San Diego, CA).

*Preparation of single cell suspension:*

Animals were sacrificed at 8 weeks post-operatively under isofluorane anesthesia. Prior to dissection, the right ventricle was cannulated with a 23-gauge needle and perfused with 20-30mL of 37 °C Hank's Balanced Salt Solution (HBSS; Life Technologies, San Diego, CA) containing 5% fetal bovine serum (FBS; Life Technologies, San Diego, CA), 10mM HEPES (Life Technologies, San Diego, CA), and 0.2% heparin (Life Technologies, San Diego, CA). The liver, spleen, and proximal jejunum were dissected and placed in RPMI 1640 (Life Technologies, San Diego, CA; spleen and jejunum) or HBSS containing 5% FBS and 10mM HEPES (liver) on ice. All animal procedures were performed in accordance with institutional guidelines provided

by the Animal Care and Use Committee at Brigham and Women's Hospital under an approved study.

Single-cell suspensions of liver, spleen, and proximal jejunum tissue were prepared using a solution of Liberase TL (Roche, Penzberg, Germany; 200 ng/mL) and DNAse I (Sigma-Aldrich, St. Louis, MO; 0.15 mg/mL). Digestion was halted with the addition of the corresponding FBS-containing media and the lysates strained through a 70uM nylon cell strainer (Celltreat, Pepperell, MA). Cell suspensions were then centrifuged at 400g for 7 minutes, at which time red blood cell lysis was performed by resuspension of the cell pellet in 2mL of ACK lysis buffer (Life Technologies, San Diego, CA) and room-temperature incubation for 2 minutes. RBC lysis was not performed on liver samples due to high cell death rates. Lysis was halted using 3 volumes of RPMI media and cells centrifuged and resuspended in 1 mL of RPMI or HBSS. Cells were counted manually using a hemocytometer and 0.4% trypan blue stain (Life Technologies, San Diego, CA). Cells were then aliquoted into 96-well polypropylene cell culture plates at a density of 1 million cells per well and centrifuged at 200g for 5 minutes.

*Mass cytometry:*

For mass cytometry applications, cells were resuspended in a cell staining buffer (CSB) containing CyTOF-grade PBS (Fluidigm, San Francisco, CA), 0.625% bovine serum albumin (Fluidigm, San Francisco, CA), and 25mg sodium azide (Fluidigm, San Francisco, CA) and stained for viability with 100 uM cisplatin (Fluidigm, San Francisco, CA) for 2 min at room temperature. Cells were washed again in CSB and then blocked with mouse Fc block (BioLegend, San Diego, CA). Staining for extracellular markers was then performed for 30 min at room temperature using heavy metal-tagged antibodies produced and validated by the BWH

CyTOF core facility (**Table 1**). Cells were then fixed and permeabilized using permeabilization buffer (eBioscience, San Diego, CA) and stained for the intracellular marker anti-Arg1-161Dy for 30 min at room temperature. Cells were then washed in CSB and resuspended in 1.6% paraformaldehyde (Thermo Scientific, Waltham, MA) in PBS, incubated for 10 min at room temperature. Cells were subsequently washed and stained with iMaxPar Intercalator-Ir (Fluidigm, San Francisco, CA), washed in CyTOF-grade water and resuspended in water at a density of 100,000 to 1,000,000 cells/mL. Cells were strained through 35 um filters (BD Biosciences, Bedford, MA) and suspended in EQ Four Element Calibration beads (Fluidigm, San Francisco, CA). Samples were subsequently analyzed using a CyTOF-Helios mass cytometer (Fluidigm, San Francisco, CA).

Data analysis was performed using the Cytobank software suite (Cytobank, Mountain View, CA). High-dimensional cytometry data analysis was performed using the visualization of t-distributed stochastic neighbor embedding (viSNE) method. viSNE is an algorithm that reduces high-parameter data, in this case the 32 CyTOF antibody signals associated with each cell analyzed, down to two dimensions for easy visualization and rapid exploratory data analysis of any data type.[57] Phenotyping of immunologic subsets was performed using the gating strategy shown in **Figure 1**. Briefly, macrophages were identified as CD45+CD11b+F4/80+ cells, while dendritic cells were identified as CD45+CD11c+MHCII+. B cells were identified as being CD45+CD19+. Subsets of the macrophage pool were further evaluated by their expression of MHCII, Ly6C, and the M2 marker Arginase 1 (Arg1). B cell subsets were evaluated for their expression of CD21, CD23, and IgM. Differential recruitment of these immune cell populations in SG and sham-operated mice was assessed by unpaired, two-tailed Student's T-test ($p = 0.05$) using the percentage of CD45+ leukocytes bearing a given phenotype. Assessment of Arg1 and IgM protein staining was

made using the mean intensity of antibody staining as measured by CyTOF and evaluated by unpaired, two-tailed Student's T-test ($p = 0.05$).

**IV. Results:**

In order to assess the efficacy of SG in a murine model for the promotion of improved glycemic control, OGTT was performed 2-weeks post-operatively in both lean and obese animals undergoing SG and sham surgery. Both lean-sham ($n = 7$) and lean-SG ($n = 8$) animals maintained a fasting serum glucose of 120-150mg/dL, while obese animals demonstrated higher baseline serum glucoses, with obese-sham animals ($n = 10$) averaging >200 mg/dL and obese-SG animals ($n = 9$) averaging significantly lower at 150-200 mg/dL (**Figure 2**). In all four groups, serum glucose increased dramatically from baseline following an oral glucose challenge and returned to baseline by 120 min. Both lean-SG and obese-SG animals exhibited significantly decreased serum glucose levels 30- and 60-min post-challenge as compared to their sham counterparts. In both groups, SG-treated animals returned to their baseline serum glucose by 60 min, whereas sham-treated animals took 120 min to return to baseline.

Weight loss was evaluated in both lean and obese animals following SG and sham surgery (**Figure 3**). Both lean-SG ($n=4$) and lean-sham animals ($n = 5$) exhibited significant weight loss in the perioperative period as a percentage difference of their pre-operative weight (assessed on POD -2 for all animals), with lean-SG animals losing significantly more weight in the first 10 post-operative days compared to sham. By POD14, however, lean-SG and lean-sham animals had an insignificant difference in weight loss, and this trend continued through POD 36. In obese animals, both SG and sham surgery resulted in similar weight loss in the first four post-operative days. After POD4,

however, obese-SG animals exhibited significantly more weight loss through POD 36 compared to obese-sham animals.

In order to determine whether SG alters the systemic immune milieu, CyTOF analysis was performed on single-cell suspensions of splenic tissue from lean and obese animals treated with SG or sham surgery using a panel of antibodies capable of distinguishing T-, B-, NK, and myeloid lineage cells (**Table 1**). Using the viSNE algorithm, these data were visualized on two axes using the algorithm-generated variables tSNE1 and tSNE2 (**Figure 4**). In lean-sham animals, viSNE representation shows a dense population cluster in the left upper quadrant that is significantly diminished following SG. A similar population in the obese-sham group (left lower quadrant) appears to disappear completely in the obese-SG group. In both lean and obese animals, this population of cells exhibits high CD19 and CD21 expression, suggestive of B-cell lineage. Obese-SG animals further demonstrate a dense population cluster in the right lower quadrant of the viSNE plot that is not seen in obese-sham populations. This population of cells strongly expressed the myeloid lineage marker CD11b, as well as macrophage/dendritic cell markers such as F4/80, MHCII, and CD11c. Unlike in the spleen, viSNE analysis of the liver and jejunum of obese and lean animals did not demonstrate clear patterns of difference.

Given this qualitative evidence supporting a change in systemic B-cell and myeloid compartments following SG, we performed gated analysis (**Figure 1**) to assess changes in these immune cell populations and phenotype. In lean animals, SG was associated with a 32% decrease in systemic $CD21^{+}CD23^{+}$ follicular B-cells in the spleen (**Figure 5**), without significant changes in the total $CD19^{+}$ B cell pool, or in $CD21^{+}CD23^{-}$ or $CD23^{+}CD21^{-}$ subsets. In obese animals, a 33.5% decrease in $CD21^{+}CD23^{+}$ follicular B-cells was observed in SG-treated animals compared to controls, as well as a 28% decrease total $CD19^{+}$ B cells (**Figure 5**).

B cell populations were further evaluated in the local immune milieu of the proximal jejunum and the liver. In the proximal jejunum of obese-SG animals, a significant decrease in both total $CD19^+$ B cells (55%, $p = 0.0069$) and $CD21^+CD23^+$ follicular B cells (63%, $p = 0.0089$) was observed as compared to obese-sham animals (**Figure 6**). Interestingly, lean animals had markedly smaller jejunal B cell populations across experimental groups and did not exhibit a significant difference in B cell accumulation following SG (**Figure 6**). Analysis of hepatic B cell populations in both lean and obese animals revealed no differences between SG and sham-treated animals (**Figure 7**). Subgroup analysis of B cells from lean and obese animals did not reveal any significant differences in the expression of CCR6 or CCR7 on B cells in the spleen, liver, or jejunum of any of the experimental groups (data not shown).

Based on initial viSNE screening data, myeloid cell populations were further analyzed. Total macrophage and dendritic cell populations did not differ consistently between obese-sham and obese-SG groups in the proximal jejunum and liver; however, the spleen exhibited a significant increase in the $CD11c^-MHCII^-F4/80^+$ macrophage population (**Figure 8**). Interestingly, these macrophages predominantly expressed intermediate-to-low levels of the macrophage activation marker Ly6C. A trend was observed towards increased recruitment following SG in other myeloid compartments, including CD11c-MHCII+ macrophages, CD11c+ macrophages, plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs), however, these differences did not reach statistical significance in the obese group (**Figure 8**). In lean animals, a trend towards increased $CD11c^-MHCII^-F4/80^+$ macrophage accumulation was observed in the jejunum, but this did not reach statistical significance. Other myeloid cell populations in lean animals did not exhibit any significant changes in any of the three organs examined.

In addition to increased $CD11b^{+}CD11c^{-}MHCII^{-}F4/80^{+}$ splenic macrophage recruitment and/or polarization, splenic $CD11b^{+}$ myeloid cells exhibited a broad trend towards increased expression of Arginase1, a marker of alternative, or M2, macrophage activation (**Figure 9**). This trend reached statistical significance in subgroup analysis of $CD11c^{-}$ and $CD11c^{+}$ macrophages, pDCs and cDCs, with an increase in Arg1 protein expression as high as 40-fold, as measured by mean metal intensity (MMI). This trend was further seen in the proximal jejunum of obese-SG animals with similar increases in Arg1 expression across all myeloid groups evaluated (**Figure 9**). No significant differences in Arg1 expression were observed in myeloid cell lineages between lean-SG and lean-sham animals in the jejunum, liver, or spleen.

**V. Discussion:**

Our study demonstrates that murine sleeve gastrectomy is capable of improving glycemic control, as measured by 2-week post-operative oral glucose tolerance testing, in both lean and obese mice. This improvement in glycemic control occurs independent of significant weight loss at POD 14 in both lean and obese animals. Moreover, while obese-SG animals exhibited significant weight loss at POD 36, lean-SG animals did not despite both groups being fed ad libitum. This dissociation between improvements in glycemic control, which occurred independent of an animal's diet, and significant durable weight loss, which only occurred in animals fed HFD, is particularly significant because it lends support to observations in human patients that SG results in rapid post-operative demand for anti-hyperglycemic agents.[18] Other rodent models of sleeve gastrectomy using similar surgical approaches in obese animals have previously been described, with comparable demonstration of improved glycemic control as measured by OGTT, fasting glucose levels, and insulin sensitivity testing.[58, 59, 60, 61, 62]

Interestingly, our study design is markedly different from prior animal studies, which have largely utilized pair-feeding models in which sham-treated animals are fed a comparable caloric load as SG-treated animals to account for altered feeding behaviors as a result of surgery. Human studies of SG have similarly compared SG-treated patients either to other operative models, such as laparoscopic Roux-en-Y gastric bypass, or to intensive dietary interventions – essentially a pair-feeding intervention.[63] We believe that, by performing SG in mice fed to hunger on a normal chow diet, we can more accurately assess for weight loss-independent effects of surgery since these animals will more faithfully replicate whatever changes SG induces in feeding behavior and metabolic activity than a control group who's weight loss is orchestrated by the investigators through dietary manipulations, especially since such interventions have been shown to be less effective than surgery in human populations.[64] As such, we believe that our lean-SG model offers significant advantages over other experimental models in identifying meaningful, weight-independent cellular and physiologic alterations following bariatric surgery.

To our knowledge, no one has yet evaluated the immunologic impact of surgery on the systemic or local immune milieu much less the dependence of any such changes on weight loss, although many studies have examined the role of immune cells in the underlying pathophysiology of obesity and T2DM. Several murine models of diet-induced obesity (DIO) using a high-fat rodent chow have been developed and used to evaluate immunologic phenotypes, and these studies have established roles for chronic, low-grade systemic and tissue inflammation, including in the visceral adipose tissue (VAT), liver, brain, and intestines, as well as the production of cytokines, such as TNFα, IL-1β, IL-6, and interferon γ, in the pathogenesis of T2DM in obesity.[54,65] Innumerable cell type-specific changes associated with DIO have been documented in rodents and humans, including enlargement of the macrophage and DC pools, $CD8^+$ and Th1 T cell recruitment, and decreased regulatory T cell

populations.[54] Interestingly, a specific role for B lymphocytes and macrophages in the pathogenesis of T2DM in murine diet-induced obesity models has been demonstrated. In mice fed a high-fat diet, classically polarized $CD11c^+$ macrophages infiltrate the VAT in as little as 1 week after starting the diet, heralding B cells infiltration of the VAT 6-12 weeks after initiation of the diet.[54, 66] Studies by Winer et al. have further demonstrated that B-2 cells, a B cell subtype that includes mature follicular B cells, in HFD-fed animals undergo class switching to IgG, produce less anti-inflammatory IL-10, and produce more pro-inflammatory IL-8.[67] Most significantly, Winer et al. have demonstrated that B cell-deficient mice fed HFD exhibit better glycemic control than wildtype animals, and adoptive transfer of both B-2 cells and IgG from DIO animals to B cell-deficient animals worsens metabolic parameters.[67] These findings clearly implicate B-2 cells, and specifically B-cell dependent IgG production, in the pathogenesis of diet-induced obesity

Given the paucity of data on the immunologic effects of bariatric surgery in either humans or mice and a wealth of cell types implicated in the pathogenesis of obesity-induced T2DM, we elected to make use of the CyTOF platform for single cell analysis of murine tissues to facilitate analysis of the greatest number of cell types. This platform is largely based on traditional flow cytometry principles, allowing for the analysis of cells individual cells using multiple labeled antibodies to cell surface and intracellular antigens. Unlike flow cytometry, however, CyTOF utilizes heavy metal-tagged antibodies, rather than fluorescent antibodies, which allows for analysis across a greater number of channels without concern for spectral overlap that plagues large flow cytometry experiments. In our antibody panel, this allows for the simultaneous assessment of the innate and adaptive immune cell compartments, as well as more detailed phenotyping of specific cell types of interest.

Using the CyTOF platform, we have demonstrated that $CD21^+CD23^+$ follicular B-cell populations, a subcategory of the B-2 cells discussed by Winer et al, are significantly altered at both

the systemic level, as demonstrated by single-cell analysis of splenic tissue, and at the local level, as demonstrated by analysis of proximal jejunal tissue, in both lean and obese mice treated with SG. Follicular B cells typically reside in secondary and tertiary lymphoid organs and constitute the majority of mature, splenic B cells. These cells, as their name suggests, typically reside in lymphoid follicles, expressing a diverse B cell receptor (BCR) repertoire, and participate in CD40-CD40L dependent signaling to promote effective primary immune responses, affinity maturation, and antibody class switching.[68] Follicular B cells are also capable of producing a diverse array of cytokines, including pro-inflammatory Th1 polarizing cytokines such as IFNγ, IL-6, IL-12 and TNF-α.[69] Many such cytokines meanwhile, most notably TNF-α and IL-6 have been implicated in the pathogenesis insulin resistance and have been shown to decrease following bariatric surgery in humans.[70] Thus, the significant decrease in this population in both local and systemic immune compartments may implicate a number of immune signaling pathways, for instance through regulation of antigen-antibody responses or cytokine-dependent signal amplification.

Our findings demonstrating an increase in local and systemic Arg-1-expressing macrophages following SG in obese animals provide an intriguing corollary to the decreased prevalence of follicular B cell populations. Although distinctions between M1, or classically-activated, and M2, or alternatively-activated, macrophages remains an area of intense study, imbalances or shifts in macrophage polarization have been implicated in a number of inflammatory diseases, including obesity, T2DM, and metabolic syndrome.[45] Broadly speaking, M1 cells in numerous tissues, including adipose and the pancreatic islets, are thought to be polarized by both dietary pro-inflammatory mediators, such as free fatty acids, and by chemokine and cytokine signaling. These cells are activated to express MHC class II and Ly6C on the cell surface, as well as pro-inflammatory pathways such as the NLRP3 inflammasome and IL-1b, TNF-α, and NO production.[71] These pro-inflammatory

mediators serve to amplify polarization signals and activate of NF-κB transcriptional products and other signaling cascade regulators (including SOCS, ERK, JNK, IKK, and PKC) capable of interfering with beta cell function in the pancreas and signaling downstream of the insulin receptor in peripheral tissues.[72]

In the conventional model of M2 macrophage polarization, activation by IL-4 and IL-13 induces an upregulation of arginase-1 (Arg1), IL-10, and TGF-β, leading to tissue regeneration and repair. In our study, we observed a significant increase in arginase-1 expression across multiple myeloid cell types, including macrophage and DC lineages, which would suggest a broad trend towards M2 polarization of cells across the entire myeloid lineage in both the local and systemic immune compartments. Significantly, this change was coupled with an increase in $CD11c^{-}MHCII^{-}$ $Ly6c^{-}F4/80^{+}$ macrophages in obese-SG animals, but not lean-SG animals. These macrophages are generally thought to represent monocyte-derived cells that have transitioned from a pro-inflammatory monocyte ($Ly6C^{+}$) to a patrolling anti-inflammatory monocyte-macrophage ($Ly6C^{-}$). Thus, these data point to a systemic increase in the number of anti-inflammatory monocytes bearing low antigen presenting capacity (MHCII) and elevated M2 marker expression only in obese post-surgical animals. An increase in the number of these cells, coupled with their increased Arg-1 expression, appear to represent a shift in the systemic myeloid compartment towards an anti-inflammatory innate immune phenotype. Notably, unlike alterations in the systemic B cell compartment, these alterations in myeloid cell accumulation and polarization appear to be weight-dependent, suggesting that gastric surgery alone does not induce such effects, but that instead there is some cellular or physiologic aspect of inflamed obese state that is necessary.

It should be observed, however, that Arg1 is a single marker of macrophage polarization, and we did not include additional M2 markers or M1 markers, such as iNOS, as a CyTOF-compatible

antibody was not available. Moreover, the broad increase in Arg1 expression across multiple myeloid lineages, including both macrophages and dendritic cells, is unusual and could simply represent off-target staining, though if this were the case we might expect higher background staining in other cell populations or in the lean animals, which was not observed.

Several notable limitations to this study are also worth discussing. Murine models of bariatric surgery are resource intensive and require substantial surgical expertise, factors that substantially limited the number of animals that could be included in this study. Repeating this study with additional animals may provide a clearer picture, especially with respect to smaller myeloid population subsets for which substantial variability in population size was observed. Moreover, all of the above studies were performed in wild-type animals and we did not attempt to determine whether the observed immune cell changes are necessary or sufficient to affect the resolution of T2DM following bariatric surgery. Future study will seek to determine whether B cell changes in SG mice cause improved glycemic control following adoptive transfer of B cells or humoral components such as IgG from SG-treated mice. Such a study would also offer the opportunity to evaluate the effects of follicular B cells on macrophage polarization. Additional assessment of the cytokine milieu would also be beneficial, both to assess the production of specific cytokines (including TNF$\alpha$, IFN$\gamma$, IL-6, IL-10) as a function of weight and surgical treatment in whole tissue and serum, as well as to identify cell types responsible for cytokine production. Lastly, it will be essential to correlate these findings with peripheral blood samples from human SG patients to assess for post-operative changes in B cell populations, and potential downstream modulators, as compared to pre-operative baseline samples.

## VI. Summary:

We have herein presented a unique murine surgical model for the evaluation of sleeve gastrectomy and its weight loss-dependent and weight loss-independent effects on glycemic control using non-pair-fed lean and obese animals treated with sleeve gastrectomy. Using this model, we have demonstrated that durable improvements in glycemic control occur in both lean and obese mice treated with SG, independent of the induction of durable weight loss. Using the novel CyTOF platform, we have further shown that significant, weight loss-independent decreases occur in follicular B cell populations in the spleen. By contrast, a comparable decrease in jejunal follicular B cell accumulation, and an increase in splenic $CD11c^{-}MHCII^{-}$ macrophage populations occurs only in obese animals fed a high fat diet. Similarly, obese animals, but not lean animals, exhibited significantly higher Arg1+ myeloid cell expression, which may point to a trend towards M2 anti-inflammatory macrophage polarization following SG in obese animals. Together these results demonstrate that SG is associated with significant alterations in both the systemic and local immune milieu in both weight loss-dependent and -independent fashions, suggesting that further evaluation of immunologic pathways affected by SG may represent valuable pharmacologic targets for the treatment of T2DM.

**VII. Tables and Figures:**

| Antigen | Clone | Conjugate metal |
|---|---|---|
| CD45 | 30-F11 | 141Pr |
| CD45R/B220 | RA3-6B2 | 142nd |
| CD21/35 | 2e9 | 143Nd |
| CD5 | 53-7.3 | 144Nd |
| CD4 | RM4-5 | 145Nd |
| CD11c | N418 | 146Nd |
| Ly6G | 1A8 | 148nd |
| CD19 | 6D5 | 149Sm |
| LY6C | HK1.4 | 151Eu |
| CD3 | 145-2C11 | 152Sm |
| CD335/NKp46 | 29A1.4 | 153Eu |
| CD80 | 16-10A1 | 154Sm |
| IgM | rmm1 | 156gd |
| T-bet | 4B10 | 158gd |
| CD23 | B3B4 | 159tb |
| CD11b | M1/70 | 160Gd |
| Arginase 1 | Polyclonal | 161DY |
| FoxP3 | FJK-163 | 162Dy |
| NK1.1 | PK136 | 163DY |
| CD8a | 53-6.7 | 164DY |
| CD103 | 2E7 | 166Er |
| CD25/IL-2Rα | 3C7 | 174Yb |
| CD64 | x54-5/7.1 | 168Er |
| CCR6 | 29-2L17 | 169Tm |
| ROR-γt | AFKJS-9 | 170Er |
| GATA3 | TWAJ | 172Yb |
| CD69 | H1.2F3 | 173YB |
| F4/80 | BM8 | 174YB |
| IgD | 11-26c.2a | 175Lu |
| CD197/CCR7 | 4B12 | 176YB |
| MHCII (IA/IE) | M5/114.15.2 | 209Bi |

**Table 1.** Antibodies used for CyTOF analysis of mouse spleen, liver, and proximal jejunum. All antibodies were prepared and validated by the BWH Mass Cytometry Core.

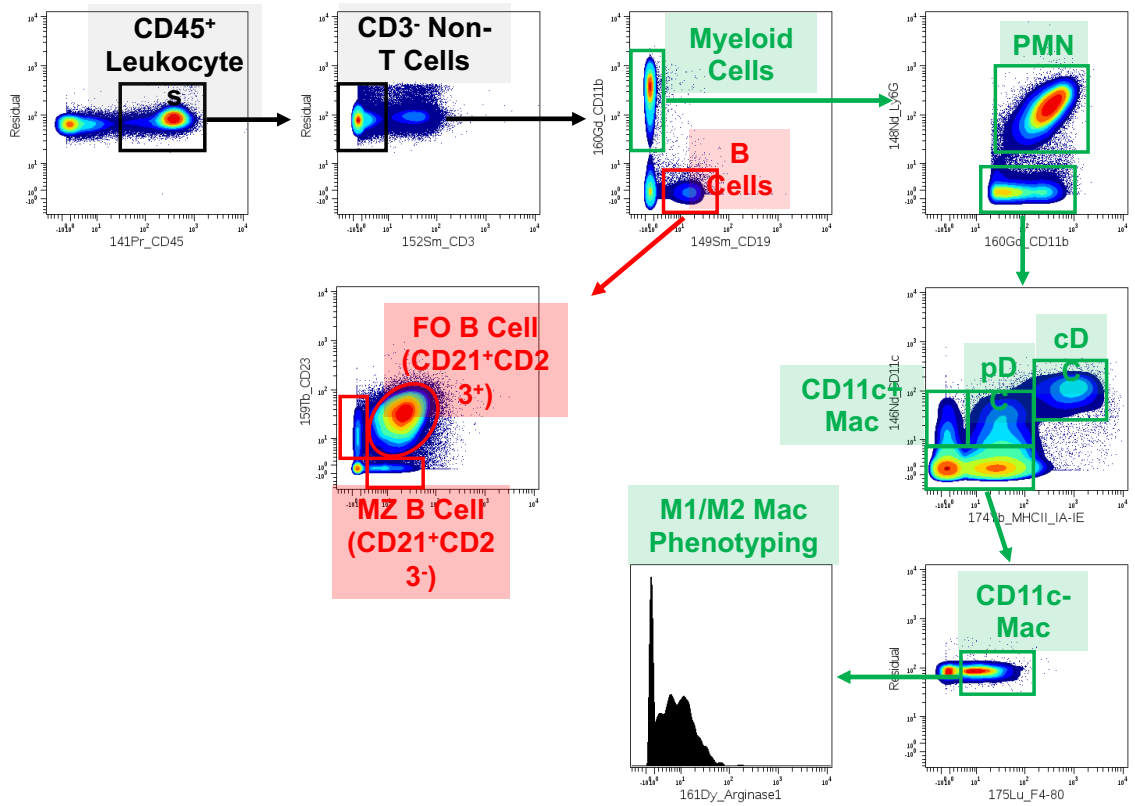


**Figure 1.** Gating scheme for analysis of myeloid and B-cell populations by CyTOF mass cytometry.

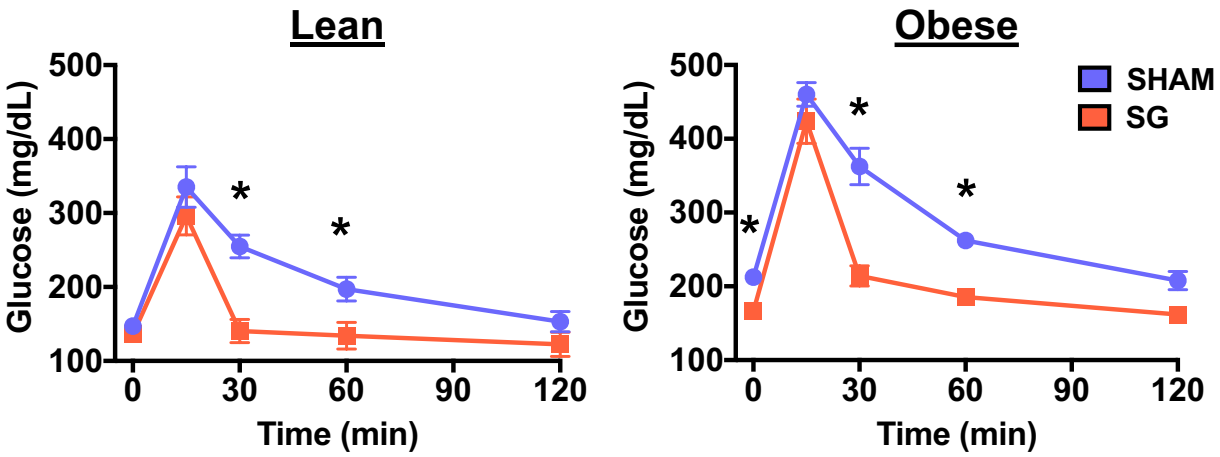


**Figure 2.** Oral glucose tolerance testing (OGTT) demonstrates improved glucose management, as measured by serum glucose levels 30 and 60 min after oral glucose challenge, in both lean and obese animals after SG as compared to sham. * = $p < 0.05$.

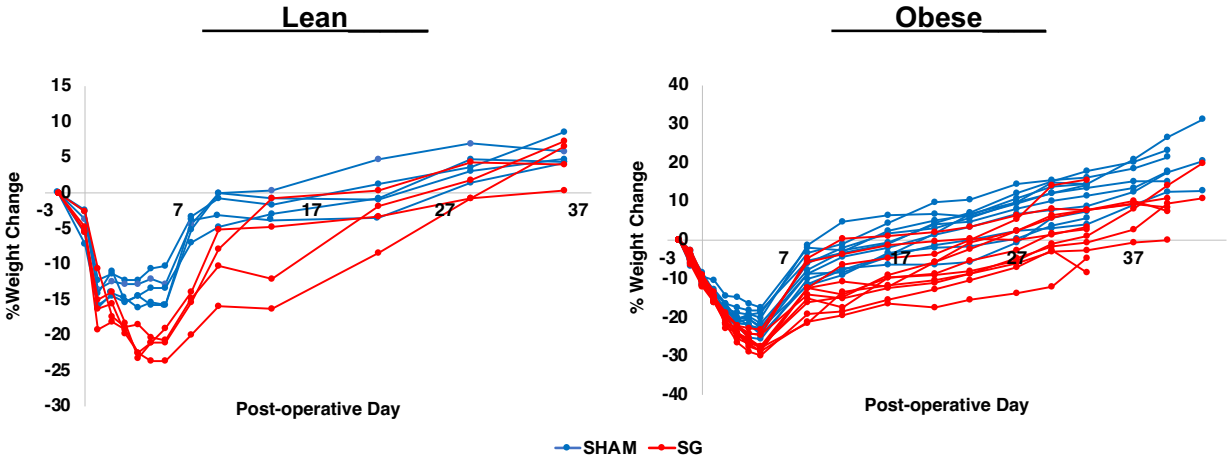


**Figure 3.** Post-operative weight change in lean and obese mice that underwent SG (red) or sham surgery (blue). Weight changes are expressed as a percentage difference from the pre-operative (POD -2) weight. Differences in weight loss between lean-SG and lean-sham mice did not reach statistical significance. Obese-SG mice, however, lost significantly more weight than obese-sham mice beginning on POD 4 and exhibited significantly lower weights thereafter as measured by student's T-test ($p < 0.05$).

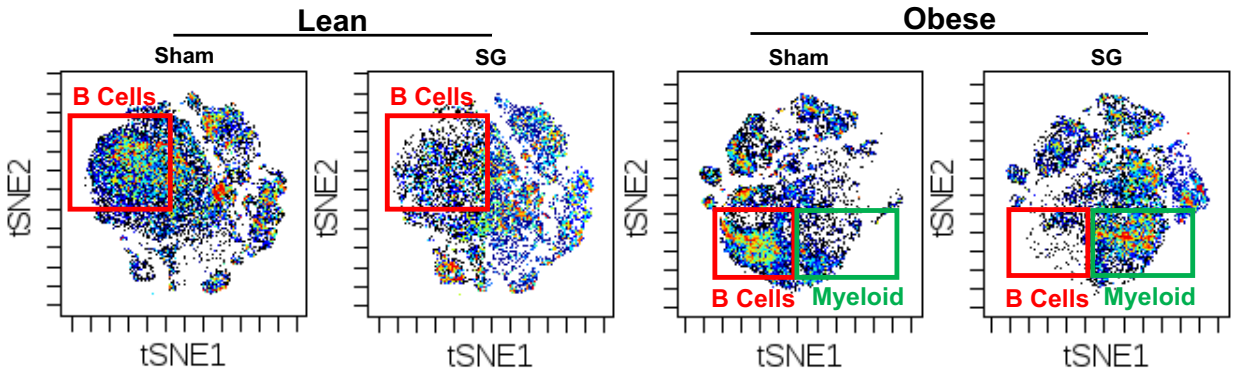


**Figure 4.** Multivariate viSNE screening analysis demonstrates qualitative alterations in the splenic immune milieu, demonstrated by representative plots, after SG in both lean and obese animals. Subset analysis demonstrates decreased cell counts in regions with high CD19 and CD21 signal intensity corresponding to B cells (Red boxes). Obese animals also demonstrate an increase in cell population in a region with high CD11b expression corresponding to myeloid cells (Green boxes).

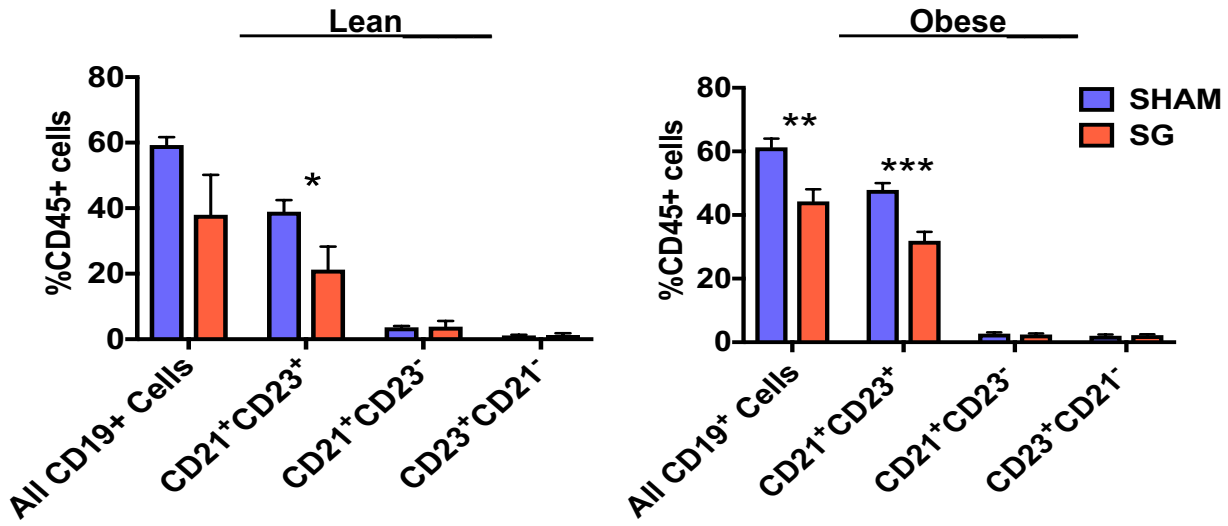


**Figure 5.** Mice that undergo SG (red) have fewer splenic $CD21^{+}CD23^{+}$ Follicular B cells than sham (blue), independent of diet and weight. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

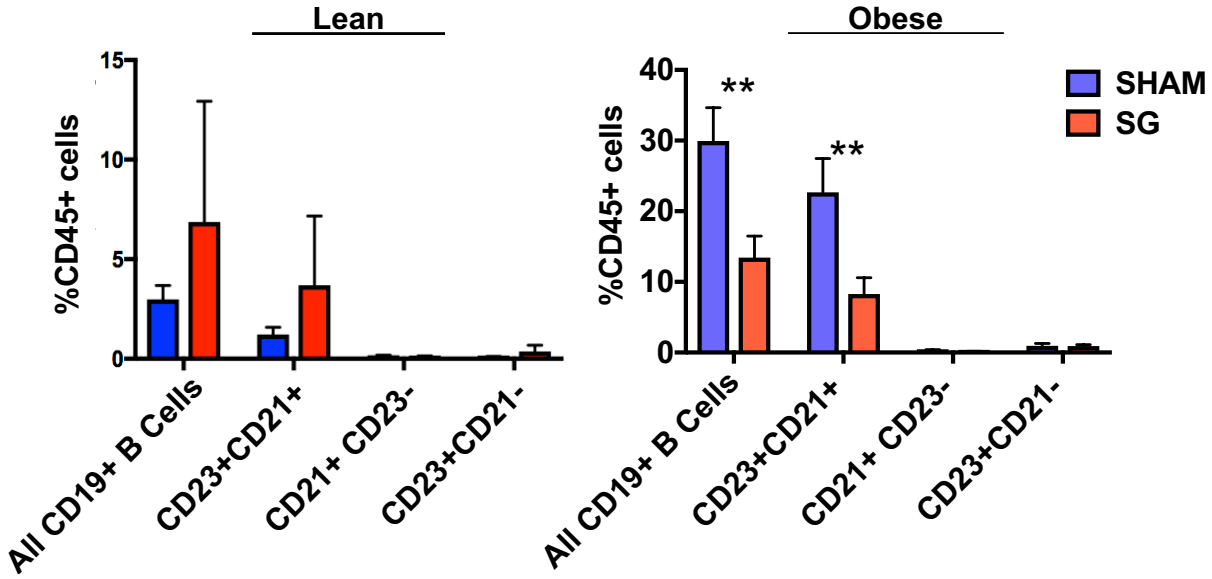


**Figure 6.** SG induces a decrease in jejunal total and follicular B cells in obese, but not lean, mice. ** = $p < 0.01$

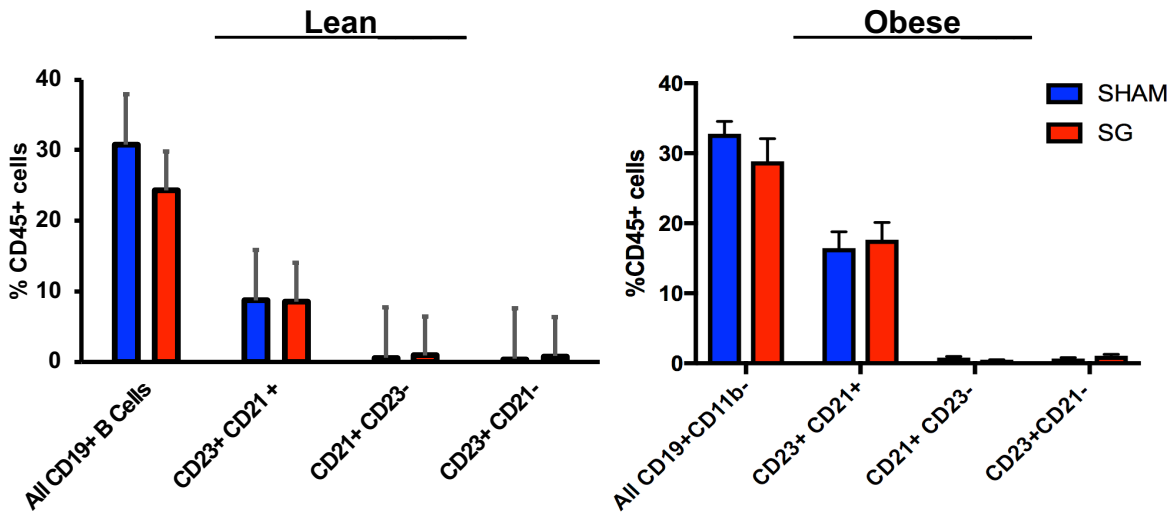


**Figure 7.** Hepatic B cell populations did not differ significantly in lean and obese animals treated with SG or sham surgery.

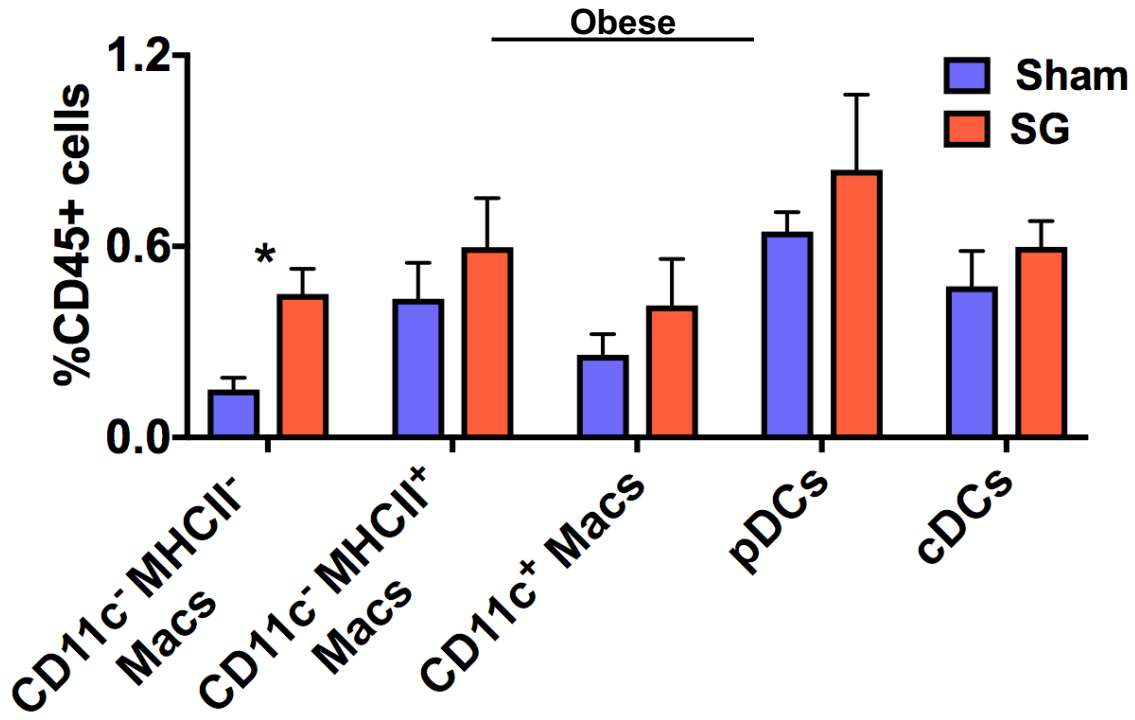


**Figure 8.** Obese-SG mice exhibit a significant increase in $CD11c^{-}MHCII^{-}$ splenic macrophages compared to sham-treated obese mice. * = $p < 0.05$

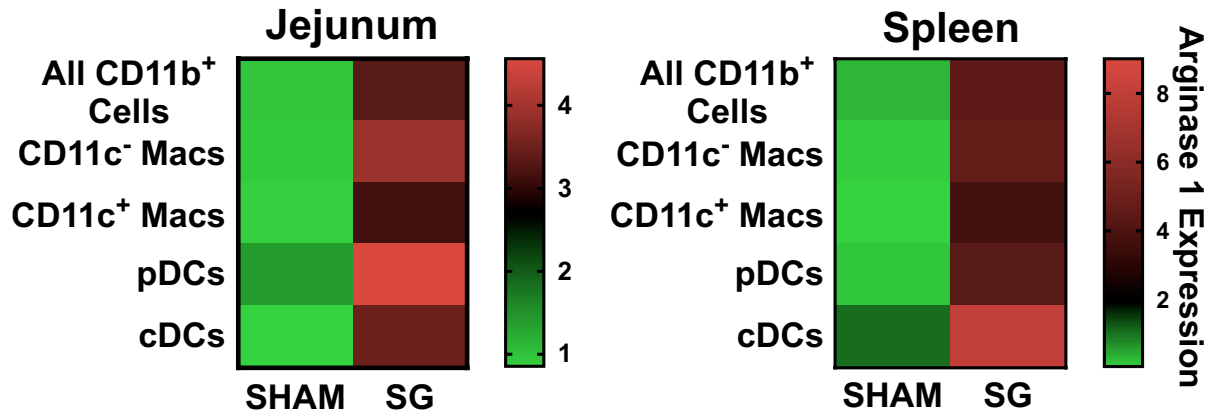


**Figure 9.** SG in obese mice is associated with increased Arg1 expression as measured by mean metal intensity (MMI) across all $CD11b^+$ myeloid cell (Jej: p = 0.022; Spleen: p = 0.06) sub-types, including $CD11c^-$ macrophages (Jej: p = 0.031; Spleen: p = 0.021) and $CD11c^+$ macrophages (Jej: p = 0.0118; Spleen: p = 0.029), pDCs (Jej: p = 0.015; Spleen: p = 0.015)) and cDCs (Jej: p = 0.005; Spleen: p = 0.034).

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