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Acute high-intensity interval exercise reduces human monocyte Toll-like receptor 2 expression in type 2 diabetes

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Abstract

Acute highintensity interval exercise reduces human monocyte Toll-like receptor 2 expression in type 2 diabetes. Am J Physiol Regul Integr Comp Physiol 312: R529 -R538, 2017. First published January 25, 2017; doi:10.1152/ajpregu.00348.2016.-Type 2 diabetes (T2D) is characterized by chronic low-grade inflammation that contributes to disease pathophysiology. Exercise has anti-inflammatory effects, but the impact of high-intensity interval training (HIIT) is not known. The purpose of this study was to determine the impact of a single session of HIIT on cellular, molecular, and circulating markers of inflammation in individuals with T2D. Participants with T2D (n 10) and healthy age-matched controls (HC; n 9) completed an acute bout of HIIT (7 1 min at ~85% maximal aerobic power output, separated by 1 min of recovery) on a cycle ergometer with blood samples obtained before (Pre), immediately after (Post), and at 1 h of recovery (1-h Post). Inflammatory markers on leukocytes were measured by flow cytometry, and TNF- was assessed in both LPS-stimulated whole blood cultures and plasma. A single session of HIIT had an overall anti-inflammatory effect, as evidenced by 1) significantly lower levels of Toll-like receptor (TLR) 2 surface protein expression on both classical and CD16 monocytes assessed at Post and 1-h Post compared with Pre (P 0.05 for all); 2) significantly lower LPSstimulated TNF- release in whole blood cultures at 1-h Post (P 0.05 vs. Pre); and 3) significantly lower levels of plasma TNF- at 1-h Post (P 0.05 vs. Pre). There were no differences between T2D and HC, except for a larger decrease in plasma TNF- in HC vs. T2D (group time interaction, P 0.05). One session of low-volume HIIT has immunomodulatory effects and provides potential antiinflammatory benefits to people with, and without, T2D.

Keywords

monocyte, human, diabetes, reduces, type, exercise, interval, high-intensity, acute, expression, 2, receptor, toll-like

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Acute high-intensity interval exercise reduces human monocyte toll-like receptor 2 expression in type 2 diabetes

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Key Words: inflammation, tumor necrosis factor-α, innate immunity, leukocyte, CD14+ monocyte

Abbreviations: T2D, type 2 diabetes; HIIT, high-intensity interval training; LPS, lipopolysaccharide; TLR, toll-like receptor; CD, cluster of differentiation; TNF, tumor necrosis factor; IL, interleukin; VO_2, oxygen consumption; VCO_2, carbon dioxide output; HC, healthy controls; HR, heart rate; RER, respiratory exchange ratio; ECG, electrocardiogram; MFI, median fluorescence intensity; RPE, rating of perceived exertion; FMO, fluorescence minus one

Abstract

Type 2 diabetes (T2D) is characterized by chronic low-grade inflammation that contributes to disease pathophysiology. Exercise has anti-inflammatory effects but the impact of high-intensity interval training (HIIT) is not known. PURPOSE: To determine the impact of a single session of HIIT on cellular, molecular, and circulating markers of inflammation in individuals with T2D. METHODS: Participants with T2D (n=10) and healthy age-matched controls (HC; n=9) completed an acute bout of HIIT (7 X 1-min @ ~85% maximal aerobic power output, separated by 1-min recovery) on a cycle ergometer with blood samples obtained before (Pre), immediately after (Post), and at one-hour of recovery (1-h Post). Inflammatory markers on leukocytes were measured by flow cytometry, and tumor necrosis factor (TNF)-α assessed in both lipopolysaccharide (LPS)-stimulated whole blood cultures and plasma. RESULTS: A single session of HIIT had an overall anti-inflammatory effect, as evidenced by: i) significantly lower levels of toll-like receptor (TLR) 2 surface protein expression on both classical and CD16+ monocytes assessed at Post and 1-h Post compared with Pre ($p<0.05$ for all); ii) significantly lower LPS-stimulated TNF-α release in whole blood cultures at 1-h Post ($p<0.05$ vs. Pre); and iii) significantly lower levels of plasma TNF-α at 1-h Post ($p<0.05$ vs. Pre). There were no differences between T2D and HC except for a larger decrease in plasma TNF-α in HC vs. T2D (group x time interaction, $p<0.05$). CONCLUSIONS: One session of low-volume HIIT has immunomodulatory effects and provides potential anti-inflammatory benefits to people with, and without, T2D.

Introduction

Chronic low-grade inflammation, characterized by increases in basal leukocyte numbers, circulating pro-inflammatory cytokines and/or acute phase reactants, is implicated in the pathogenesis of obesity, insulin resistance, and type 2 diabetes mellitus (T2D; (45). While the underlying cause of inflammation has not yet been fully elucidated, studies have shown elevation in surface protein expression of toll-like receptors (TLRs; (10)) and augmented release of pro-inflammatory cytokines by immune cells isolated from patients with T2D compared to age-matched normoglycemic controls (11), correlating altered immune cell phenotype and function with the inflammatory pathology in T2D. TLRs are conserved pattern-recognition receptors that recognize a variety of exogenous and endogenous pathogens to coordinate innate immune responses (26). Increased TLR2 and TLR4 expression and the resulting pro-inflammatory environment are associated with a cluster of cardiometabolic risk factors, including insulin resistance, T2D and atherosclerosis (10).

In addition to elevated TLRs, there is also evidence that monocyte subsets may be skewed towards a more pro-inflammatory profile in T2D (14). Monocytes can be categorized as classic, intermediate, and non-classical with the use of immunofluorescence analysis to determine cell surface expression of CD14 and CD16 (60). CD14++/CD16- “classical” monocytes are regarded as anti-inflammatory whereas CD16+, i.e. intermediate and non-classical, monocytes are considered as pro-inflammatory (4). CD16+ monocytes produce higher levels of tumor necrosis factor (TNF)-α compared to classical monocytes when stimulated with the same concentration of bacterial lipopolysaccharide (LPS) and other microbial ligands (4). CD16+ monocytes also show a blunted production of the anti-inflammatory cytokine interleukin IL-10 (5). Additionally, CD16+ monocytes are reported to have elevated surface expression of TLR2 and TLR4

compared to classical monocytes (20), further supporting the notion that CD16+ monocytes have a "pro-inflammatory" phenotype.

Exercise improves metabolic health and is a frontline therapy for the treatment and prevention of T2D (9). One potent systemic benefit of exercise is thought to be its anti-inflammatory effects (37). Some of the anti-inflammatory effects of chronic exercise are likely attributable to a reduction in adipose tissue (3) but there is also growing evidence that acute exercise, in the absence of weight loss, can directly impact immune cell phenotype and alter systemic inflammatory mediators (for review see (37)). In addition to benefits on glucose control and cardiorespiratory fitness (48), intervention studies report that exercise training can reduce the level of circulating pro-inflammatory markers, such as C-reactive protein and TNF-α (25, 29). An increase in circulating IL-6 following acute exercise is well-established (43) and is followed by the appearance of anti-inflammatory factors IL-1 receptor antagonist (IL-1RA), IL-10, and soluble TNF receptor (42). For this reason, exercise-induced elevations in circulating IL-6 are generally considered to be anti-inflammatory in nature (43). The ability of exercise to reduce monocyte TLRs is another hypothesized mechanism through which acute exercise may create a systemic anti-inflammatory milieu (16). Most studies have shown reduced monocyte TLR2 and TLR4 expression after acute endurance exercise (19) but there are reports of increased monocyte TLRs immediately and 1 h following prolonged strenuous exercise (60 km cycling ergometer time trial) (5). The influence of exercise on TLR expression on other distinct immune cells, including granulocytes/neutrophils, has not been adequately studied but our initial studies show that short-term exercise training can reduce TLR expression on neutrophils in addition to monocytes in individuals with obesity (47), suggesting a systemic impact of exercise for lowering leukocyte TLRs. Research also shows that exercise training can lead to a reduction in

the ratio of CD16+ "pro-inflammatory" monocytes to classical monocytes (58) suggesting that exercise might promote skewing towards a more anti-inflammatory monocyte profile; but this has not been tested in T2D.

High intensity interval training (HIIT) has gained recent attention as a time-efficient exercise strategy for improving cardiometabolic health, providing a unique physiological stimulus compared to traditional exercise (18). Several studies have shown potent glucose lowering and cardiovascular health benefits of HIIT (27, 35, 36) and a recent meta-analysis concluded that HIIT was superior to traditional continuous exercise for improving insulin sensitivity and glucose control (24). These findings highlight the potential utility of HIIT as a therapeutic exercise strategy in T2D but the impact of HIIT on inflammation in T2D has not, to our knowledge, been studied. There are speculations that vigorous exercise may be pro-inflammatory in people with cardiometabolic disease (28), even though empirical evidence showing that HIIT promotes inflammation is lacking. Further understanding of the inflammatory impact of HIIT in T2D is needed before this exercise strategy can be promoted for providing anti-inflammatory benefits.

The primary purpose of this study was to examine the impact of a single bout of HIIT on indicators of cellular and systemic inflammation in people with T2D. We examined: 1) leukocyte numbers and expression of TLR2 and TLR4 on classical monocytes, CD16+ monocytes, and CD16+ granulocytes (i.e., neutrophils); 2) *ex vivo* endotoxin-stimulated cytokine secretion in whole blood cultures as an index of innate immune cell activation; and 3) circulating TNF-α, to test the hypothesis that acute HIIT would promote anti-inflammatory effects in T2D. An age-matched normoglycemic healthy control group (HC) was included to help ascertain whether the presence of T2D influenced the immunomodulatory effects of acute HIIT.

Materials and Methods

Study Design and Participants

Ten T2D patients and nine age-matched normoglycemic controls were recruited for this two-group time-series study. T2D participants were diagnosed by a physician according to Canadian Diabetes Association criteria, based on haemoglobin A1C ≥6.5, fasting plasma glucose ≥7.0 mmol/l, and/or 2-h oral glucose tolerance test glucose ≥11.1 mmol/l. All T2D participants were enrolled in the Kelowna Diabetes Program (23) and had an A1C value <8.0% [mean(SD) = 6.5(0.7)]. T2D patients were screened for any cardiovascular abnormalities and cleared for vigorous exercise by a cardiologist via a 12-lead electrocardiogram (ECG) stress test prior to baseline fitness testing. Descriptive characteristics are presented in Table 1. Informed consent was obtained from all subjects prior to the study, which was approved by the UBC Clinical Research Ethics Board (H14-01636). Participants underwent baseline fitness testing using a ramp protocol (15 W/min) on an electronically-braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to determine peak power output (defined as the highest Watts achieved) and peak oxygen uptake (VO_{2PEAK}). Expired gas was collected via a mouthpiece (7600 Series V2 Mask, Hans Rudolph, Shawnee, KS) and oxygen uptake (VO_2) and carbon dioxide output (VCO_2) were determined by a metabolic cart (Parvomedics TrueOne 2400, Salt Lake City, Utah, USA), which was calibrated with a 3.0 L syringe and gases of known concentration prior to each test. Participants were instructed to pedal at a constant rate above 50 rpm for the duration of the test, which was stopped when participants could not maintain this cadence and/or volitional exhaustion. VO_{2PEAK} was defined as the highest 30-second average VO_2. Heart rate was monitored continuously (Polar Heart Rate Sensor H1, Polar, Kempele, Finland) and maximal heart rate (HR) was defined as the highest value attained during the test. Criteria for verifying

maximal exertion were as follows: a peak HR of at least 90% of age-predicted maximal HR (based on 220 – age) and a peak respiratory exchange ratio (RER) of at least 1.15 (38). All participants met these criteria.

Participants with T2D were participating in a 12-week exercise trial (ClinicalTrials.gov Identifier: NCT02251301) and completed four cycling HIIT familiarization sessions involving 4-6 X 1-min intervals prior to the acute exercise trial, which was done across two weeks in order to introduce them to HIIT, ensure no abnormal blood pressure or HR responses to HIIT, and build up to the exercise protocols for testing days. Age-matched normoglycemic controls self-reported completing 150-300 minutes of light-to-moderate physical activity per week (e.g., walking, golfing) but were not participating in any structured exercise training prior to the acute exercise trial. Sample size was calculated to detect an expected 30-50% reduction in TLR2 (19) and/or TLR4 (19, 41) described previously on CD14+ monocytes using means and standard deviations for median fluorescence intensity (MFI) of CD14+ TLR2 and TLR4 obtained from previous work in our lab (n=25 T2D patients). Using the sample size calculator G Power (version 3.1), 10 participants were needed at 80% power with α set at 0.05 assuming a moderate correlation (r=0.5) among repeated measures.

Acute Exercise Trial

All participants refrained from exercise for 48 hours prior to the acute exercise trial. T2D participants maintained their normal medication schedule throughout the study, including the day of the acute exercise trial. Subjects warmed up on the cycle ergometer at 30 W for four minutes before completing a HIIT session that was based on previously published protocols (35, 47) and consisted of 7 X 1-min intervals at 85% peak power output with 1-min rest periods at 15% peak power output in between. A 3-min cool down was completed after the final interval. HR data

were collected continuously by 12-lead ECG and ratings of perceived exertion (RPE; CR-10; (6)) were assessed during the final 10 seconds of each interval. Exercise began at either 11:00am or 4:00pm four hours postprandial and water was provided *ad libitum* throughout.

Blood Samples

Prior to the acute exercise trial, an indwelling 21-gauge venous catheter (BD Nexiva, Sandy, UT) was inserted into an antecubital vein and kept patent with sterile saline. Venous blood samples were taken before (Pre), immediately after (Post) and 1 h after (1-H post) the exercise session. These time points were chosen to be consistent with previous studies showing exercise-induced reductions in TLRs (5, 19, 41, 52). Blood was collected into vacutainers containing EDTA and kept at room temperature (for whole blood culture experiments) or on ice (for all other parameters) before further analysis. A portion of the blood collected was centrifuged at 1550g for 15 minutes at 4°C with plasma frozen at -80°C for later analysis of TNF-α via MagPIX assay (High-sensitivity T-cell HSTCMAG-28SK, Millipore, Massachusetts, USA), as we have previously described (47). The remainder of the blood was used for whole blood cultures and flow cytometry.

Whole Blood Cultures

Whole blood cultures were prepared by diluting blood 10 times in serum-free RPMI media (Sigma) supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml) containing 5 mM glucose and seeding cells in 12x75 mm polystyrene culture tubes at 540 µl per well as we have described previously (47). At each time point, one culture tube was left unstimulated and one was stimulated with 10 ng/ml bacterial lipopolysaccharide (LPS, from *Escherichia coli* 055:B5; L6529, Sigma). Supernatants were collected after 4 h of incubation at 37°C in 5% CO_2 for

analyses of TNF-α production via MagPIX assay (Human Cytokine/Chemokine Magnetic Bead Panel HCYTOMAG-60K) according to the manufacturer's instructions.

Flow Cytometry

FcR blocking reagent (Cat no. 130-059-901, Miltenyi Biotec, Bergisch Gladbach, Germany) was added to 90 µl of whole blood and allowed to incubate for 10 minutes at 4°C in the dark. This was followed by addition of conjugated antibodies specific for human CD14 (Vioblue®, Cat no. 130-094-364, Miltenyi Biotec), CD16 (FITC, Cat no. 130-091-244, Miltenyi Biotec), TLR2 (PE, Cat no. 130-099-016, Miltenyi Biotec), and TLR4 (APC, Cat no. 130-096-236, Miltenyi Biotec). Samples were then incubated for 10 minutes at 4°C in the dark. Finally, 1 ml of red blood cell lysis buffer (Cat no. 120-001-339, Miltenyi Biotec) was added to the samples and a final incubation step of 15 minutes at room temperature in the dark was administered. Immediately prior to flow cytometer analysis, 2 µl of propidium idodide (PI) (Cat no. 130-093-233, Miltenyi Biotec) was added to each sample for dead cell exclusion. Samples were analyzed on a MACSQuant® Analyzer 10 flow cytometer. 10,000 monocytes, identified by scatter profile, were counted in each sample. Bank instrument settings were used to account for any drift in laser strength over time. Compensation was performed prior to analysis to control for any spillover among fluorochromes. Flow cytometry data was analyzed with MACSQuantify™ Version 2.6 (Miltenyi Biotec).

CD14+/CD16- (i.e., classical monocytes), CD14+/CD16+ monocytes (i.e., CD16+ monocytes), CD16+ neutrophils were identified via a hierarchical gating strategy. Specifically, cells that stained positive for PI were first excluded from analysis and then cells were characterized as CD14+/CD16-, CD14+/CD16+, or CD14-/CD16+ populations (Figure 1; Panel A). The CD14+/CD16- and CD14+/CD16+ populations were then confirmed to be monocytes (Figure 1;

Panel B & C), and the CD14-/CD16+ population was confirmed to be neutrophils (Figure 1; Panel D) via characteristic scatter profile. TLR2 and TLR4 median fluorescence intensity (Figure 1; Panels E & F, respectively) were then determined on each of the cell types (classical monocytes, CD16+ monocytes, and neutrophils) with fluorescence minus one (FMO) controls used to determine gating on positive and negative populations. Monocytes, neutrophils, and lymphocytes were identified by their characteristic scatter profiles and total leukocyte number was calculated by addition of the three sub-populations. If cell populations had less than 300 total events, TLR expression was not analyzed due to insufficient events.

Insert Figure 1. Here

Statistical Analysis

Statistical analyses were performed using R (46). Statistical outliers were objectively removed from analysis using interquartile range with a multiplier of 2.2 based off the method by Hoaglin and Iglewics (22). Briefly, the 25^{th} and 75^{th} percentiles were determined and added to the interquartile range multiplied by 2.2 to calculate the "lower" and "upper" limits. Based off these limits, values that fell outside were deemed to be outliers and removed from the analyses. Normality was assessed using a Shapiro-Wilk test. Non-normal data was log or square-root transformed in order to reduce skewness. A mixed 2-factor ANOVA was used with time as a within subject factor and T2D status as a between subject factor to analyze differences in variables in response to exercise. Significance was set at $p<0.05$. Significant main effects of time were probed with Fisher LSD post-hoc tests with groups collapsed whereas interactions were probed with Fisher LSD post-hoc tests across time within groups.

Results

Participant Characteristics

T2D participants (n=5 males, n=5 females) had a higher body mass, lower VO_{2PEAK}, and lower $Watt_{PEAK}$ compared to age-matched HC (n=4 males, n=5 females) (Table 1). All participants completed the HIIT session with no issues. There were no differences in mean percent maximal HR (T2D: 81.4 ± 8.9%; HC: 83.8 ± 6.4%, p=0.52) or mean RPE (T2D: 5 ± 2; HC: 5 ± 2, p=0.46) measured during exercise between T2D and controls. Baseline carbohydrate, protein, fat, or energy intake assessed by 24-hour dietary recall did not differ between T2D and HC (data not shown).

Insert Table 1. Here

Leukocyte Numbers

The impact of a single session of HIIT on blood leukocyte numbers is presented in Table 2. There was a main effect of time for total leukocyte concentration ($p<0.001$; n=9 HC, n=10 T2D). The total number of leukocytes in the blood increased immediately after exercise ($p<0.001$, Post vs. Pre) and then declined at one hour following exercise ($p<0.001$, 1-H Post vs. Post). There was also a main effect of group (p=0.03) with T2D participants having a higher total leukocyte count than HC. There was a main effect of time ($p<0.001$; n=9 HC, n= 10 T2D) for classical monocyte numbers. Classical monocyte numbers were elevated immediately following exercise (Post) compared to before exercise (Pre) ($p<0.001$) and decreased one-hour post exercise (1-H Post) compared to pre-exercise (p=0.04, 1-H Post vs. Pre) and post-exercise ($p<0.001$, 1-H Post vs. Post). There was also a main effect of time for $CD16^+$ monocytes (p=0.004; n=8 HC, n= 10 T2D). $CD16^+$ monocyte numbers were elevated immediately post-exercise compared to both pre-

exercise (p=0.01, Post vs. Pre) and one-hour post exercise (p=0.005, 1-H Post vs. Post). A main effect of time was detected for neutrophil numbers (p<0.001; n=9 HC, n=10 T2D) with post-hoc tests indicating an increase immediately post-exercise compared to both pre-exercise (p<0.001) and one-hour post-exercise (p<0.001). There was also a main effect of group (p=0.02) with T2D displaying higher numbers of neutrophils than the HC. There were no group X time interactions for any of the leukocyte subsets analyzed (all p>0.05).

Insert Table 2. Here

Toll-like Receptor 2

A significant main effect of time was found for TLR2 expression on classical monocytes (p=0.01; n=7 HC, n=10 T2D). TLR2 expression on classical monocytes was decreased by ~16 % Post (p=0.007, Post vs. Pre) and by ~15% at 1-H Post (p=0.03, 1-H Post vs. Pre) (Figure 2A). There was no effect of group for TLR2 expression on classical monocytes (p=0.38) and no interaction effect (p=0.72). TLR2 expression on $CD16^+$ monocytes showed a similar main effect of time (p=0.007; n=8 HC, n=8 T2D) with post-hoc tests revealing a significant decrease of 18% Post (p<0.001, Post vs. Pre) and by ~11% 1-H Post (p=0.04, 1-H Post vs. Pre) (Figure 2B). There was no effect of group (p=0.7) or interaction (p=0.65) for TLR2 expression on $CD16^+$ monocytes. An acute session of HIIT had no effect on $CD16^+$ neutrophil TLR2 expression (p=0.11; n=8 HC, n= 10 T2D); however, there was a main effect of group with T2D expressing ~35% higher TLR2 than HC participants at all timepoints (p<0.001; Figure 2C).

Insert Figure 2. Here

Toll-like Receptor 4

There were no effects of time (p=0.56; n=9 HC, n=8 T2D), group (p=0.11), or interaction (p=0.60) for TLR4 expression on classical monocytes (Figure 3A). There were no significant effects of time (p=0.22; n=8 HC, n=7 T2D) nor was there an interaction (p=0.4) for TLR4 expression on $CD16^+$ monocytes; however, there was a main effect of group with T2D having ~15% higher TLR4 expression compared to HC participants across all timepoints (p=0.049; Figure 3B). There were no effects of time (p=0.25; n=8 HC, n=10 T2D) nor was there an interaction (p=0.93) for TLR4 expression on $CD16^+$ neutrophils. There was a main effect of group with T2D participants displaying ~10% higher TLR4 expression compared to HC on CD16+ neutrophils (p=0.02; Figure 3C).

Insert Figure 3. Here

Whole Blood Cultures

Absolute Cytokine Concentration

There were no effects of group for LPS-stimulated TNF-α release (p=0.12; n=9 HC, n=8 T2D) nor was there an interaction (p=0.76). There was a main effect of time for LPS-stimulated TNF-α release ($p<0.001$) with post-hoc tests revealing a ~20% decrease 1-H Post (p=0.02) compared to Pre. TNF-α release was also significantly lower (by ~33%) 1-H Post compared to immediately Post-exercise (p=0.001; Figure 4A). Unstimulated TNF-α release was largely undetectable and unchanged at all time points (data not shown; n=3 HC, n=4 T2D).

Leukocyte Corrected Cytokine Release

When corrected for total leukocyte numbers, there was a main effect of time (p=0.03; n=9 HC, n=9 T2D) for LPS-stimulated TNF-α release, with a ~20% decrease seen at 1-H Post compared

to Pre (p=0.03 vs. Pre) as well as a main effect of group with T2D releasing ~39% less TNF-α than HC (p=0.02) but no interaction (p=0.78; Figure 4B). Unstimulated leukocyte-corrected TNF-α release was largely undetectable and unchanged at all time points (data not shown; n=3 HC, n=4 T2D).

Insert Figure 4. Here

Plasma Cytokines

There was a group x time interaction for plasma TNF-α (p=0.02; n=6 HC, n=10 T2D). Visual inspection of Figure 5 suggests a larger decrease after exercise in HC. Post-hoc analysis revealed an ~14% decrease in T2D (p=0.04 vs. Pre) and a 44% decrease in HC (p=0.005) 1-H Post compared to Pre.

Insert Figure 5. Here

Discussion

This study shows that, in both T2D and HC, one bout of HIIT significantly reduces TLR2 expression on classical and CD16+ monocytes measured immediately after and at 1-h recovery from exercise. This was accompanied by small but significant reductions in both TNF-α production from LPS-stimulated whole blood cultures and in circulating plasma TNF-α. Overall, this suggests an anti-inflammatory effect of acute HIIT.

Effects of Exercise on TLRs

TLRs propagate an innate immune response to multiple ligands (including endotoxin, free fatty acids, and glucose) that may be elevated in T2D and it is theorized that higher TLR expression may drive chronic low-grade inflammation in T2D (10-12). One of the proposed cellular

mechanisms underlying the anti-inflammatory effect of exercise is a reduction in TLR expression (37). A reduction in cell surface TLR2 and TLR4 has been demonstrated after both acute bouts of exercise and longer duration training studies (41, 52, 55). The majority of the studies investigating the effect of acute exercise, however, tend to utilize relatively long duration exercise protocols lasting ≥90 minutes (5, 19, 33, 41, 52). In addition to reductions in cell-surface expression of TLRs, recent evidence also points to an upregulation of genes involved in the negative regulation of TLR signalling in whole blood cultures following a single bout of exercise (1). Exercise-induced reductions in TLR expression and signalling may be of particular relevance to inflammation in T2D because mechanistic studies have found that hyperglycemia can increase TLR2 and TLR4 expression in monocytes (10, 11) and both TLR2 (7) and TLR4 (51) are implicated in the pathogenesis of insulin resistance. We found that, on both classical and CD16+ monocytes, one bout of HIIT reduced TLR2 expression, which is in agreement with previous work using longer duration exercise bouts (19, 33, 41). There were no differences in the response between groups, suggesting that HIIT had equal impact on monocyte TLR2 reduction in T2D participants and HC. In contrast to previous work demonstrating a fairly consistent reduction in TLR4 after prolonged (>1 h) moderate-to-vigorous exercise (5, 19, 33, 41) we did not see any changes in TLR4. This may suggest that HIIT is not sufficient stimulus to reduce TLR4 and may have a preferential effect on TLR2. It is also possible that we did not detect an effect on TLR4 expression due to the timing of the blood measurements although we feel that is unlikely as past studies have observed changes at the timepoints chosen (5, 19, 41, 52). Given the previous research, it is reasonable to speculate that TLR4 may be more sensitive to exercise duration when compared to TLR2. As our primary purpose was to examine the impact of acute HIIT in T2D we unfortunately did not include a comparison to prolonged continuous exercise,

which we felt was largely impractical for patients with T2D. Indeed, T2D patients often cannot complete prolonged continuous exercise without rest breaks or sufficient acclimatization to the exercise (40, 57). The precise physiological mechanisms responsible for reductions in TLR expression in response to exercise have not been elucidated (for review see (16)). It is possible that the observed reduction in monocyte TLR2 expression after exercise is a consequence of receptor shedding, internalization, and/or suppression of gene expression. Matrix metalloproteinase (MMP) activation appears to be responsible for shedding of TLR2 from immune cells, which leads to an increase in soluble TLR2 (34). Acute exercise, which has been shown to increase circulating levels of MMP-9 (49) could promote TLR2 shedding but definitively testing this hypothesis in humans remains difficult. Internalization of TLRs is thought to occur following ligand binding where the TLR complex is recruited into lipid rafts and targeted to the golgi apparatus (59). Many TLR agonists have been shown to increase during exercise, including free fatty acids and heat shock proteins (2, 13, 56) which could potentially be involved in this mechanism of TLR down regulation. It is also possible that the reduction in TLR2 expression on classical and CD16+ monocytes observed Post and 1-H Post were due to the addition of a different population of cells into circulation than those observed at Pre (e.g., monocytes that were previously in the marginated pool) that may have expressed lower levels of surface TLR2. However, as the goal of this study was to determine the impact of a single bout of HIIT on TLR2 and TLR4 expression, the mechanism behind this effect was not investigated.

The majority of studies in the literature have investigated the role of exercise on TLR expression in monocytes. A novel aspect of this study was the characterization of TLR2 and 4 expression on neutrophils (CD16+ granulocytes). Neutrophil TLR2 is implicated in cytokine expression and superoxide production (31) while neutrophil TLR4 plays a crucial role in cell survival (50).

Although we observed a higher level of TLR2 and TLR4 on neutrophils in T2D compared to HC, there was no effect of exercise on neutrophil expression of either TLR2 or TLR4. These findings suggest that the impact of exercise on TLRs may be specific to monocytes.

Acute HIIT led to an expected increase in monocyte, neutrophils, and lymphocytes measured immediately after exercise (i.e., leukocytosis). Exercise-induced leukocytosis following a bout of high-intensity exercise is a well-established phenomenon, which has been demonstrated in both continuous and interval type exercise (15, 17, 21). Neutrophils and monocytes are thought to be mobilized primarily from the marginal pool and possibly bone marrow (15, 21, 44) whereas lymphocytes are likely recruited from the spleen and other lymphoid organs, as well as the lungs and the walls of high-endothelial venules (32, 39). This effect is dependent on exercise-induced elevations in circulating epinephrine and cortisol (39). Leukocyte numbers returned to baseline levels 1-h after exercise, which is in contrast with steady-state exercise where sustained leukocytosis has been shown to occur for up to two hours into recovery (21). Even though T2D participants had higher total leukocytes, there were no apparent group differences in the impact of acute HIIT on leukocyte numbers suggesting that T2D and HC respond similarly to this type of exercise. There were no effects of acute HIIT on the number or % of CD16+ monocytes, which suggests that acute vigorous exercise performed as HIIT does not impact the proportion of the main circulating monocyte subsets.

Cytokine Response

Interestingly, there was a small, yet statistically significant, reduction in plasma TNF-α 1-h after exercise in both T2D and HC. While both groups displayed lower levels of plasma TNF-α one hour into recovery, the reduction appeared more pronounced in the HC group (group X time interaction effect). This reduction in circulating TNF-α could be interpreted as an anti-

inflammatory effect of acute HIIT, although the mechanisms are not clear. In attempts to better understand the impact of acute HIIT on cytokine secretion from leukocytes, we performed parallel whole blood culture experiments in both unstimulated and LPS-stimulated conditions. Unstimulated whole blood culture TNF-α secretion was largely undetectable and there were no differences between groups or across time. In examining both absolute and leukocyte-corrected LPS-stimulated cytokine secretion in the whole blood cultures, the results tended to match the changes in plasma TNF-α such that LPS-stimulated TNF-α release was lower at 1 h recovery from acute HIIT. Taken together, the reduction in plasma TNF-α and LPS-stimulated whole blood culture TNF-α at 1 h recovery support an anti-inflammatory effect of an acute bout of HIIT in T2D and HC participants.

Limitations

Most previous studies examining the anti-inflammatory mechanisms of acute exercise, including monocyte TLRs and LPS-stimulated cytokine release, have used prolonged continuous moderate-to-vigorous exercise protocols (5, 19, 33, 41, 52). Due to the increasing popularity and utility of HIIT for improving cardiometabolic health in T2D and the unlikelihood that previously inactive older adults with T2D would perform ≥1 hour of moderate-to-vigorous intensity exercise, we focused on time-efficient HIIT in this study and unfortunately cannot directly compare HIIT to previous work involving more traditional endurance-oriented exercise.

In this study, we did not observe universally higher TLR expression in T2D compared to HC, which is inconsistent with previous findings by Dasu et al. (10), but is in line with work from other groups (30). It is possible that we did not detect any baseline differences in TLR expression due to the fact that the T2D participants in our study were not newly diagnosed and were taking

glucose lowering medications (Table 1). Indeed, it has been shown that the commonly prescribed T2D medication metformin can decrease TLR4 expression on human monocytes (61).

Similar to previous research (53, 54), we used LPS to stimulate whole blood cultures to examine blood leukocyte cytokine secretion in response to a standard inflammatory insult. Although TLR2 has been shown to be involved in monocyte responses to LPS (8, 50), TLR4 is regarded as the main LPS sensing receptor. Given that we saw reductions in TLR2 on monocytes following exercise, and higher TLR2 on neutrophils in T2D, stimulation of cultures with more pure TLR2 ligands such as PamCSK4 or peptidoglycan may have provided more insight into the functional responses of these cells following receptor downregulation.

Although we examined leukocyte numbers, phenotype, and function in response to acute HIIT it is not possible to examine or track inflammatory markers in immune cells that have infiltrated tissues (e.g., adipose, skeletal muscle, blood vessels) in vivo in human studies. Future work is needed to determine if the changes in monocyte TLR2 and cytokine secretion are also paralleled in tissue macrophages.

It is also worth noting that plasma TNF-α concentrations were not corrected for plasma volume shifts. The logic for this is to report plasma TNF-α concentrations that better represent the changing environment that the circulating leukocytes were exposed to.

It is important to note that the T2D participants had completed a brief familiarization period prior to the acute exercise trial. This involved four sessions of cycling HIIT (4-6 X 1-min intervals at ~80% maximal HR, RPE of ~5/10). This was deemed necessary to ensure the T2D participants could complete 7 X 1-min interval sessions, were accustomed to this type of vigorous exercise, and did not experience any abnormal HR or blood pressures responses to HIIT. This

familiarization amounted to a very low volume of exercise, but the results may not generalize to T2D participants completely naïve to HIIT. Both the T2D and HC participants refrained from any exercise for 48 hours prior to the acute trials but the HC participants did not complete the four cycling HIIT familiarization sessions as they were already habitually active for 150-300 minutes per week and completing such a low volume of familiarization HIIT was deemed unnecessary. The HC was leaner and more fit but was included in order to assess what the response to HIIT would be in healthy older adults without the potential complications of obesity or other comorbidities. Additionally, the exercise trials took place four hours postprandial in order to standardize the timing of exercise after a meal.

Perspectives and Significance

This study indicates that, in older adults with and without T2D, one bout of low-volume HIIT can reduce TLR2 expression, but not TLR4 expression, on monocytes. Acute low-volume HIIT had no discernable effect on neutrophil TLR2 or TLR4 expression. A single session of HIIT also led to reductions in both circulating and ligand-induced TNF-α. Taken together, these results indicate that HIIT is an efficient exercise stimulus for inducing cellular and molecular anti-inflammatory effects. As there was no indication of a pro-inflammatory effect of HIIT on the parameters measured in this study in either T2D patients or age-matched healthy controls, HIIT may be a suitable option for ameliorating the chronically elevated levels of inflammation implicated in T2D pathophysiology. Whether the anti-inflammatory effects induced by individual bouts of HIIT can culminate over time to improve health and impede the pathogenesis of T2D and its complications remains to be determined.

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Conflict of Interest/Financial Disclosure Statement: All authors declare no conflicts of interest and have nothing to disclose.

Table 1.

Participant Characteristics

Characteristic	Type 2 diabetes	Healthy controls	P-value
Weight (kg)	99.6 ± 17.0	71.2 ± 13.6	<0.001
Height (cm)	169.4 ± 11.8	168.8 ± 7.4	0.89
BMI	34.8 ± 5.9	24.8 ± 3.6	<0.001
Age (years)	57.9 ± 5.4	55.8 ± 9.0	0.53
VO_{2peak} (ml/kg/min)	18.9 ± 4.0	31.4 ± 4.5	<0.001
$Watt_{peak}$ (Watts)	147.6 ± 34.0	189.4 ± 43.1	0.03
Metformin only (n)	7	0	NA
Sulfonylurea + GLP1 Agonist	1	0	NA
SGLT2 Inhibitor + GLP1 Agonist	1	0	NA
DPP4 Inhibitor	1	0	NA

Note. Data are means ± standard deviation. Type 2 diabetes; n=5 males, n=5 females. Healthy controls; n=4 males, n=5 females

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Table 2.

Leukocyte Response to an Acute Bout of High-Intensity Interval Training (HIIT)

Cell Type	Type 2 diabetes			Healthy controls			P-value		
	Pre	Post	1-H Post	Pre	Post	1-H Post	Group	Time	Group X Time
Classical Monocytes x 10^5 /ml	3.2 ± 0.40	4.5 ± 0.58*	3.1 ± 0.29*$^{\#}$	3.3 ± 0.83	4.2 ± 1.6*	3.1 ± 0.92$^{\#}$	0.63	<0.001	0.24
CD16+ Monocytes x 10^5 /ml	0.18 ± 0.12	0.27 ± 0.19*	0.18 ± 0.11$^{\#}$	0.15 ± 0.08	0.17 ± 0.11*	0.09 ± 0.04$^{\#}$	0.3	0.004	0.17
Neutrophils x 10^5 /ml	30.6 ± 6.2	44.1 ± 11.8*	32.2 ± 5.8$^{\#}$	25.3 ± 7.6	32.1 ± 11.2*	24.4 ± 5.4$^{\#}$	0.02	<0.001	0.21
Lymphocytes x 10^5 /ml	15.9 ± 4.2	28.0 ± 7.3*	16.8 ± 4.7$^{\#}$	15.6 ± 3.9	26.1 ± 10.4*	15.1 ± 3.4$^{\#}$	0.57	<0.001	0.64
CD16+ Monocytes (% of total Monocytes)	4.5 ± 3.5	5.4 ± 3.7	5.3 ± 3.0	4.8 ± 2.4	4.3 ± 2.0	3.5 ± 1.8	0.48	0.62	0.08
Total Leukocytes x 10^5 /ml	50.3 ± 7.9	75.1 ± 18.3*	52.6 ± 8.3$^{\#}$	42.8 ± 13.1	58.9 ± 18.6*	43.5 ± 7.8$^{\#}$	0.03	<0.001	0.77

464 *Note*. Data are means ± standard deviation. Type 2 diabetes; n=5 males, n=5 females. Healthy controls; n=4 males, n=5 females.
465 *Fisher LSD post-hoc vs. Pre (time main effect, $p<0.05$). $^{\#}$Fisher post-hoc vs. Post (time main effect, $p<0.05$).

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Figure 1. Gating strategy for analysis of surface toll-like receptor (TLR) 2 and 4 on monocytes and neutrophils. Cells that stained positive for PI were first excluded from analysis. Cells are first gated on expression of CD14+/CD16- (classical monocytes), CD14-/CD16+ (CD16+ neutrophils), or both CD14+/CD16+ (CD16+ monocytes) (Panel A). Cell type is then confirmed via characteristic forward and side scatter profile for classical monocytes, CD16+ monocytes, and neutrophils (Panels B, C, and D, respectively). Cell surface TLR2 and TLR4 expression were then measured on each cell type (i.e., classical monocytes, CD16+ monocytes, and CD16+ neutrophils), FMO controls are displayed in red (Panels E and F).

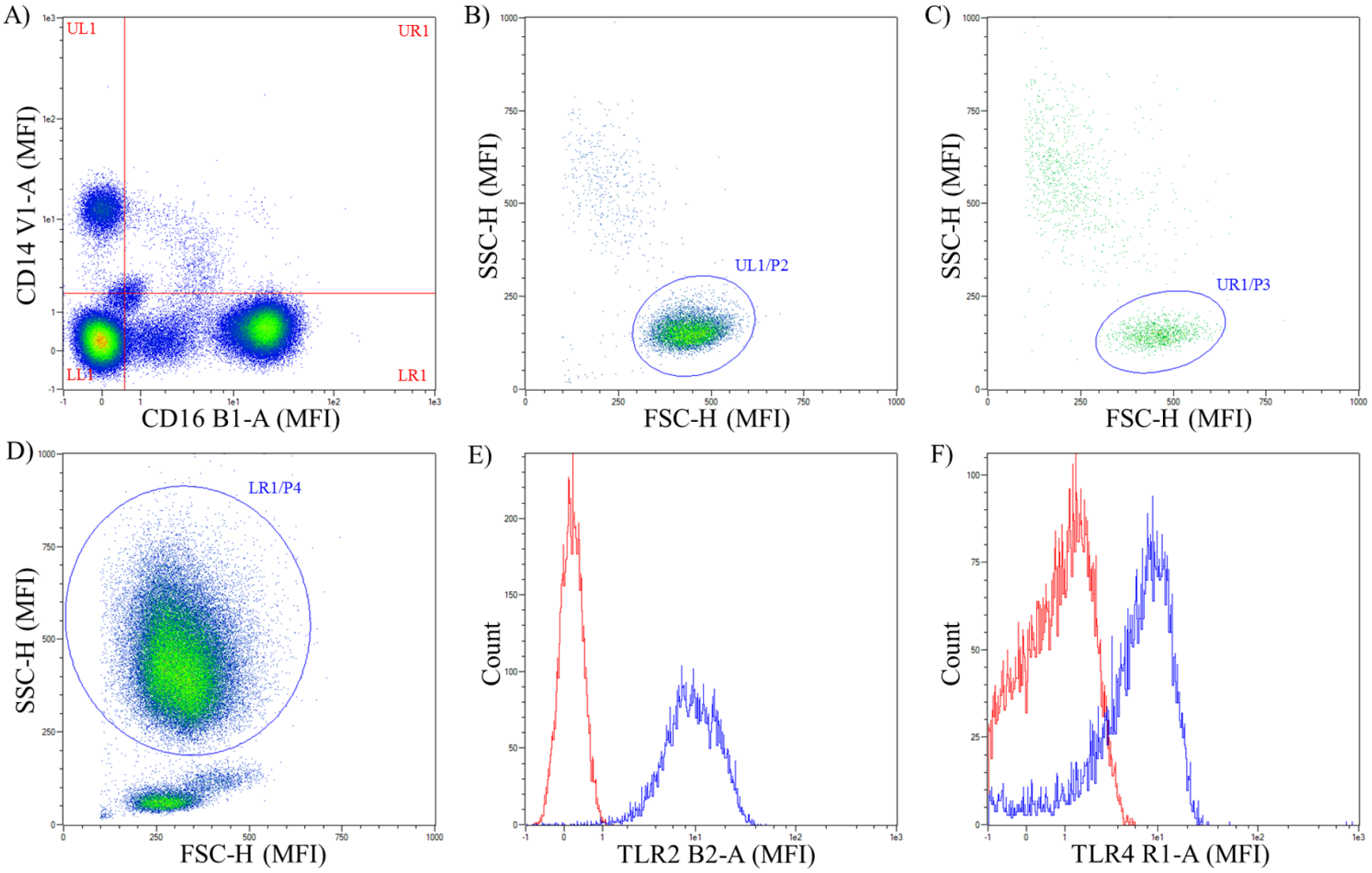
Figure 2. Toll-like receptor 2 expression on CD14+/CD16- classic monocytes, CD16+ monocytes, and CD16+ neutrophils in response to an acute bout of high-intensity interval training (HIIT). Blood samples were obtained before (Pre), immediately after (Post), and one hour following (1-H Post) a single session of HIIT involving 7 X 1-min @ 85% peak power output and TLR2 median fluorescence intensity (MFI) was measured by flow cytometry on CD14+/CD16- monocytes (A), CD16+ monocytes (B), and CD16+ neutrophils (C). Group means are denoted by dotted (Healthy Controls) or solid (Type 2 Diabetes) horizontal lines. Repeated measures ANOVA revealed a significant main effect of time for classical monocytes and CD16+ monocytes (all $p<0.05$). *$p<0.05$ vs. Pre (Fisher LSD post-hoc). †A main effect of group was also detected for CD16+ neutrophils ($p<0.05$)

Figure 3. Toll-like receptor 4 expression on CD14+/CD16- classic monocytes, CD16+ monocytes, and CD16+ neutrophils in response to an acute bout of high-intensity interval training (HIIT). Blood samples were obtained before (Pre), immediately after (Post), and one hour following (1-H Post) a single session of HIIT involving 7 X 1-min @ 85% peak power output and TLR4 median fluorescence intensity (MFI) was measured by flow cytometry on

CD14+/CD16- monocytes (A), CD16+ monocytes (B), and CD16+ neutrophils (C). Group means are denoted by dotted (Healthy Controls) or solid (Type 2 Diabetes) horizontal lines. †Repeated measures ANOVA revealed a significant main effect of group for CD16+ monocytes and CD16+ neutrophils (both $p<0.05$).

Figure 4. Whole blood culture TNF-α concentration from 4-H supernatants stimulated with 10 ng/ml lipopolysaccharide (LPS) in response to an acute bout of high -intensity interval training (HIIT). Blood samples were obtained before (Pre), immediately after (Post), and one hour following (1-H Post) a single session of HIIT involving 7 X1-min @ 85% peak power output and absolute TNF-α (A) and leukocyte concentration corrected TNF-α (B) secretion in whole blood cultured in the presence of 10 ng/ml LPS was measured. Supernatants were collected after four hours in culture and TNF-α was measured by Magpix ELISA. Group means are denoted by dotted (Healthy Controls) or solid (Type 2 Diabetes) horizontal lines. Repeated measures ANOVA revealed a significant main effect of time for absolute TNF-α concentration ($p<0.05$) and a significant main effect of group for leukocyte corrected TNF-α concentration (†$p<0.05$). *$p<0.05$ vs Pre (Fisher LSD post-hoc). #$p<0.05$ vs Post (Fisher LSD post-hoc).

Figure 5. Circulating plasma TNF-α concentration in response to an acute bout of high -intensity interval training (HIIT). Blood samples were obtained before (Pre), immediately after (Post), and one hour following (1-H Post) a single session of HIIT involving 7 X 1-min @ 85% peak power output and TNF-α in plasma samples was measured by Magpix ELISA. Groups means are denoted by dotted (Healthy Controls) or solid (Type 2 Diabetes) horizontal lines. Repeated measures ANOVA revealed a significant group x time interaction for TNF-α concentration ($p<0.05$). ‡$p<0.05$ vs. Pre within each group (Fisher LSD post-hoc).


A)
UL1
UR1
LL1
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CD14 V1-A (MFI)
CD16 B1-A (MFI)
B)
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SSC-H (MFI)
FSC-H (MFI)
C)
UR1/P3
SSC-H (MFI)
FSC-H (MFI)
D)
LR1/P4
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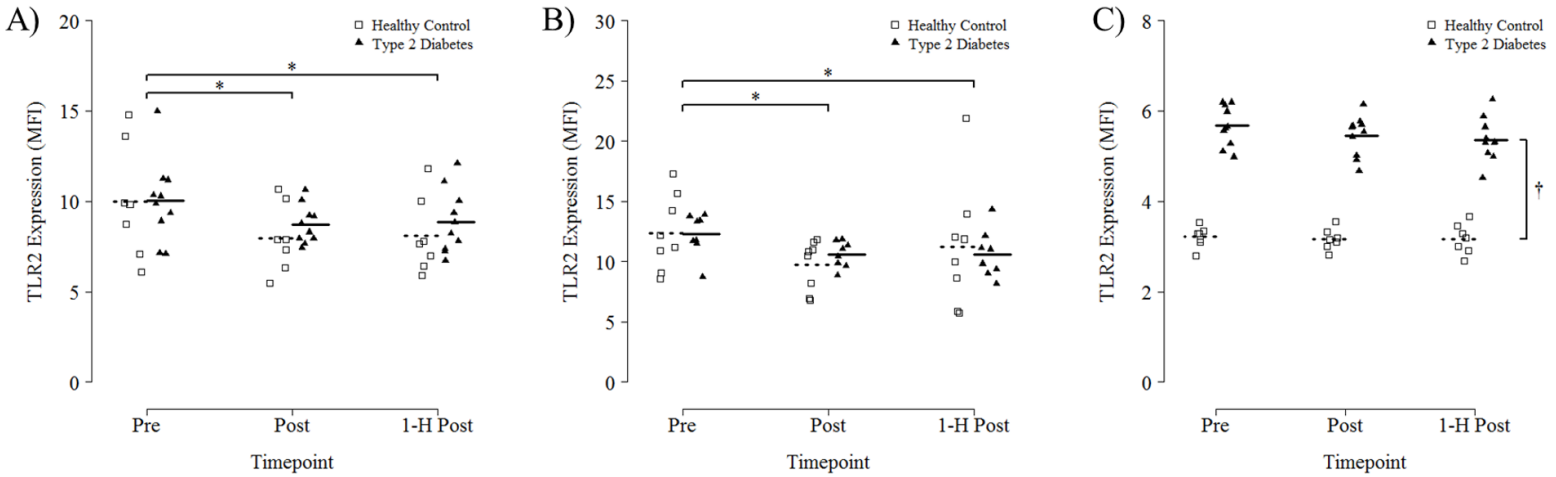
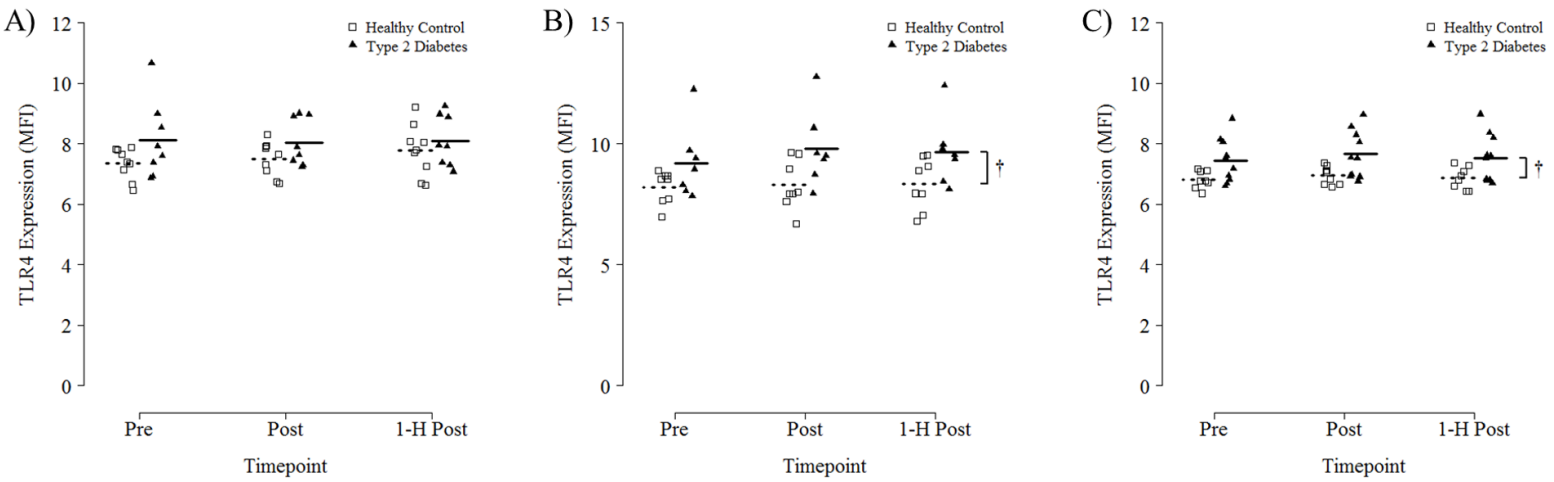
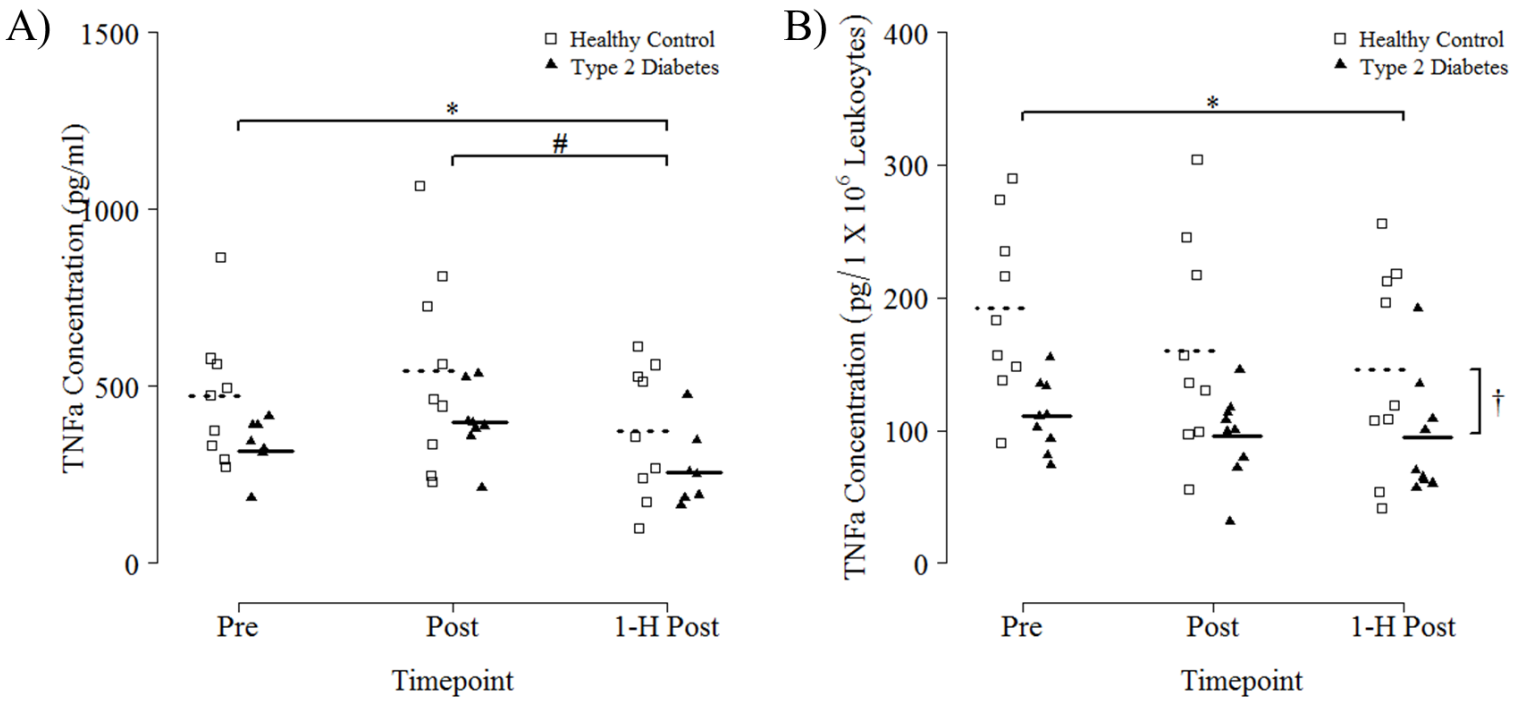
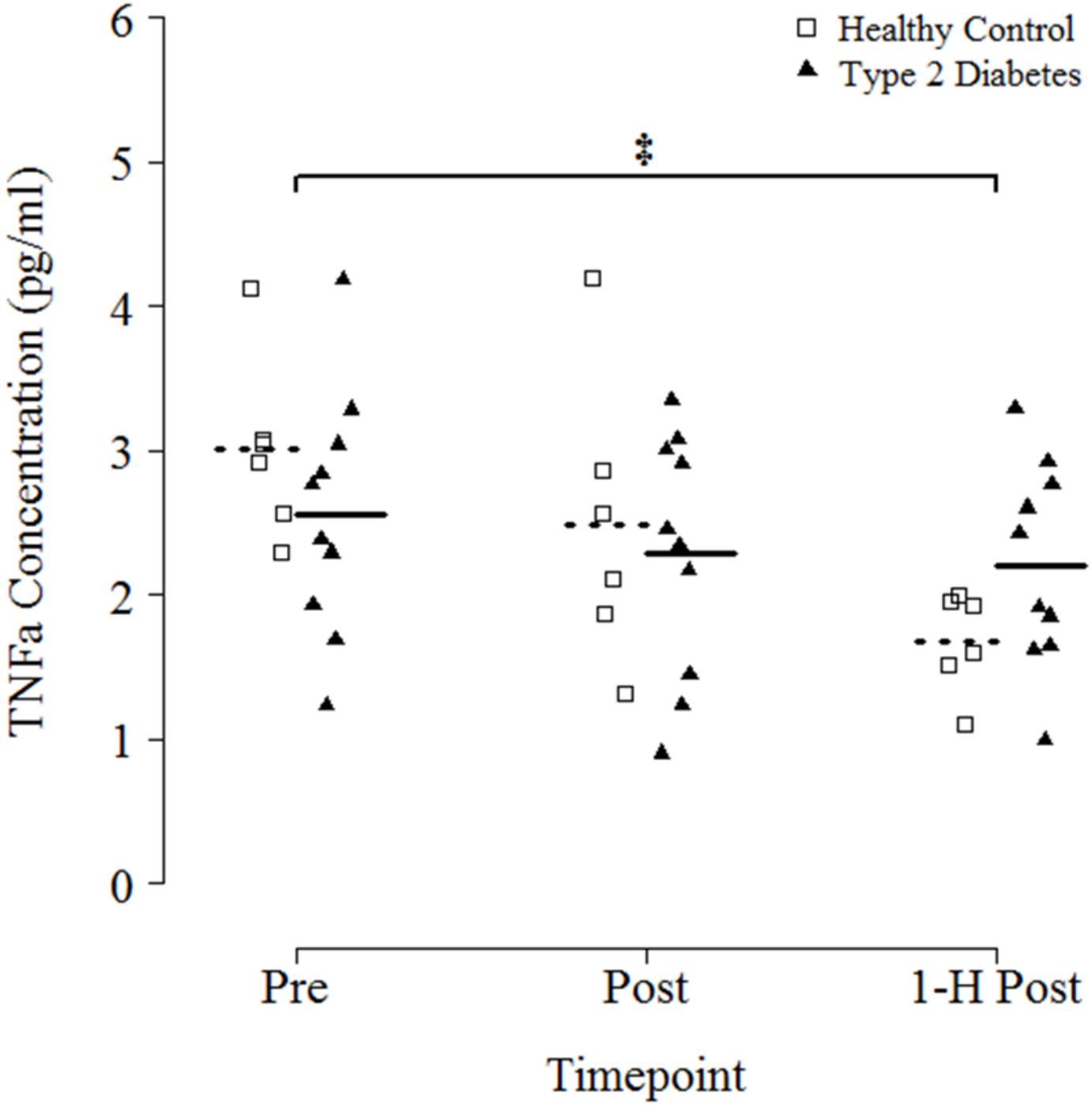

A)
B)
C)
Healthy Control
Type 2 Diabetes
TLR2 Expression (MFI)
Timepoint
Pre
Post
1-H Post
*
†


A)
B)
C)
Healthy Control
Type 2 Diabetes
TLR4 Expression (MFI)
Pre
Post
1-H Post
Timepoint
†


A)
1500
1000
500
0
TNFa Concentration (pg/ml)
Healthy Control
Type 2 Diabetes
*
#
Pre
Post
1-H Post
Timepoint
B)
400
300
200
100
0
TNFa Concentration (pg/1 X 10^6 Leukocytes)
Healthy Control
Type 2 Diabetes
*
†
Pre
Post
1-H Post
Timepoint


Healthy Control
Type 2 Diabetes
TNFa Concentration (pg/ml)
0
1
2
3
4
5
6
‡
Pre
Post
1-H Post
Timepoint