The mobilisation of early mature CD56^{dim}CD16^{bright} NK cells is blunted following a single bout of vigorous intensity exercise in Type 1 Diabetes

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ABSTRACT

Type 1 diabetes (T1D) is a T cell mediated autoimmune disease that targets and destroys insulin-secreting pancreatic beta cells. Although T cell mediated, a number of other immune cells are also critically involved in co-ordinating the events leading to T1D. Specifically, innate subsets play an important role in the pathogenesis of T1D. NK cells are one of the first cell types to infiltrate the pancreas, causing damage and release of beta cell antigens. Previous work in our group has shown differential mobilisation of highly differentiated CD8$^+$ T cells during vigorous intensity exercise in T1D compared to a control cohort. Here, we aimed to explore exercise-induced mobilisation of other cell types involved in T1D pathogenesis. In this study, we investigated the effects of a single bout of vigorous (80% predicted VO_2 max) intensity exercise on innate cell mobilisation in T1D and control participants. T1D (N=12, mean age 33.2yrs, predicted VO_2 max 32.2 ml.kg.min^{-1}, BMI 25.3 kg.m^{-2}) and control (N=12, mean age 29.4yrs, predicted VO_2 max 38.5 ml.kg.min^{-1}, BMI 23.7 kg.m^{-2}) male participants completed a 30-minute bout of cycling at 80% predicted VO_2 max in a fasted state. Peripheral blood was collected at baseline, immediately post-exercise, and 1 hour post-exercise. NK cell subsets mobilised during vigorous intensity exercise in both control and T1D participants. However, mature NK cells, defined as the CD56^{dim}CD16^{bright} subset, displayed a lower percentage increase following vigorous intensity exercise in T1D participants (Control: 185.12%, T1D: 97.06%). This blunted mobilisation was specific to early mature NK cells (KIR$^+$) but not later differentiated NK cells (KIR$^+$CD57$^+$). Myeloid lineage subsets mobilised to a similar extent in both control and T1D participants. In conclusion, vigorous exercise mobilises innate immune cells in people with T1D albeit to a different extent to those without T1D. This mobilisation of innate immune cells provides a mechanistic argument to support exercise in people with T1D where it has the potential to improve surveillance for infection and to modulate the autoimmune response to the beta cell.

Key Words: Exercise, Physical activity, Type 1 Diabetes, Innate immunity, Natural Killer cells

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INTRODUCTION

Type 1 Diabetes (T1D) results from beta cell destruction caused by autoreactive T cells. Pancreatic lymphocyte infiltration results in a hallmark inflammation within the pancreas referred to as insulitis (57). In the initial stages of insulitis, a mixed leukocyte population including CD4$^+$ T cells, CD8$^+$ T cells, natural killer (NK) cells, Dendritic cells (DC), B cells, and macrophages infiltrate the pancreas (49, 67, 69, 81). Trafficking of CD45$^+$ cells from lymphoid organs to the pancreas, specifically to the region of insulitis, has been demonstrated by cell tracking in mice (49). CD4$^+$ T cell, CD8$^+$ T cell, and B cell populations are seen to migrate into the pancreas. NK cells and B cells make up much lower proportions of pancreatic lymphocyte populations (67). However, NK cells are one of the first cell types to infiltrate the pancreas, even before autoreactive T cell infiltration occurs (2, 8, 29, 64). The presence of NK cells results in damage and release of beta cell antigens. Myeloid lineage subsets then present beta cell antigens to autoreactive T cells and thus begins the autoreactive cascade. As demonstrated by these mixed immune populations infiltrating the pancreas in T1D, a range of immune cell subsets are involved in the pathogenesis of T1D.

T1D is an immune mediated disorder and the primary treatment is insulin replacement. However insulin replacement does not achieve the level of diabetes control required to protect patients from the complications of this condition (44). Whilst significant effort has been invested in trying to prevent and cure T1D, little progress has been made to date (75). Exercise has clear immunomodulatory effects which could unlock the potential for immunotherapy of T1D (43). However, to do so, we need to understand the effects of exercise on the immune system in T1D. Previous work in our group has shown differential mobilisation of highly differentiated CD8+ T cells during vigorous intensity exercise in T1D, a subset highly involved in the pathogenesis of T1D (23). This study however did not address the effect of exercise on innate immune cells. Because innate immune cells are some of the first cells to infiltrate the pancreatic islet in T1D, we also wanted to characterise the effect of exercise on innate immune cell mobilisation in people with T1D.

Innate cells in T1D

NK cells home to the diabetic pancreas before T cell infiltration or beta cell destruction occurs. NK cells have been

detected from day 1 in the pancreatic infiltrate of the non-obese diabetic (NOD) mouse, before inflammation becomes established (8, 64). A high percentage of NK cells are also detected at weeks 4-5 in the NOD pancreas, i.e. the prediabetic stage. Both NK cells and T cells were then detected in later weeks 9-10 (8). Furthermore, NK cells were present in the pancreas of NOD-Rag (no B or T cells) mice indicating NK cells infiltrate the pancreas independent of T cells and established inflammation, and is believed to be the first event in insulitis (8).

NK cell infiltration into the pancreas drives T1D onset. Increased intra-pancreatic NK cells accelerated the onset of diabetes in NOD mice and NK cell associated cytokines were hyper-expressed in the pancreas from mice with accelerated diabetes onset (2). In addition to this, intra-pancreatic NK cells influence diabetogenic T cell function. Activated NK cells within the pancreas of NOD mice produce large amounts of IFN-γ, thus promoting effector function of diabetogenic $CD4^+$ T cells (29). Furthermore, depletion of NK cells has prevented T1D in animal models, supporting their involvement in T1D pathogenesis (2, 30, 64).

NK cells within the islet have a distinct phenotype, differing to those found in the spleen and peripheral blood (74). Pancreatic NK cells are in an activated state indicted by increased expression of CD25, CD69, and PD-1, coupled with down-regulation of CD62 ligand (CD62L). Pancreatic NK cells also express higher killer cell lectin-like receptor subfamily G member 1 (KLRG1) than splenic NK cells, indicating their high proliferation state (74), and lower natural-killer group 2-member D (NKG2D) expression, a natural cytotoxicity receptor, was also reported (74).

Myeloid subsets, DC and macrophages, can also be detected within the pancreas during insulitis in diabetic donors (54, 69). DCs (defined as lineage negative, $HLA\text{-}DR^+$ (48)) are present in the islets of diabetic donors and have a clear role in the pathogenesis of T1D (69, 81). DC present beta cell antigens to autoreactive T cells, driving T cell trafficking to the pancreas (78). In NOD mice, DCs are efficient antigen presenting cells (APC). DCs effectively stimulated GAD, a beta cell autoantigen, reactive T cell proliferation in *in vitro* co-cultures (52). In another study, plasmacytoid DCs (pDC) were shown to present immune complexes to $CD4^+$ T cells more efficiently than conventional DCs (cDCs), suggesting a possible pathogenic role of pDCs in T1D onset (3).

Macrophages have also been implemented in the pathogenesis of T1D. Macrophages are recruited to the pancreas by $CD4^+$ T cells (13). One week after adoptive transfer of $CD4^+$ T cell into NOD.SCID (no B or T cells) mice, macrophages were detected in pancreatic infiltrates. Diabetogenic $CD4^+$ T cells recruit macrophages through C-C Motif Chemokine Ligand (CCL)1 secretion. This interacts with and recruits activated $CCR8^+$ macrophages (13). Macrophage infiltration is also mediated through CCL2 expression on beta cells. CCL2 promotes recruitment of macrophages from the bone marrow to the islets. Furthermore, CCL2 receptor inactivation prevents macrophage recruitment (54).

Acute exercise and innate cells

A significant amount of work on the effects of exercise on immunity has been undertaken in non-T1D cohorts. Acute exercise causes significant immune cell mobilisation, of which NK cells are the most exercise responsive lymphocyte subset (35, 56, 73). Fully differentiated $CD8^+$ T cells are the next most significantly mobilised cell subset (12, 79), with lower levels of mobilisation among $CD4^+$ T cells and B cells (12, 34, 72).

NK cells are phenotypically identified as $CD3^-CD56^+$ (21, 68). NK cells can be further divided based on their CD16 expression into functionally different subsets; $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56d^{im}CD16^{dim}$ and $CD56^{bright}CD16^-$ (65). During maturation, NK cells become $CD56^{dim}$, and lose NKG2A expression (7). This is followed by increased killer immunoglobulin receptor (KIR) expression, with a gradual increase in CD57 (7). CD57 is a marker of highly mature, highly differentiated NK cells, and is expressed by highly cytotoxic $CD56^{dim}CD16^{bright}$ NK cells (47). Lack of Inhibitory C-type lectin receptor A (NKG2A) and expression of KIRs independently correlated with reduced proliferation, and co-expression of CD57 was associated with a completely abolished proliferative response to cytokines. This is evidenced by $KIR^+CD57^-NKG2A^-$ proliferate more than $KIR^+CD57^+NKG2A^-$.

NK cells are the most responsive lymphocyte subset to acute exercise due to their high beta-adrenergic receptor expression resulting in their preferential intensity-dependant mobilisation in response to adrenaline during acute exercise (5, 25, 50, 82). Of the NK cells mobilised, $CD56^{dim}CD16^{bright}$ NK cells and those with a highly differentiated phenotype ($CD57^+$ KIR^+ $NKG2A^{lo}$) are preferentially redeployed and demonstrate the largest increase post-exercise (6, 76). This is followed by a larger decrease below baseline during the recovery period. Following exercise, $CD56^{bright}$ subsets return to baseline levels, whereas $CD56^{dim}$ decrease below baseline levels (6, 76). There have also been reports of IL-2Rβ ($CD122^+$) and IL-2Rα ($CD25^+$) NK cells increasing following exercise. CD25 is expressed on $CD56^{bright}$ NK cells in comparison to CD122 which is constitutively expressed on all naïve NK cells (56, 73).

DCs also increase in the peripheral blood in response to physical stress (9, 24, 38, 60). In particular, there is an increase in DCs expressing adhesion molecules CCR5 and CD62L. Circulating DCs also display reduced toll-like receptor (TLR) responsiveness after acute exercise, as evidenced by a less pronounced upregulation of activation markers, HLA-DR and CD86. Therefore this indicates the mobilisation of DCs which may be less prone to drive inflammatory processes following exercise (24). In a recent study by Brownet al., (2018), 9 healthy males completed a 20-minute cycling bout at 80% VO_2 max. DCs were reported to increase by 150% following exercise. In this study, there was a preferential mobilisation of plasmacytoid DCs (pDC) ($CD303^+$) over than myeloid DCs (mDC) ($CD303^-$) during exercise. Within the mDC subsets, $CD1c^-CD141^-$ cells showed the largest exercise-induced mobilisation, with a stepwise pattern observed for

$CD1c^+CD141^-$, $CD1c^+CD141^+$, $CD1c^-CD141^+$ cells. It was also reported that $CD205^-$ mDC, DCs capable of recognising apoptotic and necrotic cells, were the most exercise responsive. All DC subsets returned to resting levels within 30 minutes following exercise cessation (9).

Other mononuclear cells also respond to exercise. Monocytes mobilise in an exercise intensity-dependent manner, with mature monocytes ($CD14^{low}$) increasing the most (33, 71, 80). Granulocytes also increase, with the majority of these being neutrophils. Neutrophils increase immediately post-exercise and fall below baseline during the 1 hour post-exercise recovery period, this is followed by an increase 2 hours post-exercise referred to as the "second wave" (10, 46, 66). Vigorous acute exercise also increases hematopoietic stem and progenitor cells (HSPC) post-exercise (4, 27).

Acute exercise and T1D

To date, there is a limited amount of research investigating the effects of exercise in people with T1D. This research is predominantly focused in two areas. First, exercise training in T1D has been shown to mediate improvements in beta cell function through increased insulin content and insulin secretion (43, 58, 59). We have previously hypothesised that an exercise training programme has the potential to modulate beta cell loss in people newly diagnosed with T1D (59). We have tested this hypothesis in a pilot randomised controlled trial (43, 58). This study showed that beta cell function, when corrected for the changes in insulin sensitivity that accompany physical exercise, appears to be preserved in people with T1D. Furthermore, exercise training in streptozotocin-induced T1D mice significantly increased insulin content and insulin secretion compared to sedentary mice (39).

Second, exercise modulates immunity in T1D (18, 23, 61). Two studies show that exercise training in NOD mice reduced immune cell infiltration into the pancreas and insulitis. These are the only two exercise studies in a model of T1D to demonstrate the modulatory effects of exercise on islet immunity (18, 61). Recently, our group has shown that acute vigorous intensity exercise causes intensity-dependant lymphocytosis in T1D. However, we observed an impaired mobilisation of highly differentiated $CD8^+$ EMRA T cells during vigorous intensity exercise in T1D (23). These are amongst the subsets which are directly involved in the pathogenesis of T1D. Furthermore, these subsets express high levels of the beta-2-adrenergic receptor and mobilise in response to adrenaline during acute exercise. This has led us to hypothesise that the adrenaline response during vigorous exercise may be impaired in T1D. Reduced beta-adrenergic sensitivity of lymphocytes in T1D has been reported previously, resulting in a dampened adrenaline response (31, 41, 77). During acute exercise, increased beta-adrenoceptor density and sensitivity of lymphocytes is noted in healthy participants. However, patients with congestive heart failure (CHF) who exhibited reduced beta-adrenoceptor density and sensitivity, displayed a blunted lymphocyte increase following acute exercise (51). Therefore, a similar effect may be seen in T1D and may impact exercise-induced lymphocytosis of exercise responsive subsets.

In this study, we describe the effects of vigorous intensity exercise on innate cell subsets in T1D, with a particular focus on the most exercise responsive subset, NK cells. We also investigated the adrenaline response during exercise in T1D to gain insight into the mechanisms of any differential lymphocyte mobilisation between T1D and controls.

METHODS

Participants

Ethical approval was granted by the Preston Research Ethics Committee (REC) for this study. Twelve controls and twelve T1D participants were recruited. All participants were male and between 16-65 years of age. Male only participants were chosen to minimise differences in immune cell phenotypic and functional capacity evident in females due to higher oestrogen levels (19, 36, 45). Participant baseline characteristics are reported in Table 1. T1D participants had a clinical diagnosis of T1D, were on basal bolus insulin regime or insulin pump therapy, competent in carbohydrate content estimation of meals, were willing to test glucose through capillary testing, and were able to recognise hypoglycaemic symptoms before blood glucose fell to 3.9mmol/L. Participants did not have a history of cardiac disease or other significant illness that would prevent attendance at the study site. All T1D participants did not have active proliferative diabetic retinopathy, autonomic neuropathy, or history of severe hypoglycaemia requiring third party assistance within the 3 months prior to the study. Discrepancies in participant numbers for the outcome measures presented in this study are due to low sample volume or missing reagents on the day of an individual's visit. Exact number of participants for each outcome measure can be found in Table and Figure legends.

Experimental design

Participants had one enrolment visit, where baseline demographics and anthropometric assessment were carried out (Table 1). These visits were undertaken in the NIHR/Wellcome Trust Clinical Research Facility at the University of Birmingham. Blood pressure and heart rate data were collected following 10min rest and using an Omron Professional Blood Pressure monitor. All equipment, including those for measuring height and weight are regularly calibrated for accuracy as per CRF protocol. During the enrolment visit, each participant completed a non-fasted incremental sub-maximal (85% HRmax) cycle ergometer test to calculate their predicted VO_2 max. This was used to calculate workload and heart rate for the subsequent exercise visits to adjust for individual fitness (16). The enrolment visit and exercise visits were separated by a minimum of one week. Participants were asked to abstain from vigorous exercise 24 hours prior to each exercise visit. Participants were also required to record a food diary for the 24 hours prior to each exercise visit. Participants were advised to use these diaries to ensure that the same foods were consumed in the 24 hours prior to each exercise bout. The exercise visits started at 8.30am for all participants and consisted of a thirty-minute bout of cycling at 80% predicted VO_2 max. An initial fasting blood sample was taken for each participant once the cannula was inserted. The participant was then allowed to rest for a further 20 minutes before preparing

Table 1 Baseline Characteristics of T1D and control participants

	[1]Control	[2]T1D
	mean±SD	
Age (years)	28.8±4.6	33.2±9.7
Weight (Kg)	74.5±8.7	80.8±15.6
Height (cm)	155.6±54.8	177.63±7.3
BMI (kg/m2)	23.5±2	25.3±3.9
Waist circumference (cm)	86.3±6.5	90.3±12.3
Hip circumference (cm)	90.1±7	94.8±8.6
Chest Circumference (cm)	94.1±3.8	98.6±12.4
Waist-hip ratio	0.95±0.03	0.94±0.06
Body fat (%)	16.6±4.8	21.1±6.2
HbA1c*		65±12.4
Disease duration*		13.5±8.9
VO_2 Max	38.5±5.4	32.2±9.3
CMV index	0.42±0.47	0.51±0.5
Glucose (mmol)	5.24±0.48	8.91±3.15
Heart Rate (bpm)	68.1±8.12	74.4±14.37
Systolic BP (mmHg)	123.3±8.31	128.9±18.77
Dystolic BP (mmHg)	71.7±10.9	78.6±10.63
Number of cigarettes (per wk)#		
0	10	11
1-5	2	1
Alcohol intake (units per wk)#		
0	2	3
1-5	3	4
6-10	4	1
11-20	1	3
21-40	2	0
Job related PA (min/wk)	325±470	557±1221
Transportation PA (min/wk)	313±237	240±190
House Maintenance (min/wk)	129±122	135.45±129
Sport and Leisure PA (min/wk)	140±86	285.5±283
Light PA (min/wk)	42.5±45.2	81±91
Moderate PA (min/wk)	7.5±18.7	64.5±132
Vigorous PA (min/wk)	90±97	140±12
Time sitting (hrs/wk)	48±18	39.73±12
Stress score (1 year)	4.33±3.3	4.5±4.3
Stress score (1 month)	10.25±8.1	11.83±10.3
Stress score (visit 1)	7.36±9.86	5.67±9
Stress score (visit 2)	4.18±3	4±5

Mean and standard deviation values for baseline characteristics in control and T1D participants.

Note:
number of participants
[1]controls n=12
[2]T1D n=12

for the acute exercise bout. Fasting blood samples were collected intravenously at immediately pre-exercise, immediately post-exercise, and 1 hour post-exercise. Pre and post-exercise samples were taken whilst the participant was sitting on the cycle ergometer and sampling was strictly timed using a stopwatch. Timing for the immediately post-exercise sample was crucial because lymphocytes egress from peripheral blood within minutes of exercise cessation (70). At all 3 visits, all participants completed an international physical activity questionnaire (IPAQ) (37) and perceived stress questionnaires; the life scale events questionnaire (17), perceived stress scale (20), the undergraduate stress questionnaire (22), self-perceived health status (53), and the Pittsburgh sleep quality index (11).

Sample processing

All blood samples were processed under identical conditions using the same laboratory reagents and apparatus. Blood samples for immunophenotyping analysis were taken in lithium heparin vacuette tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany) and placed on a roller at room temperature to ensure constant mixing of the blood sample until processing. All sample processing was initiated within 2 hours of blood-draw. Haematological measures were conducted on 25µl of whole blood using an automated coulter counter (ABX Micros ES 60, HORIBA Medical). Relative cell number (cells/µl) of immune cell subsets was then calculated from this.

Whole blood staining

The whole blood staining protocol was optimised prior to the start of the study. The protocol was adapted from the Clinical Immunology Service, University of Birmingham. Red blood cells were lysed by preparing whole blood in 4ml aliquots and washed with 16ml Ammonium Chloride lysis buffer (16g Ammonium Chloride (326372, Sigma-Aldrich, Dorset, UK), 2g sodium hydrogen carbonate (S/4240/60, Fisher scientific Ltd, Loughborough, UK), 0.2g EDTA (E5134, Sigma-Aldrich, Dorset, UK), and 2L ddH2O). The sample was centrifuged at 1000g for 5 minutes. Pelleted cells were resuspended in 10mls RPMI-1640 (R0833, Sigma-Aldrich, Dorset, UK) (supplemented with 2% FBS) and centrifuged at 1000g for 5 minutes. Cells were then counted and resuspended to a concentration of $1x10^6$ cells/ml. Cells were stained with appropriate antibodies listed below and incubated in dark at 4°C for 20 minutes. Stained cells were fixed with 500µl 1X BD FACS lysing solution (containing 14% formaldehyde) (349202, BD Biosciences, Wokingham, UK) and incubated in dark at 4°C for a further 15 minutes. Fixed cells were washed (centrifuged at 1000g for 5 minutes) in 2ml phosphate-buffered saline (PBS). Pelleted cells were resuspended in 500µl PBS and stored at 4°C until flow cytometry analysis. The stability of fixed stains was assessed and confirmed that cells could be stored up to 24 hours at 4°C before flow cytometry analysis. All samples were analysed using BD LSR Fortessa X-20. Parent populations (i.e. lymphocytes) were selected based on their size on FSC/SSC dot plots. Doublets were omitted by selecting the linear population shown on FSC-A/FSC-H dot plots prior to recording. Events to record were set to 100,000 within the parent singlet population gate. Compensation was carried out monthly using compensation beads and single stained cells. A negative control (unstained whole blood) was run for each experiment.

Innate cell subset analysis

Two multicolour flow cytometry panels were designed to enable phenotypic analysis of leukocyte subsets using the following mAbs; **Panel 1 (NK cells):** anti-CD3 PE-Cy7 (UCHT1), anti-CD16 PE-CF594 (3G8), anti-CD18 (LFA-1) APC (6.7), anti-CD25 PE (M-A251) anti-CD56 BV510 (NCAM16.2), anti-CD57 BB515 (NK-1), anti-CD122 BV421 (131411), anti-CD158a (KIR) BV711 (HP-3E4), Live/Dead

fixable viability stain 780 APC-Cy7. **Panel 2 (Dendritic cells and monocytes):** anti-CD11c BV510 (B-ly6), anti-CD14 BV711 (MPhiP9), anti-CD16 PE-CF594 (3G8), CD123 BV421 (7G3), HLA-DR BV786 (G46-6), Lineage cocktail 2 (CD3 – SK7, CD14 – MoP9, CD19 – SJ25C1, CD20 – L27, CD56 – NCAM16.2) FITC, Live/Dead fixable viability stain 780 APC-Cy7.

Data analysis

FlowJo version 10 (FlowJo LLC, Oregon) was used to analyse flow cytometry data. Doublets were removed using FSC-A versus FSH-H. Dead cells positive for the viability stain were removed, and lymphocytes were gated based on size on SSC-A versus FSC-A dot plot. NK cells were selected as $CD3^-$ and further selected on $CD56^{+}/CD16^{+/-}$ expression as follows: $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56^{bright}CD16^-$, $CD56^{dim}CD16^{dim}$, and $CD56^-CD16^+$ (Figure 1). Cell surface expression of CD25, CD57, CD122, KIR, NKG2A, and LFA-1 was examined on the two most common NK cell subsets; $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$. Mature NK cells were $CD56^{dim}CD16^{bright}$ and defined as early mature (KIR^+) and highly-differentiated ($KIR^+CD57^+/NKG2A^{lo}$). t-SNE analysis was performed on concatenated samples from each time-point

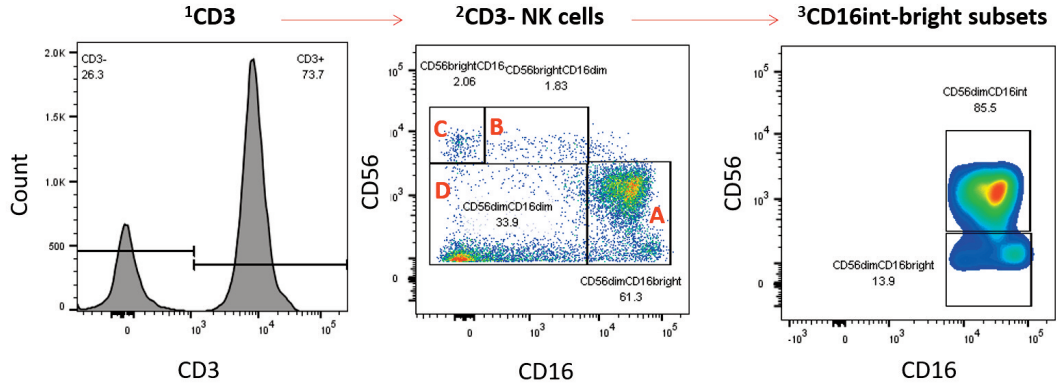

Figure 1. Representative flow cytometry gating strategy for NK cell subpopulations. CD3 negative lymphocytes -> CD56+ NK cells. NK cell subsets: A. CD56dim CD16bright, B. CD56bright CD16dim, C. CD56bright CD16-, D. CD56dim CD16dim. CD56dim CD16bright can be divided into CD16intermediate and CD16bright

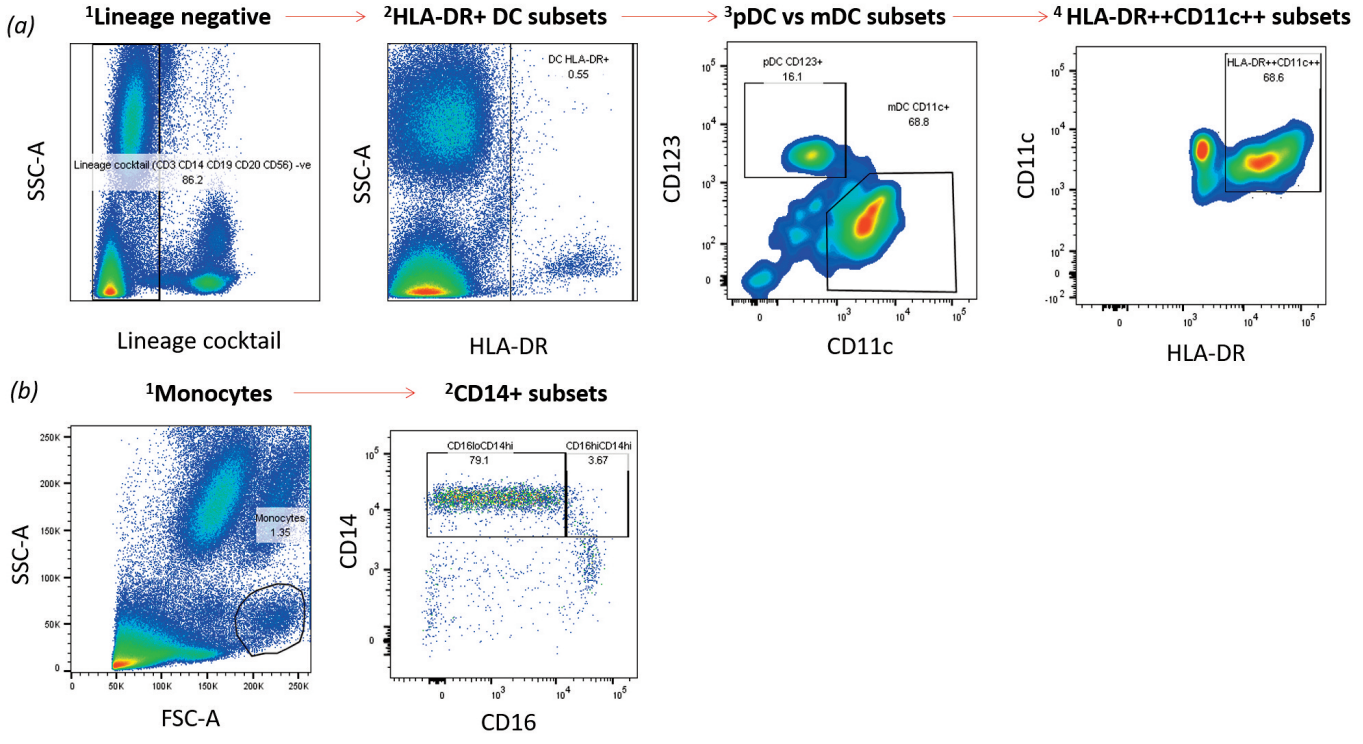

Figure 2. Representative flow cytometry gating strategy for DC and monocyte populations (a)Lineage negative -> HLA-DR+ DC subsets-> plasmacytoid vs myeloid DC subsets-> HLA-DR++CD11c++ DC subsets (b)Monocytes ->CD16loCD14hi and CD16hiCD14hi monocyte subsets.

during vigorous intensity exercise in control and T1D participants. A down sample population of $CD3^{-}CD56^{+}$ NK cells was selected up to 15,000 events. t-SNE analysis was run using phenotypic markers of NK cell subsets (CD56, CD16, CD57, KIR). Cell surface markers which are not core phenotypic markers and with variable expression level (CD25, CD122, LFA-1) were applied once the t-SNE plot was calculated.

Dendritic cells were selected as Lineage- 2 (CD3-, CD14-, CD19-, CD20-, CD56 -) and further selected on HLA-DR expression. Further subdivisions of dendritic cells were selected as $CD11c^{+}$ (myeloid) and $CD123^{+}$ (plasmacytoid) (Figure 2). DC subsets during vigorous exercise only is shown in T1D due to missing data. Monocytes were selected based on size on FSC-A/SSC-A and further selected on $CD14^{high}/CD16^{high/lo}$ expression (Figure 2).

Plasma isolation and adrenaline measurement

Blood was collected in EDTA vacuette tubes (454209, Greiner Bio-one GmbH, Frickenhausen, Germany) and immediately placed on ice. The blood was centrifuged at 1500RPM for 10 minutes at 4°C. The plasma (top layer) was removed using sterile pipette tips and placed in sterile Eppendorf tubes. Aliquots were made to avoid deterioration of the serum by freeze-thaw cycles. Plasma was stored at -80°C until use. Adrenaline (pg/mL) was measured at pre-exercise, post-exercise, and 1 hour post-exercise plasma samples using EPI (epinephrine/adrenaline) ELISA kit (E-EL-0045, Elabscience, Texas, USA).

Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM, Chicago) and GraphPad Prism version 7 (GraphPad Software, California). Firstly, normality tests were performed on all data using Q-Q plots in SPSS. Data which was not normally distributed was logged and normality tests were repeated, confirming all subsets to have normal distribution. Main effects of exercise are described as changes over time. Changes immediately post-exercise and 1 hour post-exercise are compared to baseline values and are reported in tables for each group under the heading "contrast". P values were reported as sphericity assumed however where Mauchly's test of sphericity was violated, i.e. $p \leq 0.05$, Greenhouse-Geisser corrected value was used. Student T-tests were performed on baseline characteristics. The p values, F values, and degrees of freedom (df) are reported in tables as [F = (df, df error) value, p-value]. Data are presented in tables as mean ± standard deviation (SD). P values ≤0.05 were considered significant. Significantly mobilised subsets in both control and T1D groups, that also demonstrated a blunted egress immediately post-exercise in T1D group, are highlighted in the results tables in bold.

RESULTS

Participants anthropometric and physiological characteristics are shown in Table 1. No statistically significant differences between groups were found for anthropometric and physiologic characteristics. The statistical analysis for innate cell subpopulations during vigorous intensity exercise are summarised in Tables 2-5.

Reduced NK cell mobilisation in T1D participants following vigorous intensity exercise compared to control participants

As previously described, NK cells are the most sensitive subset to mobilisation during acute exercise. In this study, NK cells significantly mobilised during vigorous intensity exercise in both the control (p=0.021) and T1D (p=0.005) group, significantly increasing immediately post-exercise in both groups independently (T1D: p=0.044, control: p=0.021). However, the magnitude of the response immediately post-exercise was higher in control participants (T1D: 100.49%, control: 174.13%) (Table 2).

NK cells, in particular mature CD56dimCD16bright subsets, display blunted mobilisation during vigorous intensity in T1D

NK cell subsets were defined using the classical cell surface markers CD56 and CD16 as shown in Figure 2; $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$, and $CD56^{bright}CD16^{-}$. The mean, standard deviation, and statistical analyses are displayed in Table 2.

Table 2. NK cell subset mobilisation during vigorous intensity exercise in T1D and control participants

Subset	[1]Controls T1	[1]Controls T2 mean±SD	[1]Controls T3	[b]Time	[a]Δ%	[2]T1D T1	[2]T1D T2 mean±SD	[2]T1D T3	[b]Time	[a]Δ%	[c]Time (overall)	[c]Time*Group
Total NK cells (CD56+)	164.21±101.76	450.18±345.08	119.25±72.19	**$F_{(1.1, 7.1)}$= 8.584, p=0.021**	174.13	183.28±62.26	367.47±173	154.55±40.17	**$F_{(2, 8)}$= 11.212, p=0.005**	100.49	**$F_{(1, 11.4)}$= 14.696, p=0.002**	$F_{(1, 11.4)}$= 0.118, p=0.748
- $CD56^{dim}CD16^{bright}$	72.85±34.95	207.70±106.46	53.35±43.13	**$F_{(1.1, 7.5)}$= 27.545, p=0.001**	185.12	104.38±47.47	205.69±120.49	78.18±51.31	**$F_{(2, 12)}$= 3.929, p=0.049**	97.06	**F(1.4, 18.2)= 22.237, p<0.001**	$F_{(1.4, 18.2)}$= 2.113, p=0.159
- $CD56^{bright}CD16^{dim}$	3.63±3.14	5.62±4.52	3.28±2.55	**$F_{(2, 14)}$= 10.682, p=0.002**	54.79	3.35±1.32	5.31±2.83	2.45±0.82	$F_{(2, 12)}$= 3.340, p=0.070	58.34	**$F_{(1.4, 17.9)}$= 12.477, p=0.001**	$F_{(1.4, 17.9)}$= 0.662, p=0.473
- $CD56^{dim}CD16^{dim}$	14.19±12.58	30.29±26.43	11.93±9.884	**$F_{(1.2, 8.2)}$= 8.822, p=0.015**	113.78	14.81±6.47	20.51±12.72	20.60±22.54	$F_{(1.1, 6.3)}$= 0.385, p=0.689	38.54	**$F_{(2, 26)}$= 5.679, p=0.009**	$F_{(2, 26)}$= 2.657, p=0.089
- $CD56^{bright}CD16^{-}$	59.03±52.93	93.16±109.78	40.12±41.74	**$F_{(2, 14)}$= 13.011, p=0.001**	57.81	71.46±48.75	162.2±201.27	84.84±86.38	**$F_{(2, 12)}$= 6.493, p=0.012**	126.98	**F(2, 26)= 17.607, p<0.001**	$F_{(2, 26)}$= 0.471, p=0.630

Mean, standard deviation, and statistical analysis of NK cell sub populations for control and T1D participants during vigorous intensity exercise. Within subject's effect shown displayed under "time" and between subject's effects over time displayed under "time*group". Significant results highlighted in bold changes over time were statistically significant for both groups independently i.e. p values <0.05 were considered significant, and the percentage increase was considerably blunted in the T1D (<50 Δ% compared to control highlighted in red).

Note:

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=8, [2]T1D n=7

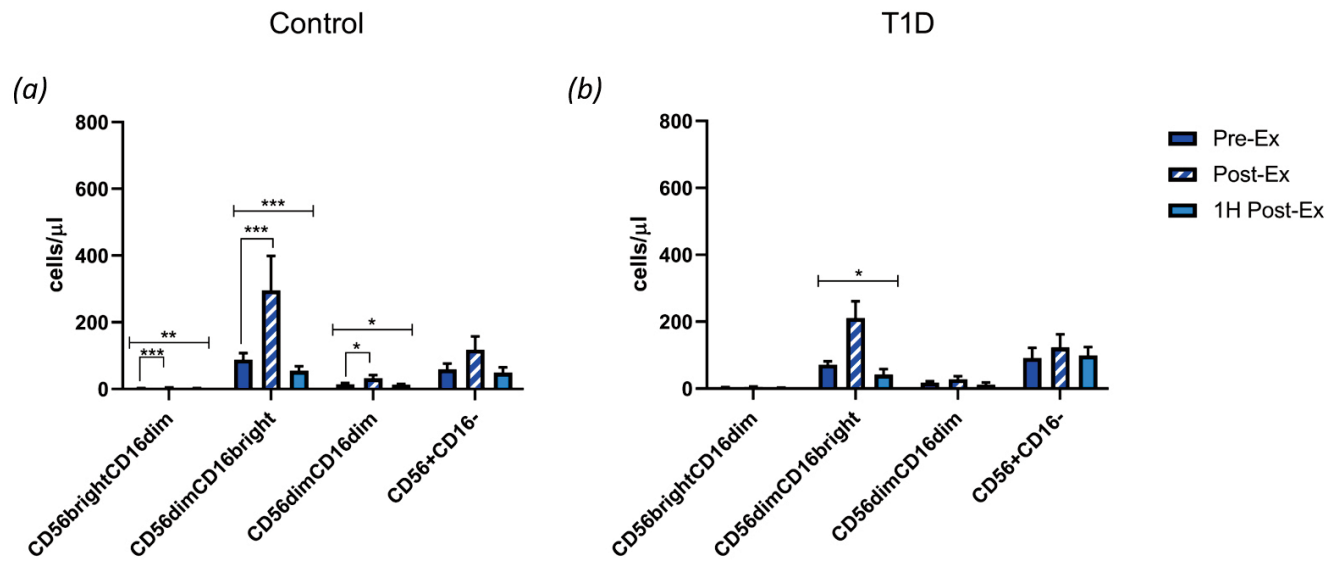

Figure 3. NK cell subsets during moderate and vigorous intensity exercise in control and T1D participants (a) NK cell subsets during vigorous exercise in control participants (b) NK cell subsets during vigorous exercise inT1D participants. Error bars represent SEM.
Note: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Typically, $CD56^{dim}CD16^{bright}$ NK cells demonstrate the largest response to acute exercise. This is further supported by the results of our study (Figure 3); the percentage increase of $CD56^{dim}CD16^{bright}$ NK cells is a minimum of 3-fold higher than that of the other subsets. $CD56^{dim}CD16^{bright}$ NK cells mobilised significantly during vigorous intensity exercise in both groups independently (T1D: p=0.049, controls: p=0.001) (Figure 3a and 3b). However, $CD56^{dim}CD16^{bright}$ NK cells significantly increased immediately post-exercise in the control group only (p=0.001). Furthermore, the magnitude of response post-exercise was much larger in control participants (T1D: 97.06%, control: 185.12%) (Table 2).

Interestingly, $CD56^{dim}CD16^{bright}$ NK cells were the only subset to significantly mobilise in the T1D group during vigorous intensity exercise, but to a lesser magnitude than the control group (Figure 3b). In the control group, $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$ all significantly mobilised during vigorous intensity exercise (Figure 3a).

Early mature $KIR^{+}CD56^{dim}CD16^{bright}$ NK cell subsets drive the blunted mobilisation during vigorous intensity exercise in T1D

$CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD56^{dim}$ NK cells are the most studied NK cell subpopulations. For this reason, we used a number of cell surface markers to define differentiation and migratory status of these NK cell subpopulations. CD57 and KIR can be used to define NK cells with a highly differentiated phenotype, with KIR being expressed first followed by co-expression with CD57 as NK cells reach maturation (6, 76). LFA-1 is an adhesion molecule involved in NK cell migration. Lastly, NK cells expressing both CD25 (IL-2Rα), also a marker of activation, and CD122 (IL-2Rβ/IL-15R), involved in NK cytokine signalling, have been shown to migrate during exercise previously (56, 73). The mean, standard deviation, and statistical analyses are displayed in Table 3.

Although a small subset, we measured all of the above surface markers on $CD56^{bright}CD16^{dim}$ cells. The results and statistical analysis are displayed in Table 3. $CD56^{bright}CD16^{dim}$ expressing KIR, albeit a small sub-group, are the only subset to mobilise in both groups during vigorous exercise (Control p=0.013, T1D p=0.045), with a similar percentage increase immediately following exercise in both groups. This subset may represent NK cells reaching early maturity as they begin to express KIR. However, we have focused mainly on the larger NK subpopulation $CD56^{dim}CD16^{bright}$ and our findings our described below.

All $CD56^{dim}CD16^{bright}$ NK cell subsets expressing the above surface markers, apart from LFA-1, significantly mobilised during vigorous intensity exercise in both groups. $LFA\text{-}1^{+}$ $CD56^{dim}CD16^{bright}$ NK cells did however significantly mobilise in the control group during vigorous intensity exercise (Table 3).

$CD56^{dim}CD16^{bright}$ NK cells make up the largest proportion of NK cells with a highly differentiated phenotype as defined by expression of KIR and CD57, whilst lacking NKG2A expression. As described previously, during NK cell maturation they lose NKG2A and gain KIR expression. This is followed by a gradual increase in CD57 expression as they become highly differentiated (7, 65). $KIR^{+}CD56^{dim}CD16^{bright}$ NK cells retain proliferative capacity, however $KIR^{+}CD57^{+}CD56^{dim}CD16^{bright}$ NK cells have a reduced proliferative capacity. It is well supported that mature, highly differentiated NK cells mobilise dramatically during acute exercise and we see this in both the control and T1D group in this study. Here we describe the differential mobilisation of early mature (KIR^{+}) and highly differentiated ($KIR^{+}CD57^{+}NKG2A^{lo}$) $CD56^{dim}CD16^{bright}$ NK cells in control and T1D participants.

$KIR^{+}CD56^{dim}CD16^{bright}$ NK cells significantly mobilised during vigorous intensity exercise in the control and T1D group

Table 3. CD56^{bright}CD16^{dim} and CD56^{bright}CD16^{dim} NK cell subset mobilisation during vigorous intensity exercise in T1D and control participants

Subset	T1	[1]Controls T2 mean±SD	T3	[b]Time	[a]Δ%	T1	[2]T1D T2 mean±SD	T3	[b]Time	[a]Δ%	[c]Time (overall)	[c]Time*Group
CD56dimCD16bright												
LFA-1+	48.56±35.22	141.14±114.30	41.14±33.83	**$F_{(1,7.3)}$= 13.541, p=0.007**	190.63	70.92±49.46	118.81±73.34	56.46±52.87	$F_{(2,12)}$= 2.674, p=0.110	67.53	**$F_{(1.5,20.1)}$= 14.133, p<0.001**	$F_{(1.5,20.1)}$= 2.880, p=0.074
CD25+	21.97±30.85	84.88±141.73	8.87±9.28	**$F_{(1.1,7.5)}$= 18.108, p=0.003**	286.20	38.03±28.55	140.89±137.34	15.90±19.26	**$F_{(2,10)}$= 12.677, p=0.012**	270.47	**$F_{(1.2,13.7)}$= 30.596, p<0.001**	$F_{(1.2,13.7)}$= 0.483, p=0.623
CD122+	81.21±82.74	318.74±410.99	34.01±32.59	**$F_{(2,14)}$= 7.416, p=0.006**	292.49	115.08±122.30	306.72±355.35	42.81±42.69	**$F_{(2,10)}$= 12.227, p=0.002**	166.53	**$F_{(2,24)}$= 17.600, p<0.001**	$F_{(2,24)}$= 1.169, p=0.328
KIR+	56.60±31.70	165.51±100.50	24.35±13.53	**$F_{(2,14)}$= 15.068, p<0.001**	192.40	62.55±87.75	108.98±123.43	14.14±14.16	**$F_{(2,10)}$= 14.695, p=0.001**	74.24	**$F_{(2,24)}$= 30.349, p<0.001**	$F_{(2,24)}$= 0.529, p=0.596
KIR+CD57+	25.5±17.56	59.55±27.99	2.67±1.41	**$F_{(2,4)}$= 70.295, p=0.001**	133.56	11.85±10.47	75.11±84.69	2.90±3.22	**$F_{(2,6)}$= 10.081, p=0.012**	533.88	**$F_{(2,10)}$= 29.023, p<0.001**	$F_{(2,10)}$= 0.748, p=0.498
(KIR+CD57+)LFA1+	18.93±17.73	50.13±33.60	2.89±1.37	**$F_{(2,4)}$= 83.110, p=0.001**	164.81	10.47±12.21	6.04±3.12	0.50±0.18	$F_{(2,4)}$= 4.669, p=0.090	42.34	**$F_{(2,8)}$= 27.895, p<0.001**	$F_{(2,8)}$= 1.891, p=0.213
CD56brightCD16dim												
LFA-1+	1.88±2.83	2.88±3.92	1.30±1.49	$F_{(2,12)}$= 2.383, p=0.134	52.99	1.73±1.88	1.97±2.74	1.23±1.16	$F_{(2,10)}$= 0.616, p=0.559	13.80	$F_{(2,22)}$= 1.110, p=0.347	$F_{(2,22)}$= 1.850, p=0.181
CD25+	0.39±0.55	0.66±1.12	0.15±0.20	$F_{(1,8.3)}$= 1.516, p=0.254	67.27	0.19±0.25	0.53±0.67	0.19±0.29	$F_{(1,5.1)}$= 3.266, p=0.130	173.64	$F_{(1.1,14.1)}$= 2.583, p=0.129	$F_{(1.1,14.1)}$= 0.259, p=0.638
CD122+	2.74±2.12	4.58±2.96	2.14±1.69	**$F_{(2,10)}$= 12.033, p=0.002**	66.92	3.27±2.01	6.93±5.30	2.77±2.19	$F_{(1.1,5.5)}$= 3.546, p=0.069	112.19	**$F_{(1.2,11.6)}$= 7.843, p=0.014**	$F_{(1.2,11.6)}$= 0.704, p=0.439
KIR+	1.56±1.89	2.66±2.69	1.12±1.31	**$F_{(2,12)}$= 8.546, p=0.005**	70.18	0.43±0.29	0.88±0.59	0.42±0.36	**$F_{(2,10)}$= 4.312, p=0.045**	105.95	**$F_{(2,22)}$= 10.687, p=0.001**	$F_{(2,22)}$= 0.496, p=0.616
CD57+	0.087±0.05	0.15±0.15	0.06±0.04	$F_{(2,6)}$= 1.148, p=0.378	66.92	0.03±0.05	0.15±0.06	0.07±0.08	$F_{(2,6)}$= 3.893, p=0.082	424.26	$F_{(2,12)}$= 2.684, p=0.109	**$F_{(2,12)}$= 4.712, p=0.031**

Mean, standard deviation, and statistical analysis of CD56^{bright}CD16^{dim} and CD56^{bright}CD16^{dim} NK cell subset for control and T1D participants during vigorous intensity exercise. Within subject's effect shown displayed under "time" and between subject's effects over time displayed under "time*group". Significant results highlighted in bold changes over time were statistically significant for both groups independently i.e. p values <0.05 were considered significant, and the percentage increase was considerably blunted in the T1D (<50 Δ% compared to control highlighted in red).

Note:
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Results were analysed using multiple regression analysis in control and T1D groups combined.
Vigorous: [1]Controls n=8, [2]T1D n=6

(p<0.001, p=0.001 respectively). However, the percentage increase immediately following vigorous intensity exercise was blunted in the T1D group (Control 192.4%, T1D 74.24%).

The fully differentiated KIR^{+}CD57^{+} subset significantly mobilised during vigorous intensity exercise in the control and T1D group (p=0.001, p=0.012 respectively). Furthermore, highly differentiated NK cells (KIR^{+}CD57^{+}) with a migratory capacity (LFA-1^{+}) significantly mobilised during vigorous intensity exercise in the control group (p=0.001), but this was not significant in the T1D group.

In summary, highly differentiated, mature KIR^{+}CD57^{+} NK cells increase dramatically following vigorous intensity exercise in both the control and T1D groups. However, early mature KIR^{+} NK cells display a blunted increase following vigorous intensity exercise in the T1D group and this may drive the overall blunted mobilisation of CD56^{dim}CD16^{bright} NK cells observed.

Dimensionality reduction analysis of NK cell subsets supports observed blunted mobilisation of early mature KIR^{+}CD56^{dim}CD16^{bright} NK cell subsets in T1D

Dimensionality reduction using t-Distributed Stochastic Neighbour Embedding (t-SNE) algorithms is a powerful analysis technique that allows visualisation of multiple interactions on a 2D scale. t-SNE plots are formed using phenotypic markers to cluster similar populations. Markers which can change expression, for example adhesion molecules, can be assessed once the populations are defined. Here we have used t-SNE to observe changes within the CD56^{+} NK profile pre, immediately post, and 1 hour post vigorous intensity exercise (Figure 4). The NK cell subsets CD56^{dim}CD16^{bright} (blue), CD56^{bright}CD56^{dim} (green), CD56^{dim}CD16^{dim} (pink), and CD56^{bright}CD16^{-} (orange) are represented in t-SNE plots in Figure 4. The CD56^{dim}CD16^{bright} cluster is further divided into CD56^{dim}CD16$^{int\text{-}bright}$ (light blue) and CD56^{dim}CD16$^{bright+}$ (dark blue) to further delineate the

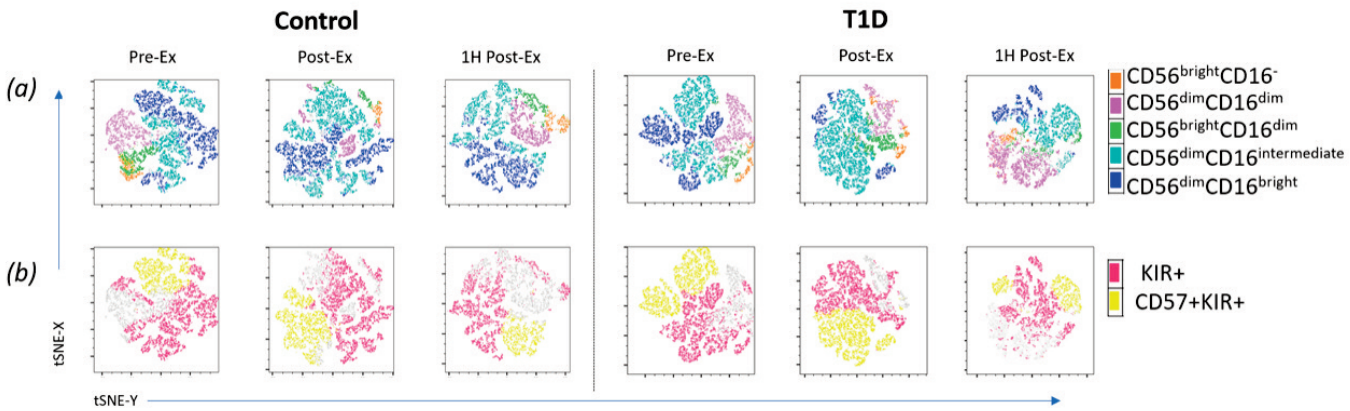

Figure 4. Representative concatenated t-SNE plots for NK cells during vigorous intensity exercise in control and T1D participants (a) NK cell populations CD56/CD16: CD56^{bright}CD56^{dim} (green), CD56^{dim}CD16^{dim} (pink), CD56^{bright}CD16^{-} (orange), CD56^{dim}CD16$^{int\text{-}bright}$ (light blue) and CD56^{dim}CD16$^{bright+}$ (dark blue) (b) maturity markers KIR/CD57: KIR^{+}NK cells (pink), CD57^{+}KIR^{+} NK cells (yellow)

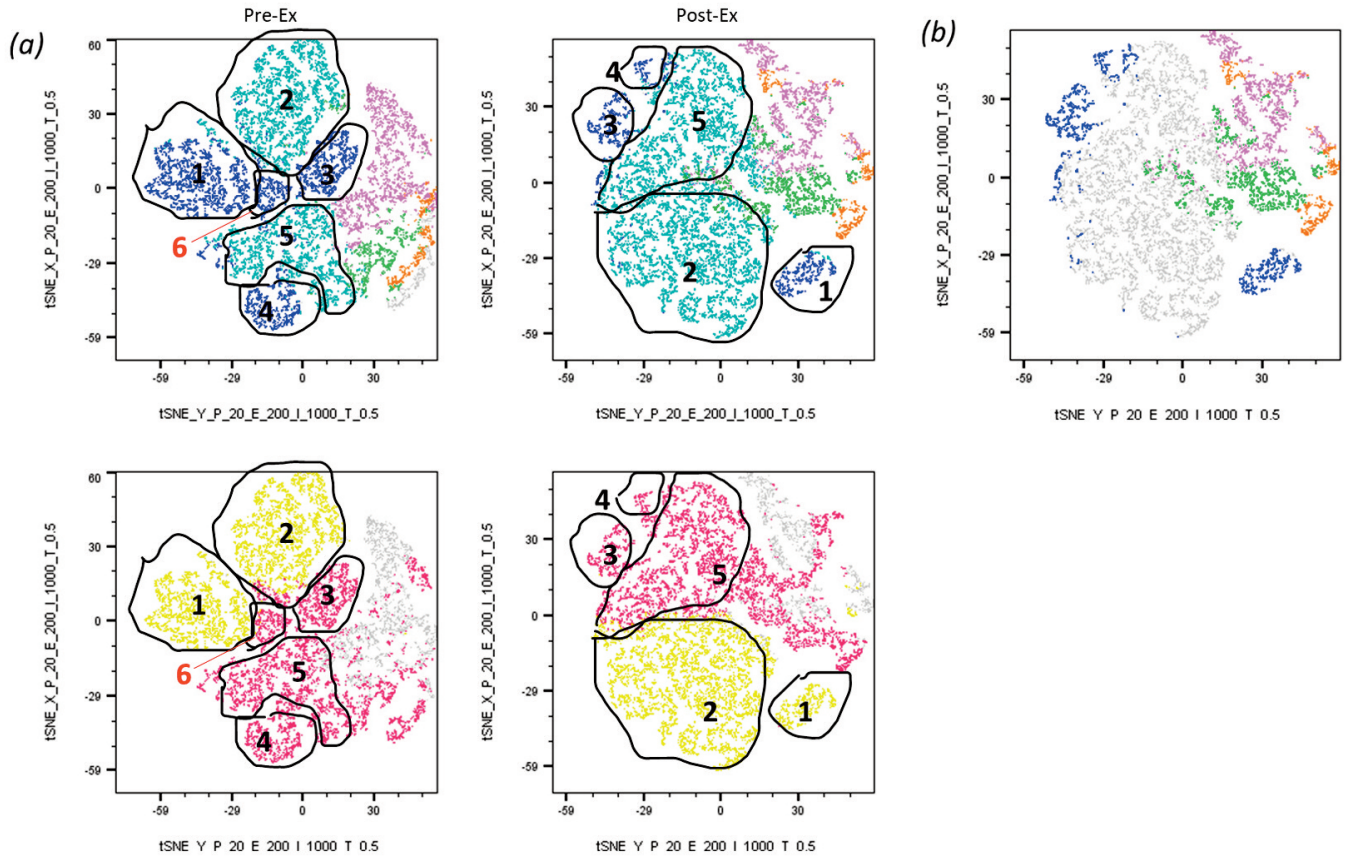

Figure 5. (a) Cluster definition (b) Confirms dark blue clusters not hidden behind light blue clusters.

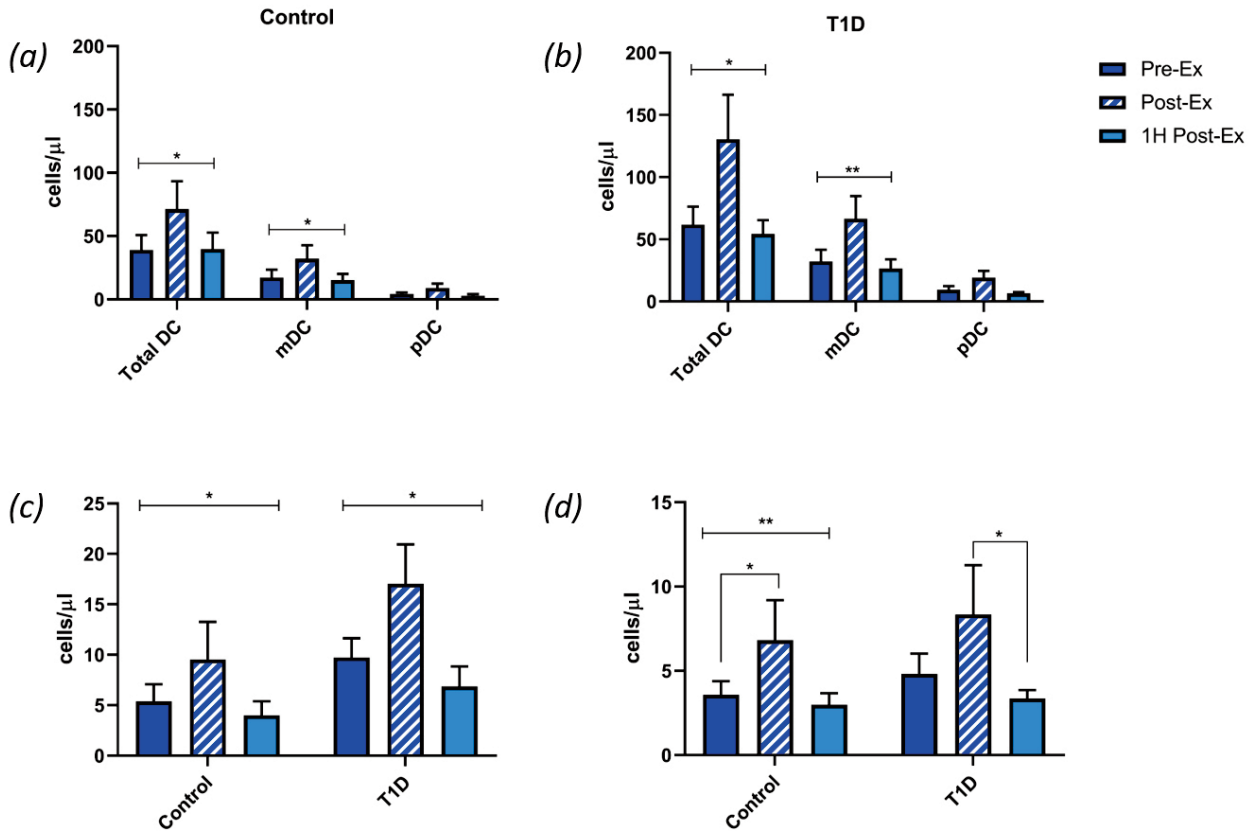

Figure 6. Dendritic cell subsets and monocytes during vigorous intensity exercise in control and T1D participants (a) DC subsets in control participants (b) DC subsets in T1D participants (c) HLA-DR^{hi} CD11c^{hi} mDC subsets during vigorous exercise in control and T1D participants (d) Total monocytes during vigorous exercise in control and T1D participants. Error bars represent SEM.
Note: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

changes observed within this subset during vigorous intensity exercise (Figure 4a).

At baseline, the combined CD16^{bright} subsets (blue) make up the bulk of the NK cell subsets in both groups (Figure 4a). Immediately post vigorous exercise, the CD56^{bright}CD16^{-} (orange), CD56^{bright}CD16^{dim} (green), and CD56^{dim}CD16^{dim} (pink) subsets are reduced, as the CD16^{bright} subsets (blue) have increased at this time point. Comparing the control group to the T1D group at this time point, it is obvious that the CD56^{dim}CD16$^{int\text{-}bright}$ (blue) subset is present post-exercise, but a proportion of the CD56^{dim}CD16^{bright} NK cells (blue) are absent, supporting the blunted effect reported above. Following recovery, 1 hour post-exercise, NK subsets return almost to normal in the control group, but the CD16^{bright} subset (blue) has only marginally returned to baseline levels in the T1D group. Here, the CD56^{dim}CD16^{dim} subsets (pink) represents are larger proportion of the NK cell subsets in the T1D group.

In Figure 4b, the pink clusters represent the early mature KIR^{+}NK cells and the yellow clusters represent the late mature CD57^{+}KIR^{+} NK cells. As expected, these populations reside mainly within the CD56^{dim}CD16^{bright} subsets (blue) shown in Figure 4a. Immediately post vigorous exercise, it is obvious that a proportion of KIR^{+}CD56^{dim}CD16^{bright} NK cluster is absent from the T1D group as shown by the appearance of an incomplete t-SNE plot. To further explain this, there are 6 clusters belonging to CD56^{dim}CD16^{bright} populations in the baseline sample (Figure 5). Four small clusters belong to the CD56^{dim}CD16^{bright} subset, one of which is CD57^{+}KIR^{+} (cluster 1 – yellow) and it is evident in the post-exercise t-SNE plot that there are only three clusters present, to which this missing cluster belongs to one of the three KIR^{+} populations identified at baseline (cluster 6). Both CD57^{+}KIR^{+} clusters (clusters 1 and 2) in yellow are present in the post-exercise plot, however cluster 1 which belongs to the CD56^{dim}CD16^{bright} subset, is dramatically reduced post-exercise in T1D (Figure 5a). At 1 hour post-exercise, KIR^{+} and CD57^{+}KIR^{+} NK cells return almost to normal in the control group, with a small decrease in CD57^{+}KIR^{+} NK cells as expected. This is also observed for the T1D group.

Using t-SNE algorithms to visualise changes in NK cell subsets during vigorous intensity exercise further highlights that the blunted increase of CD56^{dim}CD16^{bright} NK cells immediately post vigorous exercise is driven by a lack of early mature KIR^{+}CD56^{dim}CD16^{bright} NK cells. This dimensionality reduction analysis supports the findings using conventional flow cytometry gating analysis and enumeration of the populations during vigorous intensity exercise.

Myeloid lineage subsets mobilise similarly in control and T1D participants

The mean, standard deviation, and statistical analyses for dendritic cell (DC) and monocyte subsets are displayed in Table 4. Total DC mobilised during vigorous intensity exercise in both control (p=0.049) and T1D (p=0.027) groups, and to a similar magnitude in both groups (Figure 6a and 6b). Within the DC subsets, mDC preferentially mobilised during vigorous intensity exercise. mDC mobilised during vigorous intensity exercise in both control (p=0.045) and T1D (p=0.008) groups, and to a similar magnitude in both groups. This was driven by HLA-DR^{hi} CD11c^{hi} mDC, in both control and T1D groups (p=0.033, p=0.012 respectively). pDC did not significantly mobilise in either group. However, there was an evident percentage increase immediately following vigorous exercise in both groups.

Total monocytes significantly mobilised during vigorous intensity exercise in the control group (p=0.006). This was not found to be significant in the T1D group. However, there was an evident increase immediately post vigorous exercise.

Systemic adrenaline concentration during exercise is similar between groups

The adrenaline concentration during vigorous intensity exercise in control and T1D participants is shown in Figure 7.

Table 4. Myeloid subset mobilisation during vigorous intensity exercise in T1D and control participants

Subset	[1]Controls T1	[1]Controls T2 mean±SD	[1]Controls T3	[b]Time	[a]Δ%	[2]T1D T1	[2]T1D T2 mean±SD	[2]T1D T3	[b]Time	[a]Δ%	[c]Time (overall)	[c]Time*Group
Dendritic Cells (HLA-DR+)	38.84±37.82	71.26±65.78	39.76±38.96	**$F_{(2, 14)}$= 3.775, p=0.049**	83.5	58.81±36.54	122.29±90.70	52.1±25.40	**$F_{(2, 10)}$=5.315, p=0.027**	107.9	**$F_{(2, 24)}$= 9.978, p=0.001**	$F_{(2, 24)}$= 2.213, p=0.131
myeloid DC	17.29±19.6	32.02±32.39	15.30±14.46	**$F_{(2, 14)}$= 3.896, p=0.045**	85.2	30.48±21.38	61.75±42.62	24.83±17.20	**$F_{(2, 10)}$=8.105 p=0.008**	102.6	**$F_{(2, 24)}$= 13.293, p=0.000**	$F_{(2, 24)}$= 2.207, p=0.132
mDC(HLADR^{hi}CD11c^{hi})	5.39±5.06	9.52±11.2	3.98±4.20	**$F_{(2, 12)}$= 4.592, p=0.033**	76.8	9.04±4.66	16.22±8.99	6.49±4.51	**$F_{(2, 10)}$=7.213 p=0.012**	79.3	**$F_{(2, 22)}$= 11.899, p=0.000**	$F_{(2, 22)}$= 0.416, p=0.665
plasmacytoid DC(CD123+)	4.19±3.40	9.15±8.43	2.82±3.12	$F_{(1, 5.03)}$= 2.782, p=0.109	118.5	8.41±6.50	17.39±11.76	5.91±2.40	$F_{(1, 3)}$= 2.288, p=0.183	106.9	**$F_{(2, 16)}$= 5.342, p=0.017**	$F_{(2, 16)}$= 1.095, p=0.358
Monocytes	3.57±2.57	6.81±7.15	2.98±2.05	**$F_{(2, 14)}$= 7.436, p=0.006**	90.8	4.52±3.07	8.19±7.18	3.17±1.21	$F_{(2, 10)}$=2.212, p=0.160	81.1	**$F_{(2, 24)}$= 5.058, p=0.015**	$F_{(2, 24)}$= 1.305, p=0.290
CD16^{hi}CD14^{hi} monocytes	0.15±0.15	0.18±0.18	0.09±0.12	$F_{(2, 14)}$= 2.064, p=0.164	18.7	0.25±0.18	0.36±0.30	0.20±0.19	$F_{(2, 10)}$=1.165, p=0.351	40.5	$F_{(2, 24)}$= 2.642, p=0.092	$F_{(2, 24)}$= 0.525, p=0.598
CD16^{lo}CD14^{hi} monocytes	1.33±1.17	2.16±1.67	1.29±1.23	$F_{(2, 14)}$= 2.769, p=0.097	62.6	1.78±1.31	3.16±3.25	1.46±0.76	$F_{(2, 10)}$=1.435, p=0.283	77.7	$F_{(2, 24)}$= 2.976, p=0.070	$F_{(2, 24)}$= 0.968, p=0.394

Mean, standard deviation, and statistical analysis of myeloid subset for control and T1D participants during vigorous intensity exercise. Within subject's effect shown displayed under "time" and between subject's effects over time displayed under "time*group". Significant results highlighted in bold changes over time were statistically significant for both groups independently i.e. p values <0.05 were considered significant.

Note:
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Results were analysed using multiple regression analysis in control and T1D groups combined.
Vigorous: [1]Controls n=8, [2]T1D n=6

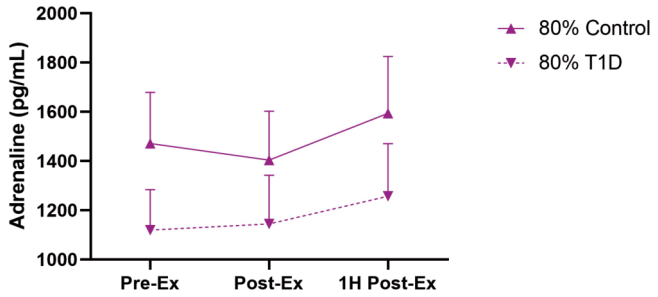

Figure 7. Adrenaline response during vigorous intensity exercise in control and T1D participants Total adrenaline concentration (pg/mL) during vigorous exercise in control and T1D participants. Error bars represent SEM. Control = 12, T1D = 12

Whilst there is a trend for lower adrenaline levels in T1D, this is not statistically significant. Nonetheless, these experiments need to be repeated with larger patient numbers, but we cannot currently exclude the possibility that lower basal adrenaline release in T1D participants may have an impact on the blunted NK cell mobilisation observed. It is notable that the baseline adrenaline concentration is abnormally elevated and therefore may mask the expected increase post-exercise in both groups (26, 28, 42).

DISCUSSION

This study has, for the first time, characterised the effects of acute exercise on the mobilisation of innate cell subsets in people with T1D. First, we aimed to investigate the effects of vigorous intensity exercise on innate cell subsets in T1D, with a particular focus on the most exercise responsive subset, NK cells. Second, we aimed to investigate the adrenaline response during exercise to gain insight into the mechanisms of differential lymphocyte mobilisation in T1D.

In this study we have demonstrated mobilisation of NK cell subsets in both control and T1D participants. Our findings are supported by previous studies also showing a preferential mobilisation of NK cells during acute exercise (5, 25, 50, 82). However, we show for the first time that this effect was blunted in the T1D participants. A strength to our study is that we have carried out a detailed characterisation of NK cell subsets during exercise including $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$, and $CD56^{bright}CD16^{-}$. We found that all NK subpopulations $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$ and $CD56^{bright}CD16^{-}$ significantly mobilised during vigorous intensity exercise in controls. In T1D, only $CD56^{dim}CD16^{bright}$ NK cells significantly mobilised during vigorous intensity exercise. As demonstrated in previous studies, $CD56^{dim}CD16^{bright}$ NK cells demonstrate the largest response to acute exercise. The percentage increase of $CD56^{dim}CD16^{bright}$ NK cells is a minimum of 3-fold higher than that of the other subsets in our study. As seen with total NK cells, the magnitude of the response immediately following vigorous exercise was blunted in T1D participants.

Both $CD122^{+}$ (IL-2Rβ/IL-15R) and $CD25^{+}$ (IL-2Rα) $CD56^{bright}CD16^{dim}$ NK cells significantly mobilised during vigorous intensity exercise in control participants. However, only $CD122^{+}$ $CD56^{bright}CD16^{dim}$ NK cells subsets significantly mobilised during vigorous intensity exercise in T1D participants. Previous reports have shown mobilisation of $CD122^{+}$ and $CD25^{+}$ NK cells following exercise in healthy cohorts (56, 73). We have for the first time demonstrated this in T1D during vigorous intensity exercise. We have also demonstrated in our study that $CD56^{bright}CD16^{dim}$ NK cells with a migratory capacity ($LFA\text{-}1^{+}$) significantly mobilise following vigorous intensity exercise in the control group.

It has been well established that mature $CD56^{dim}CD16^{bright}$ NK cells with a highly differentiated phenotype mobilise dramatically during acute exercise (6, 76). In this study, we have examined the mobilisation of both early mature (KIR+) and highly differentiated ($KIR^{+}CD57^{+}$) NK cells during acute exercise. As expected, highly differentiated NK cells significantly mobilised during vigorous intensity exercise in the control and T1D group. Early mature NK cells (KIR^{+}) exhibited a similar effect. However, the percentage increase immediately following vigorous intensity exercise was blunted in the T1D group. In summary, highly differentiated, mature $KIR^{+}CD57^{+}$ NK cells increase dramatically following vigorous intensity exercise in both the control and T1D groups. However, the observed blunted increase of $CD56^{dim}CD16^{bright}$ NK cells is driven by early mature KIR^{+} subset. In this study we have used t-SNE visualisation to assess changes in NK cell subsets during vigorous intensity exercise to further delineate the evident blunting following vigorous exercise in T1D. Most of the data in the field of exercise immunology is presented in absolute numbers because changes in the proportions of populations are difficult to detect. The t-SNE plots allow us to identify and easily visualise subtle changes in subgroups that may not be obvious by directly looking at the proportion. Here we have further divided the $CD56^{dim}CD16^{bright}$ NK subsets into $CD56^{dim}CD16^{int\text{-}bright}$ and $CD56^{dim}CD16^{bright}$. Thus, further highlighting that the blunted increase in $CD56^{dim}CD16^{bright}$ NK cells is driven by the early mature KIR^{+} subgroup, specifically those within the $CD56^{dim}CD16^{bright}$ clusters. To further increase the strength of this powerful tool, more parameters need to be included in the phenotyping panels. Inclusion of more phenotypic markers such as other NK inhibitory and activation receptors may identify more subpopulations. In our study, multiple clusters reside within the mature NK cell populations suggesting some phenotypic differences within this subgroup. This could identify new exercise sensitive NK cell populations in future investigations on the effects of exercise on immune parameters in healthy and disease states. Although much work has been done to identify populations mobilised during exercise, deeper immunophenotyping analysis is necessary to broaden the scope of analysis and in particular, the investigations of exercise in disease states which have a skewed immune phenotype. Within the myeloid subsets, total DC mobilised during vigorous intensity exercise in both groups, and to a similar magnitude. Of the DC subsets, activated $HLA\text{-}DR^{hi}CD11c^{hi}$ mDC, preferentially mobilised during vigorous intensity exercise in both groups. Total monocytes significantly mobilised during vigorous intensity exercise in the control group. However, an increase in total monocytes immediately following exercise was evident in the T1D group. Previous studies have

also shown DC and monocyte mobilisation during acute exercise in healthy cohorts (9, 24, 33, 38, 60, 71, 80). In particular, a recent study demonstrated preferential mobilisation of pDCs during exercise in a healthy cohort (9). However, in our study we see a preferential mobilisation of mDC. This could possibly be due to differences in the phenotypic surface markers used; in our study we used CD11c to identify mDC and CD123 to identify pDC. In the aforementioned study, CD303- was used to identify mDC and $CD303^+$ to identify pDC. Additionally, the exercise bout lasted 20 minutes in the aforementioned study, whereas the acute exercise bout last 30 minutes in our study. This highlights how small differences in study design can impact on the variation of findings between research groups. The mechanisms through which differential mobilisation of lymphocyte subsets during exercise in T1D need to be defined to further understand the implications of acute exercise in T1D. NK cells are the most responsive lymphocyte subset to acute exercise due to their high beta-adrenergic receptor expression resulting in their preferential intensity-dependant mobilisation in response to adrenaline during acute exercise (5, 25, 50, 82). As seen in our previous work, exercise sensitive subsets including highly differentiated $CD8^+$ T cells which respond to adrenaline are also blunted. This leads us to the premise that the blunted effect is due to an impaired adrenaline response during vigorous intensity exercise in T1D (23). We measured systemic adrenaline during vigorous intensity exercise in both control and T1D participants. In this study, we found no statistical significant differences in systemic adrenaline between groups during exercise.

As a result, there are a number of points to consider surrounding the adrenaline response in our cohorts. The pre-exercise adrenaline concentration is abnormally elevated in both groups. Resting baseline adrenaline would be expected to be approximately 50-200pg/ml (26, 28, 42). However, in our study we found baseline adrenaline concentrations above 1000pg/ml. This is comparable to expected post-exercise adrenaline concentrations (42). Psychological stress is an important factor to consider when measuring adrenaline as it can result in dramatic increases in systemic adrenaline (26). Although we aimed to reduce psychological stress by taking an initial resting baseline sample 30 minutes prior to the pre-exercise sample, the stress of being in laboratory conditions may have caused an increase in adrenaline at this time point. In future studies, it may be better to increase the resting time or to have the participant sitting on the bike for a short period of time before taking the pre-exercise blood sample. Beginning with an elevated adrenaline level may have disguised the expected increase post-exercise in both groups and therefore makes it difficult to delineate differences in adrenaline release between control and T1D groups. Furthermore, although we aimed to recruit participants with similar activity levels, it is worth considering that the adrenaline response is different between trained and untrained individuals (40). Nonetheless, the lower basal adrenaline release in the T1D cohort may influence the blunted NK cell mobilisation observed in our study. Therefore, these experiments need to be repeated with larger patient numbers to fully elucidate the mechanism of differential mobilisation of certain lymphocyte subsets following vigorous intensity exercise in T1D. On the other hand, reduced beta-adrenergic sensitivity of lymphocytes in T1D has been reported previously, resulting in reduced adrenaline responses (31, 41, 77). Reduced beta-adrenoceptor density and sensitivity, causes a blunted lymphocyte increase during acute exercise (51). Therefore, exercise-induced mobilisation of exercise responsive subsets including highly differentiated $CD8^+$ T cells and early mature NK cells may be blunted due to a reduced lymphocyte beta-adrenoceptor density and sensitivity in T1D.

Early mature KIR^+ and highly differentiated mature KIR^+CD57^+ NK cells are important cytotoxic populations involved in immune surveillance. Both subsets are highly cytotoxic, however early mature $KIR^+CD56^{dim}CD16^{bright}$ are still proliferative compared to KIR^+CD57^+ NK cells. The blunted increase in early mature KIR^+ NK cells may be indicative of an increased risk of cancer associated with T1D (15, 62). Although a blunted increase is evident, there is still mobilisation of a proportion of early mature KIR^+ and complete mobilisation of highly mature KIR^+CD57^+ NK cells. Therefore, repeated bouts of acute vigorous intensity exercise may have positive implications in reducing the risk of cancer in T1D, particularly those with long-standing T1D. This is supported by previous studies investigating exercise training in cancer models in which immune surveillance was increased and tumour growth was reduced (63). NK cell infiltration into tumours was increased in trained mice. In this study, it was reported that adrenaline and IL-6 were imperative for NK cell mobilisation, redistribution, and activation to control tumour growth (63).

Preliminary data presented here provides evidence to investigate exercise training in T1D. Exercise-induced improvements in T1D following exercise training have been shown in NOD mice (61). NOD mice that were trained for 20 weeks demonstrated reduced immune cell infiltration into the pancreas and therefore a reduction in insulitis (61). NK cells are one of the first immune subsets to infiltrate the pancreas before diabetes onset, therefore reduced insulitis may be in part through a reduction in NK cell infiltration. Exercise training also acts as a preventative measure for atherosclerosis in people at risk such as people with T1D (1, 55). Together, this provides a strong basis to investigate exercise training in T1D to reduce pancreatic NK cell infiltration, reduce atherosclerosis risk, and increase immune surveillance. Respectively, these benefits may translate into a number of clinically relevant outcomes. Firstly, exercise may modulate the natural history of autoimmune T1D. We have recently published the results of a pilot clinical trial to explore whether physical exercise reduces the rate of beta cell loss in people newly diagnosed with T1D (58). Whilst immune changes were not reported as part of this trial, the results do suggest that exercise has the potential to protect beta cells in new-onset patients. The trial needs to be taken forward to a formal and adequately powered clinical trial for beta cell preservation. However, the innate immune cell changes we report here do provide mechanistic support for physical exercise modulating immune cell behaviour in T1D. Second, innate immune cells play an important role in infection and increasing the responsiveness of these cells is one approach to control this (32). People with T1D are at significantly increased risk of hospitalised infection (14) and the ability of exercise to mobilise

innate immune cells may be beneficial in the surveillance and management of this risk. In summary therefore, this study provides important immune based evidence to support the encouragement and provision of exercise to people with T1D. However, whilst our findings do demonstrate that vigorous exercise increases the mobilisation of innate immune cells in T1D, the mobilisation is not as significant as in people without diabetes. It may be therefore that exercise will need to be of higher intensity, longer duration or of a different form to achieve the mobilisation observed in non-T1D. Although this is the first study of its kind, it is important to note that participant numbers in our study were relatively low. This has largely contributed to the lack of statistical significance between groups. However, this study offers insight into exercise related changes in T1D compared to healthy participants. It provides a basis for future investigation into the effects of exercise on NK cells in T1D and this is imperative in moving research in this field forward.

In conclusion, mobilisation of NK cell subsets is observed in control and T1D groups following vigorous intensity exercise. However, $CD56^{dim}CD16^{bright}$ NK cells display a blunted increase following vigorous intensity exercise in T1D and this was driven by impaired mobilisation of an early mature KIR^{+} subset. This may be due to differences in lymphocyte beta-adrenergic receptor density in T1D. Our findings have implications for immune surveillance in T1D and supports the need for studies to explore whether vigorous intensity exercise can modulate the autoimmune response in T1D.

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Disclosures

Michelle Curran is now an AstraZeneca employee and is part of the AstraZeneca postdoc program.

Abbreviations

BMI	Body Mass Index
BP	Blood Pressure
CHF	Congestive Heart Failure
DC	Dendritic Cell
ddH2O	Double-distilled water
EDTA	Ethylenediaminetetraacetic Acid
EMRA	Effector Memory re-expressing CD45RA
EXTOD	Exercise for Type One Diabetes
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FMO	Fluorescence Minus One
FSC-A	Forward Scatter-Area
FSC-H	Forward Scatter-Height
HbA1c	Haemoglobin A1c
HSPC	Hematopoietic Stem and Progenitor Cells
KIR	Killer Immunoglobulin Receptor
KLRG1	Killer cell Lectin-like Receptor subfamily G member 1
mAb	Monoclonal antibody
mDC	Myeloid DC
NK	Natural Killer
NKG2A	Inhibitory C-type lectin receptor A
NOD	Non-Obese Diabetic
O2	Oxygen
PBS	Phosphate Buffer Saline
pDC	Plasmacytoid DC
REC	Research Ethics Committee
SSC-A	Side Scatter- Area
SSC-H	Side Scatter- Height
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Th1	Type 1 helper
Th2	Type 2 helper
TLR	Toll-like Receptor
tSNE	t-Distributed Stochastic Neighbor Embedding
UHBFT	University Hospitals Birmingham NHS Foundation Trust
WTCRF	Wellcome Trust Clinical Research Facility

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