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Doctoral Thesis

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Doctoral Thesis

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Co-mentor: Assoc. Prof. Tamara Turk Wensveen, MD, PhD

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SVEUČILIŠTE U RIJECI

MEDICINSKI FAKULTET

Dora Gašparini

UTJECAJ ŠEĆERNE BOLESTI TIPA 2 I NJEZINOG LIJEČENJA NA FUNKCIJU LIMFOCITA

Doktorski rad

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Komentorica: izv. prof. dr. sc. Tamara Turk Wensveen

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Rad je izrađen na Zavodu za histologiju i embriologiju Medicinskog fakulteta Sveučilišta u Rijeci pod mentorstvom izv. prof. dr. sc. Felixa Wensveena i u Centru za dijabetes, endokrinologiju i kardiometabolizam Specijalne bolnice Thalassotherapia Opatija pod komentorstvom izv. prof. dr. sc. Tamare Turk Wensveen, a u sklopu poslijediplomskog sveučilišnog znanstvenog (doktorskog) studija „Biomedicina“ pri Medicinskom fakultetu Sveučilišta u Rijeci. Rad je izrađen u sklopu projekata Hrvatske zaklade za znanost izv. prof. dr. sc. Felixa Wensveena (IP-2016-06-8027, IP-CORONA-2020-04-2045) i Potpore Sveučilišta u Rijeci (19-41-1551) te projekta izv. prof. dr. sc. Tamare Turk Wensveen „Utjecaj šećerne bolesti tipa 2 i njezinog liječenja na funkciju limfocita“ Hrvatske zaklade za znanost (IP-2020-02-7928).

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SUMMARY

Objectives: Systemic chronic low-grade inflammation plays a pivotal role in the development of type 2 diabetes mellitus (T2DM) and its comorbidities including atherosclerosis, diabetic kidney disease, and liver steatosis. However, the impact of T2DM on the inflammatory state of the immune system is incompletely understood. The primary objective of this study is to explore whether T2DM is associated with a pro-inflammatory profile within the antiviral arm of the immune system and whether this can be reversed through antidiabetic treatment.

Materials and Methods: After signed informed consents were collected, we isolated peripheral blood mononuclear cells from both individuals with T2DM and control subjects. Additionally, a subgroup of patients who achieved target glycated haemoglobin A_{1c} below 7.0% underwent blood collection before and six months after the optimisation of their antidiabetic treatment. We conducted a comprehensive analysis of cytotoxic lymphocyte phenotype, proliferation and cytokine production using multiparametric flow cytometry.

Results: Our findings reveal a significant increase in the production of tumour necrosis factor-α by $CD8^+$ T cells and Granzyme B by natural killer (NK) cells and γδ T cells in patients with diabetes compared to the control group. These observations showed a positive correlation with both age and diabetes duration. In a subset of patients with poorly controlled diabetes, we optimised their antidiabetic treatment regimen, subsequently repeating the analysis three months after successfully lowering their blood glucose levels. Remarkably, optimal antidiabetic treatment demonstrated the capacity to reduce cytokine production by $CD8^+$ T cells, NK cells, and γδ T cells.

Conclusion: Cytotoxic immune cells undergo functional alterations in the context of diabetes, potentially contributing to the development and exacerbation of inflammation-driven diabetic complications. Lowering of blood glucose levels was associated with an amelioration of the hyper-inflammatory profile of these cells. Our findings underscore the potential for optimal antidiabetic treatment to revert these immune changes, offering promising avenues for managing diabetes-related inflammation.

Key words: CD8-Positive T-Lymphocytes; Cytokines; Diabetes Mellitus, Type 2; Glucagon-Like Peptide-1 Receptor; Inflammation; Sodium-Glucose Transporter 2 Inhibitors; Tumour Necrosis Factor-alpha.

SAŽETAK

Cilj istraživanja: Sustavna kronična upala niskog stupnja igra ključnu ulogu u pojavi šećerne bolesti tipa 2 i povezanih komorbiditeta poput ateroskleroze, dijabetičke nefropatije i steatoze jetre. Međutim, utjecaj šećerne bolesti tipa 2 na upalno stanje imunološkog sustava nije u potpunosti razjašnjen. Glavni cilj ovog istraživanja je otkriti je li šećerna bolest tipa 2 povezana s proupalnim profilom unutar antivirusnog kraka imunološkog sustava i može li antihiperglikemijsko liječenje poništiti taj utjecaj.

Materijali i metode: Nakon prikupljanja potpisanih informiranih pristanaka, izolirali smo mononuklearne stanice periferne krvi osoba sa šećernom bolesti tipa 2 i kontrolnih ispitanika. Usto, podskupina bolesnika, koja je postigla ciljni glikirani hemoglobin A_{1c} niži od 7.0%, podvrgnuta je prikupljanju krvi prije početka i šest mjeseci nakon optimizacije njihovog antidijabetičkog liječenja. Proveli smo opsežnu analizu citotoksičnih limfocita, točnije njihovog fenotipa, proliferacije i proizvodnje, korištenjem multiparametrijske protočne citometrije.

Rezultati: U bolesnika sa šećernom bolesti tipa 2 zabilježili smo značajan porast u proizvodnji čimbenika tumorske nekroze α iz $CD8^+$ limfocita T te granzima B iz prirodnih stanica ubojica (engl. *natural killer*, NK) i γδ limfocita T u usporedbi s kontrolnom skupinom. Ova su opažanja u pozitivnoj korelaciji s dobi i trajanjem šećerne bolesti. U podskupini bolesnika s loše kontroliranom šećernom bolesti, optimizirali smo njihov antihiperglikemijski režim liječenja te ponovili analizu tri mjeseca nakon uspješnog snižavanja razine glukoze u krvi. Zanimljivo je da je optimalno antihiperglikemijsko liječenje pokazalo sposobnost smanjenja proizvodnje citokina iz $CD8^+$ limfocita T, stanica NK i γδ limfocita T.

Zaključak: Citotoksične imunološke stanice prolaze kroz funkcionalne promjene u kontekstu šećerne bolesti, potencijalno pridonoseći razvoju i pogoršanju dijabetičkih komplikacija izazvanih upalom. Naša otkrića naglašavaju potencijal optimalnog antihiperglikemijskog liječenja u poništavanju ovih imunoloških promjena, pritom nudeći inovativan način upravljanja upalom povezanom sa šećernom bolesti.

Ključne riječi: CD8-pozitivni limfociti T; Citokini; Čimbenik tumorske nekroze-alfa; Inhibitori prijenosnika natrija i glukoze 2; Receptor glukagonu sličnog peptida 1; Šećerna bolest, tip 2; Upala.

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. The burden of type 2 diabetes mellitus: challenges and solutions

Diabetes mellitus, a chronic metabolic disease marked by a reduced ability to control blood glucose homeostasis, has been referred to as the pandemic of the 21st century. It affects approximately 537 million adults worldwide, equivalent to 10.5% of the global adult population [1]. The prevalence of diabetes is projected to escalate to 643 million by 2030 and 784 million by 2045 [1]. Consequently, more than 6.5 million people are estimated to die every year from diabetes or hyperglycaemia-induced comorbidities [1]. Diabetes is associated with a large number of micro- and macrovascular complications, including chronic kidney disease, ischemic heart disease and diabetic retinopathy. Nevertheless, many aspects of diabetes and its comorbidities are still poorly understood.

Type 2 diabetes mellitus (T2DM) stands as the most prevalent form of diabetes, largely attributed to the widespread occurrence of obesity and other related risk factors [2]. To advance the development of more precise and effective treatments, a deeper comprehension of the pathophysiology of T2DM and its complications is imperative. Initially, a triad of three key factors for diabetes development were identified, which later expanded to an ominous octet of eight factors [3, 4]. Now, we understand that impaired ability to control glycemia is a result of interconnected pathology in eleven different organ systems [5] but the main underlying mechanism of T2DM-induced hyperglycaemia stems from diminished glucose uptake due to insulin resistance within the liver, muscles and visceral adipose tissue.

As per the most recent guidelines established by the American Diabetes Association (ADA)[6], the diagnosis of T2DM is determined when a patient exhibits a fasting plasma glucose concentration (FPG) of ≥ 7.0 mmol/l, a glycated haemoglobin A_{1c} (HbA_{1c}) $\geq 6.5\%$, or a blood glucose value (2HG) of ≥ 11.1 mmol/l two hours after the initiation of oral glucose tolerance test. Unlike FPG and 2HG values, HbA_{1c} percentage is less subject to daily changes as it represents a weighted average of blood glucose levels over approximately three months, corresponding to the lifespan of a red blood cell. This stability makes HbA_{1c} a routine marker for disease control and treatment efficacy monitoring. The primary objective of antidiabetic treatment is to lower blood glucose levels below 'target' thresholds [7], which, in clinical practice, is typically defined as an HbA_{1c} of 7%. Nevertheless, these treatment goals are frequently individualised and tailored to the patient's physical condition and risk profile [7].

The management of newly diagnosed T2DM typically commences with lifestyle modifications and the initiation of metformin as the first-line medication [7]. If metformin alone fails to achieve the target HbA_{1c} levels, add-on therapy should be administered. Currently, a diverse array of therapeutic options for T2DM treatment are available, and the selection for each patient hinges on a multitude of factors, including

age, disease duration, and the presence of comorbidities (**Figure 1**). Moreover, non-clinical considerations such as financial or administrative factors can also be of key importance. For instance, sodium-glucose co-transporter-2 inhibitors (SGLT-2i) are the preferred choice for patients with heart failure (HF) and chronic kidney disease. Glucagon-like peptide-1 receptor agonists (GLP-1RA) are better suited for those with atherosclerotic cardiovascular disease (ASCVD) and obesity, defined as body mass index (BMI) above 30 kg/m^2 [7]. The first-line antidiabetic treatment regimens, incorporating SGLT-2i and/or GLP-1RA alongside metformin, have been extensively studied for their beneficial effects on cardiovascular and renal outcomes in large-scale randomised clinical trials. SGLT-2 inhibitors, such as empagliflozin and dapagliflozin, act through a decrease in glucose reabsorption in the proximal tubules causing an increase in urinary glucose excretion and subsequent caloric loss. Additionally, the associated osmotic diuresis, resulting from increased glucose excretion, contributes to a reduction in blood pressure. On the other hand, GLP-1RA, such as semaglutide, liraglutide and dulaglutide, stimulate insulin secretion, suppress glucagon release, slow gastric emptying, and promote a sense of satiety, collectively contributing to improved glycaemic control, reduced postprandial glucose levels, and potential benefits in body weight management. Despite significant differences in their mechanisms of action, both medication classes demonstrate a comparable potential for cardiorenal protection. So far, randomised clinical trials have shown a significant reduction in major adverse cardiovascular events (defined as a composite of non-fatal myocardial infarction, non-fatal stroke and cardiovascular death), as well as a decrease in cardiovascular and all-cause mortality in patients treated with these medications [8-13]. Interestingly, SGLT-2i provide protection for individuals with heart failure and chronic kidney disease, both in the presence and absence of diabetes [14-17]. In a systematic review and meta-analysis, GLP-1RA have been shown to reduce hospital admission for heart failure and the composite kidney outcome [18]. Nevertheless, as dedicated trials assessing kidney or heart failure outcomes with GLP-1RA have not yet been completed, this class of medications, unlike SGLT-2i, currently lacks additional indications for heart failure and chronic kidney disease. Although conclusive evidence supporting additional benefits from the combination of SGLT-2i and GLP-1RA is lacking, current guidelines and recommendations advocate the addition of an SGLT-2i after a GLP-1RA or vice versa [7]. This approach is encouraged for patients with or at high risk of ASCVD and those with chronic kidney disease (**Figure 1**) considering that the combination therapy is highly likely to provide further outcome reduction.

The focus of current antidiabetic treatment, therefore, extends beyond glucose control to encompass additional health benefits like weight management or reducing cardiovascular risk (**Figure 1**). The United Kingdom Prospective Diabetes Study [19], a large-scale randomised trial focused on individuals with T2DM and the assessment of antidiabetic therapies, has unearthed a profound revelation: a mere 1% reduction in HbA_{1c} levels is intricately linked to a notable decrease in the risk of various critical health outcomes. These encompass a 37% reduction in microvascular disease, a 12% lower risk of stroke, a 16% decrease in HF

incidence, and a 14% lower risk of myocardial infarction. Furthermore, this 1% decrease in HbA_{1c} translates into a 14% reduction in all-cause mortality among T2DM patients, accompanied by a remarkable 21% decrease in diabetes-related mortality. Nonetheless, it is imperative to acknowledge that despite these significant findings from large-scale randomised clinical trials, information regarding the impact of glucose-lowering therapies on certain overlooked complications, particularly those related to immunological defects among diabetes patients, remain insufficient to this day.

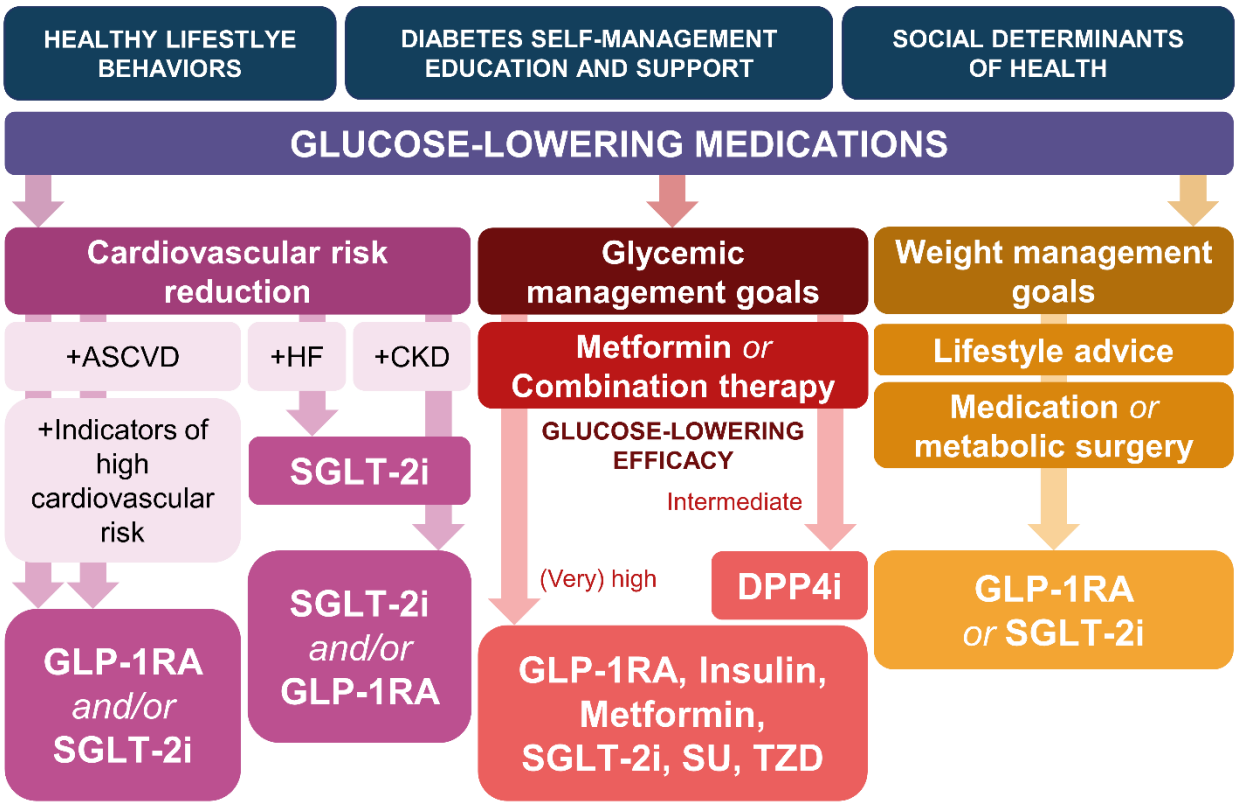

Figure adapted from: 9. Pharmacologic Approaches to Glycemic Treatment: Standards of Care in Diabetes-2024. Diabetes care. 2024;47:S158-78.[7]

Figure 1. Type 2 diabetes mellitus treatment algorithm

ASCVD, atherosclerotic cardiovascular disease; DPP-4i, dipeptidyl peptidase-4 inhibitors; GLP-1RA, glucagon-like peptide-1 receptor agonists; HF, heart failure; SGLT-2i, sodium-glucose co-transporter 2 inhibitors; SU, sulfonylureas; TZD, thiazolidinediones.

1.2. Diabetes mellitus as a risk factor for infection

An often underestimated aspect of diabetes is its detrimental impact on the immune system. Several uncommon infectious diseases, such as emphysematous pyelonephritis, malignant otitis externa, mucormycosis, and Fournier's gangrene, are notably prevalent among individuals with T2DM [20]. Equally significant, numerous studies have highlighted the increased susceptibility of diabetes patients to infections. A landmark prospective study conducted across primary care facilities, involving 6,712 patients with T2DM and 18,911 control subjects over one year, explored their susceptibility to infection [21]. The results revealed that individuals with T2DM had an elevated risk of contracting lower respiratory tract infections, urinary tract infections, bacterial skin and mucosal infections, as well as fungal infections. In 2011, a comprehensive meta-study comprising of 97 prospective cohort studies further demonstrated that T2DM is associated with a significant increase in infection-related mortality, even when excluding for pneumonia [22]. Importantly, T2DM not only increases susceptibility to infections but also influences the progression, duration and complication risks of infections. A comparative study of bloodstream infections in 71 diabetes patients and 252 non-diabetic counterparts found that the former group experienced prolonged stays in intensive care, longer mechanical ventilation, and a greater rate of renal or hepatic failure [23]. Moreover, a systematic review of 13 observational studies established that individuals with T2DM face an elevated risk of developing tuberculosis (TB) compared to healthy controls [24]. Given the increased susceptibility of T2DM patients to infections, the ADA guidelines recommend vaccination against influenza, *S. pneumoniae* and hepatitis B virus in addition to routine vaccines [25]. These findings from epidemiological studies provide compelling evidence of the increased susceptibility to infections in patients with T2DM.

This became strikingly evident during the emergence of the novel coronavirus disease (COVID-19) pandemic. Some of the underlying pathological mechanisms of diabetes intersect with those of COVID-19, leading to an amplified susceptibility and increased severity of COVID-19 among patients with diabetes [26-28]. Notably, diabetes was identified in a third of patients with a composite outcome measure encompassing intensive care unit admission, the need for mechanical ventilation, and death [26]. Among individuals afflicted by both COVID-19 and diabetes, the risks of severe pneumonia, an uncontrolled inflammatory response, and a hypercoagulable state associated with dysregulation of glucose metabolism are notably increased [29].

1.3. Proposed mechanisms of diabetes-induced immune dysregulation

Multiple factors contribute to the dysregulated immune response to pathogens in individuals with T2DM (**Figure 2**), but hyperglycaemia stands out as a pivotal mediator in this scenario. Research has

consistently shown that the level of HbA_{1c} is positively correlated with both the duration and severity of infections caused by various pathogens. In a large retrospective case-control study including more than 34,000 patients with pneumonia and more than 342,000 controls over an eight-year period, patients with T2DM exhibited a significantly higher risk of pneumonia (relative risk, RR 1.23; 95% Confidence interval, CI 1.19–1.28) [30]. Notably, the risk was significantly higher for T2DM patients with an HbA_{1c} level exceeding 9.0% (RR 1.60, 95% CI 1.44–1.76) compared to those maintaining HbA_{1c} levels below 7.0% (RR 1.22, 95% CI 1.14–1.30) [30]. A similar study involving 4,748 patients with type 1 diabetes mellitus (T1DM) and 12,954 controls across a 14-year follow-up period unveiled a significantly higher incidence of infections in individuals with T1DM compared to the control group, with infection rates positively correlated with HbA_{1c} percentage [31]. Although various other studies echo these findings, some are limited by inadequate statistical power or insufficient documentation of key metabolic parameters [20]. Nevertheless, a consistent pattern emerges, suggesting a direct relationship between glycaemic levels and the susceptibility of diabetes patients to infections.

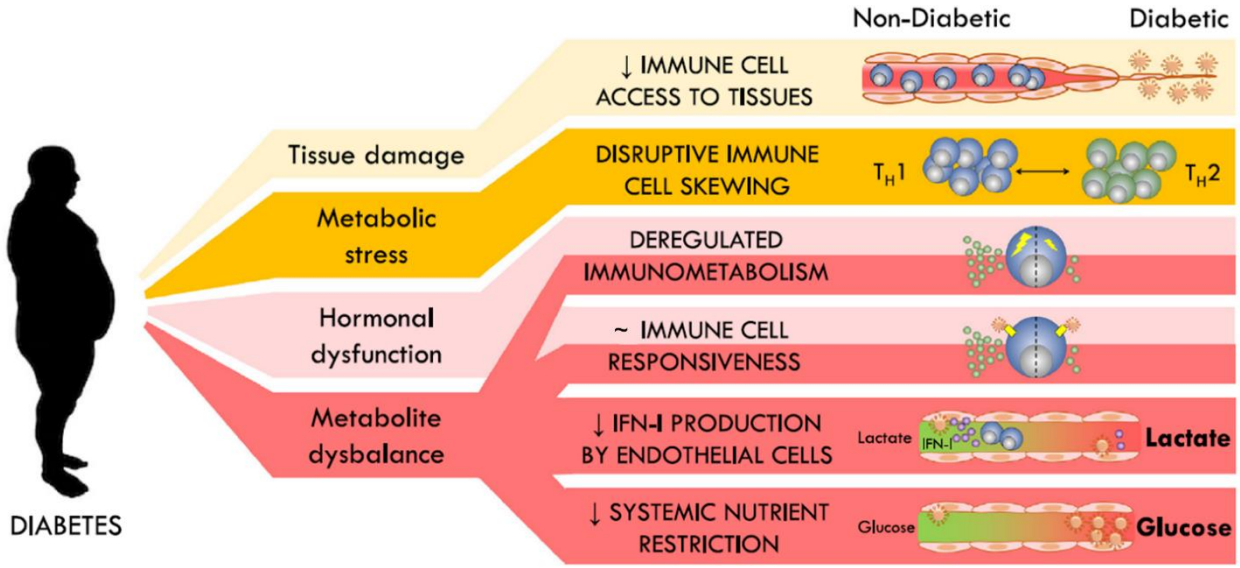

Figure from: Turk Wensveen T, Gašparini D, Rahelić D, Wensveen FM. Type 2 diabetes and viral infection; cause and effect of disease. Diabetes Res Clin Pract. 2021;172:108637. [74]

Figure 2. Negative impacts of type 2 diabetes mellitus on immunological control of viral infection

IFN, interferon; T_H, T helper.

The precise manner in which diabetes increases the susceptibility to infection remains elusive, but it has been associated with an impaired immune response. Numerous potential mechanisms of immune dysregulation in the context of T2DM have been proposed, albeit primarily based on studies involving

murine models. In experiments involving diabetic mice induced through the destruction of pancreatic β-cells using streptozotocin (STZ), it was observed that these animals exhibited reduced capacity to activate macrophages upon TB infection leading to delayed recruitment of neutrophils and dendritic cells, alongside reduced levels of pro-inflammatory cytokines [32, 33]. Furthermore, research revealed that STZ-induced diabetes impaired T cell-mediated immune responses to skin allografts, although the precise molecular mechanism remained unclear [34].

Human studies in this regard are both scarce and offer less definitive conclusions. Notably, acute changes in blood glucose levels have demonstrated an influence on the numbers of circulating $CD4^+$ and $CD8^+$ T cells [35]. One study noted that individuals with T2DM exhibited reduced cytotoxic potential in $CD8^+$ T cells and natural killer (NK) cells upon TB infection, as they produced lower levels of Granzyme B and Perforin and displayed decreased degranulation potential [36]. Conversely, a separate study found that the cytotoxicity of cells from T2DM patients remained intact, but the production of cytokines by $CD4^+$ and $CD8^+$ T cells was diminished [37]. The exact impact of hyperglycaemia on immune cells remains elusive as certain studies even reported increased cytokine production by $CD4^+$ T cells of T2DM patients with TB infection after *in vitro* stimulation [38, 39]. Furthermore, T2DM affects the responsiveness of innate immune cells. Granulocytes isolated from T2DM patients were observed to undergo apoptosis mediated by neutrophil extracellular traps, thereby impairing the process of wound healing [40, 41]. Additionally, the production of pro-inflammatory cytokines, such as interleukin (IL)-2 and IL-6, was found to be impaired in peripheral blood mononuclear cells (PBMCs) stimulated under hyperglycaemic conditions [42]. Moreover, hyperglycaemia was demonstrated to directly facilitate the replication of several pathogens [43, 44], further affecting the ability of the immune system to combat infections under such conditions. In summary, the dysregulation of the immune cells leads to inadequate responses to infections, resulting in an overall impairment of the immune response. Diabetes-induced immune dysregulation can be attributed to four pivotal factors: metabolite disbalance, hormonal disruption, hyperinflammation, and tissue damage.

1.3.1. Metabolite disbalance

While we have significant knowledge about which immunological processes are impacted by hyperglycaemia, the precise molecular mechanisms underlying these effects remain less clear. In immune cells, there is a close connection between metabolism and function. In their resting state, immune cells primarily rely on oxidative phosphorylation for their energy requirements. However, upon activation, particularly $CD8^+$ T cells and pro-inflammatory M1 macrophages shift their metabolism towards glycolysis to generate adenosine triphosphate and channel metabolites into the pentose-phosphate pathway [45]. When the metabolic control of immune cells is disrupted, it profoundly affects their functionality. For instance,

$CD8^+$ T cells that fail to increase glycolytic metabolism struggle to mount an effective effector response [46, 47], while memory cell formation is significantly reduced when these cells cannot activate oxygen-dependent metabolism [48]. One way through which hyperglycaemia impairs normal immune cell function is by disturbing immuno-metabolism [49].

Beyond glucose, T2DM also disrupts the balance of numerous other carbon-based metabolites involved in the defence against infections. During illness, our immune system orchestrates a controlled reduction in appetite and nutrient intake, shifting our metabolism into a fasting state [50]. This transition involves an increased utilisation of fatty acids and ketone bodies for sustenance. However, many pathogens prefer glucose metabolism to meet their metabolic demands. For example, cytomegalovirus actively induces glycolytic metabolism in host cells to facilitate its replication, a process hindered when glucose uptake is prevented [51, 52]. The replication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is enhanced in the presence of high glucose levels [53]. Nonetheless, due to the persistent hyperglycaemia characteristic of T2DM even under fasting, this strategy of depriving pathogens of glucose becomes ineffective. Furthermore, fasting metabolism has recently been discovered to prime non-immune cells to activate their innate cellular defences against infections. All nucleated cells can produce type I interferons (IFNs) upon infection, a potent alarm signal that attracts immune cells to the infection site. Defects in type I IFN production in humans are typically associated with lethal infections in early life [54]. SARS-CoV-2 leads to severe pathology in a significant number of patients because it delays the production of type I IFNs [55]. In response to feeding, most glucose is taken up by muscles and rapidly converted into lactate [56]. Consequently, fasting results in a reduction in systemic lactate levels. Recent research has shown that lactate inhibits type I IFN production in response to infection by inhibiting retinoic acid-inducible gene 1 proteins that detect viral infections. Notably, patients with T2DM exhibit elevated blood lactate levels [57, 58]. As a result, the innate ability of cells to recruit immune cells following viral infection is diminished.

1.3.2. Hormonal dysfunction

In addition to blood nutrient levels, hormonal disbalance contributes to immune dysregulation in patients with diabetes. Individuals with T2DM often exhibit elevated blood insulin and leptin levels, particularly shortly after diagnosis [59, 60]. While these hormones can stimulate the immune system, the effectiveness of the immune response relies on its ability to adapt to specific pathogens. Different pathogens trigger distinct transcriptional profiles in key innate immune cells like dendritic cells, which play a crucial role in activating T cells. For example, respiratory syncytial virus (RSV) infection leads to high levels of type I and type III IFNs, whereas SARS-CoV-2 infection results in increased IL-6 expression [61]. In diabetes, dendritic cell differentiation becomes skewed, causing reduced expression of costimulatory

molecules CD80 and CD86 [62], whilst promoting the development of plasmacytoid dendritic cells that produce type I IFNs [63]. Several studies suggest that hormonal irregularities can disrupt the balance between T-helper 1 (T_H1) and T-helper 2 (T_H2) activity, impairing the immune response against viruses such as RSV and cytomegalovirus [64, 65]. Therefore, the immuno-stimulatory effects of insulin and leptin in T2DM may not be beneficial, as they lead to skewed immune responses and reduced efficiency.

1.3.3. Chronic low-grade inflammation

In individuals with T2DM, changes in the cytokine environment from altered interactions between organs responsible for maintaining metabolic balance and their tissue-resident immune cells. Patients with T2DM typically exhibit elevated pro-inflammatory cytokine levels in their bloodstream [66], indicating chronic low-grade inflammation, primarily originating in visceral adipose tissue due to excess fat accumulation [67]. In obesity, adipocytes experience cellular stress, detected by innate immune cells like NK cells, which respond by releasing pro-inflammatory cytokines, most notably IFN-γ [68]. This triggers a shift in macrophages from an anti-inflammatory M2 to a pro-inflammatory M1 phenotype [69]. Activated adipose tissue macrophages further fuel local inflammation and immune cell recruitment, resulting in a continuous release of cytokines into the bloodstream. T2DM exacerbates cytokine-induced damage during infections such as Influenza and COVID-19 [70, 71]. Additionally, the pro-inflammatory environment in patients with T2DM is associated with abnormal clot formation and hypercoagulation, contributing to the elevated mortality risk in COVID-19 [72, 73].

1.3.4. Tissue damage

Pathological microvascular changes are a defining feature of diabetes, disrupting organ function in those reliant on microcirculation, such as the kidneys, retina, and peripheral nervous system. These microvascular alterations, including delayed vascularisation, reduced blood flow, diminished innate immunity, decreased growth factor production, and psychological stress factors, impair the local immune response. This defect often becomes apparent in the skin of the extremities of individuals with T2DM, where it leads to chronic infections, ulcers, and delayed wound healing [74]. However, microvascular damage negatively impacts a normal immune response in basically all tissues.

1.4. Inflammation as the key driver of diabetic complications

Prolonged and excessive metabolic stress in patients with diabetes can trigger harmful inflammatory responses, potentially giving rise to autoinflammatory diseases. While the precise manifestations may vary

between organs, a common underlying pattern emerges in tissues affected by metabolic stress. Elevated nutrient exposure induces adipocyte hypertrophy; however, beyond a certain saturation point, these adipocytes lose their ability to store additional lipids, leading to the expression of stress signals in patients with obesity. This, in turn, initiates the release of cytokines and chemokines, subsequently leading to the recruitment and proliferation of immune cells [75]. In contrast, microvascular alterations occurring in the retina and peripheral neurons of patients with diabetes lead to hypoxia, which has been proposed as a trigger of the immune response [75]. The inflammasome, particularly the nucleotide-binding domain leucine-rich–containing family pyrin domain–containing-3 (NLRP3) inflammasome, plays a central role in detecting these alterations and responds to increased levels of glucose, fatty acids, cholesterol, uric acid, and hypoxia [76]. Activation of NLRP3 inflammasome requires two steps: priming and activation. The priming step (signal 1) implies induction of the nuclear factor kappa b (NF-κB)-mediated NLRP3 and pro-IL-1β expression, and the activation step (signal 2) consists of promoting NLRP3 inflammasome assembly and caspase-1-mediated IL-1β and IL-18 secretion [77]. Depending on the tissue involved, the inflammatory cascade induced by metabolic stress results in different pathological conditions. Notably, age has recently been acknowledged as a significant contributing factor that amplifies the inflammatory cascade in patients with chronic conditions.

Age-induced inflammation, also referred to as inflammageing, contributes to tissue dysfunction, diminishes cellular repair processes, and facilitates the development of age-related diseases such as cardiovascular disease, chronic kidney disease and cancer [78]. Fransceschi et al. [79] initially conceptualised inflammageing as a component of the two-hit theory in the development of age-related diseases. A persistent inflammatory background, driven by continuous antigenic load and stress, was proposed as the first hit, requiring a second hit (genetic factors) for the manifestation of specific inflammatory-driven conditions [79]. Notably, metabolic dysregulation appears to fuel inflammageing, as evidenced by the association between higher levels of inflammatory markers in patients with type 2 diabetes and an increased vascular- and all-cause age-adjusted mortality risk [80]. Importantly, targeted interventions aiming to mitigate these inflammatory pathways may improve cardiovascular outcomes in patients with type 2 diabetes [81, 82]. Increased inflammageing is observable among several diabetes-related inflammatory disorders, such as Alzheimer's disease and polycystic ovary syndrome. However, a precise characterisation of the impact of type 2 diabetes on inflammageing-associated factors is currently lacking.

1.4.1. Inflammation and macrovascular complications of diabetes

Nearly two centuries ago, atherosclerosis was recognised as an inflammatory process, an insight later substantiated by contemporary research [83]. Recent studies have revealed that atherosclerosis shares

a common thread of metabolic stress-induced inflammation with T2DM. Myeloid cells, with the IL-1 system serving as a pivotal sensor for metabolic shifts, are at the core of inflammation in ASCVD [75]. In the early stages of atherosclerosis, monocytes adhere to the endothelial surface and infiltrate arterial walls, where they can transform into foam cells accumulating around endothelial lesions [84]. This process is initiated by the NLRP3 inflammasome, which activates in response to crystalline cholesterol and saturated fatty acids. Substituting saturated fats with monounsaturated fats reduces IL-1β priming and secretion, as well as the subsequent modulation of monocyte chemotaxis and adhesion [85]. The study of IL-1β inhibitor Canakinumab (Canakinumab Anti-Inflammatory Thrombosis Outcomes or CANTOS study) has further underscored the significance of IL-1β in ASCVD [86, 87]. Other cytokines, such as IL-1α and IL-6 [75], also contribute to atherosclerosis progression, and ongoing clinical trials will shed further light on this complex interplay. Diabetes seems to further aggravate the systemic inflammatory response in these patients, thus leading to increased pathology and worse clinical outcomes.

1.4.2. Inflammation and microvascular complications of diabetes

Inflammation is a well-established player in a wide range of diabetic complications, contributing significantly to the development of conditions such as diabetic retinopathy and macular oedema. Chronic retinal inflammation persists throughout the disease's progression, fuelled by key cytokines like IL-1β, IL-6, IL-8, and tumour necrosis factor-alpha (TNF-α), as well as the adhesion of monocytes and their chemotaxis [88-91]. Hypoxia and hyperglycaemia further exacerbate these processes, resulting in damage to retinal blood vessels, disrupting the blood-retinal barrier and culminating in macular oedema and retinal neovascularization. Notably, vascular endothelial growth factor (VEGF) serves as a critical mediator in the development of diabetic retinopathy, induced by factors such as hypoxia, IL-1β, IL-6, insulin and IGF-1 [92, 93]. The involvement of cytokines helps explain the elevated expression of VEGF in diabetic macular oedema, even in the absence of overt hypoxia [93].

In diabetic nephropathy, we observe changes in innate immunity, where TLR4 is notably overexpressed in the kidneys of patients with T2DM. This overexpression correlates positively with HbA_{1c} levels but negatively with renal function [94]. Additionally, the NLRP3 inflammasome activation of IL-1β plays a significant role in sensing metabolic stress in the diabetic kidney [95-98].

Diabetic polyneuropathy is a condition with significant clinical implications, including diabetic foot ulcers and the risk of amputation. Paradoxically, research on its underlying causes has remained limited, with traditional classifications considering diabetic neuropathy as non-inflammatory, in contrast to conditions like Guillain-Barré syndrome or demyelinating neuropathy [99]. However, recent studies have

unearthed compelling connections between inflammation and diabetic neuropathy [99, 100], although detailed mechanistic insights in this area are still lacking.

Diabetes triggers the immune system in various ways, thus contributing to pathology of many of its comorbidities. However, to date most attention has been given to NLRP3-mediated inflammation, focused around the cytokine IL-1β [66, 85]. However, animal studies suggest that the antiviral immune system also plays a key role in the pathophysiology of diseases associated with T2DM [32-34, 101-103]. However, how T2DM impacts these cells in humans is mostly unknown.

In the light of recent studies proving the remarkable efficacy of SGLT-2i and GLP-1RA in diverse diabetic complications, it is unclear whether antidiabetic therapy also has a beneficial impact on the inflammatory profile of immune cells in patients.

2. RESEARCH GOALS

The ultimate goal of this project is to elucidate whether antidiabetic therapy improves $CD8^+$ T cell, γδ T cell and NK cell dysregulation in the context of T2DM. This study will provide valuable new insights in lymphocyte dysregulation, an underappreciated aspect of T2DM. More importantly, it will allow clinicians to include the immunological risk profile of patients as an additional parameter when considering their best treatment options.

Based on scarce epidemiological data from the literature and our previous research in animal models of obesity and diabetes, we hypothesize that glycemia has an effect on lymphocyte function in the context of diabetes. We will investigate whether we can confirm this data by comparing flow cytometry parameters of lymphocyte phenotype and function of patients with T2DM and non-diabetic control subjects. Using this information, we will identify key surface markers and cytokines that define lymphocyte dysregulation in patients with T2DM. Finally, we will test whether reduction of glycemia also restores normal lymphocyte function in patients with T2DM. Our overall research question can therefore be formulated as follows:

How does glycemia in patients with T2DM impact lymphocyte function and is this effect reversible?

In order to answer the main research question, three specific research goals were set:

1. To determine a baseline for lymphocyte function using cells of healthy volunteers.

2. To determine the nature of lymphocyte dysregulation in patients with T2DM.

3. To determine whether reduction of glycemia restores normal lymphocyte function in patients with T2DM.

3. SUBJECTS AND METHODS

3.1. Subjects

3.1.1. Subject recruitment and eligibility criteria

Patients with T2DM were recruited from the Centre of Diabetes, Endocrinology and Cardiometabolism, the diabetes outpatient clinic at the Special Hospital Thalassotherapia Opatija, Croatia, between November 2020 and October 2023. The diagnosis of T2DM was made according to the latest ADA guidelines [6]. Based on medical history, clinical examination and initial laboratory evaluation, exclusion criteria were defined to omit patients with the presence of the following immune-modulating factors (**Table 1**, **Figure 3**): diagnosis of T1DM or latent autoimmune diabetes in adults (based on decreased C-peptide levels or positive autoantibodies to glutamic acid decarboxylase or islet antigen 2), acute infection or recent vaccination (up to 4 weeks prior), recurrent urinary tract infection, chronic inflammatory disease, active malignant disease, disorders with an impact on the hypothalamus-pituitary-adrenal (HPA) axis, anti-inflammatory or immunosuppressive medication use, advanced liver or kidney disease. Patients taking insulin or more than two oral antidiabetic drugs were not included in the study. Participants who fulfilled all exclusion criteria but did not meet the criteria for diabetes diagnosis [6] were included in the control group (n=30) (**Figure 3**). All participants were over 18 years of age and signed informed consent before inclusion. In a subcohort of the Diabetes group, patients eligible for antidiabetic treatment modification including SGLT-2i and/or GLP-1RA as add-on therapy to metformin were followed-up for another 6-9 months. If they reached the target HbA_{1c} value of <7.0% after 3 months of modified antidiabetic treatment, blood collection was repeated at the 6-month time point. If the glycaemic target was not reached after 3 months, the treatment regimen or dosing were adjusted and the follow-up period was prolonged to 9 months according to the study diagram (**Figure 4**). If the glycaemic target was not reached 6 months after the antidiabetic treatment modification, if premature treatment discontinuation due to adverse effects occurred or if the patient was lost to follow-up, then the blood collection for flow cytometry analysis was not repeated at the 6-month time point.

Table 1. Inclusion and exclusion criteria of the study

Inclusion criteria	Exclusion criteria
1. Male and female gender	1. Patients with chronic inflammatory diseases (e.g. RA, IBD, COPD)
2. Age 18-79 years	2. Patients with latent autoimmune diabetes in adults
3. Clinical diagnosis of type 2 diabetes mellitus according to American Diabetes Association guidelines [6]	3. Patients taking anti-inflammatory or immunosuppressive drugs (e.g. corticosteroids)
	4. Patients with advanced liver disease (clinically evident signs of liver cirrhosis or LSM >12 kPa)
4. $HbA_{1c} \geq 7.0\%$ on current therapy	5. Patients with chronic kidney disease of stages G3b and worse, defined as estimated glomerular filtration rate <45 ml/min per $1.73m^2$
	6. Patients with acute infection/vaccinated in the past month
	7. Patients with C reactive protein >10 mg/l
	8. Patients with recurrent urinary tract infection
	9. Patients with active malignant disease
	10. Patients with diseases that impact the HPA-axis (e.g. Addison's disease, Cushing syndrome, etc.)
	11. Patients taking insulin or more than two oral antidiabetic drugs

COPD, Chronic obstructive pulmonary disease; HbA_{1c}, Haemoglobin A_{1c}; HPA, Hypothalamus-pituitary-adrenal axis; IBD, Inflammatory bowel disease; LSM, Liver stiffness measure; RA, Rheumatoid arthritis.

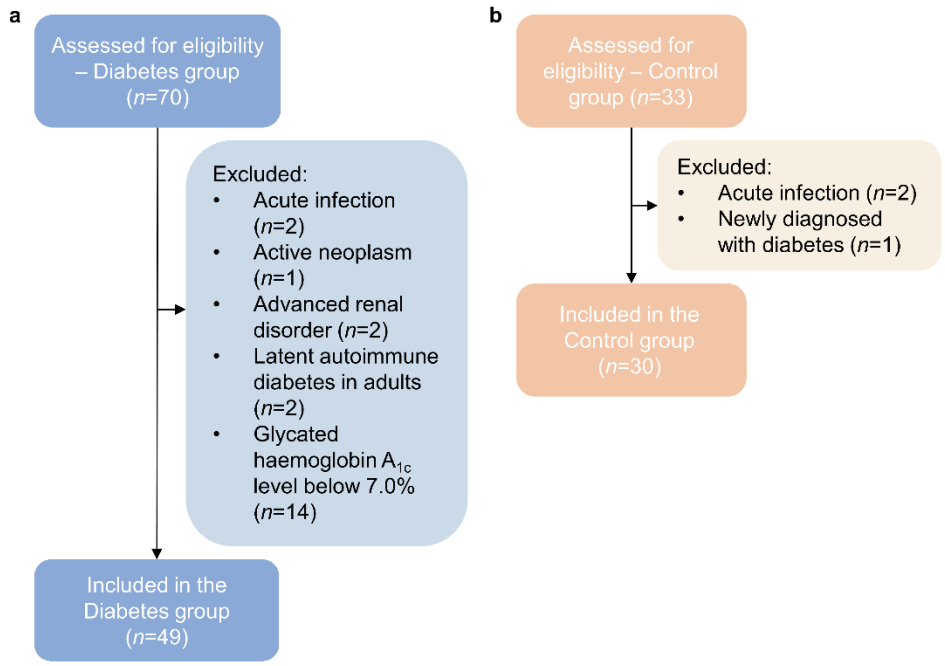

Figure 3. Flowchart of the study participants

Two groups of participants were enrolled in the study after initial evaluation: (a) a group of participants with type 2 diabetes mellitus poorly controlled on current treatment (Diabetes group), and (b) a group of nondiabetic participants (Control group).

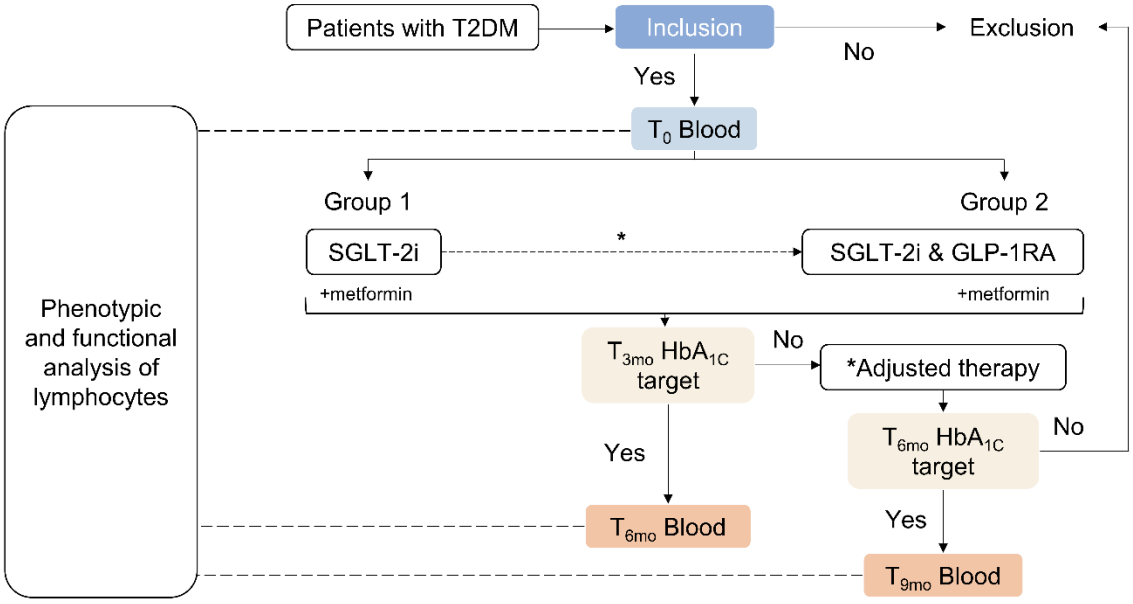

Figure 4. Design of the follow-up part of the study

GLP-1RA, glucagon-like peptide-1 receptor agonists; HbA_{1c}, haemoglobin A_{1c}; mo, month; NA, not applicable; SGLT-2i, sodium-glucose co-transporter-2 inhibitors; T2DM, type 2 diabetes mellitus.

3.1.2. Ethical considerations

The ethics committee of the Institutional Review Board at Thalassotherapia Opatija approved this research under number 01-000-00-503/2-2020. We conducted the research in accordance and agreement with the International Conference on Harmonization Good Clinical Practice Guidelines and with the Declaration of Helsinki.

3.2. Materials

3.2.1. Buffers and cell culture mediums

Roswell park memorial institute medium (RPMI) 1640

RPMI medium (PAN-Biotech GmbH, Aidenbach, Germany), 3-10% Foetal calf serum (FCS; PAN-Biotech GmbH, Aidenbach, Germany), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.2; PAN-Biotech GmbH, Aidenbach, Germany), 2 mM L-glutamine (PAN-Biotech GmbH, Aidenbach, Germany), 10^5 U/l Penicillin (PAN-Biotech GmbH, Aidenbach, Germany), 0.1 g/l Streptomycin (PAN-Biotech GmbH, Aidenbach, Germany), β-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri, United States).

Cell culture freezing medium

70% RPMI medium (Pan Biotech), 20% FCS (Pan Biotech), 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Missouri, United States).

Fluorescence-Activated Cell Sorting (FACS) medium

Phosphate-buffered saline (PBS), 1% Bovine serum albumin (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 0.1% sodium azide (NaN_3) (Sigma-Aldrich, St. Louis, Missouri, United States), 1 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, Missouri, United States).

3.2.2. Antibodies, chemicals and recombinant proteins

Antibodies were purchased from suppliers as indicated in **Table 2**.

Table 2. List of antibodies used in experiments

Antibody (clone)	Source	Identifier
Anti-human CD3 monoclonal antibody Alexa Fluor™ 700 (OKT3)	eBioScience, Inc., San Diego, California, United States	56-0037-42
Anti-human CD8a monoclonal antibody APC-eFluor™ 780 (OKT8)		47-0086-42
Fixable Viability Dye eFluor™ 506		65-0866-14
Anti-human CD45RA monoclonal antibody SB645 (HI100)		64-0458-42
Anti-human CD45RO monoclonal antibody PE-eFluor™ 610 (UCHL1)		61-0457-42
Anti-human CD27 monoclonal antibody FITC (LG.7F9)		11-0271-82
Anti-human CD57 monoclonal antibody PE-Cyanine7 (TB01)		25-0577-42
Anti-human CD28 monoclonal antibody PE (CD28.2)		12-0289-42
Anti-human IFN-γ monoclonal antibody FITC (4S.B3)		11-7319-82
Anti-human TNF-α monoclonal antibody PE-Cyanine7 (MAb11)		25-7349-82
Anti-human IL-2 monoclonal antibody APC (MQ1-17H12)		17-7029-82
Anti-human Granzyme B monoclonal antibody PE (N4TL33)		12-8896-42
Anti-human Granzyme B monoclonal antibody eFluor™ 450 (N4TL33)		48-8896-42
Anti-human CD16 monoclonal antibody APC-eFluor™ 780 (CB16)		47-0168-42
Anti-human CD56 monoclonal antibody PE (NCAM)		12-0567-42
Anti-human CD337 monoclonal antibody eFluor™ 450 (AF29-4D12)		48-3379-42
Anti-human CD314 monoclonal antibody PerCP-eFluor™ 710 (1D11)		46-5878-42
Anti-human Perforin monoclonal antibody APC (dG9)		17-9994-42
Anti-human CD69 monoclonal antibody SB780 (FN50)		78-0699-42
Anti-human TCRα/β monoclonal antibody PE-eFluor™ 610 (IP26)		61-9986-42
Anti-human CX3CR1 monoclonal antibody APC (2A9-1)		17-6099-42
Anti-human IL-17A monoclonal antibody APC (eBio64DEC17)		17-7179-42
Anti-human KIR2D monoclonal antibody FITC (NKVFS1)	Miltenyi Biotec, Bergisch Gladbach, Germany	130-092-687
Anti-human NKG2A monoclonal antibody APC (REA110)		130-113-563
Anti-human NKG2C monoclonal antibody PE-Vio®615 (REA205)		130-123-037
Anti-human TCR Vδ1 monoclonal antibody PerCP-Vio®770 (REA173)		130-120-440
Anti-human TCR Vδ2 monoclonal antibody PE (REA771)		130-111-010
Anti-human CD197 monoclonal antibody Brilliant Violet™ 421 (G043H7)	BioLegend, San Diego, California, United States	353208

Clones indicated in parentheses. APC, allophycocyanin; CX3CR1, CX3C motif chemokine receptor 1; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; KIR, killer-cell immunoglobulin-like receptor; NKG2A, natural killer group 2 member A; NKG2C, natural killer cell group 2 isoform C; PE, phycoerythrin; PerCP, peridinin chlorophyll-A protein; SB, super bright; TCR, T cell receptor; TNF, tumour necrosis factor.

3.3. Methods

3.3.1. Laboratory evaluation

After peripheral blood collection was performed, laboratory parameters were measured in the diagnostic laboratory of Thalassotherapia Opatija and Clinical Hospital Centre Rijeka as a part of their standard panels (**Table 3**). Immunochemical parameters were measured using commercially available assays (Roche Diagnostics GmBH, Basel, Switzerland) on the Cobas® 8000 analyser series cobas® e801 analytical unit (Roche Diagnostics International, Ltd, Rotkreuz, Switzerland). Complete blood count was determined by an automated haematology analyser. Standard urinalysis was supplemented by protein content measurement.

Insulin indices were calculated from laboratory parameters. To assess fasting plasma insulin (FPI) relative to FPG levels, Homeostatic model assessment of insulin resistance (HOMA-IR) and β-cell function (HOMA-β), as well as Quantitative insulin-sensitivity check index (QUICKI) were determined.

$$\text{HOMA-IR} = \frac{\text{FPI} \times \text{FPG}}{22.5} \qquad \text{HOMA-}\beta = \frac{20 \times \text{FPI}}{\text{FPG} - 3.5} \qquad \text{QUICKI} = \frac{1}{\log \text{FPI} + \log \text{FPG}}$$

Table 3. List of analysed blood parameters

Blood parameters	Blood tube type
• Inflammatory marker: C reactive protein • Renal panel: urea, creatinine • Liver panel: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamic carboxylase • Lipid panel: low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total cholesterol, triglycerides • Metabolic panel: fasting plasma glucose, insulin, C-peptide • Serology: glutamic acid decarboxylase autoantibodies, islet antigen-2 autoantibodies	BD Vacutainer® Serum Separation Tubes
• Complete blood count • Glycated haemoglobin A_{1c}	BD Vacutainer® K_2EDTA Tubes

K_2EDTA, dipotassium ethylenediaminetetraacetic acid.

3.3.2. Anthropometric evaluation

Weight and height were measured using a high-precision digital column scale with a stadiometer (Seca, Hamburg, Germany). Anthropometric parameters, including BMI, body fat and skeletal muscle mass, were measured by Tanita MC 780-P MA scale (Tanita Corp.) based on bioelectrical impedance analysis technology at Thalassotherapia Opatija. A standard cloth tape measure was used to record waist and hip circumference in cm.

3.3.3. Immune cell isolation protocols

3.3.3.1. Isolation of peripheral blood mononuclear cells

Peripheral venous blood was collected between 7 and 9 a.m. into EDTA tubes from individuals who previously fasted for at least 10 hours. Sulfonylurea derivatives, dipeptidyl-peptidase-4 inhibitors and metformin were omitted 24–48h before the blood collection. PBMCs were isolated from full blood by density gradient centrifugation using Histopaque 1077 Density Gradient Medium (Sigma-Aldrich, St. Louis, Missouri, United States) or Lymphoprep (ProteoGenix, Schiltigheim, France) within 6-8 hours of blood collection. The blood was transferred to a 50-ml conical centrifuge tube containing PBS in a 1:1 ratio. Blood diluted with PBS was then divided into half and each half is carefully layered onto 15 ml of Histopaque®-1077 in two 50-ml conical centrifuge tubes per study participant. The tubes were then centrifuged at 400×g (~2100 revolutions per minute, rpm) for 30 minutes at room temperature. After centrifugation, the buffy coat containing PBMCs was transferred into a clean 50-ml conical centrifuge tube containing 30 ml of PBS in a 1:3 ratio. The suspension was centrifuged at 300×g (~1500 rpm) for 15 minutes at 4°C, after which the supernatant was discarded and the pellet resuspended in 30 ml of PBS. Then, the suspension was centrifuged at 100×g (~700 rpm) for 10 minutes at 4°C, after which the supernatant was discarded and the pellet was resuspended in 4 ml of the freezing medium, consisting of FCS (Corning, Inc., Corning, New York, United States) and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Missouri, United States), and 3 vials per study participant were cryopreserved at -80°C and later transferred to liquid nitrogen until use.

3.3.4. Cell counting

The number of cells per millilitre of the cell suspension was determined by Corning® Cell Counter (CytoSMART Technologies, Eindhoven, Netherlands) automated cell counter. Only live cells were counted with the use of trypan blue, vital stain that selectively colours dead cells blue. Fifteen microliters of cell

suspension were mixed with 15 µl trypan blue (1:1) and 10 µl of this mix was applied to the slide. The software would make an approximate cell count per millilitre of the cell suspension based on three to five visual fields.

3.3.5. Flow cytometry

The results of phenotypic and functional cell assays were obtained by flow cytometry using a FACSVerse (BD, Franklin Lakes, New Jersey, United States) or FACSAria IIu (BD, Franklin Lakes, New Jersey, United States) and analysed using FlowJo software (FlowJo LLC, Ashland, Oregon, United States).

3.3.5.1. Cell surface staining

Phenotypic lymphocyte analysis was performed by fluorophore-labelled antibodies that are specific to certain cellular markers. For flow cytometry, the single cell suspension of PBMCs was prepared according to the protocols described above. For the analysis of surface markers, labelled antibodies were diluted in FACS medium (40 µl) containing specific antibodies. After 30 min of incubation at +4°C and in the dark, the cells were washed with FACS medium, centrifuged for 5 minutes at 300×g and resuspended in 50 µl of intracellular fixation buffer (eBioScience, Inc., San Diego, California, United States). After 30 minutes of incubation at room temperature, the cells were washed with 100X permeabilisation buffer (eBioScience, Inc., San Diego, California, United States), centrifuged for 5 minutes at 300×g and resuspended in 120 µl of 100X permeabilisation buffer (eBioScience, Inc., San Diego, California, United States). The samples were stored up to seven days at +4°C and in the dark until flow cytometry analysis was performed.

3.3.3.2. Cytoplasmic intracellular cell staining

For cytoplasmic intracellular staining (IFN-γ, TNF-α, IL-2, IL-17A, Granzyme B; eBioscience, Inc., San Diego, California, United States), a kit for fixation and permeabilisation of cells was used according to the manufacturers' instructions (eBioscience, Inc., San Diego, California, United States). After cell surface staining, the cells were first fixed for 30 min at room temperature with Foxp3 fixation and permeabilisation mix (Concentrate to Diluent 1:3 ratio) and then washed in 100X permeabilisation buffer (eBioscience, Inc., San Diego, California, United States). After centrifugation, cells were incubated in 40 µl of antibody mix for 30 minutes at +4°C. Cells were then washed, centrifuged at 300×g for 5 minutes and resuspended in FACS buffer for flow cytometry analysis on the same day.

3.3.3.3. Carboxyfluorescein succinimidyl ester labelling

Carboxyfluorescein succinimidyl ester (CFSE) labelling was performed according to the manufacturer's protocol. Cells that were in the medium with FCS were washed three times with PBS to remove proteins from the medium. Cells were resuspended in PBS and the volume was adjusted in order to achieve concentration of maximally 10^7 cells/ml. Cells were mixed with CFSE (Sigma-Aldrich, St. Louis, Missouri, United States) diluted in PBS in ratio 1:1 resulting with the final concentration of 2.5 μmol/l CFSE. Cells with CFSE were incubated for 10 min at 37°C in dark after which they were washed 2x with 1 ml of 10% RPMI.

3.3.3.4. Viability dye

Viability dye was used at a dilution factor of 1:150 in FACS medium together with cell surface staining antibodies. Cells were stained for 30 minutes at +4°C. eBioscience™ Fixable Viability Dye eFluor™ 506 was used for all stainings.

3.3.6. *In vitro* stimulations

PBMCs were cultured in RPMI 1640 medium (PAN-Biotech), supplemented with 10% FCS (PAN-Biotech) and 2-ME (Sigma-Aldrich, St. Louis, Missouri, United States). For cytokine production analysis after *in vitro* stimulation, one million cells per well in Cellstar® U-bottom 96 well plate (Greiner Bio-One, Kremsmünster, Austria) were stimulated for 4h with 20 ng/ml Phorbol myristate acetate (PMA, Sigma-Aldrich, St. Louis, Missouri, United States) and 1 ng/ml Ionomycin (Sigma-Aldrich, St. Louis, Missouri, United States) in the presence of Brefeldin A (eBioscience, Inc., San Diego, California, United States). For proliferation analysis after *in vitro* stimulation, 500,000 cells per well in Cellstar® U-bottom 96 well plate (Greiner Bio-One, Kremsmünster, Austria) were stimulated for 72 and 96h with T Cell Activation and Expansion Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturers' instructions and 120h with 100 ng/ml recombinant human IL-15 (PeproTech, Inc., Rocky Hill, Connecticut, United States).

3.3.7. Cytokine array

A bead-based immunoassay LEGENDplex™ Human Anti-Virus Response Panel (13-plex) with V-bottom Plate (BioLegend, San Diego, California, United States) was used to determine blood serum concentration of cytokines according to manufacturers' instructions. Data analysis was performed using Qognit software (Qognit Inc., San Carlos, California, United States).

3.3.8. Quantification and statistical analysis

Unless otherwise noted, data are presented as mean ± standard deviation (SD) or standard error of mean (SEM). For qualitative variables, Fisher or χ^2 test were used to determine the statistical significance of differences between study (sub)groups. Depending on the quantitative data distribution, statistical significance of differences between two independent groups was determined either by unpaired t-test or Mann-Whitney test, while paired t-test or Wilcoxon test were used for repeated measures. Spearman correlation analysis and regression model analysis were used to determine the nature of associations between clinical and immunological parameters in each group of study participants. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, La Jolla, California, United States). Statistically significant differences were considered with *p* values of <0.050 (*p<0.050, **p<0.010 and ***p<0.001).

4. RESULTS

4.1. Baseline characteristics of study participants

This study was conducted to investigate how T2DM affects the immune system. To accomplish this, two groups of patients were carefully selected, with similar overall characteristics but differing in their T2DM status. Special attention was given to ensuring that both groups had low complication rates to eliminate the influence of tissue inflammation on the immune system. The study included 49 patients with T2DM, diagnosed in accordance with ADA guidelines [6], and 30 individuals who tested negative for T2DM and had no prior history of the condition. These two groups were referred to as the Diabetes group and the Control group, respectively (**Figure 3**). To further refine the Diabetes group, three specific exclusion criteria were applied: (1) HbA_{1c} levels below 7.0%, (2) patient-reported instances of high glucovariability or hypoglycaemic episodes within the past 6 months, and (3) a positive screening result for latent autoimmune diabetes of adults, as determined by C-peptide levels and titres of islet cell autoantibodies (**Figure 3**). Additionally, to eliminate potential factors that could impact the immune system, participants with active infections, neoplasms, or advanced renal disorders (defined by an estimated glomerular filtration rate or eGFR <45 ml/min per 1.73 m^2) were excluded (**Figure 3**). Furthermore, blood collection was postponed for 4-6 weeks for patients with a recent history of infection or vaccination.

The demographic composition of the study groups was similar (**Table 4**). **Table 4** also provides a comparison of other baseline characteristics. In the Diabetes group, the mean HbA_{1c} level was 8.4% (**Table 4**). Approximately one-third of the participants had been diagnosed with T2DM within the two years preceding the study, while the remainder had an average duration of 10 years with the condition (**Table 5**). Non-drug-naïve patients had been on antidiabetic treatment regimens outlined in **Table 5** for a minimum of 3 months but discontinued them 24–48 hours prior to blood collection.

Table 4. Baseline characteristics of study participants

	Control (*n*=30)	Diabetes (*n*=49)	*p* value
Age, years	58±13 (53–68)	61±10 (57–68)	0.143
Female sex, *n* (%)	12 (40.00)	21 (42.86)	0.819
Glycated haemoglobin A_{1c}, %	5.4±0.4	8.4±1.1	<0.001
Fasting plasma glucose, mmol/l	5.6±0.6	9.6±2.1	<0.001
HOMA-insulin resistance	3.00±1.88	8.80±7.00	<0.001
HOMA-beta cell function	11.65±5.26	7.47±5.69	0.002
Quantitative insulin sensitivity check index	0.34±0.03	0.29±0.02	<0.001
Body mass index, kg/m^2	27.1±4.0	31.2±4.2	<0.001
Body fat, %	28.1±8.4	31.9±7.9	0.093
Skeletal muscle index, kg/m^2	8.0±1.0	8.8±1.2	0.014
Waist-hip ratio	0.90±0.09	0.97±0.07	<0.001
Leukocyte count, $\times 10^9$ per liter	6.4±1.7	7. 6±2.2	0.012
C reactive protein, mg/l	2.5±2.5	3.3±2.5	0.157
Triglycerides, mmol/l	1.2±0.6	1.9±1.2	0.004
HDL cholesterol, mmol/l	1.6±0.5	1.2±0.3	<0.001
LDL cholesterol, mmol/l	3.1±1.2	2.9±1.0	0.430
Total cholesterol, mmol/l	5.1±1.5	4.6±1.2	0.143
Statin use, *n* (%)	10 (33.33)	22 (44.90)	0.352
Aspartate aminotransferase, U/l	27±6	32±18	0.130
Alanine aminotransferase, U/l	27±7	42±29	0.007
Hepatic steatosis index	38.1±5.1	44.7±5.9	<0.001
Triglyceride-glucose index	4.59±0.25	5.06±0.29	<0.001
NAFLD-liver fat score	1.1±1.0	2.8±2.7	0.002
Prior/current smoking, *n* (%)	15 (50.00)	20 (40.82)	0.488
Limited physical activity, *n* (%)[a]	13 (43.33)	26 (53.06)	0.489

Data shown as mean ± SD unless otherwise specified. Age is reported as mean ± SD (interquartile range). Unpaired t-test and Fisher exact test were used to determine the statistical significance at *p* value <0.050. HDL, high-density lipoprotein; HOMA, homeostatic model assessment; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease.

[a] According to the New York Heart Association functional classification [104].

Table 5. Diabetes duration, complication rate and treatment of the Diabetes Group at inclusion

	Diabetes (*n*=49)
Short-term, *n* (%)	16 (32.65)
Long-term, *n* (%)	33 (67.35)
Disease duration, years	10±6 (6–10)
Retinopathy, *n* (%)	2 (4.08)
Polyneuropathy, *n* (%)	8 (16.33)
Nephropathy, *n* (%) [a]	
G3a	2 (6.67)
A2	10 (20.41)
Coronary heart disease, *n* (%)	11 (22.45)
Peripheral arterial disease, *n* (%)	2 (4.08)
Drug-naïve, *n* (%)	5 (10.20)
Metformin, *n* (%)	43 (87.76)
Sulfonylurea, *n* (%)	7 (14.29)
Pioglitazone, *n* (%)	4 (8.16)
Repaglinide, *n* (%)	1 (2.04)
Dipeptidyl-peptidase 4 inhibitor, *n* (%)	16 (32.65)
Sodium-glucose co-transporter 2 inhibitor, n (%)	2 (4.08)
Liraglutide, n (%)	1 (2.04)

Diabetes duration is reported as mean ± SD (interquartile range), other as indicated. [a] According to Kidney Disease Improving Global Outcomes classification based on estimated glomerular filtration rate and albuminuria [105].

4.2. Type 2 diabetes does not impact the distribution of the leukocyte compartment

In previous research, when comparing individuals with T2DM to healthy controls and using less strict criteria for excluding inflammatory conditions, variations in their overall haematological profiles have been reported [106]. As a result, we conducted an initial assessment of patients with T2DM and control subjects, which involved routine analysis of blood samples and the measurement of C reactive protein (CRP), as outlined in **Table 4**. All participants exhibited CRP levels below 10 mg/l, and no discernible distinctions were noted between the two study groups. We did observe a minor elevation in the white blood cell count among patients with T2DM when compared to the control subjects. However, a more detailed analysis of the blood count revealed a proportional increase in all types of blood cells, with a similar relative distribution within the white blood cell compartment (**Figure 5**). The cellular component of the response to chronic metabolic dysregulation characterised by the recruitment of leukocytes to the circulation is closely associated with alterations in the humoral aspect of the response.

Studies indicate that elevated serum concentrations of pro-inflammatory cytokines are linked to the risk of developing T2DM and its chronic complications [107]. Nevertheless, a few studies have reported that serum cytokine levels remained similar in both healthy controls and diabetes patients, irrespective of their glycaemic control or the presence of complications [108]. In our study, serum levels of all cytokines were in the lower part of the detection range based on cytokine array results and no significant differences were detected between study groups due to stochastic effects (**Table 6**).

Table 6. Serum concentration of cytokines in a subcohort of patients with type 2 diabetes and control subjects.

	Control (*n*=9)		**Diabetes (*n*=21)**		
Cytokine, unit	**Median**	**SEM**	**Median**	**SEM**	***p* value**
IL-1β, pg/ml	5.827	3.494	4.262	1.195	0.144
IL-6, pg/ml	3.403	1.710	1.658	0.333	0.443
IL-8, pg/ml	12.880	3.445	15.070	10.330	0.593
IL-10, pg/ml	2.791	0.922	1.633	0.221	0.161
IL-12p70, pg/ml	1.225	0.856	0.567	0.249	0.199
TNF-α, pg/ml	14.720	4.818	4.185	1.259	0.138
IP-10, pg/ml	10.860	6.856	19.380	11.580	0.089
IFN-λ1, pg/ml	30.700	5.187	32.820	13.140	0.567
IFN-λ2/3, pg/ml	27.130	10.76	27.900	13.150	0.803
IFN-α2, pg/ml	3.531	1.180	1.695	0.568	0.150
IFN-β, pg/ml	5.082	1.549	5.027	2.416	0.535
IFN-γ, pg/ml	23.080	8.446	13.210	2.447	0.227
GM-CSF, pg/ml	5.801	2.176	2.706	0.797	0.262

A bead-based cytokine array was used to measure the concentration of 13 cytokines. Mann-Whitney test was used to determine the statistical significance at p value <0.050. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon gamma-induced protein 10; TNF, tumour necrosis factor.

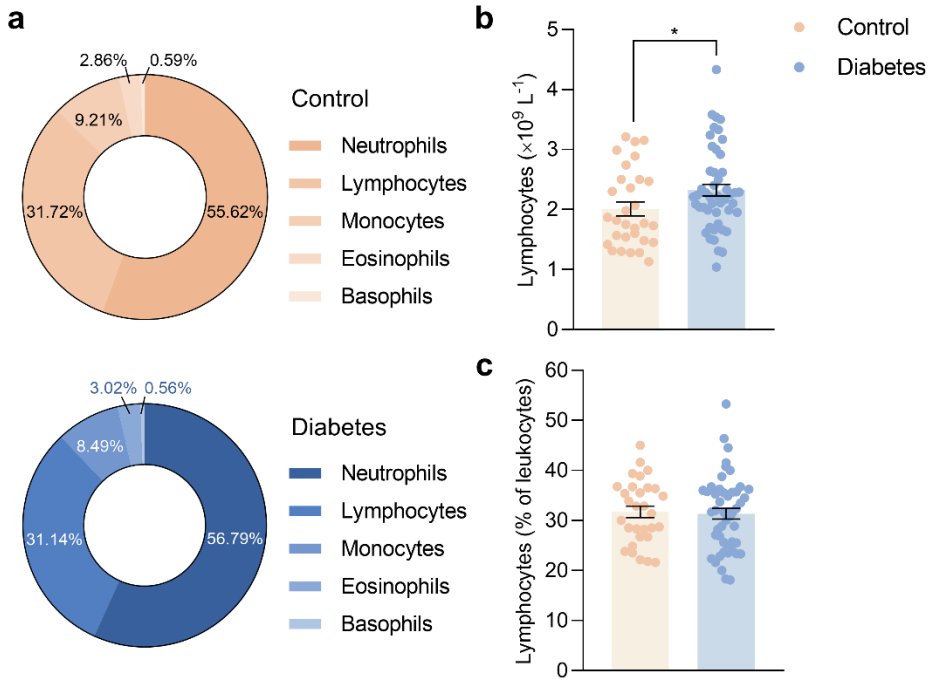

Figure 5. Blood lymphocytes of type 2 diabetes patients and control subjects show a similar distribution of subsets

Peripheral blood was collected from control subjects and patients with type 2 diabetes mellitus (T2DM). (a-c) Differential blood count was reported for both groups. (a) Representative distribution of mean relative numbers of white blood cell subtypes. (b) Absolute and (c) relative number of lymphocytes in patients with T2DM (*n*=49) in comparison to control subjects (*n*=30). An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at *p<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test.

4.3. Tumour necrosis factor-alpha production by CD8 T cells is increased in diabetes

Cytotoxic lymphocytes are specialised in targeting and eliminating non-immune cells that are under stress due to factors such as infection or oncogenic transformation. Previous *in vitro* studies have suggested that when cytotoxic lymphocytes are cultured in the presence of elevated glucose concentrations, their cytotoxic potential increases [109]. Based on this, we formulated a hypothesis that cytotoxic lymphocytes in patients with T2DM would exhibit a more responsive and pro-inflammatory profile compared to those in the control group. In humans, cytotoxic lymphocytes are predominantly composed of $CD8^+$ T cells, NK cells and γδ T cells. Therefore, our initial investigation focused on assessing the impact of T2DM on the phenotype and functionality of $CD8^+$ T cells. We found no significant difference in the proportion of $CD8^+$ T cells within the total lymphocyte population between the two study groups (**Figure 6a**). A more detailed analysis of $CD8^+$ T cell subsets in patients with T2DM revealed only a minor reduction in the percentage of

effector CD45RA$^-$ CD8$^+$ T cells, along with a slight, though statistically non-significant, increase in CD45RA (T_{EMRA}) cells (**Figure 6b-e**). Additionally, the percentage of participants with CD57$^+$ CD8$^+$ T cells and CD57$^+$ effector CD45RA$^-$ CD8$^+$ T cells, which are typically associated with cytomegalovirus infection [110], did not exhibit notable differences between the Diabetes and Control groups (**Figure 6f,g**). This suggests that individuals with T2DM have a similar history of viral infections when compared to the Control group.

To determine if the ability of CD8$^+$ T cells to undergo cell division is affected by diabetes, we exposed PBMCs labelled with CFSE to stimulation beads (anti-CD3/anti-CD28/anti-CD2) for a duration of 72 hours. We quantified the percentage of CD8$^+$ T cells that had undergone at least one round of cell division by assessing the fluorescence intensity of CFSE (**Figure 6h**). In our analysis, we found no discernible difference in the percentage of CD8$^+$ T cells undergoing proliferation upon *in vitro* stimulation when comparing patients with T2DM to control subjects (**Figure 6i**).

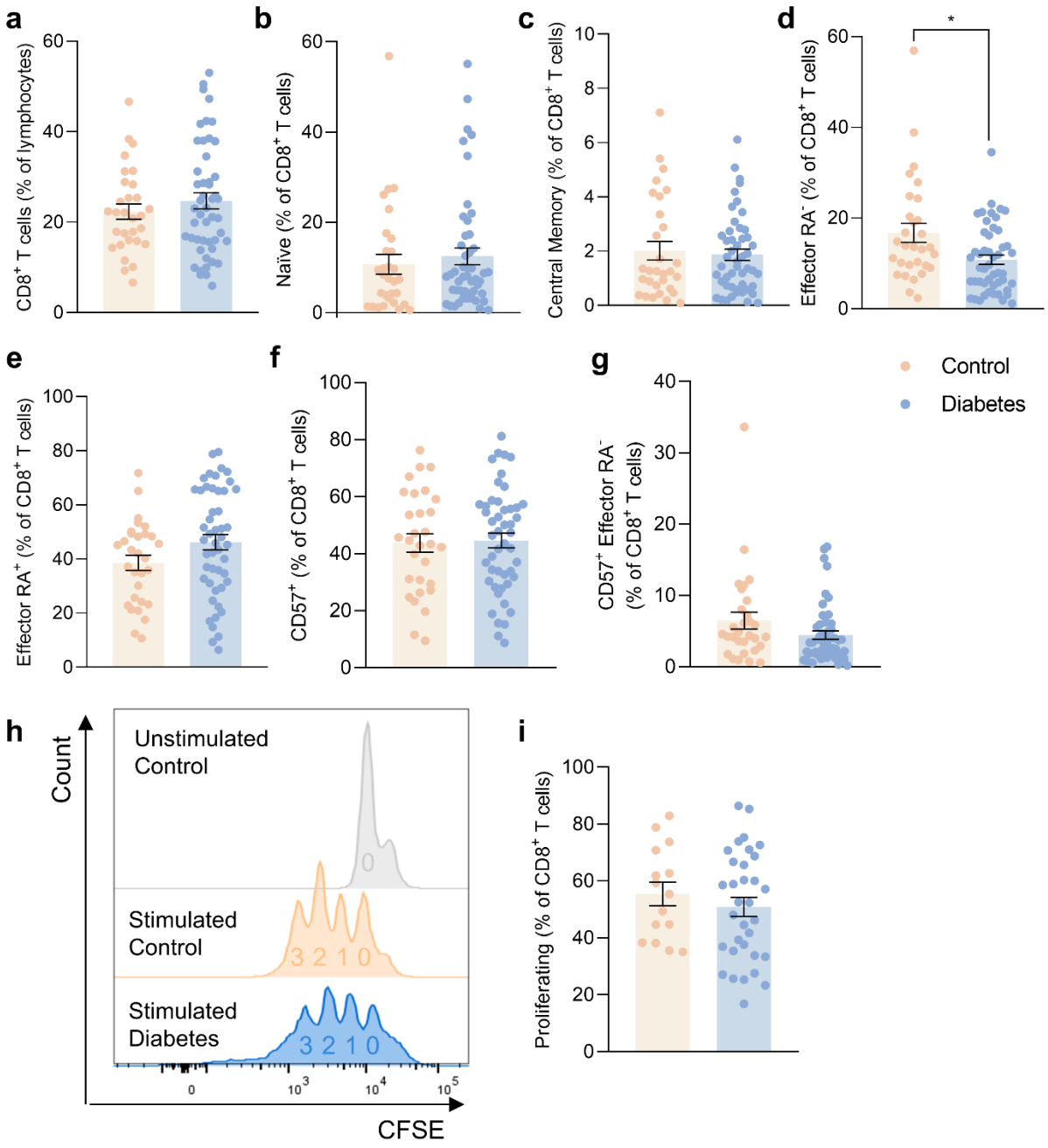

Figure 6. Type 2 diabetes is associated with minor changes in the phenotype and proliferative capacity of CD8 T cells

Peripheral blood mononuclear cells were isolated from control subjects (*n*=30) and patients with type 2 diabetes (T2DM, *n*=49). (a-g) Flow cytometry was used to analyse $CD8^+$ T cells and their subsets. Within $CD3^+CD8^+$ T cells, C-C chemokine receptor type 7 and CD45RO were used to determine naïve (Quartile 1, Q1) and central memory (Q2) $CD8^+$ T cells. In Q3 and Q4, an additional gate to determine effector $CD45RA^-$ (RA^-) and $CD45RA^+$ (RA^+, TEMRA) was used, respectively. Frequency of cell subsets based on the surface expression of CD57 on (f) $CD8^+$ T cells and (g) a subset of effector $CD45RA^-$ $CD8^+$ T cells. (h,i) Cells labelled with carboxyfluorescein succinimidyl ester (CFSE) were incubated with anti-CD3/anti-CD28/anti-

CD2 stimulation beads for 72h. (h) Representative histograms of CFSE-labelled cells, gated for live $CD3^+CD8^+$ cells. (i) Quantification of proliferating $CD8^+$ T cells in a subcohort of patients with T2DM (*n*=33) compared to control subjects (*n*=15). All the experiments were performed once unless otherwise indicated. An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at *p<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test, unless otherwise specified.

Next, we conducted an assessment of the functionality of $CD8^+$ T cells in patients with T2DM and control individuals using direct *ex vivo* stimulation assays. To investigate cytokine production by $CD8^+$ T cells, we employed intracellular flow cytometry after exposing the cells to 4 hours of standard PMA/Ionomycin stimulation in the presence of Brefeldin A (**Figure 7a-i**). Our findings revealed a noteworthy increase in the production of TNF-α by $CD8^+$ T cells in patients with T2DM when compared to the control group (**Figure 7b,e**). This trend was consistent for all $CD8^+$ T cell subsets (**Figure 7f-i**) and reached statistical significance within the naïve and effector $CD45RA^-$ $CD8^+$ T cell populations.

We formulated a hypothesis suggesting that elevated blood sugar levels in patients with T2DM might be responsible for triggering increased responsiveness in $CD8^+$ T cells. To directly explore this, we performed an *in vitro* experiment. PBMCs from control subjects were exposed to glucose concentrations representative of both physiological and hyperglycaemic conditions during a 4-hour *in vitro* stimulation. Subsequently, we measured the production of TNF-α by $CD8^+$ T cells using flow cytometry. Different glucose concentrations did not adversely affect the cell viability during the course of experiment (**Figure 7j**). What we observed was that an increase in the medium glucose concentration, shifting from 5 to 11 mmol/l, led to an increase in TNF-α production by $CD8^+$ T cells (**Figure 7k**).

Consequently, it seems that the phenotype and proliferative capacity of $CD8^+$ T cells remain relatively unaffected by T2DM, while there is a notable increase in their production of TNF-α. These findings suggest that elevated glucose levels may contribute to this phenomenon to some extent.

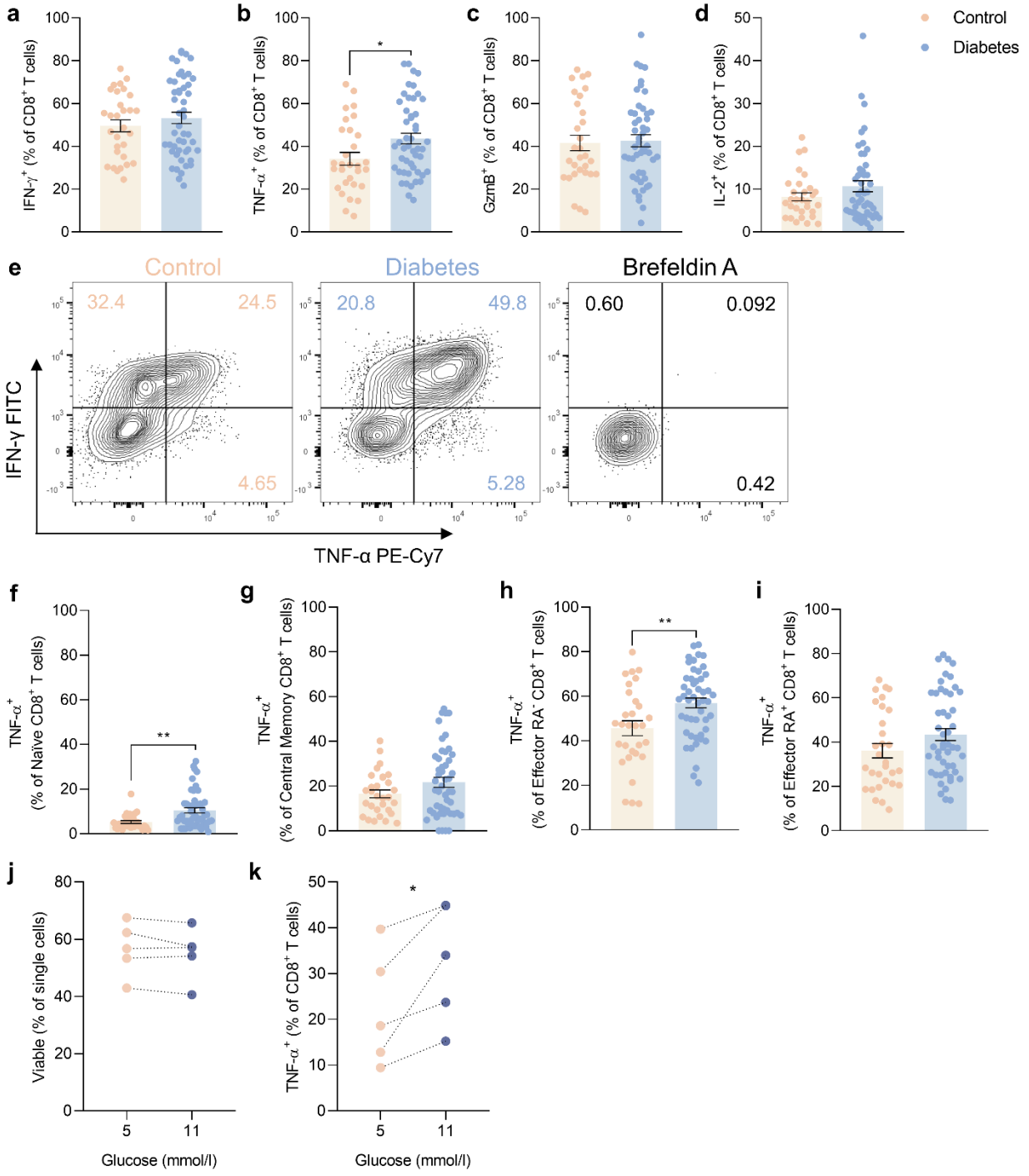

Figure 7. Type 2 diabetes is associated with increased tumour necrosis factor-alpha production by CD8 T cells

Peripheral blood mononuclear cells were isolated from control subjects (*n*=30) and patients with type 2 diabetes (T2DM, *n*=49). (a-i) Cells were stimulated with Phorbol myristate acetate (PMA)/Ionomycin in the presence of Brefeldin A for 4 hours and cytokine production was determined by intracellular flow cytometry

in $CD8^+$ T cells, and (a-d) cytokine production by $CD8^+$ T cells of patients with T2DM (*n*=49) in comparison to control subjects (*n*=30) was quantified. (e) Representative fluorescence-activated cell sorting plots of cells stained with interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), gated for live $CD3^+CD8^+$ cells. (f-i) Quantification of TNF-α production by $CD8^+$ T cell subsets of patients with T2DM (*n*=49) in comparison to control subjects (*n*=30). All the experiments were performed once. An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at $*p<0.050$, $**<0.010$ and $***<0.001$ by the Mann-Whitney test, unless otherwise specified. (j,k) Cells isolated from control subjects (*n*=5) were exposed to different concentrations of glucose during 4 hours of PMA/Ionomycin stimulation in the presence of Brefeldin A. (j) Cell viability and (k) TNF-α production was determined by flow cytometry and intracellular flow cytometry in $CD8^+$ T cells, respectively. The experiment was performed three times with similar results. Statistical significance at $*p<0.050$ was determined by paired t-test. GzmB, Granzyme B; IL, interleukin. An individual point represents one participant.

4.5. Type 2 diabetes is associated with hyperresponsive gamma delta T cells

To explore whether the increased responsiveness observed in cytotoxic cells is specific to $CD8^+$ T cells, we turned our attention to the influence of T2DM on the characteristics and function of peripheral blood gamma delta (γδ) T cells. These γδ T cells represent a relatively small subgroup of unconventional T cells that serve as a bridge between adaptive and innate immunity. Our analysis revealed no discernible distinctions in the frequency of the most common blood γδ T cell populations, which include $V\delta1^+$ and $V\delta2^+$ cells (**Figure 8a,b**), or in their respective subsets (**Figure 8c-f**).

Similar to our earlier assessment of $CD8^+$ T cells (**Figure 6h,i**), we examined the proliferative capacity of γδ T cells. This examination involved PBMCs labelled with CFSE and exposed to anti-CD3/anti-CD28/anti-CD2 stimulation beads for a duration of 96 hours. Notably, we found no significant differences in the percentage of proliferating $V\delta1^+$ and $V\delta2^+$ T cells when comparing patients with T2DM to the control group (**Figure 8g,h**).

In the subsequent functional analysis, we found that, much like $CD8^+$ T cells, $V\delta1^+\gamma\delta$ T cells in patients with T2DM exhibited higher levels of TNF-α production when compared to the control group. Furthermore, these cells showed elevated production of both IFN-γ and Granzyme B, whereas $V\delta2^+$ cells predominantly exhibited increased levels of Granzyme B production (**Figure 9a-i**). Interestingly, their production of IL-17A remained unaffected by diabetes (**Figure 9j,k**).

In summary, similar to $CD8^+$ T cells, γδ T cells in individuals with T2DM displayed a hyperresponsive cytokine profile, suggesting that they may contribute to the pro-inflammatory milieu often observed in these patients.

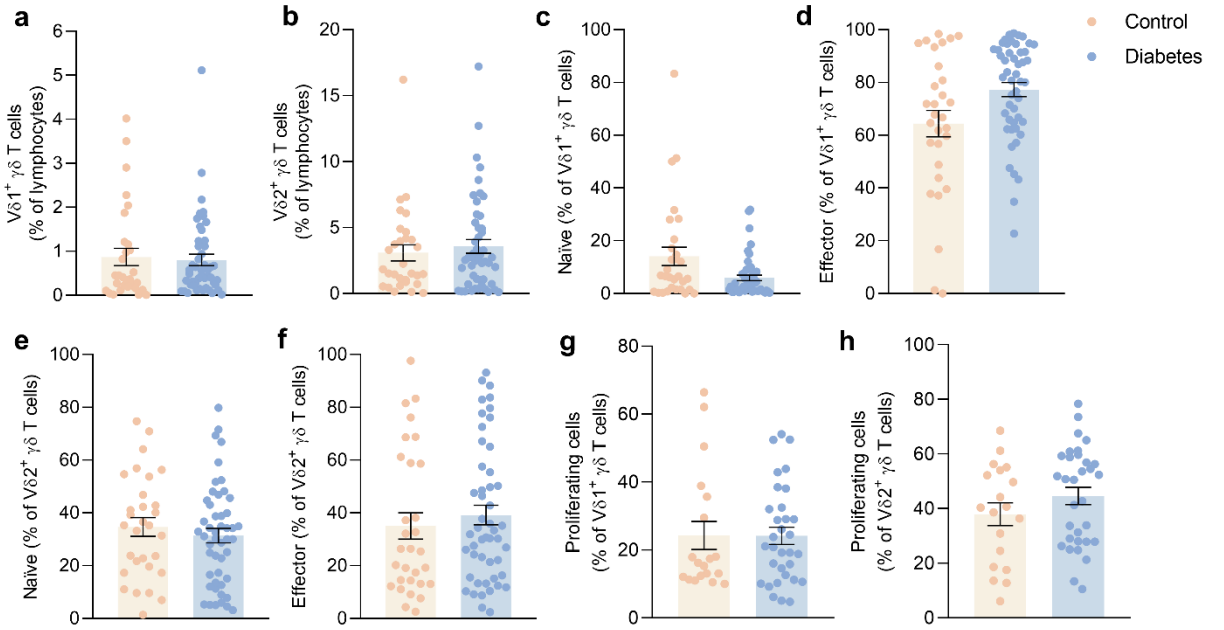

Figure 8. Gamma delta T cell phenotype and proliferative capacity are not affected by type 2 diabetes mellitus

Peripheral blood mononuclear cells were isolated from control subjects and patients with type 2 diabetes mellitus (T2DM). (a-f) Cells were analysed by flow cytometry. Quantification of (a) Vδ1$^+$ and (b) Vδ2$^+$ gamma delta (γδ) T cells and (c-f) their subsets, of patients with T2DM (*n*=49) in comparison to control subjects (*n*=30). Within cells expressing CD3 and Vδ1$^+$ or Vδ2$^+$ on their surface, CD27^{hi}CD45RA^{int}CD28$^+$CX3CR1$^-$ cells were characterised as naïve, whereas CD27^{lo}CD45RA^{hi}CD28$^-$ CX3CR1$^+$ cells were characterised as effector. (g,h) Cells labelled with carboxyfluorescein succinimidyl ester (CFSE) were incubated with anti-CD3/anti-CD28/anti-CD2 stimulation beads for 96 hours. Fluorescence intensity of CFSE was measured by flow cytometry and used to quantify the frequency of proliferating Vδ1$^+$ and Vδ2$^+$ γδ T cells in a subcohort of patients with T2DM (*n*=32) compared to control subjects (*n*=19). All the experiments were performed once. An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at *p<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test.

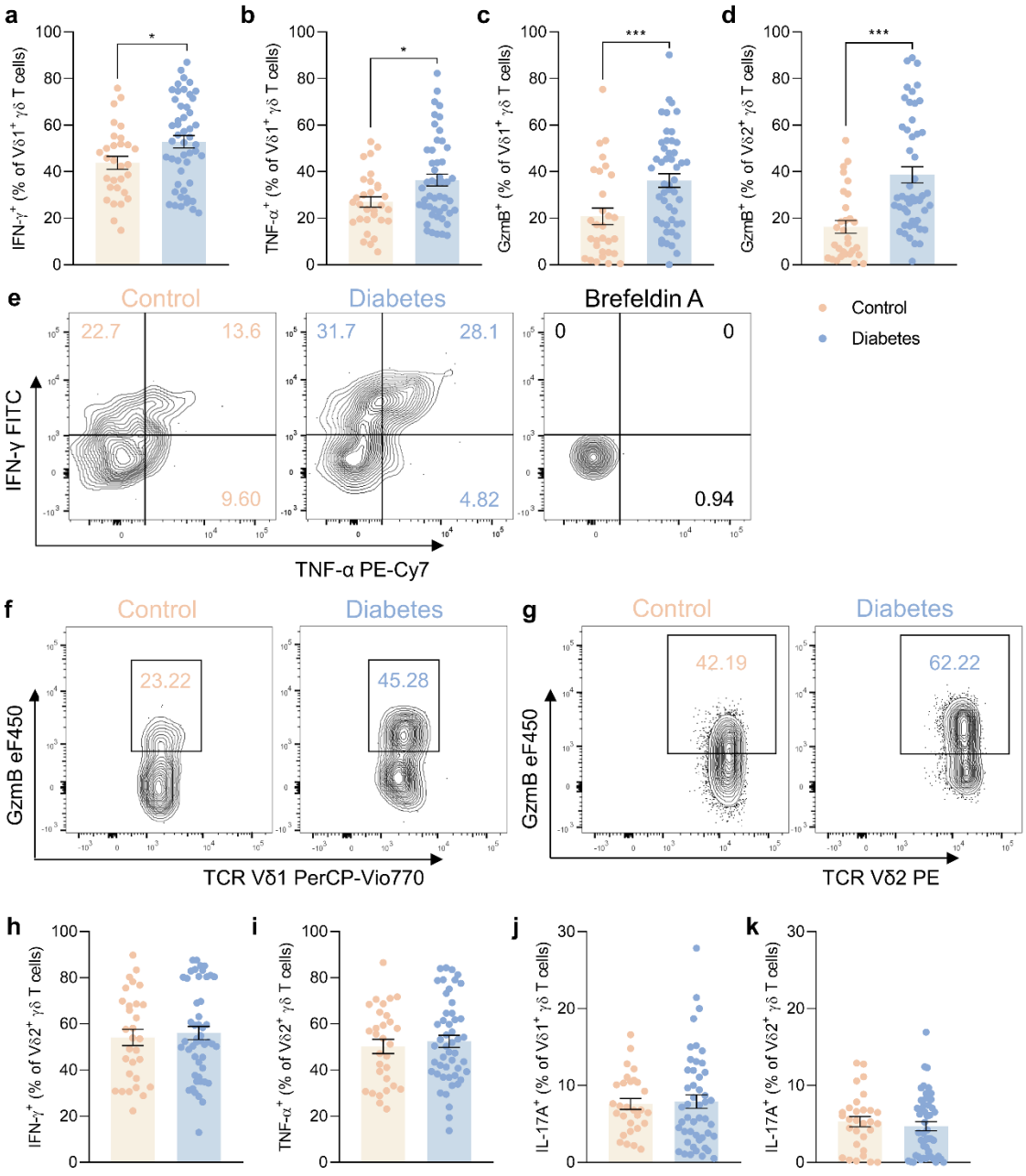

Figure 9. Gamma delta T cells of type 2 diabetes patients are hyperresponsive to in vitro stimulation

Peripheral blood mononuclear cells were isolated from control subjects and patients with type 2 diabetes (T2DM). (a-d) Cells were stimulated *in vitro* with Phorbol myristate acetate (PMA)/Ionomycin in the presence of Brefeldin A for 4 hours and cytokine production was determined by intracellular flow cytometry in Vδ1$^+$ and Vδ2$^+$ γδ T cells. Quantification of (a) interferon-gamma (IFN-γ) and (b) tumour necrosis factor-alpha (TNF-α) production by Vδ1$^+$, as well as (c,d) Granzyme B (GzmB) production by (c) Vδ1$^+$ and (d) Vδ2$^+$ γδ T cells of patients with T2DM (*n*=49) in comparison to control subjects (*n*=30). (e-g) Representative fluorescence-activated cell sorting plots of cells stained with (e) IFN-γ and TNF-α, gated for live CD3$^+$Vδ1$^+$ cells; and (f,g) GzmB, gated for live (f) CD3$^+$Vδ1$^+$ and (g) CD3$^+$Vδ2$^+$ cells. (h-k) Quantification of (h) IFN-γ and (i) TNF-α production by Vδ2$^+$, as well as interleukin-17A (IL-17A) by (j) Vδ1$^+$ and (k) Vδ2$^+$ γδ T cells, of patients with T2DM (*n*=49) in comparison to control subjects (*n*=30). All the experiments were

performed once. An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at *p<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test.

4.6. Natural killer cells of diabetes patients produce more Granzyme B than control subjects

In contrast to $CD8^+$ T cells, NK cells play a pivotal role in the early phases of antiviral immune response. Recent transcriptomic and proteomic analyses of the immune response in severe early-stage COVID-19 cases have established a connection between the functional impairment of NK cells and adverse disease outcomes [111]. Given that diabetes has been identified as a significant risk factor for increased mortality in association with COVID-19 [112], we conducted an investigation into the impact of diabetes on the phenotype and functionality of NK cells using flow cytometry. Our analysis did not reveal any noteworthy differences in the frequency of the main NK cell subsets characterised by the expression of CD56 between the study groups (**Figure 10a, b**). A more detailed examination of the more frequent $CD56^{dim}$ subgroup (**Figure 10c-f**) showed only a slight reduction in maturing and intermediate NK cells in patients with T2DM in comparison to individuals in the control group. Additionally, there was a slight, albeit statistically non-significant, concurrent increase in mature NK cells. Furthermore, we explored whether diabetes influenced the expression of NKG2D, an activating receptor that has been extensively studied in the context of viral infections [113]. In our investigation, we observed a decreased percentage of $NKG2D^+$ $CD56^{dim}$ NK cells in patients with T2DM when compared to control subjects (**Figure 10g, h**).

To assess the proliferative capacity of NK cells, which was quantified based on CFSE fluorescence intensity, we employed flow cytometry after *in vitro* stimulation of PBMCs with IL-15 for a duration of 120 hours. Corresponding to our prior observations with $CD8^+$ T and γδ T cells, we detected no significant differences in the percentage of proliferating NK cells when comparing patients with T2DM to control subjects (**Figure 10i**).

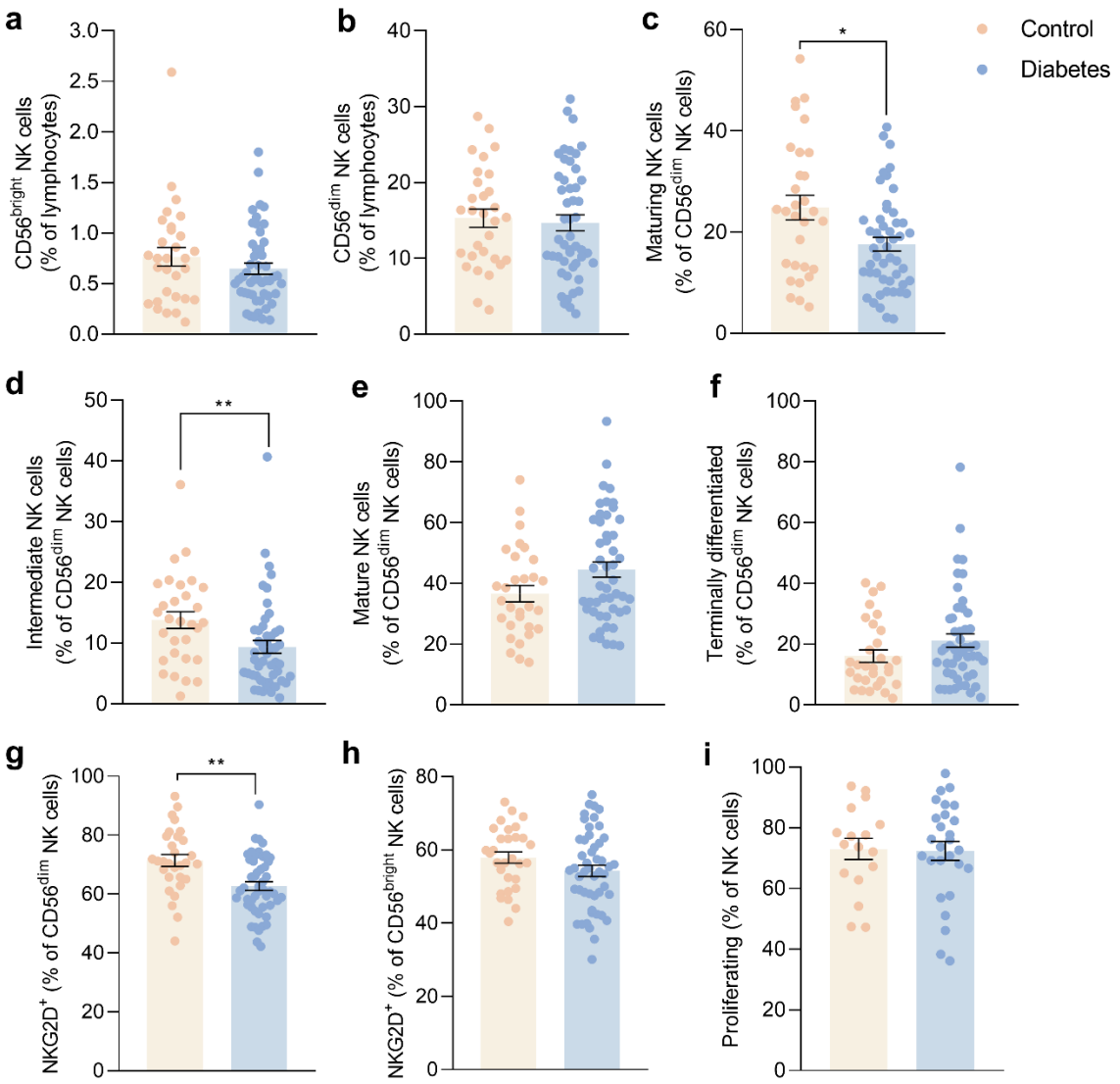

Figure 10. Natural killer cells of patients with type 2 diabetes exhibit a more mature phenotype in comparison to control subjects

Peripheral blood mononuclear cells were isolated from control subjects and patients with type 2 diabetes (T2DM). (a-h) Cells were analysed by flow cytometry. (a,b) Immune cell count for (a) $CD56^{bright}$ and (b) $CD56^{dim}$ natural killer (NK) cells, and (c-f) quantification of $CD56^{dim}$ NK cell subsets, of patients with T2DM (*n*=49) in comparison to control subjects (*n*=30). The surface expression of NK cell group 2 member A (NKG2A) and killer immunoglobulin-like receptor (KIR) 2D subtype (KIR2D) on $CD56^{dim}$ NK cells was used to determine maturing (quartile 1, Q1), intermediate (Q2) and mature (Q3) NK cells. Mature NK cells expressing CD57 represent terminally differentiated NK cells, and within the latter, a subgroup of NK group 2C (NKG2C)-expressing cells were determined (memory NK cells). (g,h) Quantification of NK cell subgroups based on the surface expression of NK cell group 2 member D (NKG2D) on (g) $CD56^{dim}$ and (h) $CD56^{bright}$ NK cells, of patients with T2DM (*n*=49) in comparison to control subjects (*n*=30). (i) Cells labelled with carboxyfluorescein succinimidyl ester (CFSE) were incubated with IL-15 for 120 hours. Fluorescence intensity of CFSE was measured by flow cytometry and used to quantify the frequency of proliferating NK cells in a subcohort of patients with T2DM (*n*=27) compared to control subjects (*n*=17).

All the experiments were performed once. An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at *p<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test.

Subsequently, we utilised intracellular flow cytometry analysis following *in vitro* stimulation of PBMCs to identify functional alterations in NK cells among patients with T2DM. In this assessment, we observed no distinctions in the production of IFN-γ and TNF-α by NK cells between study groups (**Figure 11a,b**). However, similar to γδ T cells, we did observe an increase in Granzyme B production by NK cells in patients with T2DM in comparison to those in the control group (**Figure 11c,d**).

In summary, our findings reveal that T2DM is associated with an increased pro-inflammatory profile in NK cells, potentially contributing to an aggravated response to severe infections, such as those caused by SARS-CoV-2.

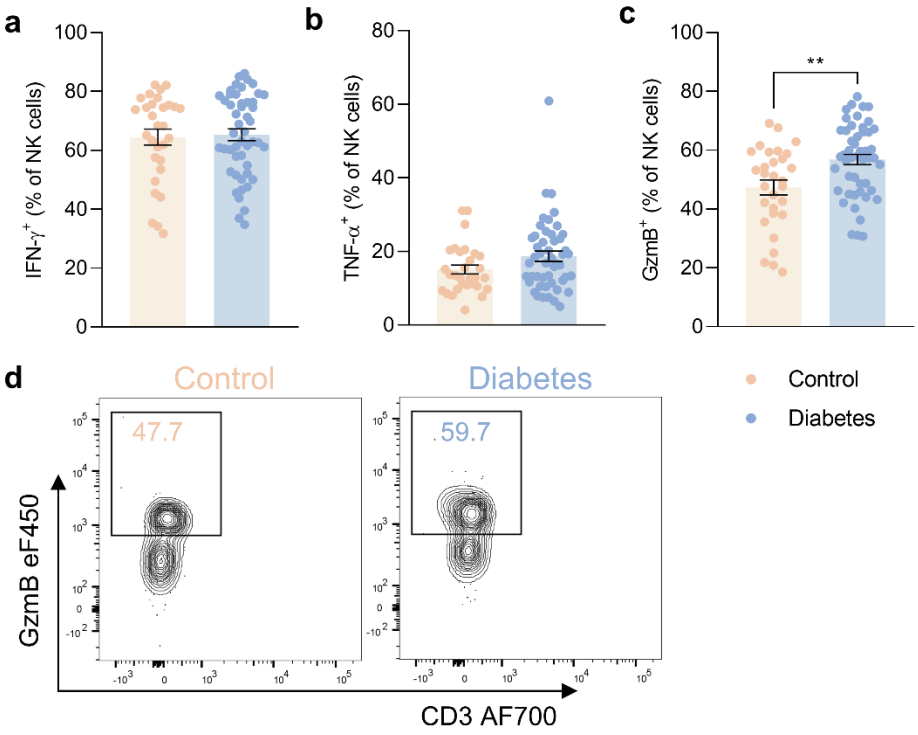

Figure 11. Granzyme B production by natural killer cells is increased in patients with type 2 diabetes

Peripheral blood mononuclear cells were isolated from control subjects and patients with type 2 diabetes (T2DM). (a-d) Cells were stimulated *in vitro* with Phorbol myristate acetate (PMA)/Ionomycin in the presence of Brefeldin A for 4 hours and cytokine production was determined by intracellular flow cytometry in NK cells. Quantification of (a) interferon-gamma (IFN-γ), (b) tumour necrosis factor-alpha (TNF-α) and (c) Granzyme B (GzmB) production by NK cells of patients with T2DM (n=49) in comparison to control subjects (n=30). (d) Representative fluorescence-activated cell sorting plots of cells stained with GzmB,

gated for live $CD3^{-}CD56^{+}$ cells. All the experiments were performed once. An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at *p<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test.

4.7. Inflammatory profile of cytotoxic lymphocytes correlates with age and diabetes duration

Next, our aim was to identify the factors associated with the observed functional changes in cytotoxic blood lymphocytes in the context of diabetes. We initially hypothesised that hyperglycaemia might be the primary driver behind the hyperresponsiveness seen in these cells. To investigate this, we conducted a correlation and regression analysis, examining TNF-α production by $CD8^{+}$ T cells in relation to HbA_{1c} levels. Surprisingly, we were unable to identify any significant associations between these parameters (**Figure 12a**). We also compared cytokine production by cytotoxic lymphocytes in patients with T2DM based on their HbA_{1c} values using a cut-off point of 8.0%, but we found no significant differences in cytokine production between these subgroups (**Figure 12b,c**). Additionally, there was no observable association between increased cytokine production and plasma glucose values relative to insulin levels, as measured by insulin indices (**Figure 12d**). These observations suggest that glycaemic levels alone do not account for the hyperresponsiveness of cytotoxic lymphocytes in patients with T2DM.

Type 2 diabetes mellitus typically occurs in individuals with obesity, a chronic metabolic condition characterised by a BMI of 30 kg/m^2 or higher, or body fat percentages exceeding 28% and 20% for females and males, respectively. Additional clinical metrics, such as waist circumference and waist-hip ratio (WHR) are also commonly used to identify patients with visceral obesity who may face a higher risk of cardiometabolic issues. In our study groups, significant disparities were evident in most anthropometric indicators of obesity (**Table 4**). Consequently, we aimed to explore the impact of obesity on lymphocyte function through subgroup analysis within the cohort of patients with T2DM. Our analysis revealed a positive correlation between TNF-α production by $V\delta1^{+}$ γδ T cells and WHR (**Figure 12e**). Nonetheless, patients with diabetes who had a BMI of 30 kg/m^2 or higher exhibited lower cytokine production by $CD8^{+}$ and γδ T cells when compared to those with normal weight and overweight individuals (**Figure 12f-h**). Conversely, the elevated TNF-α production by $CD8^{+}$ T cells could not be explained by body fat percentage in patients with T2DM (**Figure 12i**). Additionally, we observed a negative correlation between TNF-α production by $CD8^{+}$ T cells and the skeletal muscle index (**Figure 12j**). These findings suggest that having adequate skeletal muscle mass and a lower amount of visceral fat, rather than the total body fat percentage, may play a pivotal role in mitigating obesity-induced systemic inflammation in patients with T2DM.

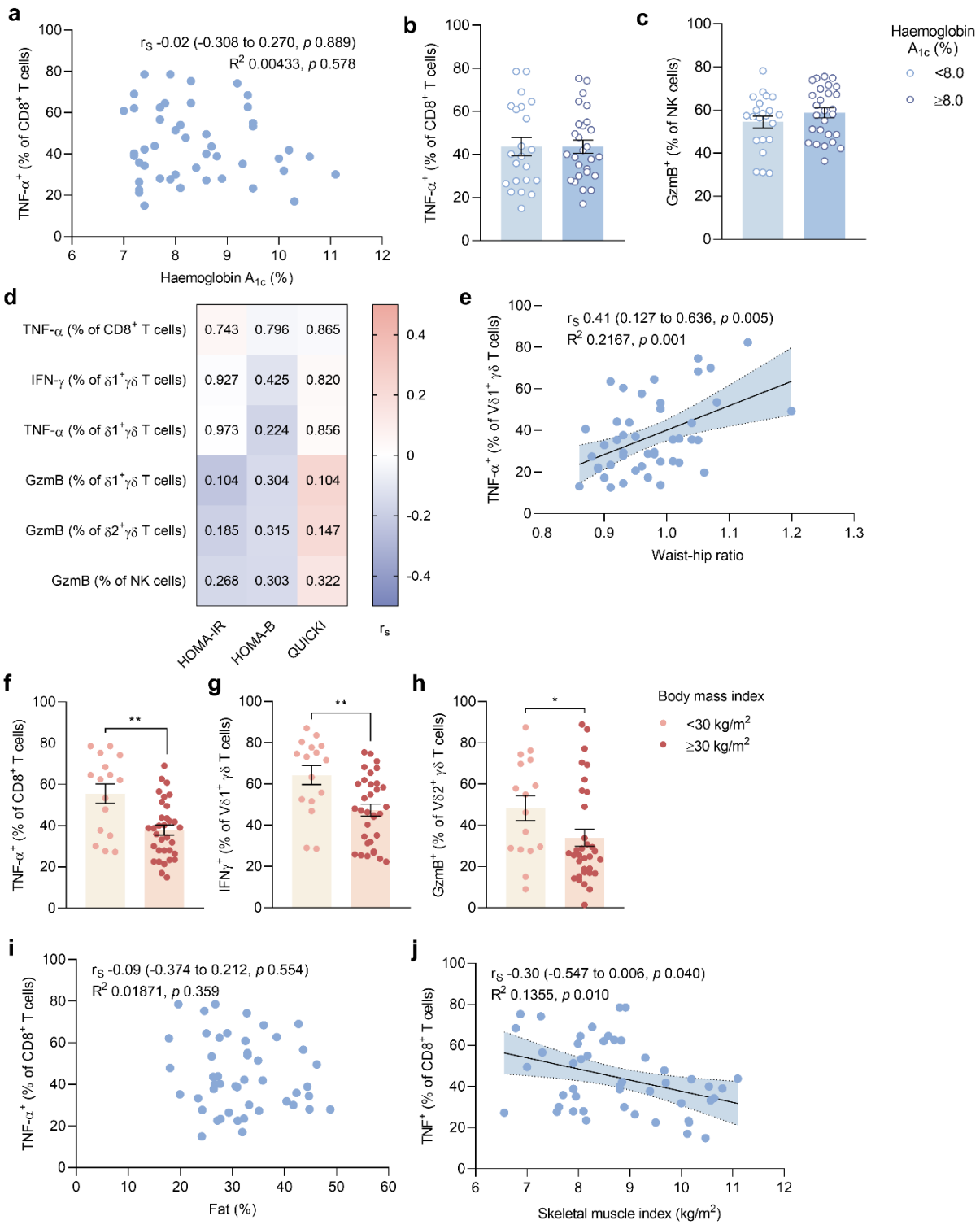

Figure 12. Adequate skeletal muscle mass and a low amount of visceral fat may attenuate systemic inflammation in patients with type 2 diabetes

Subgroup analysis, Spearman correlation and regression analysis were performed in both study groups. (a) Correlation and regression analysis between tumour necrosis factor-alpha (TNF-α) production by $CD8^+$ T

cells and glycated haemoglobin A_{1c} (HbA_{1c}) values in patients with type 2 diabetes (T2DM, *n*=49). (b,c) Comparison of (b) TNF-α production by $CD8^+$ T cells and (c) Granzyme B (GzmB) production by natural killer (NK) cells of patients with T2DM with HbA_{1c} level <8.0 (*n*=22) or ≥8.0% (*n*=27). (d) Correlation analysis between immunological and clinical parameters in patients with T2DM (*n*=49). Correlation matrix with Spearman correlation coefficient (r_s) indicated by two-colour gradient and numerically represented *p* values considered statistically significant at <0.050. (e) The association between waist-hip ratio and the percentage of TNF-α^+ V$\delta1^+$ γδ T cells of patients with T2DM (*n*=49). (f-h) Comparison of cytokine production by (f) $CD8^+$ and (g,h) gamma delta (γδ) T cells in patients with T2DM considered normal- or overweight (*n*=16) and obese (*n*=33). (i,j) The association between anthropometric parameters and the percentage of (i) TNF-α^+ $CD8^+$ T cells, and (j) TNF-α^+ V$\delta1^+$ γδ T cells, of patients with T2DM (*n*=49). An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at **p*<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test, unless otherwise specified. Regression analyses with statistically significant *p* value at <0.050 represented as mean and 95% confidence area of the best-fit line. HOMA-B, homeostatic model assessment-beta cell function; HOMA-IR, homeostatic model assessment-insulin resistance; IFN, interferon; QUICKI, quantitative insulin sensitivity check index.

As individuals grow older, their cardiometabolic risk tends to rise, often leading to a subsequent disruption in the functioning of the immune system [114]. Metabolic dysregulation has been hypothesised to trigger the persistent activation of immune cells, characterised by a pro-inflammatory and senescent phenotype, particularly in the elderly [80]. As a result, we conducted an investigation to explore the relationship between age and the observed alterations in cytokine production. Surprisingly, our subgroup and correlation analyses revealed that older patients with T2DM had higher levels of TNF-α production by $CD8^+$ T cells (**Figure 13a,b**). However, this trend did not translate to the Control group (**Figure 13c**). These findings suggest that metabolic dysregulation in patients with T2DM contributes to the creation of a systemic pro-inflammatory environment, which in turn exacerbates the age-related changes in the immune response.

We hypothesised that the hyperresponsiveness of cytotoxic lymphocytes might be more pronounced after prolonged exposure to a diabetic environment. To explore this, we compared the cytokine production by cytotoxic lymphocytes in patients who were diagnosed with T2DM within 2 years before their inclusion in the study and those who had been living with long-term T2DM. In our analysis, we observed that an extended duration of T2DM was associated with increased Granzyme B production by NK cells but did not lead to a rise in TNF-α production by $CD8^+$ T cells (**Figure 13d,e**). However, it is important to note that the duration of diabetes is sometimes underestimated in asymptomatic patients or due to irregular check-ups and delayed diagnosis. To account for this, we used renal parameters as surrogate markers for estimating diabetes duration since a glomerular filtration rate decline typically occurs in the later stages of T2DM. Our analysis unveiled a negative correlation between eGFR and TNF-α production by $CD8^+$ T cells (**Figure 13e**). Additionally, we showed that our observations were not associated with comorbidities or medication use (**Figure 14**).

Our results indicate that the duration of T2DM plays a central role in driving hyperresponsiveness among cytotoxic immune cells, significantly intensifying the process of inflammageing in these individuals.

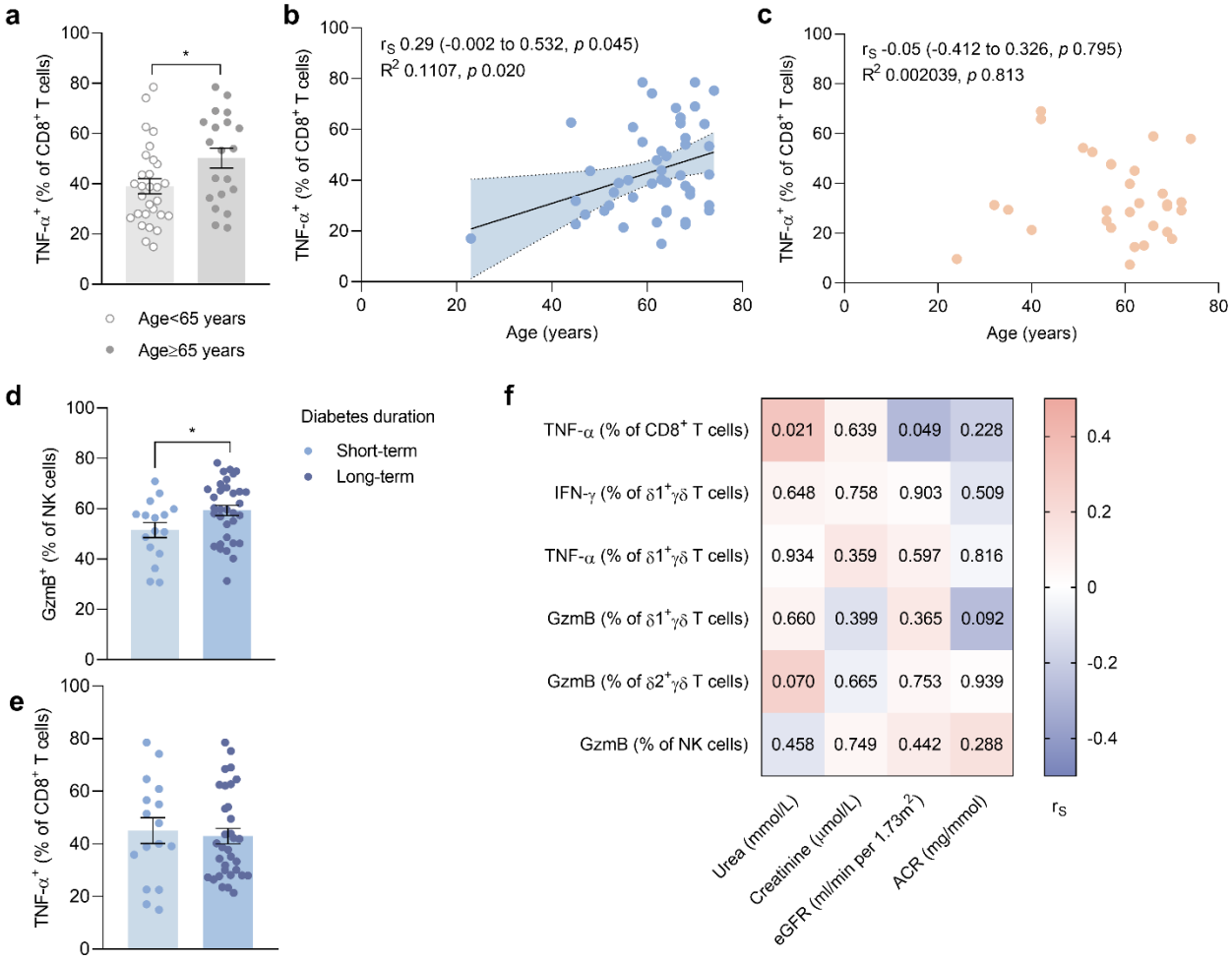

Figure 13. Hyperresponsive state of cytotoxic blood lymphocytes is associated with older age and longer diabetes duration

Subgroup analysis, Spearman correlation and regression analysis between immunological and clinical parameters were performed in both study groups. (a) Comparison of tumour necrosis factor alpha (TNF-α) production by $CD8^+$ T cells of patients with type 2 diabetes below (T2DM, *n*=29) or above the age of 65 (*n*=20). (b,c) Correlation and regression analysis between the percentage of TNF-α^+ $CD8^+$ T cells and age in (b) patients with T2DM (*n*=49) and (c) control subjects (*n*=30). Regression analyses with statistically significant *p* value at <0.050 represented as mean and 95% confidence area of the best-fit line. (d,e) Comparison of (d) Granzyme B (GzmB) production by natural killer (NK) cells, and (e) TNF-α production by $CD8^+$ T cells, in patients with short-term (*n*=16) or long-term (*n*=33) T2DM, defined as follow-up up to or longer than 2 years, respectively. (f) Correlation analysis between immunological and clinical parameters in patients with T2DM (*n*=49). Correlation matrix with Spearman correlation coefficient (r_s) indicated by two-colour gradient and numerically represented *p* values considered statistically significant at <0.050. An individual point represents one participant. Indicated are mean values ± SEM and statistical significances at *p<0.050, **<0.010 and ***<0.001 by the Mann-Whitney test, unless otherwise specified. ACR, albumin-creatinine ratio; eGFR, estimated glomerular filtration rate; IFN, interferon.

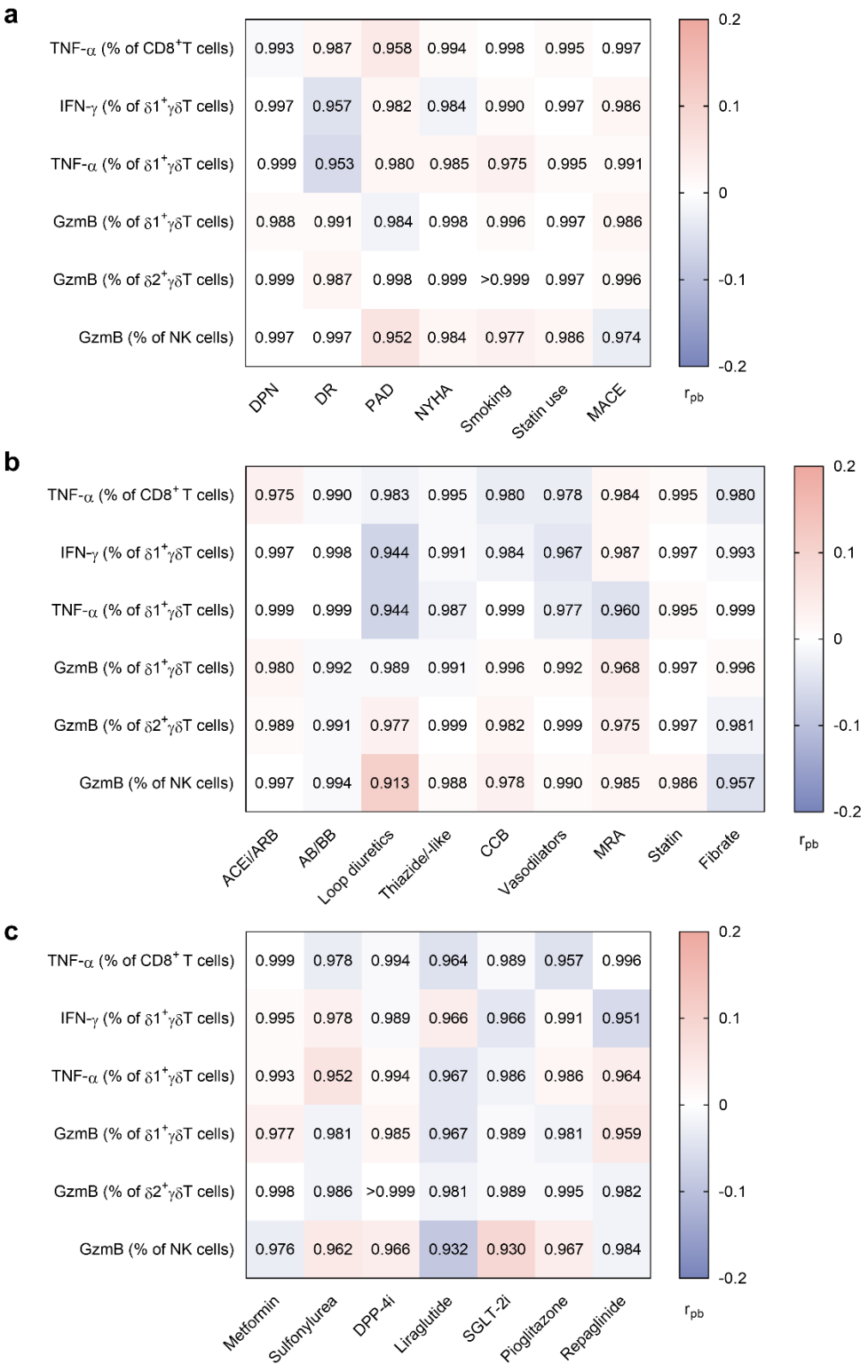

Figure 14. Functional alterations in cytotoxic lymphocytes of type 2 diabetes patients is not associated with comorbidities, cardiovascular risk factors or medication use.

Correlation analysis in patients with type 2 diabetes (*n*=49), based on the presence of (a) complications, comorbidities and cardiovascular risk factors, (b) concomitant and (c) antidiabetic medication use. Correlation matrix with Spearman (r_s) and point-biserial correlation coefficient (r_{pb}) indicated by two-colour gradient and numerically represented *p* values considered statistically significant at <0.050. AB, alpha blockers; ACEi, angiotensin-converting-enzyme inhibitors; ARB, angiotensin receptor blockers; BB, beta blockers; CCB, calcium-channel blockers; DPN, diabetic peripheral neuropathy; DPP4-i, dipeptidyl-peptidase 4 inhibitors; DR, diabetic retinopathy; GzmB, Granzyme B; IFN, interferon; MACE, major adverse cardiovascular event; MRA, mineralocorticoid receptor antagonists; NYHA, New York Heart Association; PAD, peripheral arterial disease; SGLT-2i, sodium-glucose co-transporter 2 inhibitors; TNF, tumour necrosis factor.

4.8. Optimal antidiabetic treatment reverts hyperinflammation in type 2 diabetes patients

Patients exhibiting poor control of T2DM who met the criteria for initiating SGLT-2i and/or GLP-1RA, the current drugs of choice in patients not reaching glycaemic targets on metformin or other treatment regimens, continued to the longitudinal part of the study. Following a three-month period of the newly implemented treatment regimen, a re-evaluation of their HbA1c levels was conducted. Subsequently, a comprehensive assessment, including blood collection, anthropometric measurements, and flow cytometry analysis of cytotoxic lymphocytes, was repeated at the 6-month follow-up visit for the subset of 31 patients who achieved a 3-month HbA_{1c} level below 7.0%. Clinical evaluation and laboratory assessment were used again to exclude patients with immunomodulating factors (refer to Exclusion criteria in **Table 1**).

A comparative analysis of clinical characteristics before and after reaching glycaemic targets through treatment optimisation is provided in **Table 7** and **Table 8**. As anticipated, patients not only achieved their glycaemic targets but also showed improvements in insulin sensitivity indices, effectively managed their weight and lipid profiles, and demonstrated a reduction in markers of liver inflammation (**Table 8**).

Table 7. Patient characteristics of the diabetes follow-up subcohort

	Diabetes follow-up subcohort (*n*=31)
Age, years	60±11 (54–68)
Female sex, *n* (%)	12 (38.71)
Short-term diabetes, *n* (%)	13 (41.94)
Long-term diabetes, *n* (%)	18 (58.06)
Disease duration, years	10±7 (5–10)
Retinopathy, *n* (%)	0 (0.00)
Polyneuropathy, *n* (%)	3 (9.68)
Nephropathy, *n* (%) [a]	
G3a	3 (9.68)
A2	8 (25.81)
Coronary heart disease, *n* (%)	7 (22.58)
Peripheral arterial disease, *n* (%)	2 (6.45)
Antidiabetic treatment, *n* (%)	
Metformin	31 (100.00)
Dapagliflozin	23 (74.19)
Empagliflozin	8 (25.81)
Semaglutide	14 (45.16)

Age and diabetes duration are reported as mean ± SD (interquartile range).
[a] According to Kidney Disease Improving Global Outcomes classification based on estimated glomerular filtration rate and albuminuria [105].

Table 8. Comparison of clinical characteristics before and after treatment change in patients with type 2 diabetes

	Baseline (*n*=31)	Six months (*n*=31)	*p* value
Glycated haemoglobin A_{1c}, %	8.6±1.1	6.5±0.4	<0.001
Fasting plasma glucose, mmol/l	9.7±2.2	6.8±1.0	<0.001
HOMA-insulin resistance	7.8±4.3	4.5±2.9	<0.001
HOMA-beta cell function	7.1±4.3	9.5±6.8	0.008
Quantitative insulin sensitivity check index	0.29±0.02	0.32±0.03	<0.001
Body mass index, kg/m^2	31.5±4.1	29.1±3.7	<0.001
Body fat, %	31.4±8.2	29.7±10.0	0.037
Skeletal muscle index, kg/m^2	9.0±1.2	8.4±1.2	<0.001
Waist-hip ratio	0.98±0.07	0.95±0.06	0.057
Leukocyte count, $\times 10^9$ per litre	7.7±2.0	7.7±2.1	0.867
C reactive protein, mg/l	3.1±2.3	2.0±2.4	<0.001
Triglycerides, mmol/l	1.8±1.1	1.6±0.9	0.034
HDL cholesterol, mmol/l	1.2±0.3	1.3±0.3	0.005
LDL cholesterol, mmol/l	2.8±0.9	2.2±0.9	<0.001
Total cholesterol, mmol/l	4.5±1.2	4.1±1.2	0.011
Statin use, *n* (%)	13 (41.94)	21 (67.74)	0.073
Aspartate aminotransferase, U/l	33±19	27±12	0.028
Alanine aminotransferase, U/l	45±33	29±13	<0.001
Hepatic steatosis index	45.1±6.1	41.0±5.0	<0.001
Triglyceride-glucose index	5.05±0.27	4.80±0.25	<0.001
NAFLD-liver fat score	2.6±1.9	1.6±1.6	<0.001

Data shown as mean ± SD unless otherwise specified. Paired t-test and Fisher exact test were used to determine the statistical significance at *p* value <0.050. HDL, high-density lipoprotein; HOMA, homeostatic model assessment; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease.

To address our main research question regarding the reversibility of diabetes-induced alterations in lymphocyte function, we employed intracellular flow cytometry to analyse cytotoxic lymphocytes after six months of treatment, comparing the results to baseline levels (**Figure 15a-d**). Optimal antidiabetic treatment demonstrated a remarkable reduction in IFN-γ and TNF-α, as well as Granzyme B production by $CD8^+$ T cells after direct *ex vivo* stimulation in patients with type 2 diabetes (**Figure 15a-c, e-f**). Conversely, IL-2 production by $CD8^+$ T cells in patients with type 2 diabetes exhibited no significant change before and after treatment (**Figure 15d**). These observations suggest that optimal antidiabetic treatment may effectively alleviate the hyperinflammatory state of $CD8^+$ T cells in patients with poorly-regulated type 2 diabetes.

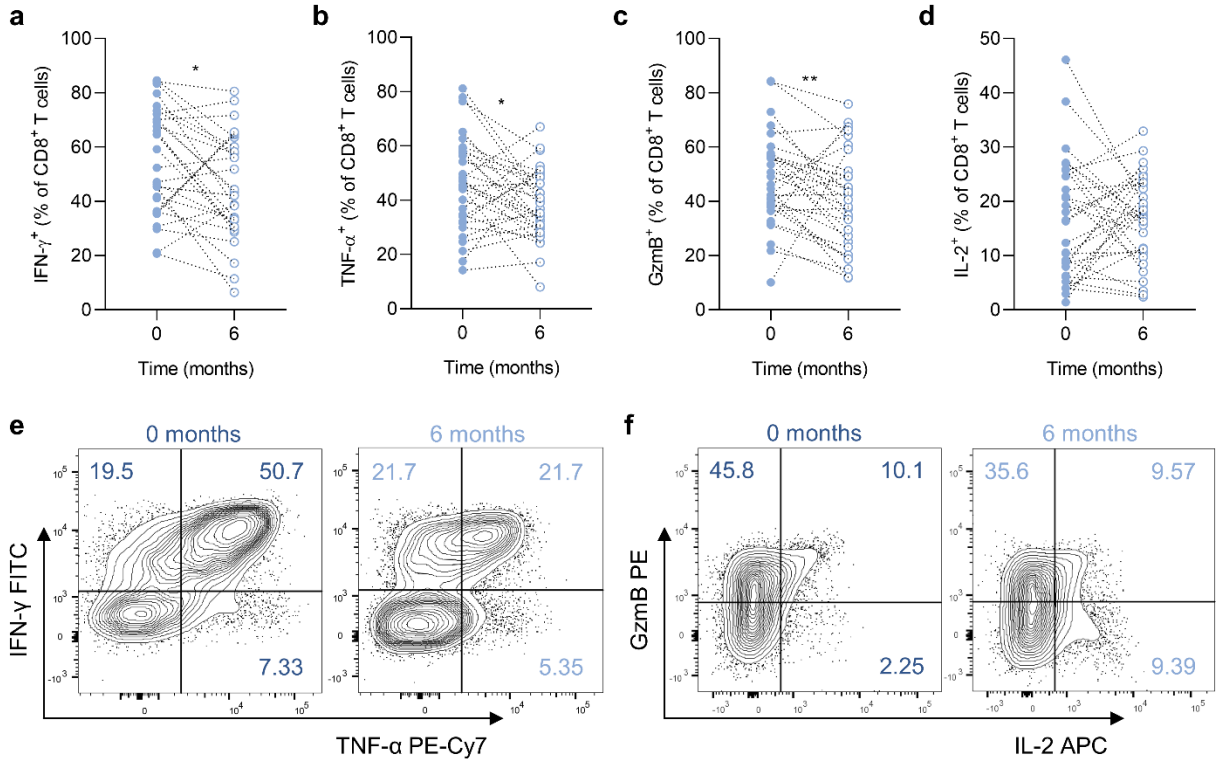

Figure 15. Optimal antidiabetic treatment reverts hyperinflammatory response of CD8 T cells

Peripheral blood mononuclear cells were isolated from patients with initially poorly-regulated type 2 diabetes mellitus (T2DM) who reached target glycated haemoglobin A_{1c} levels at two time points: before and six months after treatment optimisation. Cells were stimulated with Phorbol myristate acetate (PMA)/Ionomycin in the presence of Brefeldin A for 4 hours and cytokine production was determined by intracellular flow cytometry in $CD8^+$ T cells, and (a-d) cytokine production by $CD8^+$ T cells of patients with T2DM pre-treatment in comparison to 6 months post-treatment (n=31) was quantified. (e,f) Representative fluorescence-activated cell sorting plots of cells stained with (e) interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), and (f) Granzyme B (GzmB) and interleukin-2 (IL-2), gated for live $CD3^+CD8^+$ cells from the same patient at two time points. All the experiments were performed once. An individual point represents one participant. Indicated are statistical significances at *p<0.050, **<0.010 and ***<0.001 by Wilcoxon test.

Next, we compared the direct *ex vivo* cytokine production by γδ T cells analysed by intracellular flow cytometry. In line with $CD8^+$ T cells, we observed a consistent pattern of reduced IFN-γ and TNF-α production by both $V\delta1^+$ and $V\delta2^+$ γδ T cells after six months of optimal antidiabetic treatment (**Figure 16a-f**). In contrast, Granzyme B production by $V\delta1^+$ γδ T cells showed a slight reduction, while there was no significant change in its production by $V\delta2^+$ γδ T cells (**Figure 16g-i**). Our findings demonstrate that optimal antidiabetic treatment leads to a reduction in cytokine production by γδ T cells upon stimulation. However, the impact on suppressing Granzyme B production is less prominent compared to $CD8^+$ T cells.

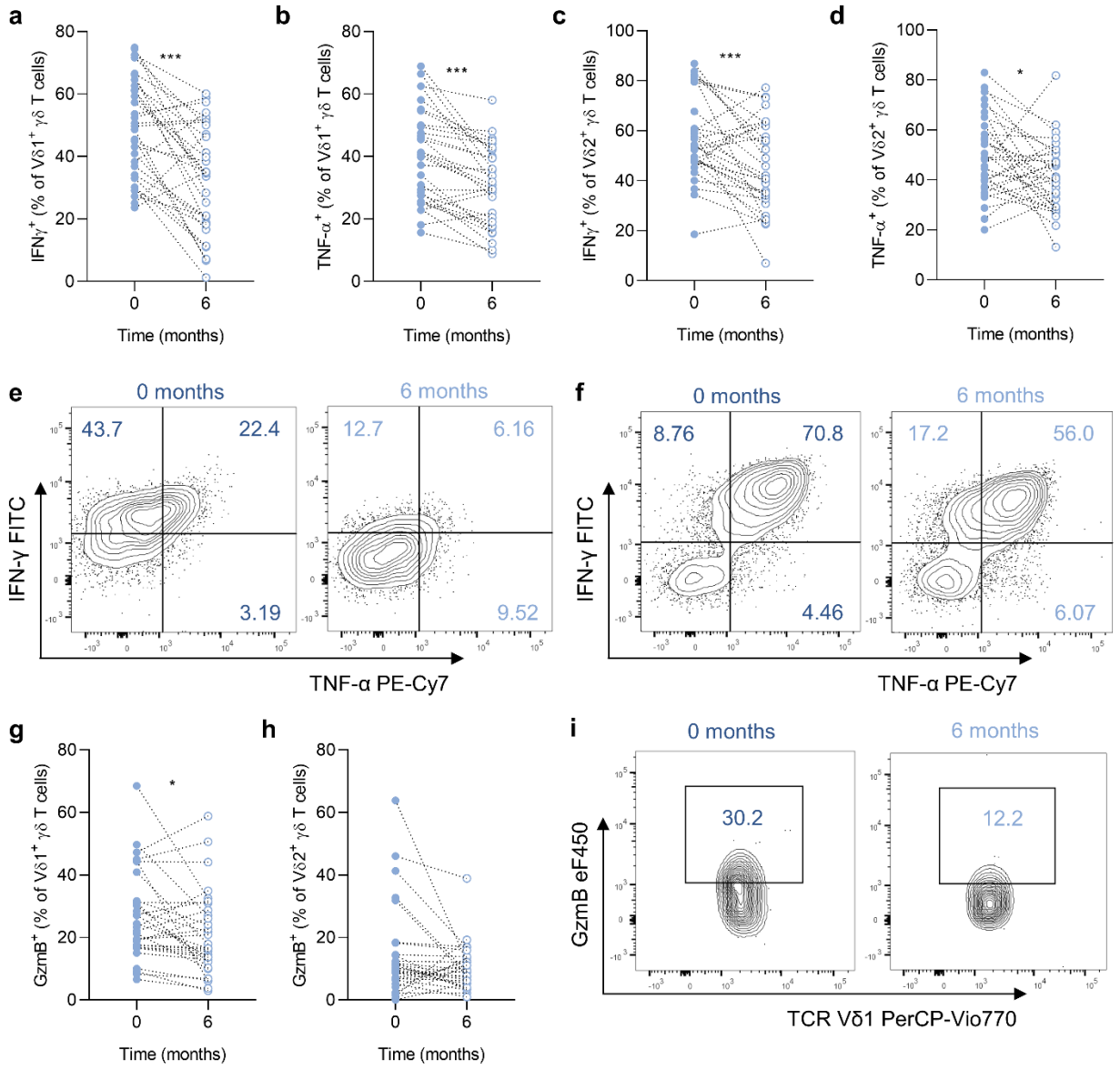

Figure 16. Hyperinflammatory state of gamma delta T cells in diabetes may be reverted by optimal antidiabetic treatment

Peripheral blood mononuclear cells were isolated from patients with initially poorly-regulated type 2 diabetes mellitus (T2DM) who reached target glycated haemoglobin A_{1c} levels at two time points: before and six months after treatment optimisation. Cells were stimulated with Phorbol myristate acetate (PMA)/Ionomycin in the presence of Brefeldin A for 4 hours and cytokine production was determined by intracellular flow cytometry in γδ T cells. (a-d) Cytokine production by γδ T cells of patients with T2DM pre-treatment in comparison to 6 months post-treatment (n=31) was quantified. (e,f) Representative fluorescence-activated cell sorting plots of cells stained with interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), gated for (e) $CD3^+V\delta1^+$, and (f) $CD3^+V\delta2^+$ cells from the same patient. (g,h) Quantification of Granzyme B (GzmB) production by (g) $V\delta1^+$ and (h) $V\delta2^+$ γδ T cells. (i) Representative fluorescence-activated cell sorting plots of cells stained with GzmB, gated for (e) $CD3^+V\delta1^+$ cells from the same patient at two time points. All the experiments were performed once. An individual point represents one participant. Indicated are statistical significances at *p<0.050, **<0.010 and ***<0.001 by Wilcoxon test.

Lastly, we investigated the alterations in cytokine production by NK cells in patients with T2DM following six months of optimal antidiabetic treatment. While no significant difference was observed in IFN-γ and TNF-α production by NK cells (**Figure 17a,b**), our results demonstrate a slight reduction in Granzyme B production by NK cells in patients with T2DM after six months of optimal antidiabetic treatment (**Figure 17c,d**).

These findings reveal the potential reversibility of diabetes-induced alterations in specific functional aspects of cytotoxic lymphocytes through optimised antidiabetic treatment, providing valuable insights for future therapeutic strategies targeting hyperinflammation in individuals with type 2 diabetes.

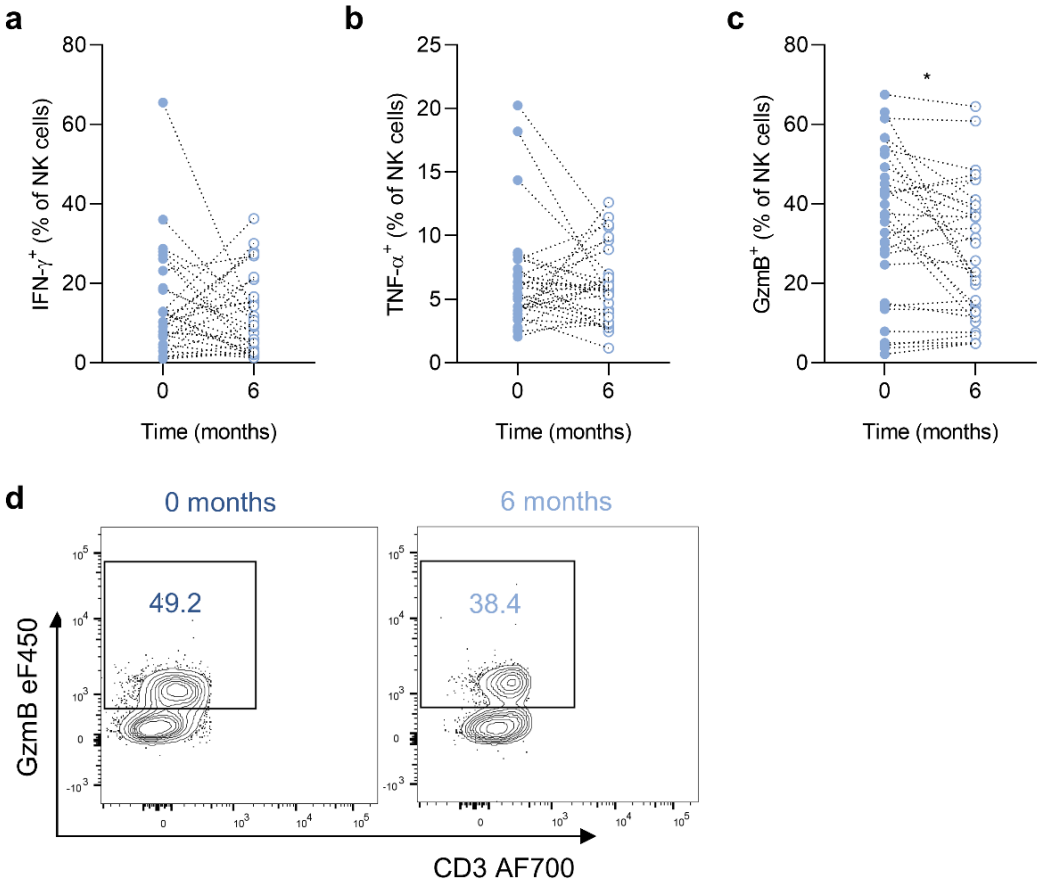

Figure 17. Natural killer cells of patients with type 2 diabetes produce less Granzyme B after six months of optimal antidiabetic treatment

Peripheral blood mononuclear cells were isolated from patients with initially poorly-regulated type 2 diabetes mellitus (T2DM) who reached target glycated haemoglobin A_{1c} levels at two time points: before and six months after treatment optimisation. Cells were stimulated with Phorbol myristate acetate (PMA)/Ionomycin in the presence of Brefeldin A for 4 hours and cytokine production was determined by intracellular flow cytometry in natural killer (NK) cells. (a-c) Cytokine production by NK cells of patients with T2DM pre-treatment in comparison to 6 months post-treatment (n=31) was quantified. (d) Representative fluorescence-activated cell sorting plots of cells stained with Granzyme B (GzmB), gated for NK cells ($CD3^{-}CD56^{+}$) from the same patient at two time points. All the experiments were performed once. An individual point represents one participant. Indicated are statistical significances at *p<0.050, **<0.010 and ***<0.001 by Wilcoxon test.

4.9. Optimal antidiabetic treatment reverts hyperinflammation independent of glucose-lowering and weight-management effects

To delve deeper into the factors contributing to the observed functional alterations in cytotoxic lymphocytes, we conducted a comprehensive subgroup, correlation and regression analysis. First, we performed a comparative analysis of treatment groups. To our surprise, no differences in immunological parameters between the patients treated with SGLT-2i and metformin (Group 1), and those receiving SGLT-2i, GLP-1RA and metformin (Group 2) were observed (**Figure 18a**). Both antidiabetic treatment regimens examined in our study appear to exert a comparable impact on the functional profile of cytotoxic lymphocytes. Subsequently, we expanded our analysis to statins, given that they were the only other class of medications introduced between baseline and the 6-month follow-up visits (**Table 8**). To assess the potential influence of statins, we compared functional changes of cytotoxic lymphocytes in a subgroup of patients introduced to statin therapy during the follow-up period with those who were not. Our analysis revealed that cytokine production by cytotoxic lymphocytes might have been affected by statin use, but this association reached statistical significance only in the case of TNF-α production by $\delta2^+$ γδ T cells (**Figure 18b**). Statin use, therefore, could be a minor contributing factor for observed changes in lymphocyte function but cannot fully explain the mitigation of hyperinflammatory response after optimal antidiabetic treatment.

After six months of antidiabetic treatment, patients with T2DM reached their glycaemic targets and remarkably improved their HbA_{1c} and FPG levels, as well as insulin sensitivity indices (**Table 8**). Subsequently, we explored the role of glycaemia in reverting immunological changes with optimal antidiabetic treatment by examining the correlation between improvements in glycaemia and absolute changes in TNF-α production by $CD8^+$ T cells (**Figure 18c,d**). We could not detect a significant association between these parameters nor between other observed immunological changes and different markers of glycaemia and insulin sensitivity (**Figure 18e**). Furthermore, we conducted a correlation analysis for the relative changes in the parameters of interest. The highest percent improvement in FPG and HOMA-B after 6 months of optimal antidiabetic treatment were associated with the highest percent reduction in TNF-α production by $\delta2^+$ γδ T cells (**Figure 18f,g**). These findings imply that improvement in glycaemia alone may not account for the observed functional changes in the antiviral arm of the immune response. Instead, it appears that the functional profile of a relatively small subset of $\delta2^+$ γδ T cells could be more responsive to shifts in glucose homeostasis during antidiabetic treatment compared to other cytotoxic lymphocytes.

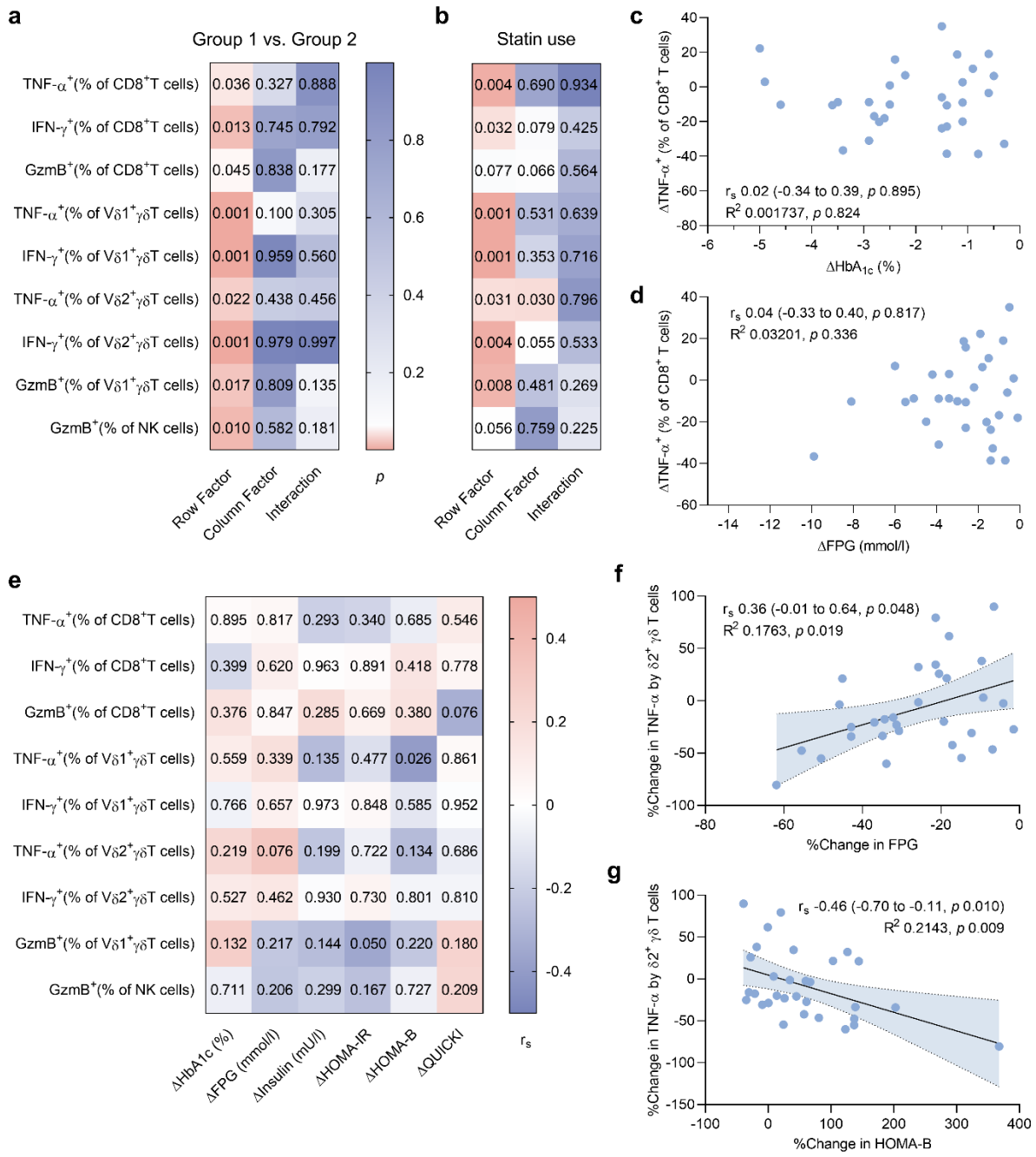

Figure 18. Beneficial effects of optimal antidiabetic treatment are only partly associated with improvement in glycaemia

Subgroup analysis, Spearman correlation and regression analysis were performed. (a,b) Comparison of immunological changes between (a) treatment Group 1 (*n*=17) and Group 2 (*n*=14) based on antidiabetic treatment received, and (b) group of patients introduced with statin (*n*=8) and those who were not (*n*=23). Two-way ANOVA was used to determine the statistical significance at *p* value <0.050. Data presented as p values for a set of row factors (Time, i.e. 0 vs. 6 months), Column factors (Treatment, i.e. Group 1 vs. Group 2) and Interaction of both factors. (c,d) Correlation and regression analysis between absolute change in

tumour necrosis factor-alpha (TNF-α) production by $CD8^+$ T cells and improvement in (c) glycated haemoglobin A_{1c} (HbA_{1c}) values, and (d) fasting plasma glucose (FPG) in patients with type 2 diabetes (T2DM) after 6 months of optimal antidiabetic treatment (*n*=31). (e) Correlation analysis between absolute changes in immunological and clinical parameters in patients with T2DM (*n*=31). Correlation matrix with Spearman correlation coefficient (r_s) indicated by two-colour gradient and numerically represented *p* values considered statistically significant at <0.050. (f) The association between percent change in FPG and homeostatic model assessment of beta cell function (HOMA-B), and the percent change of TNF-α^+ V$\delta2^+$ γδ T cells of patients with T2DM (*n*=31) after six months of optimal antidiabetic treatment. An individual point represents one participant. Regression analyses with statistically significant *p* value at <0.050 represented as mean and 95% confidence area of the best-fit line. GzmB, Granzyme B; HOMA-IR, homeostatic model assessment-insulin resistance; IFN, interferon; QUICKI, quantitative insulin sensitivity check index.

Considering the pleiotropic effects of the studied treatment regimens, encompassing improvements in multiple cardiometabolic health indicators extending beyond glucose control (**Table 8**), we performed a more in-depth analysis between observed immunological changes and changes in other laboratory parameters beyond the markers of glucose homeostasis. Since we could not fully exclude the impact of statin use on lymphocyte function, we first performed a correlation analysis between immunological parameters and lipid profile parameters (**Figure 19a**). However, our results indicate that there is no significant association between the improvement in lipid profile and the reduction in cytokine production by cytotoxic lymphocytes. Next, we addressed the improvement in markers of liver inflammation observed after six months of antidiabetic treatment. Our results showed only a weak correlation between the change in Granzyme B production by V$\delta1^+$ γδ T cells and improvement in aspartate and alanine aminotransferase levels (**Figure 19b**), while there was no significant association between other parameters.

A major favourable effect of treatment with SGLT-2i, but even more so with GLP-1RA, is weight management. To assess the effect of weight reduction and change in body composition occurring after six months of optimal antidiabetic treatment, we employed a correlation analysis for observed functional changes in cytotoxic lymphocytes. Whereas we could not detect a significant association between the majority of anthropometric indices and immunological changes (**Figure 19c**), an association between the percent change in skeletal muscle mass and IFN-γ production by V$\delta1^+$ γδ T cells was revealed (**Figure 19d**). Our findings suggest that treatment-induced changes in body weight and composition influence only a minor subset of γδ T cells.

Lastly, in line with the first part of this study, we tested whether patients with a longer disease duration might also have the biggest benefit after six months of antidiabetic treatment. Indeed, patients with a longer diabetes duration showed a greater reduction in TNF-α production by V$\delta2^+$ γδ T cells after six months of optimal antidiabetic treatment (**Figure 19e**).

In conclusion, only for γδ T cells does hyperresponsiveness to be directly associated with systemic values of some metabolic parameters. However, for most other subsets we could not identify a unique contributing factor affecting all immunological parameters after six months of optimal antidiabetic treatment. Nevertheless, we did observe a striking reduction in immune cell hyperresponsiveness for all immune cell subsets investigated. We therefore hypothesize that the regulation of immune cell function is a result of a complex interplay between multiple cardiometabolic factors and that pleiotropic effects of optimal antidiabetic treatment generate an anti-inflammatory milieu for cytotoxic lymphocytes and decrease their overall responsiveness to non-specific stimuli.

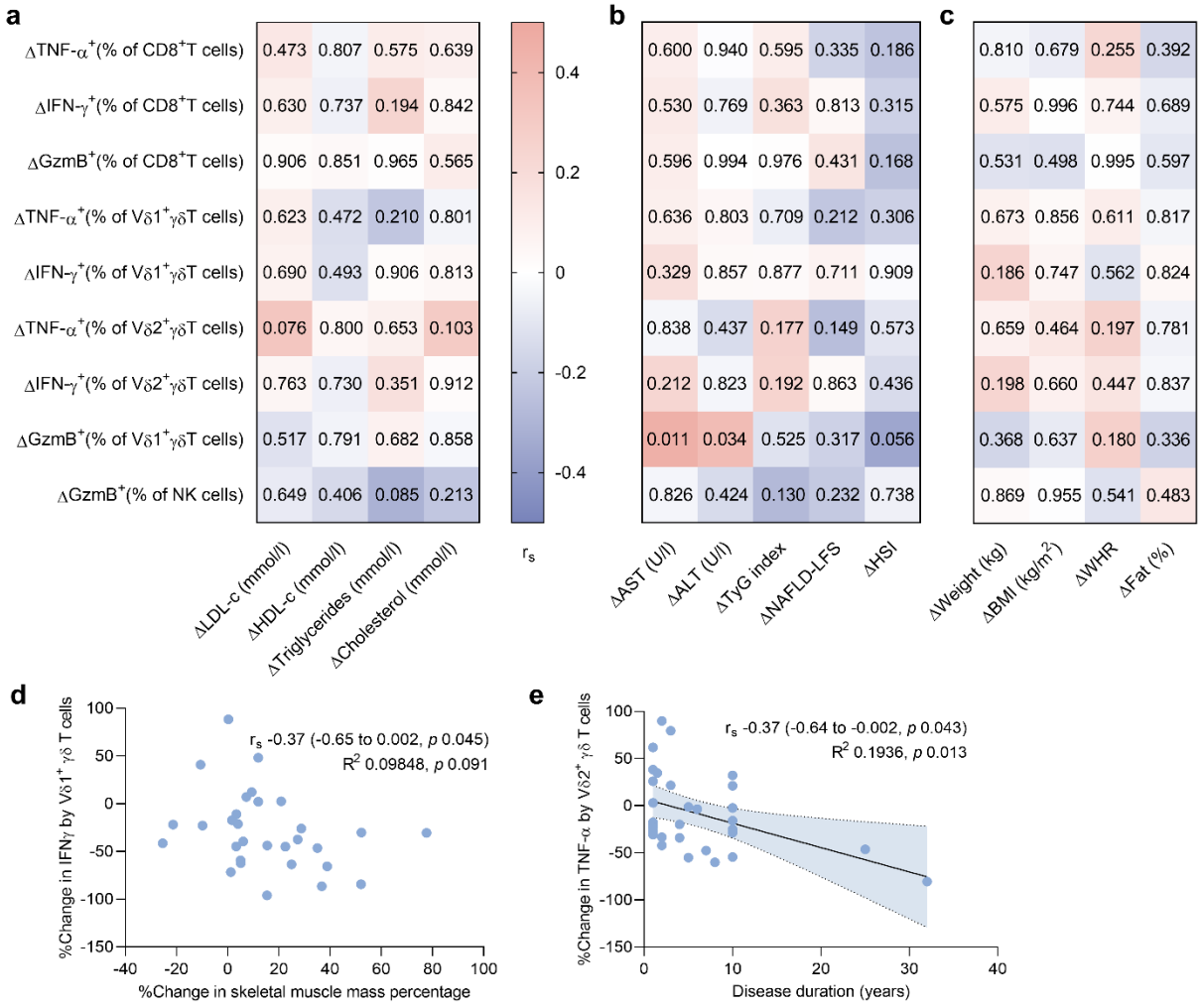

Figure 19. Patients with longer diabetes duration may benefit the most from the anti-inflammatory effects of optimal antidiabetic treatment

Spearman correlation and regression analysis were performed. (a-c) Correlation analysis between absolute changes in immunological and clinical parameters, particularly (a) lipid profile, (b) liver inflammation markers, and (c) anthropometric measurements in patients with T2DM (*n*=31). Correlation matrix with

Spearman correlation coefficient (r_s) indicated by two-colour gradient and numerically represented *p* values considered statistically significant at <0.050. (d,e) The association between (d) the percent change in interferon gamma (IFN-γ) production by $V\delta1^+$ γδ T cells and skeletal muscle mass, and (e) the percent change of TNF-α^+ $V\delta2^+$ γδ T cells and disease duration of patients with T2DM (*n*=31) after six months of optimal antidiabetic treatment. An individual point represents one participant. Regression analyses with statistically significant *p* value at <0.050 represented as mean and 95% confidence area of the best-fit line. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GzmB, Granzyme B; HDL-C, high-density lipoprotein-cholesterol; HSI, hepatic steatosis index; LDL-C, low-density lipoprotein-cholesterol; NAFLD-LFS, non-alcoholic fatty liver disease liver fat score; TyG, triglyceride-glucose; WHR, waist-hip ratio.

5. DISCUSSION

The ageing process is associated with a systemic increase in pro-inflammatory cytokines, such as TNF-α, which play a detrimental role in the development of major disabling diseases. However, the factors contributing to this effect are not well understood. This research reveals that individuals with T2DM exhibit a pro-inflammatory profile in key cytotoxic immune cells. Specifically, our findings indicate a positive correlation between elevated TNF-α production by $CD8^+$ T cells in patients with type 2 diabetes and both advanced age and prolonged diabetes duration. Furthermore, six months of optimal antidiabetic treatment, including an introduction of SGLT-2i with or without GLP-1RA, reduces the hyperresponsiveness of cytotoxic lymphocytes in patients with diabetes. This study therefore provides a deeper understanding of the aggravated immune response in patients with T2DM and sheds new light on antidiabetic medications in targeting diabetes-induced hyperinflammation.

Previous research has indicated that elevated glucose levels can amplify cytokine production by human PBMCs. When exposed to moderately increased glucose levels (8 mmol/l), monocytes demonstrated increased type I IFN production upon poly I:C stimulation [115]. Additionally, Zhu et al.[109] investigated the impact of high glucose conditions on primary human $CD8^+$ T cells stimulated by CD3/CD28 beads. Despite an increase in glucose uptake and glycolysis, they reported no alterations in the proliferation capacity of cytotoxic lymphocytes [109]. However, the study revealed enhanced killing efficiency [109]. These observations mirror our findings in cytotoxic lymphocytes isolated from patients with type 2 diabetes. Surprisingly, our analysis suggested that increased TNF-α production by $CD8^+$ T cells in the context of diabetes did not show a correlation with glycaemia per se. Esposito et al. [116] conducted a study indicating that hyperglycaemic spikes lead to a rise in plasma concentrations of IL-6, TNF-α and IL-18, possibly due to an underlying oxidative mechanism. These changes in pro-inflammatory potential could pose risks in conditions of systemic high-grade inflammation, such as severe infection with SARS-CoV-2, a disease associated with increased immune-mediated pathology in patients with type 2 diabetes [27]. However, it remains unclear whether the same mechanisms persist or if other age-associated factors contribute to chronic inflammation and the process of inflammageing in individuals with longstanding diabetes.

The length of time exposed to hyperglycaemia significantly affects the initiation and progression of all chronic complications associated with diabetes. This impact is probably due to continuous tissue damage resulting from prolonged systemic and/or local inflammation. In our study, conducted within a cohort of patients with T2DM exhibiting a relatively low complication rate, we observe that a pro-inflammatory profile of cytotoxic immune cells is associated with advanced age and a longer duration of diabetes. This effect of T2DM on the antiviral immune response is not transient. Our findings elucidate how the immune system responds to stress stimuli upregulated in metabolic disease, which is in line with previous animal

studies focusing on the visceral adipose tissue and steatotic liver [68, 102]. Diabesity, a term used to describe the combined adverse health effects of obesity and diabetes mellitus, can thus be considered as a state of chronic low-grade infection. Our findings suggest that it is not solely hyperglycaemia, but rather the duration of hyperglycaemia that influences the immune cell hyperresponsiveness associated with inflammageing. Studies propose that implementing early and intensive glycaemic control offers long-term protection against both micro- and macrovascular complications, commonly referred to as the legacy effect [117]. Targeting both glycaemia and inflammation through early intervention or selecting specific antidiabetic treatments in patients with type 2 diabetes could potentially mitigate the detrimental effects of an aggravated immune response and improve the overall prognosis.

Previous studies have proposed several potential immune-modulating mechanisms of action for SGLT-2i and GLP-1RA. However, the majority of studies have primarily focused on *in vitro* or animal models. Specifically, previous work almost exclusively investigated the effect of these medications on glomerular endothelial cells, cardiac myocytes and macrophages [118]. Empagliflozin, in animal models of both prediabetes and diabetes, has demonstrated a capacity to attenuate cardiac macrophage infiltration [119, 120]. Similarly, dapagliflozin decreases the expression of inflammatory markers, including IL-1β, IL-6 and TNF-α, independently of its glucose-lowering effects [121]. Both empagliflozin and dapagliflozin have been observed to attenuate the activation of the NLRP3 inflammasome [81, 121]. These positive effects of SGLT-2 inhibition are attributed to various cellular alterations, encompassing the mitigation of mitochondrial damage and the suppression of signalling pathways associated with inflammation, such as NF-κB pathway [122]. Conversely, studies on the mechanisms underlying the anti-inflammatory effects of GLP-1RA are even scarcer than those for SGLT-2i. In an STZ-induced model of diabetes, GLP-1RA not only prevented macrophage infiltration but also diminished oxidative stress and NF-κB activation in both kidney and cardiac tissues [123, 124]. Further *in vitro* experiments revealed the effectiveness of GLP-1RA in attenuating the release of pro-inflammatory cytokines from macrophages and reducing the production of intercellular adhesion molecule-1 in glomerular endothelial cells [123, 124]. Interestingly, no research to date has shown that anti-inflammatory effects of these medications are mediated through their target receptors but the precise underlying mechanism is still not fully understood and current knowledge remains limited to certain cell types or tissues. In our study, the broader anti-inflammatory effects, reaching beyond the limitations of renal and cardiac tissues, of the medications under investigation were demonstrated. Our findings reveal a discernible pattern of decreased cytokine production by cytotoxic blood lymphocytes after a six-month course of treatment with SGLT-2i and GLP-1RA.

Patients in this study were introduced with SGLT-2i with or without GLP-1RA, medication classes of different mechanisms of action but yielding comparable overall results, including reduction in glycaemia,

weight management, decreased markers of liver inflammation and improved lipid profile. However, none of these parameters individually correspond with reduced immune cell hyperresponsiveness, apart from disease duration and overall body composition. Also, there is no direct correlation between improvement of any of these parameters and reduced hyperresponsiveness. We hypothesise that the improved overall metabolic state observed in patients introduced with SGLT-2i with or without a concomitant GLP-1RA contributes to a diminished perception of metabolic stress in tissues. Reduction in tissue metabolic stress signals, in turn, may result in a comparable reduction of the immune cell responsiveness in both treatment groups, ultimately contributing to similar clinical outcomes.

Notably, our correlation analysis revealed that γδ T cells, as opposed to NK cells and $CD8^+$ T cells, appear to be responsive to metabolite levels. These cells make up less than 10% of PBMCs and they have a unique role in the human body by first responding to tissue stress signals and maintaining immune homeostasis in the local microenvironment [125]. In contrast to NK cells and $CD8^+$ T cells which are crucial for responding to the 'missing self' and the 'dangerous non-self', respectively, γδ T cells play a complex role in tolerating 'safe non-self' and removing the 'distressed self' [125]. The adaptable nature of γδ T cells, therefore, becomes extremely important in excessive tissue inflammation due to metabolic stress, as observed in conditions like diabesity.

Remarkably, the cytokine production by cytotoxic lymphocytes exhibited additional benefits following antidiabetic treatment, surpassing the observed distinctions between control subjects and patients with T2DM in our study. Had control subjects been selected from a cohort of healthy individuals rather than those with cardiovascular disorders, the differences in cytokine production between controls and diabetes patients could have been more pronounced. This observation might elucidate the favourable impact of SGLT-2i and GLP-1RA on cardiovascular safety, given that these medications reduce inflammatory parameters unaffected by T2DM.

In the context of the recent COVID-19 pandemic, the relevance of our discovery, which underscores the impact of specific antidiabetic medications on the antiviral arm of the immune system, becomes even more pronounced. In a study of over 12,000 COVID-19 patients, premorbid use of SGLT-2i and GLP-1RA was associated with lower mortality rates, along with a reduction in emergency room visits and hospital admissions [126]. Another study involving nearly 65,000 veterans with diabetes and COVID-19 demonstrated an inverse association between pre-admission use of a GLP1-RA, metformin, and SGLT-2i and adverse outcomes [127]. These studies underscore the anti-inflammatory properties of these medication classes, preventing excessive inflammatory cytokine production during COVID-19, while concurrently providing cardiorenal protection and improving patient outcomes. Further research should aim to clarify the precise mechanisms underlying the anti-inflammatory effects of SGLT-2i and GLP-1RA in individuals with

metabolic disorders, both preceding and following viral infections. This approach would not only enhance our comprehension of immune modulation in health and disease but also holds promise for informing therapeutic strategies targeting inflammatory pathways in diverse health conditions, paving the way for potential advancements in personalised medicine of individuals with diabetes.

Our research has certain limitations. Although it concentrates on the functional analysis of isolated PBMCs, it does not offer insights into other important components of the immune response such as tissue-specific or humoral immunity. Additionally, we recognize that our study involves a relatively small sample size, potentially impacting the generalizability of our findings given the inherent biological variability in cell populations and protein expression levels. Furthermore, our study population is exclusively Caucasian, limiting the applicability of our findings to diverse ethnic groups. Although we minimised the impact of circadian rhythm and nutrition by collecting all samples in a fasting state between 7 and 9 am, lifestyle factors such as dietary habits, exercise routine, or sleep patterns, were not collected in the study therefore their effect on the observed differences cannot be excluded. Also, the majority of participants in the Diabetes group were not drug-naïve, which may have potentially influenced our findings in comparison to control subjects. Moreover, the short longitudinal design of our study disables the extrapolation of findings to clinical outcomes or discussions on causality. Therefore, further multi-centre studies with larger and more diverse cohorts are necessary to increase the generalizability of our findings.

7. CONCLUSIONS

In conclusion, this study reveals that diabetes is associated with a hyper-responsive profile within the anti-viral arm of the immune system and establishes a correlation of these findings with age and diabetes duration. Our findings show that six months of optimal antidiabetic treatment reduces the pro-inflammatory profile of cytotoxic lymphocytes. These novel insights shed new light on complications of type 2 diabetes associated with chronic systemic inflammation and inflammageing. Moreover, our study offers a new perspective on antidiabetic medications as potential agents capable of ameliorating the hyperinflammatory milieu chronically present in individuals with diabetes.

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LIST OF ABBREVIATIONS

2HG	- Two-hour glucose
ADA	- American Diabetes Association
APC	- Allophycocyanin
ASCVD	- Atherosclerotic cardiovascular disease
BMI	- Body masss index
CFSE	- Carboxyfluoresceinsuccinimidyl ester
CI	- Confidence interval
COPD	- Chronic obstructive pulmonary disease
COVID-19	- Coronavirus disease 2019
CRP	- C reactive protein
CX3CR1	- CX3C motif chemokine receptor 1
DPP-4i	- Dipeptidyl peptidase-4 inhibitors
EDTA	- Ethylenediaminetetraacetic acid
eGFR	- Estimated glomerular filtration rate
FACS	- Fluorescence-Activated Cell Sorting
FCS	- Fetal calf serum
FITC	- Fluorescein isothiocyanate
FPG	- Fasting plasma glucose
FPI	- Fasting plasma insulin
GLP-1RA	- Glucagon-like peptide-1 receptor agonists
GM-CSF	- Granulocyte-macrophage colony-stimulating factor
GzmB	- Granzyme B
HbA_{1c}	- Glycated haemoglobin A_{1c}

HDL - High-density lipoprotein

HF - Heart failure

HOMA- β - Homeostatic model assessment of β-cell function

HOMA-IR - Homeostatic model assessment of insulin resistance

HPA - Hypothalamus-pituitary-adrenal axis

IBD - Inflammatory bowel disease

IFN - Interferon

IL - Interleukin

IP-10 - Interferon gamma-induced protein 10

KIR - Killer-cell immunoglobulin-like receptor

LDL - Low-density lipoprotein

LSM - Liver stiffness measure

mo - month

NA - Not applicable

NAFLD - Non-alcoholic fatty liver disease

NF-κB - Nuclear factor kappa b

NK - Natural killer

NKG2A - Natural killer group 2 member A

NKG2C - Natural killer cell group 2 isoform C

NLRP3 - Nucleotide-binding domain leucine-rich–containing family pyrin domain–containing-3

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate-buffered saline

PE - Phycoerythrin

PerCP - Peridinin chlorophyll-A protein

PMA - Phorbol myristate acetate

QUICKI - Quantitative insulin-sensitivity check index

RA - Rheumatoid arthritis

rpm - Revolutions per minute

RPMI - Roswell park memorial institute

RR - Relative risk

RSV - Respiratory syncytial virus

SARS-CoV-2 - Severe acute respiratory syndrome coronavirus 2

SB - Super bright

SD - Standard deviation

SEM - Standard error of mean

SGLT-2i - Sodium-glucose co-transporter-2 inhibitors

STZ - Streptozotocin

SU - Sulfonylureas

T1DM - Type 1 diabetes mellitus

T2DM - Type 2 diabetes mellitus

TB - Tuberculosis

TCR - T cell receptor

T_H - T helper

TNF - Tumour necrosis factor

TZD - Thiazolidinediones

VEGF - Vascular endothelial growth factor

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WORK EXPERIENCE

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ACTIVE PARTICIPATION ON SCIENTIFIC MEETINGS

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- Cardiology Highlights 2023 Dubrovnik – poster presentation
- Advanced Technologies and Treatments for Diabetes Conference 2023 Berlin & Online – e-poster presentation
- Annual Meeting of Croatian Immunological Society 2022 Sv. Martin na Muri – oral presentation (*Bright Sparks* session) and poster presentation
- Annual Meeting of European Association for the Study of Diabetes 2022 Stockholm – oral presentation
- Annual Meeting of European Young Endocrinologists & Scientists 2022 Zagreb – 2 poster presentations
- Annual Meeting of Croatian Immunological Society 2021 Trogir – poster presentation
- Science 4 Health 2021 Opatija – oral presentation and poster presentation
- European Congress of Immunology 2021 Online – poster presentation

AWARDS AND ACKNOWLEDGEMENTS

- First prize Poster session (Annual Meeting of Croatian Immunological Society 2023)
- Second prize Bright Sparks session (Annual Meeting of Croatian Immunological Society 2022)
- European Congress of Immunology 2021 Attendance Award
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- Dean's Award for Excellence – Class of 2019/2020
- Rector's Award for Excellence – Class of 2019/2020
- Dean's Award for Excellence in Academic Year 2017/2018
- Dean's Award for Excellence in Academic Year 2016/2017
- Dean's Award for Excellence in Academic Year 2015/2016
- Dean's Award for Excellence in Academic Year 2014/2015
- Scholarship for outstanding students from the Vrsar Municipality 2010–2023

MEMBERSHIPS

- National Societies: Croatian Immunological Society, Croatian Society for Endocrinology and Diabetology, Croatian Society for Endocrinology, Croatian Medical Association, Croatian Medical Chamber
- International Societies: European Society of Endocrinology, European Association for the Study of Diabetes

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