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1 Impact of Type 2 Diabetes on the capacity of human macrophages infected with
2 *Mycobacterium tuberculosis* to modulate monocyte differentiation through a bystander effect

3

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25

26 **SHORT TITLE:** T2DM effect on monocyte differentiation in TB

27

28 **KEYWORDS:** diabetes, macrophages, inflammation, tuberculosis, monocytes

29 **ABSTRACT**

30

31 Type 2 diabetes mellitus (T2DM) is a risk factor for the development of tuberculosis
32 (TB) through mechanisms poorly understood. Monocytes and macrophages are key
33 effector cells to control TB, but they are also subverted by *Mycobacterium*
34 *tuberculosis* (Mtb). Specifically, Mtb can induce a bystander effect that skews
35 monocyte differentiation towards macrophages with a permissive phenotype to
36 infection. Here, we evaluated whether T2DM impacts this TB aspect. Our approach
37 was to differentiate monocytes from healthy control (HC) subjects and T2DM
38 patients into macrophages (MDM), and then assess their response to Mtb infection,
39 including their secretome content and bystander effect capacity. Through flow
40 cytometry analyses, we found a lower level of activation markers in MDM from T2DM
41 patients in comparison to those from HC in response to mock (HLA-DR, CD86, and
42 CD163) or Mtb challenge (CD14 and CD80). In spite of high TGF- β 1 levels in mock-
43 infected MDM from T2DM patients, cytometric bead arrays indicated there were no
44 major differences in the secretome cytokine content in these cells relative to HC-
45 MDM, even in response to Mtb. Mimicking a bystander effect, the secretome of Mtb-
46 infected HC-MDM drove HC monocytes towards MDM with a permissive phenotype
47 for Mtb intracellular growth. However, the secretome from Mtb-infected T2DM-MDM
48 did not exacerbate the Mtb load compared to secretome from Mtb-infected HC-MDM,
49 possibly due to the high IL-1 β production relative to Mtb-infected HC-MDM.
50 Collectively, despite T2DM affecting the basal MDM activation, our approach
51 revealed it has no major consequence on their response to Mtb or capacity to
52 generate a bystander effect influencing monocyte differentiation.

53

54 **INTRODUCTION**

55

56 Type 2 diabetes mellitus (T2DM) is currently a major health issue that has reached alarming
57 levels, causing 90% of diabetes (DM) cases reported worldwide in 2019 (463 million adults)
58 ¹. In the same year, *Mycobacterium tuberculosis* (Mtb), the etiological agent of tuberculosis
59 (TB), was the leading single infectious agent responsible for 1.4 million deaths globally ².
60 T2DM is characterized by a range of metabolic, inflammatory, vascular, and other
61 physiological changes that increase TB susceptibility and worsen its pathology; the
62 combination of all these factors clearly contributes to a poor disease outcome in diabetic
63 patients ³.

64 The TB risk in patients with T2DM varies among study design and populations ranging from
65 1.16 to 7.83 fold ⁴. The global prevalence of TB-T2DM is estimated at about 15.3%, and this
66 varies among countries, ranging from 0.1% in Latvia to 45.2% in the Marshall Islands,
67 including 29.5% in Mexico ⁵. In addition to the predisposition to develop active TB, other
68 unfavorable TB outcomes are associated with comorbidity with DM, such as higher
69 incidence of cavitary lung lesions, relapse, persistence, and death ⁶. It is also thought that
70 the immune response to Mtb is affected at multiple levels in T2DM patients, but there still is
71 scarce knowledge about how this takes place ^{3, 7}. To develop intervention strategies to
72 reduce the dual burden of TB-T2DM, it is imperative to better understand the potential
73 mechanisms by which these diseases influence each other.

74 Mtb infection occurs by inhalation of bacteria that invade alveolar macrophages, causing
75 their translocation from the alveoli into the parenchyma, where the bacilli then spread to
76 interstitial monocyte/macrophages, dendritic cells, and neutrophils recruited from the
77 circulation. Subsequently, this results in the priming of a dedicated adaptive immune

78 response against Mtb ⁷. Interestingly, Mtb has adapted to the human host and evolved the
79 ability to subvert mononuclear phagocytes by modulating, for example, the activation of
80 macrophages and their biological functions. Macrophages are primordial regulators of the
81 balance of pro- and anti-inflammatory responses to ensure the control of bacterial replication
82 and limit tissue injury after pathogenic insult ^{8, 9}. In general, macrophage activation is
83 classified within a broad spectrum characterized by two opposite extremes: classic (M1, pro-
84 inflammatory) and alternative (M2, anti-inflammatory, pro-resolving) in response to
85 environmental cues or infection. The classical M1 cells display effector, pro-inflammatory
86 and type-1 immunity properties that mediate the defense against intracellular pathogens ⁸.
87 ¹⁰. Within the spectrum, there is another proinflammatory macrophage activation phenotype
88 called metabolically activated (MMe), which was described *in vivo* in adipose tissue and
89 induced *in vitro* by high glucose, insulin, and palmitate concentrations. MMe is characterized
90 by the expression of CD36, ABCA1, and PLIN2 ¹¹, but its role in infection is unknown. The
91 literature suggests that Mtb modulates the activation of macrophages, reprogramming them
92 towards the M2 spectrum to enhance its own survival. Indeed, the M2 phenotype is known
93 for being susceptible to pathogenic intracellular growth ¹². However, the underlying
94 mechanisms linking macrophage activation programs and a beneficial/harmful effect in TB
95 are not precisely determined ^{8, 13}. Additionally, monocytes (precursor cells that differentiate
96 into macrophages and dendritic cell subsets) also contribute to the alteration of the
97 macrophage compartment during Mtb infection. For instance, Lastrucci and colleagues ¹⁴
98 described that the secretome of Mtb-infected macrophages favors the differentiation of
99 human monocytes towards an M2-like activation program characterized by the
100 CD16⁺CD163⁺MERTK⁺ cell-surface phenotype and functional properties, such as high
101 protease-dependent motility, pathogen permissivity, and immunomodulatory activity, via the
102 IL-10/STAT3 axis. Moreover, they found a direct correlation of the abundance of
103 CD16⁺CD163⁺MERTK⁺pSTAT3⁺ macrophages in non-human primate lung biopsies and

104 pleural cavity of patients with TB disease severity ¹⁴. Whether T2DM affects macrophage
105 activation through monocyte differentiation remains unexplored.

106 We have previously reported that T2DM influences blood monocyte subsets in terms of
107 frequency and cell-surface markers yielding a phenotype that is preserved during their
108 differentiation towards monocyte-derived macrophages (MDM) ¹⁵. This phenotype is
109 distinguished by the lower expression of activation markers, such as HLA-DR and CD86,
110 along with the macrophage marker CD163 ¹⁵. However, we did not determine whether this
111 effect enacted by T2DM on monocytes has any consequences in the context of TB infection.
112 This is precisely what we assessed in this study. Our approach was to differentiate
113 monocytes from healthy control (HC) subjects and T2DM patients into macrophages (MDM),
114 and evaluate their response to Mtb infection, including their secretome content and
115 bystander effect capacity on monocyte differentiation.

116

117

118 **RESULTS**

119 ***Clinical characterization of human subjects***

120 To assure the differences found in the parameters of interest were due to the T2DM
121 condition, and not to other clinical aspects, we carried out a direct comparison of the clinical
122 characteristics of the participants integrating HC and T2DM individuals. As shown in table
123 1, participants from both groups had a similar age, BMI, total cholesterol, and LDL-
124 cholesterol levels. As expected based on the inclusion criteria, T2DM patients had higher
125 values for fasting glucose levels, HbA1c, triglycerides, and VLDL-cholesterol, but lower
126 HDL-cholesterol, when compared to HC controls. Of note, T2DM patients had been
127 diagnosed on average 7 years before enrollment. At the moment of recruitment patients
128 were undergoing treatment with metformin (92.8%) or metformin plus glibenclamide
129 (85.7%), but no insulin.

130

131 ***MDM from T2DM patients exhibit a lower activation status compared to controls which***
132 ***remains unchanged after Mtb infection***

133 Recently, our group reported that T2DM impacts the activation status of circulating
134 monocytes that is conserved even after differentiation towards MDM¹⁵. However, we ignore
135 whether this effect enacted by T2DM on monocyte activation plays a role in the susceptibility
136 to TB. To address this issue, we isolated circulating monocytes from HC and T2DM subjects
137 and differentiated them into MDM, which we then infected *in vitro* with Mtb H37Rv or RPMI
138 media (mock). After 18 h (overnight), we then evaluated the cell-surface expression of the
139 indicated markers, as measured by flow cytometry (Figures 1, Supplementary figure 1, 2,
140 and 3). We found that MDM from T2DM patients compared to HC exhibited a lower
141 expression of activation markers at steady-state (mock infection), such as HLA-DR and

142 CD86, and the macrophage marker CD163 (Figure 1). Upon Mtb infection, there was a
143 decrease in HLA-DR and CD86 in MDM from HC, a change not observed in cells from T2DM
144 patients. However, unlike MDM from T2DM patients, Mtb induced the expression of other
145 activation markers like CD80 and CD14 in HC (Figure 1). Of note, the expression of other
146 cell-surface markers in MDM, such as CD16, CD11b, MERTK, CD206, ABCA1 and CD36,
147 was not affected by T2DM status or by Mtb-infection (Supplementary figure 2 and 3).
148 Altogether, these results indicate that T2DM affects the activation status of MDM
149 differentiated from circulating monocytes, but fails to enact additional changes during
150 challenge with Mtb.

151

152 ***Except for a high TGF- β 1 production from T2DM-MDM, there are no major differences***
153 ***in the cytokine response to mock and Mtb infection between T2DM-MDM and HC-MDM***

154 Conditioned media (secretome) of Mtb-infected macrophages was reported to inhibit
155 monocyte differentiation towards dendritic cells ¹⁶ and skew macrophages differentiation
156 towards an anti-inflammatory phenotype favorable to the bacilli ¹⁴. In both cases, the IL-
157 10/STAT3 signaling cascade was responsible for such phenomenon. To assess the cytokine
158 profile generated by T2DM-MDM in response to mock and Mtb infection, we performed a
159 multiplex cytometric bead array (CBA) approach. From a subgroup of the subjects studied
160 for MDM marker expression, bearing the same clinical characteristic (Supplementary table
161 1), the secretomes were collected from MDM cultures for the following conditions: non-
162 infected HC (cmCTR-HC), non-infected T2DM patients (cmCTR-T2DM), Mtb-infected HC
163 (cmMTB-HC) and Mtb-infected T2DM (cmMTB-T2DM). For each subject, the corresponding
164 secretome types were generated, aliquoted, and frozen at -80°C. To characterize the
165 secretome cytokine profile, aliquots from each subject were thawed, pooled, and evaluated
166 by CBA assays. As shown in Figure 2, similar concentrations of IL-8, TNF, IL-1 β , IL-6 and

167 IL-10, were found in secretomes from non-infected cells regardless of the diabetes status
168 (cmCTR-HC vs. cmCTR-T2DM). Interestingly, the immunosuppressive TGF- β 1 was
169 significantly elevated in cmCTR-T2DM compared to cmCTR-HC (Figure 2). Upon Mtb
170 infection, except for TGF- β 1, MDM from the HC and T2DM groups secreted higher levels of
171 each evaluated cytokine in comparison to their respective non-infected conditions.
172 Notwithstanding, there was no difference in the abundance of any cytokine in question
173 between cmMTB-HC and cmMTB-T2DM. Therefore, despite the differences in the
174 expression of activation markers in T2DM-MDM induced by Mtb-infection (Figure 1), there
175 are no obvious effects of T2DM in the cytokine response to Mtb infection.

176

177 ***T2DM does not influence the bystander effect driven by Mtb infection to skew***
178 ***monocyte differentiation towards M2 macrophages***

179 As aforementioned, Lastrucci and colleagues demonstrated that Mtb infection of
180 macrophages created a bystander effect that drove monocyte differentiation towards an anti-
181 inflammatory M2-like phenotype characterized by CD16⁺CD163⁺MERTK⁺ cell-surface
182 phenotype¹⁴. To investigate whether T2DM affects this process, we used the four
183 secretomes described above to differentiate monocytes from healthy donors towards
184 macrophages. As expected, conditioning of monocytes with cmMTB-HC compared to
185 cmCTR-HC drives the differentiation of CD16⁺CD163⁺MERTK⁺ MDM. By contrast, we
186 observed no changes in the expression of these M2 markers in MDM differentiated with the
187 secretome from Mtb-infected T2DM-MDM compared to that from mock-infected T2DM-MDM
188 (Figure 3). Beyond M2 markers, we also explored a broader cell-surface marker profile
189 including M1 and MMe programs. These markers included CD14, CD11b, CD86, CD80,
190 HLA-DR, PDL-1, CD206, ABCA1, CD36 and, PLIN2 (Figures 4, Supplementary figure 4 and
191 5). As illustrated in Figure 4, there was a lower expression of CD11b and CD80 in MDM

192 differentiated with cmMTB-T2DM compared to cmCTR-T2DM. Remarkably, the secretomes
193 derived from T2DM-MDM (cmCTR and cmMTB) induced a lower proportion and median
194 fluorescence intensity (MFI) of the ABCA1 receptor when compared to those cells
195 conditioned with secretomes from the HC counterparts (Figure 4), indicating the expression
196 of this marker is driven by T2DM, but not by Mtb-infection. Of note, there was no significant
197 difference across these groups for the proportion or MFI for CD14, CD86, PDL-1, CD206,
198 CD36, and PLIN2 (Supplementary figures 4 and 5). Collectively, these results indicate that
199 T2DM does not influence the capacity of Mtb infection to control monocyte differentiation
200 towards macrophages with an anti-inflammatory program, and they also demonstrate a
201 direct effect by T2DM on the ABCA1 receptor expression independent of the TB context.

202

203 ***T2DM modulates the bystander effect driven by Mtb infection to drive monocyte***
204 ***differentiation towards macrophages that secrete high levels of IL-1 β***

205 The bystander effect by Mtb infection decreases the capacity of MDM to engage in the
206 production of proinflammatory cytokine response, such as TNF and IL-12¹⁴. Beyond the
207 assessment of cell-surface marker expression, we also investigated the effect of T2DM in
208 this aspect. Accordingly, we measured the cytokine profile secreted by MDM differentiated
209 with the four different secretomes using a CBA-based approach. As shown in Figure 5, we
210 noticed a higher production of IL-1 β in MDM differentiated with cmMTB-T2DM compared to
211 those conditioned with the other secretomes (Figure 5). Additionally, both secretomes from
212 infected MDM (cmMTB-HC and cmMTB-T2DM) produced higher levels of the anti-
213 inflammatory IL-10 compared to cells exposed to the secretome from non-infected cells (cm-
214 CTR-HC and cmCTR-T2DM) (Figure 5). Of note, there were no differences detected in the
215 cytokine context among the secretomes, including IL-8, IL-12, TNF, IL-6, and TGF- β 1
216 (Supplementary figure 6). Despite the lack of modulation of M2-like cell surface markers by

217 T2DM, these cells still produce IL-10 (M2-profile) and proinflammatory signals as IL-1 β (M1-
218 profile) in response to Mtb-infection.

219 ***T2DM does not modulate the ability of Mtb to generate a bystander effect establishing***
220 ***permissive MDM to pathogen intracellular proliferation***

221 A decrease in the binding of Mtb bacilli to monocytes and MDM in T2DM patients has been
222 previously reported ^{17, 18}. In addition, Lastrucci and colleagues also demonstrated that Mtb
223 infection generates a microenvironment conducive to permissivity to pathogen proliferation
224 in MDM ¹⁴. To explore whether T2DM influences these aspects, we challenged with H37Rv
225 Mtb the different MDM exposed to the four secretomes in question. As shown in Figure 6a,
226 although the binding capacity to Mtb by MDM exposed to cmMTB-HC was higher (albeit not
227 significant) compared to cmMTB-T2DM, there were no significant differences observed
228 among MDM from all groups. This result was consistent with the initial bacilli count based
229 on CFU analysis at day 0 of infection across all tested MDM groups (Figure 6b). On day 5
230 post-infection, we confirmed a higher CFU count in MDM exposed to cmMTB-HC in
231 comparison to those cells with cmCTR-HC. Interestingly, we observed a statistical value
232 close to the significance when compared MDM exposed to cmCTR-T2DM to those cells with
233 cmCTR-HC, suggesting that T2DM alone may hamper the MDM control of Mtb proliferation.
234 However, MDM differentiated with cmMTB-T2DM displayed a similar CFU count compared
235 to those cells with cmMTB-HC (Figure 6b). Altogether, these results indicate that T2DM does
236 not affect the bystander effect established by Mtb infection to induce susceptibility to
237 pathogen intracellular proliferation.

238

239 **DISCUSSION**

240

241 T2DM constitutes a risk factor for infection and the development of TB. However, the basis
242 of TB susceptibility in T2DM patients at the cellular and molecular level is not fully
243 understood. The immune response to Mtb is likely affected at multiple levels in T2DM
244 patients and, therefore, the study of innate immunity is an opportunity to learn about the key
245 components of such response. For this reason, in the present work, we evaluated the
246 response of MDM derived from T2DM patients to Mtb infection and the generated bystander
247 effect on monocyte differentiation towards macrophages. Collectively, we believe our study
248 makes three major contributions to the understanding of the interaction between T2DM and
249 TB in humans.

250 First, we showed the effect of T2DM on the expression of activation markers on MDM in
251 response to mock and Mtb infection, and on MDM differentiated through a bystander effect
252 generated in the context of TB. Previously, we reported the basal expression of some
253 markers in MDM from patients with T2DM ¹⁵. In this study, we extended this phenotypic
254 analysis using a battery of cell-surface markers characteristic representative of the two ends
255 of macrophage activation program spectrum (M1 and M2), and a recently proposed
256 metabolic-oriented program (MMe). Our results showed T2DM modifies in MDM the basal
257 expression levels of activation markers, such as HLA-DR and CD86 (key molecules for
258 antigen presentation), and the macrophage marker CD163 (inflammation regulator). These
259 data are in line with previous results by our group and other authors in monocytes ^{18, 19} and
260 MDM ¹⁸ from T2DM patients, and they are also consistent with the report in the human
261 monocyte cell line THP-1, where HLA-DR and CD86 expression was inhibited in cells
262 exposed to high glucose and palmitate concentrations ²⁰. Overall, these data suggest T2DM
263 for itself alters the expression of markers associated with specific functions, which would

264 translate to immune dysfunction and susceptibility to pathogens. In the context of TB, we
265 report a decrease in HLA-DR and CD86 in infected HC-MDM, which is consistent with
266 reports in monocytes and MDM from TB patients displaying low CD86 and HLA-DR
267 expression and the decreased ability of MDM to stimulate allogeneic T-cell proliferation ²¹.
268 Interestingly, the challenge of T2DM-MDM with Mtb did not change the expression of the
269 evaluated markers. In the past, we reported similar results in GM-CSF-driven MDM from
270 T2DM patients, showing no changes in CD86, CD80 and PD-L1 after Mtb H37Rv infection
271 ¹⁸. Specifically, it has been shown that monocytes ¹⁷ and MDM ¹⁸ from T2DM patients
272 displayed an impaired capacity to interact with Mtb, which could then affect downstream cell
273 activation. The expression of receptors on monocytes/MDM for the recognition of Mtb may
274 be impaired by glucose metabolism, or indirectly through increased endoplasmic reticulum
275 stress and accumulation of misfolded proteins during hyperglycemia ^{6, 22}. Therefore, these
276 mechanisms may be responsible for the decreased expression of cell-surface receptors in
277 T2DM in our present study, with less basal expression and no changes in response to Mtb
278 infection. This is consistent with similar levels of HLA-DR reported on monocytes from
279 patients with the comorbidity TB-T2DM compared to patients with TB without diabetes ²³.
280 The only net changes observed after Mtb infection in T2DM-MDM compared to HC-MDM
281 were the lower levels of CD14 and CD80 expression. Given that it would benefit the bacillus
282 to avoid the activation of a dedicated adaptive immune response, we infer that the T2DM
283 effect on the downregulation of additional molecules involved in antigen presentation could
284 translate into a significantly deficient T cell response specific for Mtb in patients with both
285 diseases.

286 With regard to the cell-surface phenotypic analyses in MDM exposed to secretomes derived
287 from mock and Mtb infection, we found an increase of CD16, CD163, and MERTK (M2-like
288 phenotype) expression on monocytes treated with cmMTB-HC, as previously described ¹⁴.

289 Interestingly, despite the similar cytokine composition between cmMTB-HC and cmMTB-
290 T2DM secretomes, healthy monocytes stimulated with cmMTB-T2DM secretome did not
291 change the expression of these M2-like markers. While it appears that the T2DM may inhibit
292 the anti-inflammatory M2 program established by the bystander effect of Mtb infection, we
293 cannot discard the lack of these cell-surface markers may be due to alternative reasons
294 such as shedding by metalloproteases present in the secretome from MDM-T2DM. T2DM
295 patients presented high levels of circulating soluble CD163 (sCD163)^{24, 25}. In addition, on
296 monocytes from T2DM patients, the levels of surface CD163 correlated inversely with insulin
297 resistance, while sCD163 correlated positively with this parameter when was evaluated in
298 the same individuals set²⁶. Concerning the receptor MERTK, its soluble form has been
299 reported to increase during chronic inflammatory disorders as cardiovascular disease²⁷ and
300 diabetic nephropathy²⁸, conditions closely related to T2DM. Future studies shall address
301 this issue by measuring the soluble levels of these receptors in T2DM and T2DM-TB
302 patients. Beyond M2 markers, we also noticed that MDM exposed to cmMTB-T2DM
303 displayed lower MFI levels of CD11b and CD80 (M1-like markers) in comparison to cmCTR-
304 T2DM, suggesting an amplification of the defective immune response to Mtb in the context
305 of diabetes. This is based on the function of CD11b as a component of complement receptor
306 3, which mediates nonopsonic binding of Mtb in macrophages²⁹, and of CD80 as a key co-
307 stimulatory molecule in antigen presentation to active T lymphocytes³⁰. Interestingly, we
308 found lower levels in the proportion and MFI of the ABCA1 receptor in MDM derived from
309 cmCTR-T2DM or cmMTB-T2DM; this seemed to be independent of the TB context. Our
310 results are consistent with a reduced expression and function of ABCA1 in leukocytes from
311 T2DM patients compared with HC³¹, and with the reported ABCA1 deficiency impairing the
312 JAK2/STAT3 pathway, resulting in an increased inflammatory status^{32, 33}. Collectively, our
313 findings highlight the effect of T2DM in the basal level of activation markers in MDM, which
314 expands to the ability of Mtb infection to downregulate cell-surface molecules participating

315 in the interaction with Mtb and antigen presentation process. In both situations, there are
316 favorable implications for Mtb, thus partially explaining why T2DM is a risk factor in TB.

317 The second major contribution of this study is the assessment of the T2DM effect in the
318 cytokine response to Mtb infection. We presented evidence of similar cytokine composition
319 in the secretomes induced by Mtb infection in MDM regardless of the diabetes status.
320 Indeed, we found Mtb infection induced the secretion of most of the tested cytokines, as
321 reported for TNF, IL-8, IL-6, and IL-10³⁴. In the diabetes context, our results are also in
322 agreement with our previous report in GM-CSF-driven MDM from T2DM patients compared
323 to HC-MDM, where the secretion of IL-6 and IL-1 β was similar at the steady-state or during
324 Mtb-infection¹⁸.

325 The secretion of TGF- β 1, however, was also driven by the T2DM condition alone given that
326 it was highly abundant in the secretome of mock-infected T2DM-MDM. These results are in
327 line with the described increase in the serum TGF- β levels at the onset of T2DM and its
328 permanent elevated abundance throughout the disease³⁵. Additionally, in HC-MDM TGF-
329 β 1 secreted levels were found higher after Mtb-infection (cmMTB-HC vs. cmCTR-HC),
330 reaching the same levels observed in the secretomes from diabetic patients during steady-
331 state. Interestingly, an excessive TGF- β 1 activity in patients with pulmonary TB, and its role
332 in macrophage deactivation and suppression of T cell response to Mtb, have been well
333 documented to affect disease outcome^{36,37}.

334 When we tested the capacity of these secretomes to establish a bystander effect on
335 monocyte differentiation, we found that the cmMTB-T2DM secretome drove the
336 differentiation of MDM that secreted high levels of the inflammatory cytokine IL-1 β . Our
337 results are consistent with the higher levels of IL-1 β reported in the blood of patients with
338 the co-morbidity TB-T2DM compared to TB or T2DM alone³⁸. Although IL-1 β is important
339 in controlling Mtb during acute infection, prolonged inflammation could contribute to

340 progressive immunopathology in the lungs and poor outcome in TB ^{39, 40}. It has been
341 suggested that the uncontrolled high levels of IL-1 β contribute to the exacerbated pathology
342 underlying TB-T2DM comorbidity compared to TB alone ^{38, 41}. In terms of anti-inflammatory
343 cytokines, we observed that MDM differentiated with the secretomes from Mtb-infected
344 MDM produce higher IL-10 levels in comparison to their mock-infected counterparts,
345 regardless of the diabetes status. These results are consistent with the increased levels of
346 IL-10 reported in serum ^{42, 43} and bronchoalveolar lavage ³⁶ of active TB patients. In fact, this
347 cytokine is considered a biomarker of TB disease severity ⁴⁴. Based on these results, future
348 studies will investigate whether IL-1 β and TGF- β 1 can be used as biomarkers in T2DM
349 patients developing TB according to disease severity.

350 The last contribution of our study is the demonstration that T2DM does not affect the capacity
351 of Mtb infection to establish permissivity in MDM to pathogen intracellular proliferation. First,
352 we showed that T2DM does not influence the capacity of MDM differentiated with the
353 secretomes derived from Mtb-infected MDM to bind/internalize the bacteria. This contrasts
354 with previous studies reporting that monocytes ¹⁷ and MDM ¹⁸ from T2DM patients display
355 an impaired capacity to interact with Mtb. This may be due to defects in complement factors
356 and expression of associated receptors for bacterial opsonization, binding of glycosylated
357 proteins to molecules that are important for Mtb recognition (e.g., antibodies, complement,
358 receptors), or inhibition of lectin binding in high concentrations of glucose causing poor
359 pathogen recognition ⁶. Of note, these studies were conducted in cells cultivated under high
360 serum concentration (20% autologous serum) ^{17, 18}. Probable reasons for the discordance
361 with our results in monocytes differentiated to macrophages under the influence of the
362 secretomes may be the use of a diluted secretome in the absence of concentrated
363 autologous serum or a direct impact of T2DM in circulating monocytes that can only be
364 assessed in cells differentiated from T2DM patients, while we used HC monocytes for

365 differentiation towards the macrophage program. Beyond the recognition and interaction
366 with the bacilli, we also found that T2DM does not affect the higher permissivity to Mtb
367 intracellular growth established in cells treated with cmMTB-HC, as reported in healthy
368 subjects ¹⁴. These results are in accordance with different studies reporting that leukocytes
369 from T2DM patients, such as whole blood cells ⁴⁵, monocytes ⁴⁶ and MDM ^{18, 45}, showed
370 similar Mtb-antimicrobial activity when compared to controls without diabetes. In addition,
371 MDM derived from TB patients showed a reduced capacity to kill Mtb that is not modified by
372 the diabetes status (patients with the comorbidity TB-T2DM) ¹⁹, suggesting that permissivity
373 of human monocytes and macrophages to Mtb growth is not exacerbated by T2DM. Future
374 studies shall address whether the high abundance of IL-1 β in MDM derived from cmMTB-
375 T2DM exposure is actually responsible for the lack of the expected exacerbation of the Mtb
376 intracellular burden. IL-1 β is a well-established antimicrobial factor that confers host
377 resistance against Mtb colonization ⁴⁷. In spite of the reported knockdown of the ABCA1 cell-
378 surface expression in THP-1 cells, which is reported to increase Mtb H37Rv survival ⁴⁸, and
379 the lower expression levels of this receptor observed in MDM treated with cmCTR-T2DM
380 and cmMTB-T2DM, still, IL-1 β may ultimately decrease the Mtb burden. Therefore, it is
381 plausible that an equilibrium on these factors may prevent the putative exacerbation of Mtb
382 proliferation in MDM by T2DM.

383 It is important to highlight that our conclusions are limited by the following different aspects.
384 First, our study was conducted with small sample size human groups; this study is meant to
385 be a pilot given that the evaluation of the bystander effect driven by TB in T2DM has not
386 been reported previously. We used secretome pools to homogenize the response of the
387 different tested individuals and, interestingly, significant differences were detected in the
388 control group as previously reported in healthy subjects in spite of our sample size ^{14, 49}.
389 Second, we used monocytes from healthy donors to evaluate the effect of secretomes from

390 MDM derived from T2DM patients. It is probably that the intrinsic characteristics imprinted
391 in cells derived by diabetes will not be reflected in monocytes from HC. Future studies will
392 assess the impact of the collected secretomes directly on monocytes derived from T2DM
393 patients. Last, our results cannot be generalized to a wide spectrum of T2DM patients, as
394 we studied subjects selected under strict inclusion criteria. Consequently, our results should
395 be seen as preliminary to future studies with larger human cohorts.

396 In conclusion, our results suggest that T2DM affects the activation of macrophages and the
397 bystander effect generated during Mtb infection influencing monocyte differentiation. In both
398 cases, there is a defective expression (starting at basal level) of important activation markers
399 implicated in the interaction with Mtb and the antigen presentation process, along with key
400 alterations in the secretion of pro- and anti-inflammatory signals in response to Mtb infection.
401 Even though the control of Mtb intracellular burden was not affected by T2DM, we propose
402 that this disease induces an abnormal function in the innate response by altering the
403 monocyte-to-macrophage differentiation program that normally promotes an efficient
404 adaptive immune response *via* antigen presentation, while worsening at the same time the
405 pathology at the site of infection by increasing inflammatory signals.

406

407 **METHODS**

408

409 **Subjects**

410 The experimental design of the clinical study was approved by the National Research
411 Committee at Instituto Mexicano del Seguro Social (IMSS) with the agreement number R-
412 2016-785-049. All participants provided their informed consent before the enrollment to the
413 study. HC subjects and T2DM patients (diagnosed according to the American Diabetes
414 Association criteria) were recruited between May 2019 to February 2020 from the hospitals
415 H.G.Z.1, Dr. Emilio Varela Luján, IMSS and H.G.26, ISSSTE, at Zacatecas City, Mexico.

416 Every participant in the study was examined by a qualified physician, who performed an
417 exhaustive clinic history focused on the presence of tuberculosis past or present. None of
418 the individuals included in our work had a past tuberculosis episode or signs or symptoms
419 of the disease. The evaluation of the participant medical history and physical examination
420 included records for height, weight, BMI, blood pressure measures, thyroid palpation,
421 cardiovascular examination; for laboratory evaluation, fasting glucose, HbA1c, hematic
422 biometry, lipid profile, serum albumin and creatinine, and estimated glomerular filtration rate
423 were assessed. Subjects with any of the following criteria were excluded from this study:
424 hypothyroidism, alcoholism, smoking, advanced retinopathy, renal failure, neuropathy,
425 infections in any organ, morbid obesity, or receiving treatment with insulin, statins, and/or
426 immunosuppressants use.

427 The buffy coats from healthy donors were obtained at the H.G.Z.1, IMSS blood bank.

428

429 **Preparation of human monocytes and monocyte-derived macrophages (MDM)**

430 The buffy coats from healthy donors were obtained at the H.G.Z.1, IMSS blood bank.
431 Monocytes from HC and T2DM were isolated by negative magnetic labeling (130-096-537;
432 Miltenyi, Bergisch Gladbach, Germany) and differentiated to macrophages (MDM) with M-
433 CSF (130-096-489; Miltenyi, Bergisch Gladbach, Germany) and autologous serum, as
434 previously described ¹⁵.

435

436 **Mtb H37Rv preparation**

437 The reference strain Mtb H37Rv was used for infection in all experiments. Mtb was grown
438 in Middlebrook 7H9 (271310; BD, Sparks, MD, USA) supplemented with 10 % ADC (212352;
439 BD, Sparks, MD, USA), and used for experiments at the time the culture was at the
440 logarithmic phase. The single cell suspension was prepared according to the reported
441 protocol ¹⁴ and bacterial concentration was determined by optical density at 600 nm. For
442 binding assays, Mtb was stained with 25 µM CFSE (C34554; Invitrogen Life technologies;
443 Eugene, OR, USA) for 30 min, using conditions standardized by our laboratory based on a
444 previous report ⁵⁰, and bacteria staining was checked by flow cytometry for each experiment.

445

446 **Preparation of conditioned media and monocyte treatment**

447 The secretomes (or conditioned media) were prepared as previously described ¹⁴, with the
448 only exception of the use of autologous serum (instead of fetal bovine serum) during
449 differentiation and infection of MDMs. The cmCTR medium was obtained from non-infected
450 macrophages from HC (cmCTR-HC) and T2DM (cmCTR-T2DM). The cmMTB medium was
451 prepared from Mtb-infected MDM at a multiplicity of infection (MOI) of three bacteria per cell
452 in RPMI 1640 (11875-119; Gibco Life Technologies, Paisley, UK) supplemented with 10%
453 autologous serum (to mimic the blood microenvironment) from HC (cmMTB-HC) and T2DM

454 patients (cmMTB-T2DM). After overnight (18 h) incubation at 37°C, culture media were
455 collected, sterilized by filtration, and several aliquots from each condition were stored at -
456 80°C (between 6.45 ± 1.67 months) until use for cytokine quantification or functional assays.
457 For conditioning, human monocytes from healthy donors were allowed to adhere for 2 h on
458 glass coverslips in the absence of serum, and then cultured for 3 days in RPMI
459 supplemented with 50% of cmCTR (-HC, -T2DM) or cmMTB (-HC, -T2DM) pools
460 respectively, 10 ng mL⁻¹ M-CSF) and 10 % human serum AB (S4190-100; Biowest, Nuaille,
461 France) to be differentiated to macrophages.

462

463 **Phenotypic analyses for cell-surface markers**

464 MDMs were recovered after recovering the supernatants using Cell Dissociation buffer
465 (13151-014; Gibco Life Technologies, Paisley, UK), and the activation markers were
466 evaluated by flow cytometry. The viability was determined using Live/dead Fixable Violet
467 Dead Cell Stain (L34964; Invitrogen Life technologies; Eugene, OR, USA) for 30 min on ice
468 and protected from light, followed by washing with phosphate-buffered saline 1X (PBS) and
469 incubated at 4°C for 15 min with heat-inactivated human serum AB to avoid nonspecific
470 binding of antibodies. The cells were then stained for 20 min at 4°C in darkness with the
471 indicated antibodies, washed with PBS, and fixed with 4% paraformaldehyde for subsequent
472 acquisition in FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). For the
473 intracellular marker PLIN2, the Fixation/Permeabilization kit (554714; BD Biosciences, San
474 Diego, CA, USA) was used.

475 The battery of antibodies for cell staining included PE anti-CD11b mAb (clone ICRF44; BD,
476 San Diego, CA, USA), Alexa Fluor 488 anti-CD14 mAb (clone MφP9; BD, San Diego, CA,
477 USA), APC-Cy7 anti-CD16 mAb (clone 3G8; BD, San Diego, CA, USA), APC anti-HLA-DR

478 mAb (clone G46-6; BD, San Diego, CA, USA), PE-Cy5 anti-CD86 mAb (clone 2331-FUN-1-
479 ; BD, San Diego, CA, USA), PE anti-CD80 mAb (clone L307.4, BD, San Diego, CA, USA),
480 PerCP-eFluor 710 anti-CD163 mAb (clone GHI/61; eBioscience, Invitrogen, San Diego, CA,
481 USA), PE anti-MERTK mAb (clone HMER5DS; Invitrogen, Carlsbad, CA, USA), APC anti-
482 CD206 mAb (clone 19.2; BD, San Diego, CA, USA), PerCP-eFluor 710 anti-CD274 mAb
483 (PD-L1, clone MIH1; eBioscience, Invitrogen, San Diego, CA, USA), PerCP-eFluor 710 anti-
484 CD36 mAb (clone NL07; eBioscience, Invitrogen, Carlsbad, CA, USA), Alexa Fluor 647
485 anti-ABCA1 mAb (clone HJ1; Novus Biologicals, Centennial, CO, USA) and anti-PLIN2 mAb
486 (clone 2C5H8; Novus Biologicals, Centennial, CO, USA) conjugated with APC (Mix-n-
487 Stain™ APC Antibody Labeling Kit -92307; Biotium, Fremont, CA, USA). The FlowJo VX
488 software (BD, Franklin, NJ, USA) was used for cytometry data analysis. The gating strategy
489 is shown in Supplementary figures 7 to 9.

490

491 **Assessment of cytokine content in the secretome pools and supernatants derived**
492 **from MDM**

493 Conditioned media aliquots were thawed, pooled for each condition, and used to
494 differentiate MDM; a fraction of the secretome employed in different experiments was used
495 to determine cytokine concentrations. After 3 days of conditioning, the supernatants were
496 recovered and evaluated for cytokine content. For all samples, the cytokines IL-8, TNF, IL-
497 12p70, IL-1 β , IL-6, IL-10 (CBA Human Inflammatory Cytokines kit, 551811; BD, San Diego,
498 CA, USA) and TGF- β 1 (Human TGF- β 1 Single Plex Flex Set, 560429; BD, San Diego, CA,
499 USA), were measured according to the manufacturer's instructions. Samples were acquired
500 using a FACS Canto II cytometer and data analyzed using FCAP array 3.0 software (BD
501 Biosciences, San Jose, CA, USA).

502

503 **Mtb association assays**

504 The challenge with CFSE-labelled Mtb was performed in MDM at a MOI of five bacteria per
505 cell, in RPMI 1640 (11875-119; Gibco Life Technologies, Grand Island, NY, USA) containing
506 10 % human serum AB for 4 h at 37°C and 5 % CO₂. Non-adhered bacteria were washed
507 with PBS 1X. Cells were collected using Cell Dissociation buffer, stained with CD11b, and
508 fixed with 4 % paraformaldehyde. The percentage of cells associated (attached or ingested)
509 with Mtb was calculated by flow cytometry data analyzed as the percentage of
510 CD11b⁺CFSE⁺ cells (Supplementary figure 10).

511

512 **Intracellular growth of Mtb in MDM**

513 MDM were infected with Mtb H37Rv at a MOI of 0.2 bacteria/cell for 4 h at 37°C and 5 %
514 CO₂, extracellular bacteria was washed away with PBS, and infected cells were lysed.
515 Lysate dilutions were plated onto Middlebrook 7H11 agar (7244A, NCM0043; Neogen,
516 Lansing, MI, USA) supplemented with 10 % OADC (211886; BD, Sparks, MD, USA) at the
517 indicated days, as previously described ¹⁴. The intracellular growth of Mtb was scored by
518 colony-forming unit (CFU) assay.

519

520 **Statistical analyses**

521 The normality of variables was calculated with the Kolmogorov-Smirnov test. Clinical
522 characteristics between HC and T2DM were compared with *t* student test for normally
523 distributed variables, and with Mann-Whitney's *U* test for non-normally distributed variables.
524 Comparisons of expression markers in MDM within the groups (HC or T2DM) with/without

525 infection were made by two-way ANOVA test and Bonferroni's post-test. For monocytes
526 conditioned with the four different secretomes, the data were compared with the Kruskal-
527 Wallis test and a Dunn's post-test assuming non-normally distributed variables due to the
528 small sample size. $P < 0.05$ was considered statistically significant. Statistical analyses were
529 performed with GraphPad Prism 8.0 (GraphPad Software Inc. San Diego, CA, USA).

530 **AUTHOR CONTRIBUTION**

531 MAVA: Investigation; Methodology; Writing-original draft. GLV: Methodology; Writing-review
532 & editing. FDA: Medical evaluation and recruitment of participants individuals. BEGC:
533 Methodology. ALS: Methodology. RGA: Supervision; Review & editing. JECD: Methodology;
534 Review & editing. OAVG: Medical evaluation and recruitment of patients. ARGV: Medical
535 evaluation and recruitment of patients. JAEM: Review & editing. CJS: Conceptualization;
536 Resources; Project administration; Supervision; Writing-original draft.

537

538 **CONFLICT OF INTEREST**

539 The authors report no financial, commercial, or other conflicts of interest relevant to this
540 article, which is the intellectual property of the authors.

541

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552

553

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- 679
- 680
- 681
- 682

683 **FIGURE LEGENDS**

684

685 **Figure 1.** Assessment of cell-surface markers on monocytes-derived macrophages (MDM)
686 from healthy control (HC) subjects and type 2 diabetes mellitus (T2DM) patients upon Mtb
687 infection. Monocytes from HC (n = 18, black circles) and T2DM patients (n = 14, white
688 circles) were differentiated into MDM, and cell-surface marker expression was determined
689 after mock- (CTR) and Mtb-infection (MTB) at a MOI of 3 bacteria per cell by flow cytometry
690 analysis. Vertical scatter plots show the median fluorescence intensity (MFI) for the indicated
691 markers, and the mean and standard deviation/median and interquartile range are
692 represented by black bars; each symbol represents an individual donor. Data were analyzed
693 by two-way ANOVA followed by Bonferroni multiple comparison test. * $P < 0.05$;
694 *** $P < 0.001$.

695

696 **Figure 2.** Measurement of the cytokine content of the secretome derived from mock- and
697 Mtb-infected T2DM-MDM. Monocytes from HC and T2DM patients were differentiated into
698 MDM and then infected with RPMI (mock, CTR) or Mtb (MOI of 3) overnight. The secretomes
699 (conditioning media) for the four different experimental conditions (cmCTR-HC, cmCTR-
700 T2DM, cmMTB-HC, and cmMTB-T2DM) were recovered and aliquoted for each subject,
701 then kept at -80°C . Secretome aliquots were thawed, pooled, and a cytometric bead array
702 (CBA) analysis was performed for the indicated cytokines. Vertical scatter plots show the
703 median concentration (pg mL^{-1}) for the indicated cytokines and the black bars the
704 interquartile range. Each symbol represents pools (technical replicates) prepared at different
705 time points from 8 HC or 9 T2DM patients. Data were analyzed by the Kruskal-Wallis test

706 followed by Dunn multiple comparison test. IL, interleukin; TNF, tumor necrosis factor; TGF-
707 β 1, transforming growth factor beta. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

708

709 **Figure 3.** Modulation of M2-like cell-surface markers in MDM differentiated with the
710 secretomes derived from mock- and Mtb-infected T2DM-MDM. Monocytes from healthy
711 donors were differentiated towards MDM with the four secretomes (cmCTR-HC, cmCTR-
712 T2DM, cmMTB-HC, or cmMTB-T2DM). After three days, the cells were collected and
713 stained for the indicated cell-surface markers to be analyzed by flow cytometry. Vertical
714 scatter plots show the **(a)** median percentage and **(b)** median fluorescence intensity (MFI)
715 for the indicated markers, and the black bars represent the interquartile range in each case;
716 each symbol represents an individual donor. Data were analyzed by the Kruskal-Wallis test
717 followed by Dunn multiple comparison test. * $P < 0.05$.

718

719 **Figure 4.** Modulation of M1- and MMe-like cell surface markers in MDM differentiated with
720 the secretome derived from mock- and Mtb-infected T2DM-MDM. Monocytes from healthy
721 donors were differentiated towards MDM with the four secretomes (cmCTR-HC, cmCTR-
722 T2DM, cmMTB-HC, or cmMTB-T2DM). After three days, the cells were collected and
723 stained for the indicated cell surface markers to be analyzed by flow cytometry. Vertical
724 scatter plots show (a) median percentage and **(b)** median fluorescence intensity (MFI) for
725 the indicated markers, and the black bars represent the interquartile range in each case;
726 each symbol represents an individual donor. Data were analyzed by Kruskal-Wallis test
727 followed by Dunn multiple comparison test. * $P < 0.05$, ** $P < 0.01$.

728

729 **Figure 5.** Measurement of cytokine secretion by MDM differentiated with secretomes
730 derived from mock- and Mtb-infected T2DM-MDM. Monocytes from healthy donors were
731 differentiated into MDM with the four secretomes (cm-CTR-HC, cmCTR-T2DM, cmMTB-HC,
732 or cmMTB-T2DM), and their supernatants were collected in order to measure the levels of
733 the indicated cytokines by a cytometric bead array (CBA). Vertical scatter plots show the
734 median concentration (pg mL⁻¹) for the indicated cytokines and the black bars represent the
735 interquartile range in each case; each symbol represents an individual donor. Data were
736 analyzed by the Kruskal-Wallis test followed by Dunn multiple comparison test. IL,
737 interleukin. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

738

739 **Figure 6.** Effect of secretomes on the association and intracellular growth of Mtb. Monocytes
740 from healthy donors were differentiated into MDM with the four secretomes (cmCTR-HC,
741 cmCTR-T2DM, cmMTB-HC, or cmMTB-T2DM), and the cells were then tested for **(a)**
742 binding assay to Mtb as measured by flow cytometry, and **(b)** control of intracellular growth
743 of Mtb as determined by colony-forming unit (CFU) assay. **(a)** Vertical plot shows the median
744 of the percentage of cells associated with Mtb, **(b)** Vertical plot shows the median of the
745 CFU counts of the intracellular level of Mtb at day 0 (left) and day 5 (right), and the black
746 bars represent the interquartile range in each case; each symbol represents an individual
747 donor. Data were analyzed by the Kruskal-Wallis test followed by Dunn multiple comparison
748 test. ***P* < 0.01.

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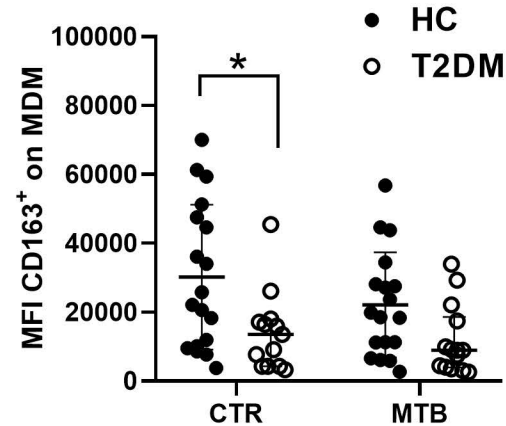
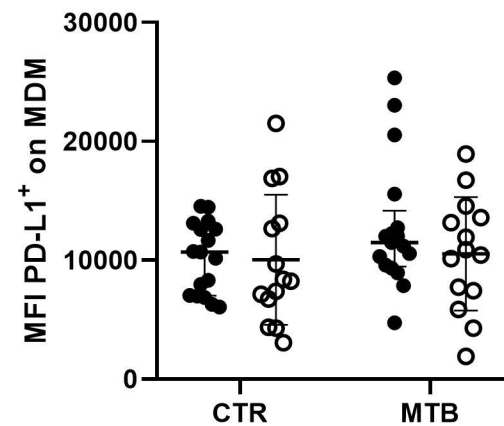
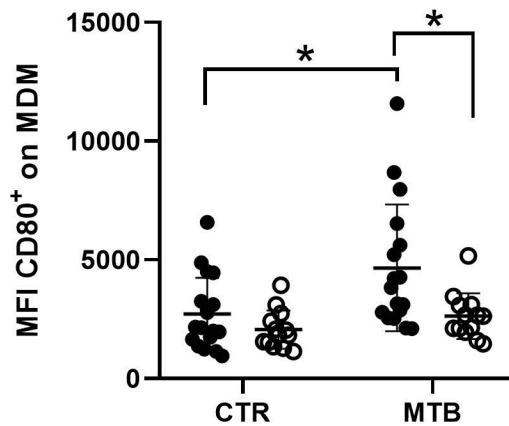
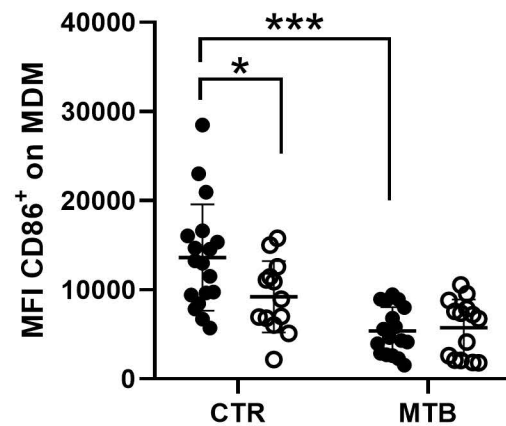
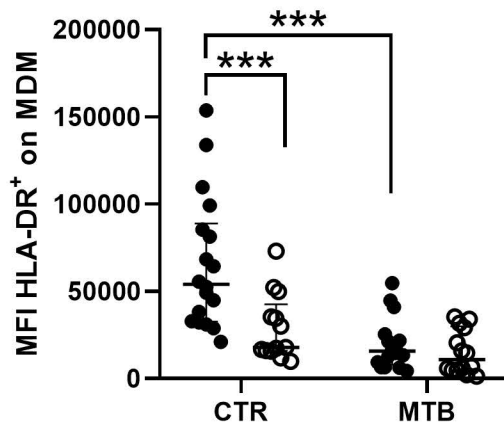
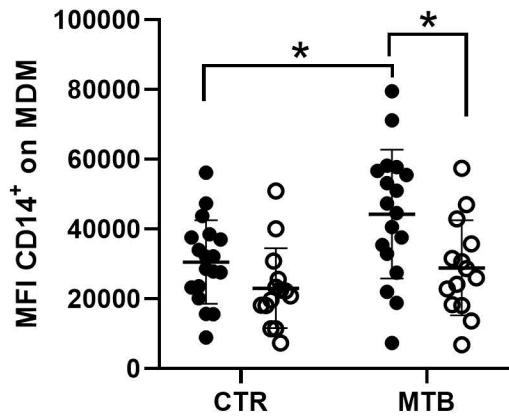
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754 **TABLES**

Table 1. Clinical characteristics of participants.

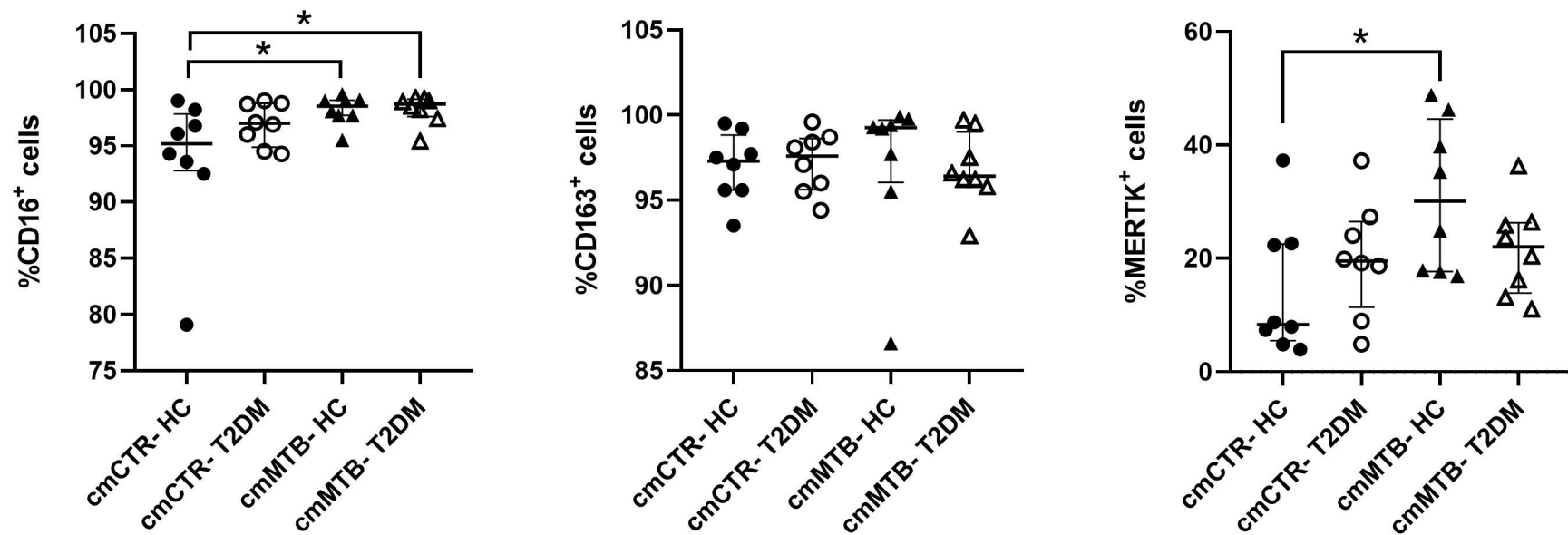
Characteristics	Healthy Control (HC) Subjects	T2DM patients	<i>P</i>
Number of cases (n)	18	14	NA
Years with T2DM diagnosis	NA	7.72 ± 5.35	NA
Age, years	42.39 ± 7.38	47.79 ± 7.61	0.053
BMI (kg/m ²)	24.73 ± 2.54	27.00 ± 4.05	0.061
HbA1c (%)	5.17 ± 0.30	10.99 ± 1.72	<0.0001***
FPG (mg/dl)	83.70 ± 11.03	247.70 ± 66.44	<0.0001***
Total cholesterol (mg/dl)	162.40 ± 42.75	185.70 ± 59.98	0.209
HDL- cholesterol (mg/dl)	52.90 ± 12.33	41.44 ± 7.74	0.006**
LDL- cholesterol (mg/dl)	105.90 ± 36.44	117.50 ± 45.88	0.461
VLDL- cholesterol (mg/dl)	20.06 ± 6.28	53.37 ± 35.28	0.0009***
Triglycerides (mg/dl) ^ξ	92.35 ± 54.15	176.50 ± 238.70	<0.0001***
Hypertension, n (%)	0 (0)	5 (35.70)	NA
Metformin, n (%)	0 (0)	13 (92.80)	NA
Glibenclamide, n (%)	0 (0)	12 (85.70)	NA
Metformin + Glibenclamide, n (%)	0 (0)	12 (85.70)	NA

t tests were performed to identify differences between groups unless specified otherwise; ^ξ: Mann-Whitney *U* test was performed for these variables; mean (± standard deviation) or ^ξ: median (± interquartile range) are shown for quantitative variables. BMI: body mass index; HbA1c: glycated hemoglobin %; HDL: high density lipoprotein; LDL: low density lipoprotein; VLDL: very low-density lipoprotein; NA: non-applicable.

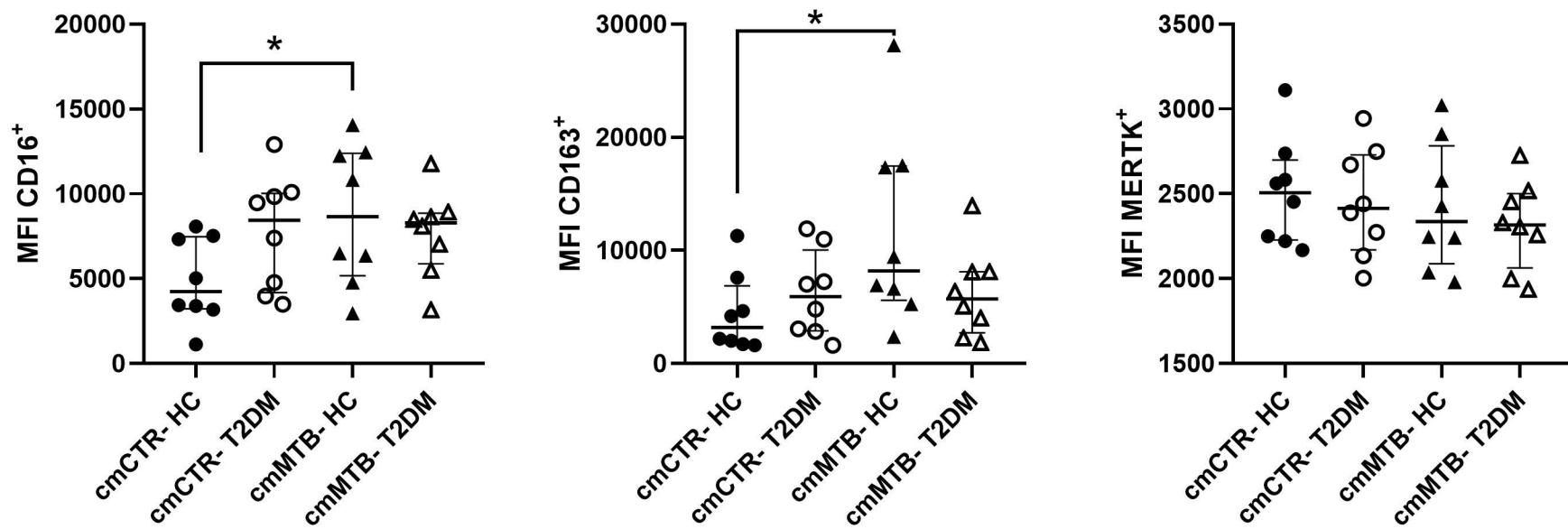


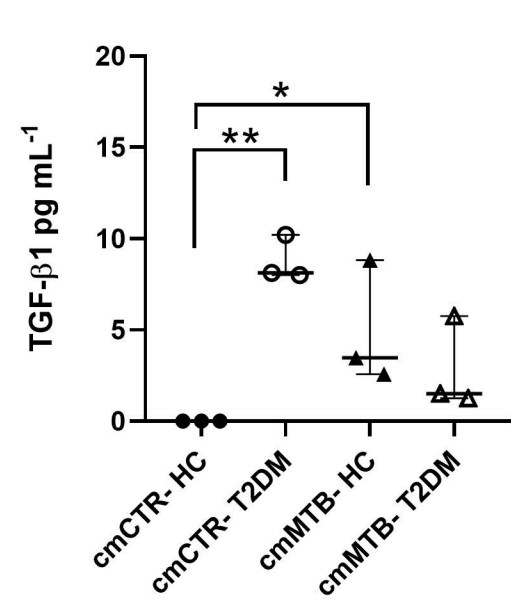
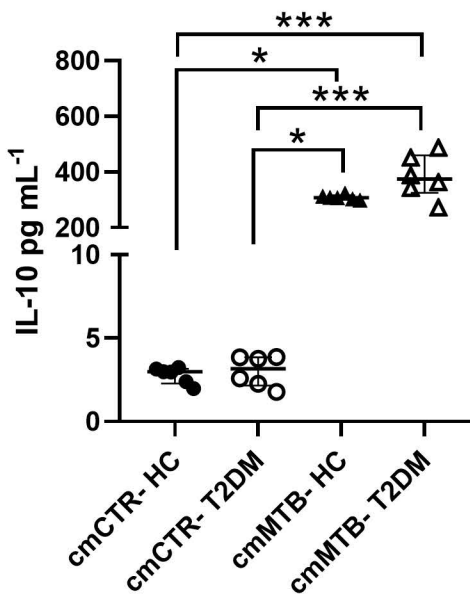
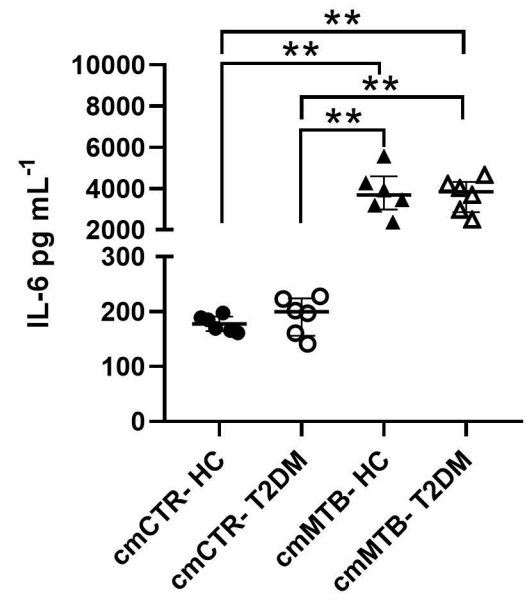
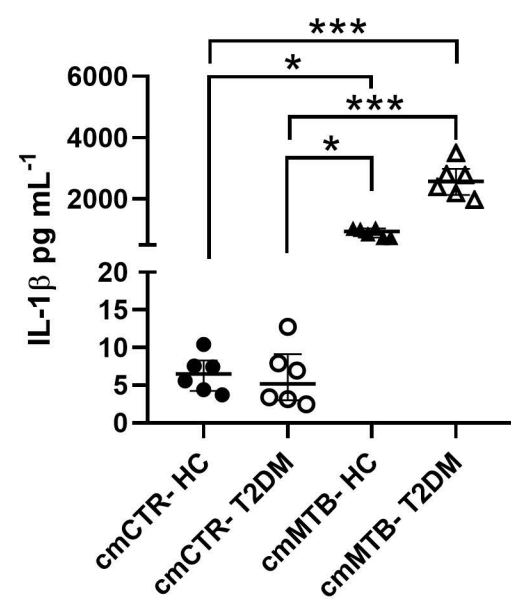
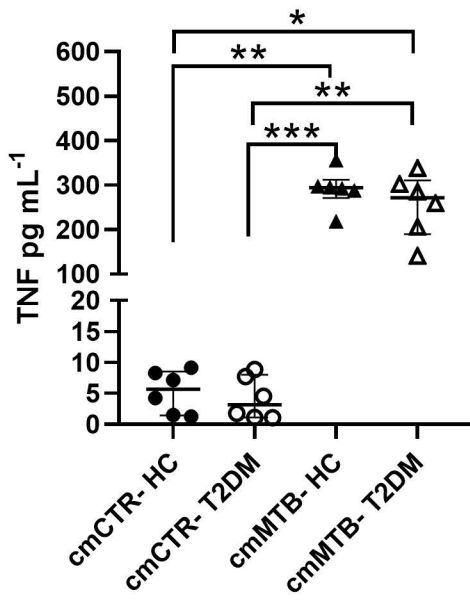
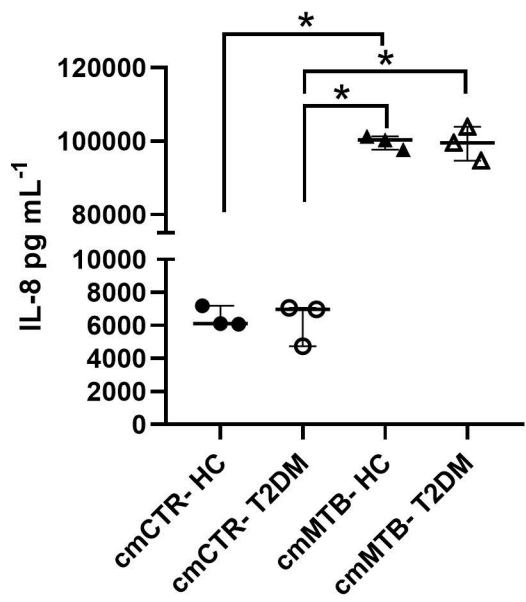
● HC
○ T2DM

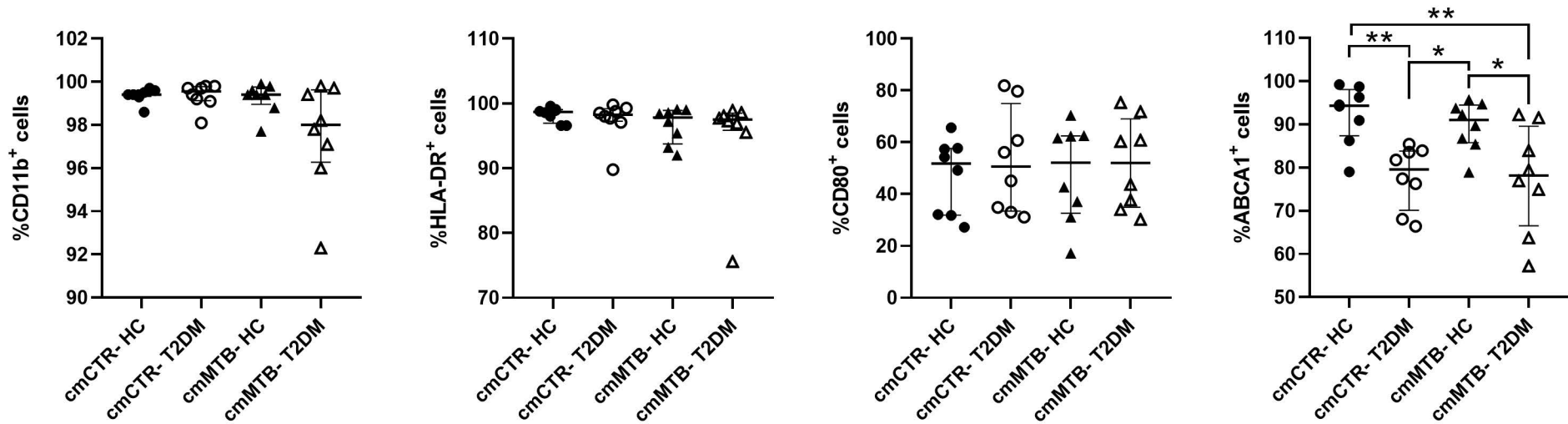
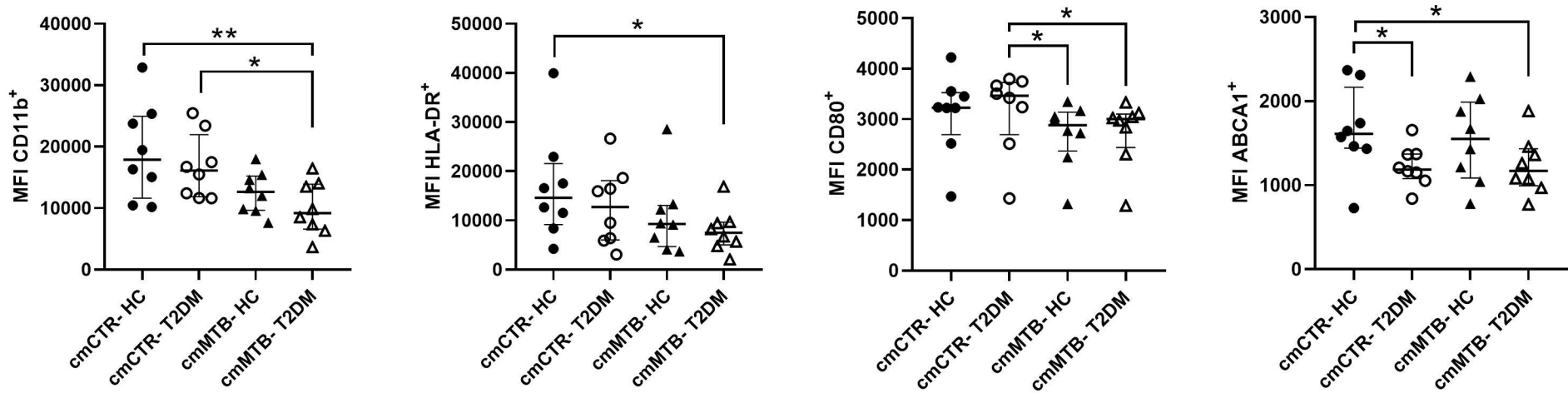
(a)

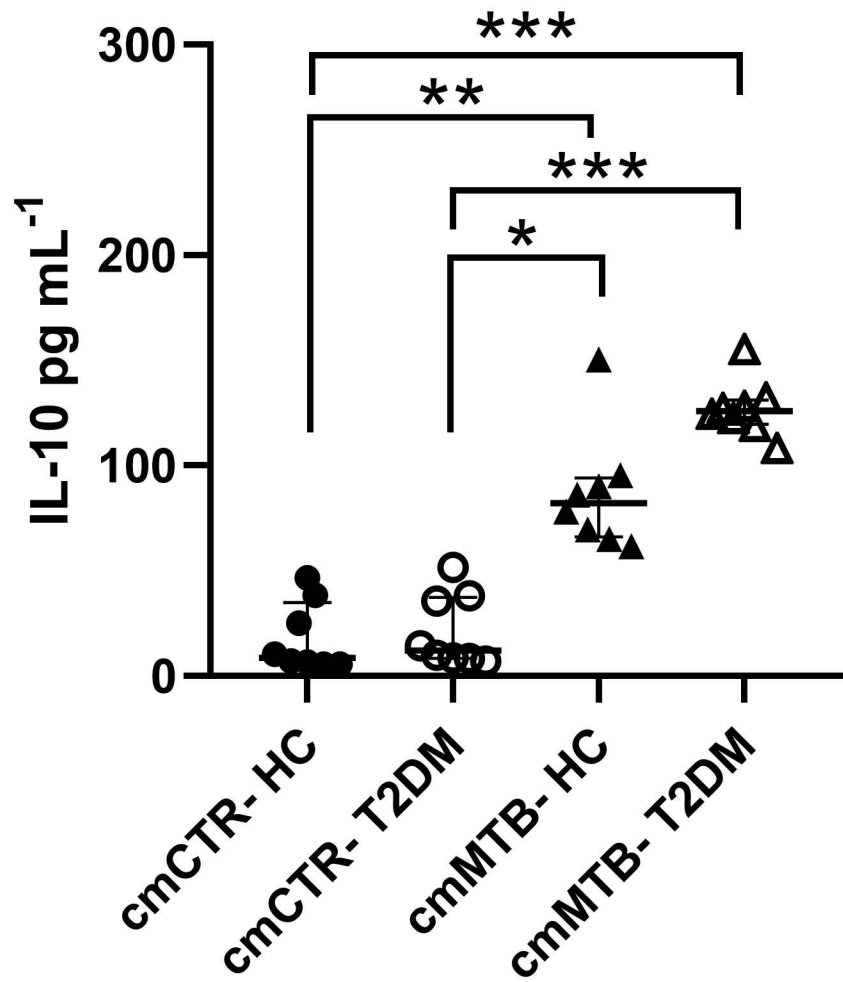
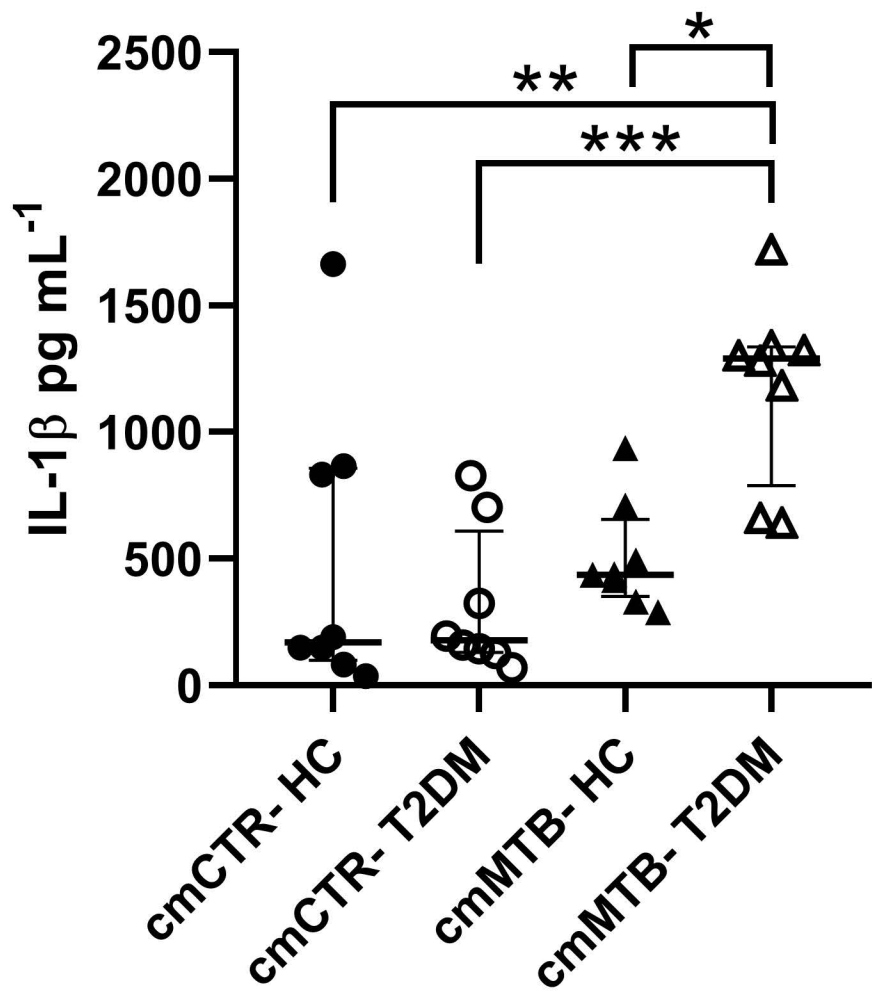


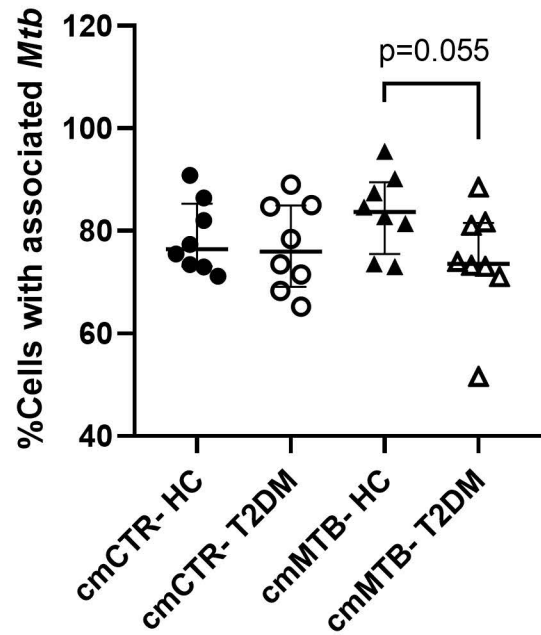
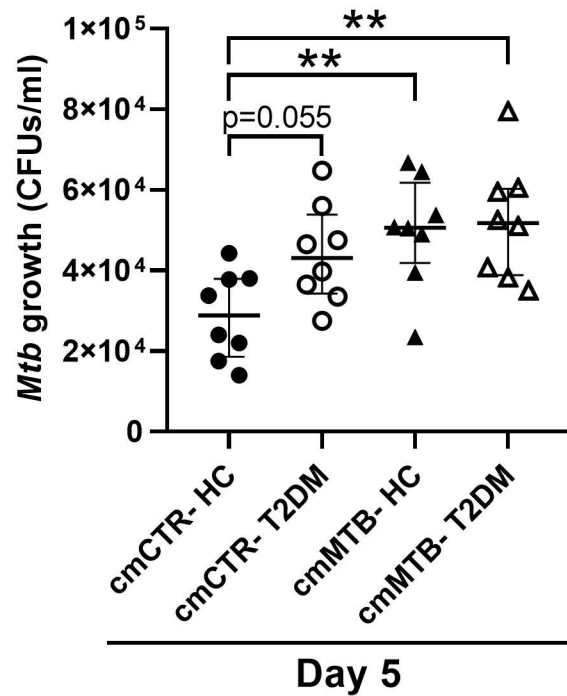
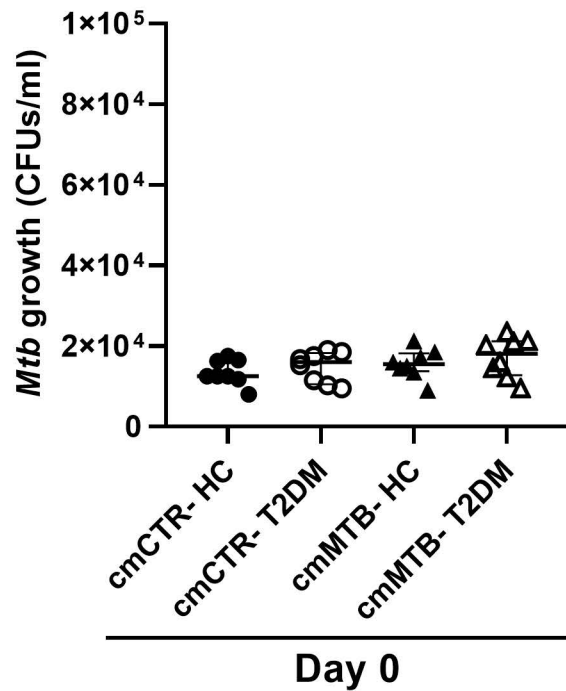
(b)





(a)**(b)**



(a)**(b)**

SUPPLEMENTARY TABLE

Supplementary Table 1. Clinical characteristics of participants for conditioned media pools.

Characteristics	Healthy Controls (HC)	T2DM patients	<i>P</i>
Numbers of cases (n)	9	8	NA
Years with T2DM diagnosis	NA	8.50 ± 4.00	NA
Age, years	47.00 ± 12.50	53 ± 12.00	0.307
BMI (kg/m ²)	24.80 ± 3.31	26.54 ± 6.47	0.113
HbA1c (%)	5.33 ± 0.51	10.54 ± 2.38	<0.0001***
FPG (mg dL ⁻¹)	82.70 ± 13.47	260.10 ± 109.70	<0.0001***
Total cholesterol (mg dL ⁻¹)	161.70 ± 54.00	188.30 ± 73.00	0.209
HDL- cholesterol (mg dL ⁻¹)	55.20 ± 17.90	42.10 ± 10.19	<0.006**
LDL- cholesterol (mg dL ⁻¹)	101.50 ± 54.30	111.50 ± 49.50	0.461
VLDL- cholesterol (mg dL ⁻¹)	19.50 ± 12.00	37.80 ± 49.50	0.0009***
Triglycerides (mg dL ⁻¹)	92.35 ± 54.15	176.50 ± 238.7	<0.0001***
Hypertension, n (%)	0 (0)	4 (50.00)	NA
Metformin, n (%)	0 (0)	6 (75.00)	NA
Glibenclamide, n (%)	0 (0)	5 (62.50)	NA
Metformin + Glibenclamide, n (%)	0 (0)	5 (62.50)	NA

Mann-Whitney *U* were performed to identify differences between groups unless otherwise specified; median (± interquartile range) are shown for all quantitative variables. BMI: body mass index; HbA1c: glycated hemoglobin %; HDL: high density lipoprotein; LDL: low density lipoprotein; VLDL: very low-density lipoprotein; NA: non-applicable.