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TITLE: Glucocorticoid treatment in newly diagnosed patients with immune thrombocytopenia switches CD14^{++}CD16^{+} intermediate monocytes from a pro-inflammatory to an anti-inflammatory phenotype.

Running title: Steroids drive an altered monocyte phenotype in ITP patients

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Summary

Immune thrombocytopenia (ITP) is thought to result from an aberrant adaptive autoimmune response, involving autoantibodies, B and T lymphocytes, directed at platelets and megakaryocytes. Previous reports have demonstrated skewed $CD4^+$ T helper subset distribution and enhanced production of pro-inflammatory cytokines such as IL-17A and IFN-γ. The role of monocytes in ITP is less widely described, but innate immune cells have a role in shaping $CD4^+$ T cell phenotypes. Glucocorticoids (GCs) are commonly used for first line ITP treatment and modulate a broad range of immune cells including T cells and monocytes. Using multiparameter flow cytometry analysis, we demonstrate expansion of intermediate monocytes ($CD14^{++}CD16^+$) in untreated newly diagnosed, ITP patients, with these cells displaying a pro-inflammatory phenotype, characterised by enhanced expression of CD64 and CD80. After 2 weeks of prednisolone treatment (1mg/kg daily), the proportion of intermediate monocytes reduced, with enhanced expression of the anti-inflammatory markers CD206 and CD163. Healthy control monocytes were distinctly different from those from ITP patients before and following GC-treatment. Furthermore, the GC-induced phenotype was not observed in patients with chronic ITP receiving thrombopoietin receptor agonists. These data suggest a role of monocytes in ITP pathogenesis and clinical response to GC-therapy.

Key words

Autoimmunity, Glucocorticoids, Steroids, Immune Thrombocytopenia, Monocyte subsets,

Introduction

Immune thrombocytopenia (ITP) is a rare (2-3/100 000) autoimmune condition characterised by a low platelet count (<100 x 10^9/L) with an increased risk of bleeding and fatigue.[1, 2] It can be categorised clinically as newly diagnosed (<3 months from diagnosis), persistent (3-12 months) or chronic ITP (>12 months).[3, 4] High dose glucocorticoids (GCs) are the recommended first-line treatment for ITP. Patients who fail corticosteroid treatment, either due to lack of response, intolerance or relapse after GC is discontinued, receive second line treatments, such as thrombopoietin receptor agonists (TPO-RA), mycophenolate mofetil (MMF) or rituximab. ITP pathogenesis is driven by B and T cells through antibody- and cell-mediated mechanisms, targeting platelets and megakaryocytes, resulting in increased consumption and decreased production of platelets.[5-7] Patients with ITP have elevated IL-17A and IFNγ producing $CD4^+$ T cells, as well as lower numbers of circulating $FOXP3^+$ Tregs.[8-10] Monocytes (MCs) play a key role in shaping the T cell response through processes of antigen presentation and cytokine production.[11] The contribution of monocytes to the development of ITP is less well understood than classical antibody and T cell-mediated autoimmunity. It is nonetheless likely that monocytes shape these adaptive immune drivers of ITP, as well as the T cell response to treatment.

Human monocytes are classified into three subsets based on their cell surface expression of CD14 (lipopolysaccharide [LPS] co-receptor) and CD16 (activatory Fc gamma receptor III) into classical (C-MCs; $CD14^{++}CD16^-$), intermediate (I-MCs; $CD14^{++}CD16^+$) and non-classical (NC-MCs; $CD14^+CD16^{++}$) monocytes.[12] In many autoimmune conditions, I-MCs are enriched[13] and in the context of inflammation drive enhanced memory T cell proliferation and inflammatory cytokine expression.[14, 15] $CD16^+$ MCs from ITP patients have been shown to specifically promote the expansion of IFN-γ^+CD4^+ T cells and concomitantly inhibit the proliferation of Tregs,[16] providing evidence that MCs shape T cell responses in ITP. However, a detailed MC phenotype in untreated, newly diagnosed patients with active ITP has not yet been described. Different MC subsets have unique abilities to shape T cells with

I-MCs from healthy controls (HC) demonstrating reduced priming of naïve T cells and reduced pro-inflammatory $CD4^+$ T cell polarisation, whilst also promoting secretion of IL-10 by regulatory T cells, implying that I-MC under homeostatic conditions attenuate $CD4^+$ T cell activity.[17]

GCs, such as prednisolone or dexamethasone, aim to suppress aberrant immune responses,[3, 4] and whilst the T cell effect of these drugs has been described,[18] the effect of GCs on MC subsets has been less well researched. GC-treatment of MCs *in-vitro* induces an expansion of $CD16^+$ MCs and induction of an anti-inflammatory monocytic phenotype, characterised by increased CD163 and CD206 expression and increased IL-10 production.[19-22] However, these studies did not investigate the *in-vivo* consequences of GC-treatment on MC subsets. Much of the published data regarding GC effect on immune cellular populations in patients with autoimmune diseases (including ITP) is limited by the heterogeneity of patients, including treatment history, disease severity and chronicity. Study of the *in-vivo* effects of GC treatment is particularly confounded by these variables.

Given previous reports in other autoimmune diseases and alongside observations in ITP, we hypothesised that the circulating $CD14^{++}CD16^+$ I-MC subset would have pro-inflammatory characteristics in untreated patients presenting acutely with active ITP, and that this would be reversed following successful GC-treatment. Here, to further elucidate the potential role of MCs in disease pathogenesis and resolution following GC-treatment, we examine the phenotype of the monocyte subsets prior to and following *in-vivo* GC-treatment in untreated newly diagnosed, ITP patients.

Materials and Methods

Participants

Regulatory approval was granted in accordance with the NHS Health Research Authority (HRA) approval at University Hospitals Bristol NHS Foundation Trust, UK. Untreated, newly diagnosed, ITP patients aged 16 years and over had peripheral blood samples taken at the point of diagnosis prior to treatment and following an average of two weeks prednisolone (1mg/kg daily) treatment, in accordance with international consensus guidelines.[3, 4] Additional patients with varying levels of disease chronicity who had received Thrombopoietin receptor agonist (TPO-RA) therapy, were also recruited (HRA ref: 15/LO/2088). Healthy control (HC) cohorts were recruited at the Bristol Eye Hospital, UHB NHS Foundation Trust (HRA ref: 04/Q2002/84,). All samples were obtained following informed written consent in accordance with the Declaration of Helsinki.

Monocyte and Platelet count

Peripheral blood was collected from patients by sterile venepuncture into ethylenediaminetetraacetic (EDTA) containing tubes. Monocyte and platelet counts were derived from the Full Blood Count (FBC) processed on the Sysmex XN-20 analyser. Samples were not analysed for anti-platelet autoantibodies.

Human cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). MCs were enriched using RosetteSep™ Human Monocyte Enrichment Cocktail (STEMCELL Technologies) according to the manufacturer's instructions.

Monocyte Culture

Isolated MCs were cultured in 24-well low-adherent plates (Corning® Costar®) with 1µM Dexamethasone (Dex) or 100ng/ml Lipopolysaccharide (LPS; Escherichia coli O55:B5)(both Sigma-Aldrich) at a density of $1x10^6$ cells/ml in complete RPMI-1640 supplemented with 10% (v/v) FCS, L-

glutamine, and penicillin/streptomycin (PAA Laboratories) for 24 hours at 37°C in a 5% humidified CO_2 incubator. Cells were harvested by incubation on ice for 15 minutes.

Phenotyping by flow cytometry

PBMCs were assessed using a panel of fluorescent-conjugated antibodies outlined in Supplemental Table I. Cultured monocytes were harvested on ice and stained with CD14, CD16, CD64, CD80, CD163 and CD206. Analysis was performed on a BD Fortessa X20 flow cytometer (BD Biosciences). FMO (fluorescence minus one) controls were used for each fluorochrome. Analysis for t-distributed stochastic neighbour embedding (t-SNE) and sequential cluster analysis was performed using FlowJo 10.6.1. The number of monocytes (identified by live, singlets expressing CD4, CD14 and HLADR) were equalised, at random, from each donor and all samples were concatenated prior to t-SNE analysis. The analysis included 11 markers outlined in Table SI and excluded CD4, SSC-A, FSC-A, FSC-H and the live/dead discriminator.

Statistical analysis

Normality of grouped data was determined, and the statistical significance was assessed by ANOVA (parametric data), Kruskal-Wallis (non-parametric data) or Friedman (paired, non-parametric data) using GraphPad PRISM software 8.2.1. Comparison between individual data sets was determined by either unpaired t-test (parametric data) or Mann-Whitney test (non-parametric data) using GraphPad PRISM software 8.2.1

Results

Intermediate MCs are expanded in untreated newly diagnosed patients with ITP

ITP patient demographics are outlined in Table I. Peripheral blood MCs from 11 untreated newly diagnosed ITP patients, presenting with a platelet count <30 $x10^9$/L, were examined by multiparameter flow cytometry to determine the MC subset phenotype prior to and following GC-treatment (Median of 13 days; range 8-26 days). MCs were gated from isolated PBMCs based on $CD4^{int}$, CD14 and $HLADR^{hi}$ as described by Abeles *et al* [23] and shown in Fig S1. Based on CD14 and CD16 expression (Fig 1A), untreated newly diagnosed ITP patients had an expansion of I-MCs compared with HC (median age 32, range 23-52, all female), with a concurrent reduction of the C-MC. The I-MC and NC-MC populations were reduced following GC-therapy, with a concurrent increase in C-MC (Fig 1A-B). The proportional change in MC subsets was not reflective of an overall change in total MC count prior to or following GC-therapy (Fig 1C). There was a significant increase in platelet count following GC-therapy (Fig 1D).

Monocytes from newly diagnosed ITP patients before and after GC-treatment have distinct phenotypes

The phenotypic differences between whole MCs from HCs and ITP patients prior to (ITP Pre-GC) and following GC-treatment (ITP Post-GC) based on multiparameter flow cytometry were visualised by generating a t-distributed stochastic neighbour embedding (tSNE) analysis and summarised in Table SII. This analysis revealed that MCs from the three cohorts were markedly different from each other (Fig 2A). Clustering analysis identified that whilst some phenotypes were shared between these cohorts, some cellular clusters were unique. For example, cluster 12 is only present in untreated newly diagnosed ITP patients, whereas cluster 6 is only observed in ITP patients following GC-therapy (Fig 2B-C). Detailed examination of these phenotypes in the MC subsets from untreated newly diagnosed, ITP patients demonstrates enhanced expression of pro-inflammatory markers CD64 on all MC subsets and CD80 on C-MCs and I-MCs, but not NC-MCs compared with HC. These pro-inflammatory markers

147 are reduced following GC-therapy on the C-MCs and I-MCs, but not on NC-MCs (Fig 3A-B, respectively).
148 CD206 and CD163 have previously been shown to be upregulated *in-vitro* and *in-vivo* following GC-
149 treatment [21]. In the ITP cohort, CD206 was upregulated on all MC subsets (Fig 3C), with CD163 being
150 specifically upregulated on both I-MCs and NC-MCs, but not C-MCs (Fig 3D) following *in-vivo* GC-
151 treatment. These data suggest that MCs from newly diagnosed ITP have a pro-inflammatory
152 phenotype and this switches to an anti-inflammatory phenotype post-treatment, with the nature of
153 this shift varying between MC subsets.

154 ***Changes in CD163 and CD206 associated with GC-therapy are not seen with TPO-RA treatment***

155 To demonstrate that the observed phenotypic changes were specifically due to GC-therapy and not
156 due to disease resolution, the peripheral blood MC phenotype of chronic ITP patients receiving TPO-
157 RA treatment (n= 9) were investigated. The platelet counts for the chronic ITP patient cohort was
158 below the normal range, although higher than the counts from the untreated newly diagnosed cohort
159 (Fig 1D) (Fig S2A). However, monocyte counts were within normal parameters (Fig S2B). Consistent
160 with the untreated newly diagnosed ITP patients, there was an increase in the proportion of I-MCs
161 and NC-MCs in ITP patients treated with TPO-RA with a concurrent decrease in C-MCs compared with
162 the concurrently analysed HC cohort (median age 27, range 22-44, all female) (Fig 4A). These post-
163 TPO-RA I-MCs did not express increased CD163 or CD206 compared with HCs (Fig 4 B-C, respectively
164 and Table SIII), suggesting that expression of these markers on I-MCs is specific to *in-vivo* GC-
165 treatment.

166 ***GC-treatment* in-vitro *drives an anti-inflammatory MC phenotype***

167 To corroborate the *ex-vivo* MC phenotype during active inflammation following diagnosis or following
168 GC-therapy, MCs from HCs (median age 32, range 23-58, all female) were treated *in-vitro* with either
169 LPS (to mimic inflammation) or dexamethasone (dex, a synthetic GC) for 24 hours under tissue culture
170 conditions. Flow cytometry analysis of *in-vitro* cultured monocytes revealed that, unlike directly *ex-*
171 *vivo* where three monocyte subsets can be detected, there are only two subsets identified: C-MC and

I-MC (Fig 5A). Therefore, further studies were focused on only these two MC subsets. Despite previous reports that GC-treatment increases MC viability,[20, 21] in this study there was good viability amongst all groups and therefore GC-treatment did not augment MC survival (Fig S3). *In-vitro* treatment with dex leads to an enrichment of the I-MCs compared with both non-treated (NT) and LPS treated MCs (Fig 5A-B). This is in contrast with the *ex-vivo* observations following *in-vivo* GC-treatment outlined in Fig 1, but is consistent with our previously reported *in-vitro* data in HCs.[17]

There is no significant increase in CD64 MFI following LPS stimulation compared with non-treated MCs (Fig 5C). However, there is a significant increase in expression of CD80 on both C-MCs and I-MCs following treatment with the inflammatory stimulus LPS compared with both non-treated and dex-treated MCs (Fig 5D). I-MCs significantly upregulate CD80 to a greater extent than C-MCs under the same treatment conditions (Fig 5D). In contrast to *ex-vivo* observations, CD206 is not significantly upregulated following dex treatment (Fig 5E). However, following dex treatment, I-MCs do have greater CD206 expression than the C-MCs in the same culture (Fig 5E). Following dex-treatment I-MCs significantly upregulate CD163 compared with non-treated and LPS treated MCs (Fig 5F). In addition, I-MCs upregulate CD163 to a greater extent following dex treatment than C-MCs, the latter of which show a non-significant increase in CD163 compared with untreated C-MCs or those stimulated with LPS (Fig 5F). Therefore, these data (summarised in table SIV) show that whilst both subsets partly mimic *ex-vivo* phenotypic changes observed in the untreated ITP cohort, the I-MCs are most changed following incubation with either dex or LPS treatment. Furthermore, monocytes isolated from newly diagnosed and chronic ITP patients and treated for 24 hours with dex, showed similar expansion of $CD16^+$ I-MCs (Fig 5G). I-MCs significantly upregulate CD163 compared with C-MCs (Fig 5H). Neither MC populations significantly upregulate CD206 (Fig 5I). This suggests that MCs from ITP patients respond similarly to HC MCs in response to dex *in-vitro*.

Discussion

Studying an untreated newly diagnosed ITP patient cohort (all patients with initial platelet count <30 $x10^9$/L) has avoided confounding factors such as heterogeneous ITP treatments (current and

198 previous), variable disease severity and chronicity. Consistent with previous reports, we observed an
199 expansion of the I-MC population in patients with active ITP, which returns to a normal proportion of
200 the peripheral monocytes following successful treatment.[24] In addition, we demonstrate that MCs
201 differ in their cell surface phenotype in untreated newly diagnosed ITP patients before and after GC-
202 treatment, and in both cases, compared to HC samples. This does however contrast with other
203 autoimmune diseases, where I-MC expansion is seen following *in-vivo* GC-treatment,[17] and suggests
204 that other factors such as different disease-specific autoimmune context may influence monocyte
205 phenotype before and after exposure to GCs. Variable disease duration and severity, or co-
206 administration of other therapies may also play a role. Recently, in a cohort of untreated ITP patients,
207 Manzano and colleagues found an increased proportion of C-MCs and decreased proportion of NC-
208 MCs, but in contrast to our results there was no change in the percentage of I-MCs.[25] However,
209 underlying differences in patient characteristics between studies, such as varying disease chronicity,
210 stability or severity of thrombocytopenia, may explain this. Concordant with our observations,
211 previous work by Liu *et al* demonstrated a reduction of CD64 (FcγRI) and consequentially reduction of
212 opsonised bead phagocytosis, following high-dose *in-vivo* dex treatment.[26] As expression of Fc
213 receptors contributes to ITP pathogenesis,[27] this suggests that reduction of FcγRs following GC-
214 therapy may help treat ITP by reducing antibody-mediated platelet phagocytosis.

215 Monocytes are the precursors of macrophages, the vast diversity of which has been described as the
216 "M1/M2 paradigm". M1-like macrophages are characterised by, but not limited to, expression of CD64
217 and CD80, with these M1 myeloid cells being pro-inflammatory and driving type I inflammatory
218 responses in adaptive immune effector cells. In contrast, M2 macrophages, often expressing CD206
219 and CD163, are wound-healing or anti-inflammatory cells.[28] Our study examines circulating
220 monocytes, which can share characteristics with these M1-like or pro-inflammatory cells and
221 conversely M2-like or anti-inflammatory cells. Specifically, we showed that prior to treatment, all
222 monocyte subsets show a pro-inflammatory, activated phenotype, and this is switched to an anti-
223 inflammatory (or more M2-like) phenotype following *in-vivo* GC therapy. This suggests that systemic

224 administration of GCs, rather than monocytes returning to a homeostatic phenotype, skews MCs to
225 be anti-inflammatory. This study clarifies that all MCs, in particular I-MCs, have the ability to be either
226 pro- or anti-inflammatory depending on the context in which they are activated, shedding light on the
227 dichotomous role of MCs in both health and disease.

228 As the *ex-vivo* anti-inflammatory phenotype (namely CD206 and CD163 upregulation) is not observed
229 with other therapeutic interventions such as TPO-RAs, we can conclude that the upregulation of these
230 molecules is due to GC-specific effects on MCs. This is further supported by previous reports of CD163
231 and CD206 upregulation in MCs treated with GCs *in-vitro*,[21, 29] and as the *in-vitro* upregulation of CD163
232 has been shown here in both newly diagnosed and chronic ITP patients, this phenotypic response to
233 GCs is apparently retained regardless of disease stage. With regard to the upregulation of CD206
234 expression following *in-vitro* dex treatment, our findings were on the margins of significance (Fig 5E;
235 p=0.0515 compared with HC), and given that CD206 regulation by GCs has previously been examined
236 in murine models or at the genomic level, [21, 29, 30] further investigation into the kinetics of CD206
237 protein expression following *in-vitro* GC-treatment in human monocytes is warranted. Consistent with
238 our findings, Manzano *et al* also demonstrated an increase in I-MCs following TPO-RA treatment, but
239 in contrast to our findings they did not observe an increase in the proportion of NC-MCs or decrease
240 in C-MCs compared with HCs[25] (this may again simply reflect differences in our patient cohorts and
241 treatment criteria). Nonetheless, there is a consistent increase in I-MCs across these studies which
242 may reflect ongoing ITP disease activity, as following successful treatment of ITP, MC subsets have
243 previously been found to return to normal proportions.[24, 25] Alternatively, increase in I-MCs may be an
244 indirect TPO-RA effect as TPO-RAs have been associated with other immune changes in B-, T- and Treg
245 cells, and altered production of anti-platelet autoantibodies.[31-33]

246 Under steady state and during acute inflammation, C-MCs are released from the bone marrow and
247 differentiate first into I-MCs and secondly NC-MCs.[34] C-MCs are relatively short-lived (~1day) and
248 differentiate into I-MCs and then the NC-MCs *in-vivo* in humans and in humanised murine models

during homeostasis and following acute inflammatory stimulus.[34] In our study, the observed expansion of the I-MC populations expressing pro-inflammatory markers is likely a persistent phenotype caused by enhanced bone marrow egressing C-MCs and the sequential differentiation into I-MCs due to sustained systemic inflammation. It is not clear whether, following GC-treatment, the phenotypic shift in the I-MCs and NC-MCs is due to suppression and phenotype change of circulating MCs or replacement of the circulating MC populations. Given that I-MCs and NC-MCs, have been shown to persist in the periphery longer than C-MCs (~4.3 days and ~7.4 days respectively) it is most likely that over the 2-week treatment period the pro-inflammatory MCs (observed prior to treatment) are being replaced by fresh bone marrow egressing C-MCs which, due to the systemic GC therapy, differentiate into the anti-inflammatory MCs observed post-GC therapy. Previous studies support this mechanism, demonstrating expansion of a CD14, CD16 and CD163 population *in-vitro* following GC-treatment, with CD163 expression being linked exclusively to GC-treatment.[17, 21, 35, 36] However, our observed reduction in the percentage of circulating $CD16^+$ MCs following GC treatment *in-vivo* has also been reported in multiple sclerosis[37] and in healthy controls.[38] This suggests that there are distinct differences with regards to CD16-expresison following GC-treatment *in-vitro* and *in-vivo*. In addition, the *ex-vivo* MC phenotype of untreated newly diagnosed ITP shows an increase in CD64 on all MC subsets, but this is not observed *in-vitro* following LPS stimulus. LPS treatment *in-vitro* is a single stimulus used to drive inflammatory myeloid cells but does not reflect the complex, multifaceted pro-inflammatory stimuli myeloid cells are exposed to *in-vivo*. Therefore, whilst *in-vitro* models are ideal for studying an individual stimulus, they do not always reflect the complexities of *in-vivo* systems.

It has been widely reported that both newly diagnosed and chronic ITP patients have elevated numbers of IFN-γ and IL-17A producing T cells.[8] In rheumatoid arthritis, MCs from inflamed joints enhance IL-17A production from autologous $CD4^+$ T cells.[39] In ITP, $CD16^+$ MCs from ITP patients promote the expansion of IFN-γ^+CD4^+ T cells [16] and separately, IL-17A production has been shown to correlate with increased $CD68^+$ myeloid cells present in bone marrow of ITP patients.[40] Alongside this, it has been shown that M1-like monocyte derived macrophages (MDMs) generated from circulating

MCs from ITP patients augment cytokine production from $CD4^+$ T cells compared with M2-like MDMs. Taken together, these data demonstrate that inflammatory myeloid cells, such as those observed in this study upon diagnosis, could contribute to enhanced $CD4^+$ T cell activity. In contrast, M2-MDMs from ITP patients differentiated in the presence of dex for seven days, similar to the post-GC MC phenotype observed in our study, had decreased CD80 and increased CD163 expression and were able to suppress $CD4^+$ T cell proliferation and cytokine production,[41] suggesting that GC-drive MCs are able to suppress $CD4^+$ T cell responses. Combined with this, newly diagnosed ITP patients treated with high-dose dexamethasone have enhanced circulating myeloid derived suppressor cells (MDSCs; $CD11b^+CD33^+HLA^-DR$) and M2-like macrophages ($CD68^+CD163^+$).[42] These data suggest that not only are the circulating peripheral MCs in ITP patients shifted to an anti-inflammatory phenotype post-GC treatment, but as they also give rise to M2-like macrophages these circulating GC-MCs may contribute to a reduction in overall immune activation and consequently support disease resolution.

Word count: 3314

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Author Contributions

ELW, MLS, LPSB PJPL and LVJ performed the research and analysed results. ELW, ADD, RWJL and CB designed the research. ELW wrote the paper; all authors reviewed and approved the final manuscript.

Conflict of Interest Disclosures

The authors have no conflicts of interest to disclose.

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Figure legends:

Fig 1. Intermediate monocytes are expanded in untreated newly diagnosed ITP patients. (A) Representative plots showing the expression of CD14 (LPS receptor) and CD16 (Fc-gamma receptor) of monocyte subsets (MC; C; classical, I; Intermediate and NC; Non-classical) from PBMCs isolated from the same untreated ITP patients (lTP Pre; left) and following 2 weeks of GC treatment (ITP Post; right). (B) Cumulative data showing the percentage of each monocyte subset in healthy controls (HC), patients with ITP prior to and following an average of 2 weeks treatment with 1mg/kg/daily prednisolone. (C) Peripheral monocyte counts and (D) platelet counts from the same untreated ITP patients and following on average, 2 weeks of GC-treatment. Dashed line represents the normal laboratory ranges for each measure. Sample size = 11 ITP patients and 10 HC mean +/- SD.

Fig 2. *Ex-vivo* tSNE Analysis of monocytes prior to and following GC-treatment in untreated ITP patients. (A) tSNE analysis of healthy controls (HC), untreated newly diagnosed ITP patients (ITP pre-GC) and paired samples from ITP patients following GC-treatment (ITP post-GC). Monocytes were conventionally gated, samples events normalised, and analysis performed based on a 11-colour flow cytometry panel outlined in Table SI. (b) Cluster analysis based was performed and each phenotypic cluster assigned a colour. (C) Heat map examining relative expression of 11-cell surface markers of each subset from (B). Sample size = 11 ITP patients and 10 HC.

Fig 3. Monocyte subset cell surface expression prior to and following GC-treatment in untreated ITP patients. Cumulative data showing **(A)** CD64 mean fluorescent intensity (MFI), **(B)** percentage CD80, **(C)** CD206 MFI and **(D)** CD163 MFI of each MC subset in healthy controls (HC), patients with ITP prior to (ITP pre) and following (ITP post) 2 weeks treatment with prednisolone. Sample size = 11 ITP patients and 10 HC, mean +/- SD.

Fig 4. Monocytes from chronic ITP patients receiving TPO-RA treatment do not express CD163 and CD206. Cumulative data showing **(A)** percentage of MC subsets, **(B)** CD206 and **(C)** CD163 mean

fluorescent intensity (MFI) expression by each MC subset in healthy controls (HC n=10), or patients with chronic ITP that had received TPO-RA (ITP/TPO-RA; n= 9), Mean +/- SD.

Fig 5. *In-vitro* induction of CD80 following LPS and CD163 following GC-treatment. **(A)** Flow cytometry examples of CD14 and CD16 expressions on monocyte subsets before and following 24h culture with either non treatment (NT), 1μM dexamethasone (dex) or 100ng/ml lipopolysaccharide (LPS). Cumulative data for **(B)** the percentage of CD16-expressing I-MCs following treatment, **(C)** CD64 MFI**, (D)** CD80 MFI **(E)** CD206 MFI and **(F)** CD163 MFI C-MC and I-MC following 24 hour treatment *in-vitro* of HC peripheral blood monocytes. **(G)** percentage of CD16-expressing I-MCs following treatment, **(H)** CD163 MFI and **(I)** CD206 MFI on C-MC and I-MC following 24 h treatment *in-vitro* of MCs from newly diagnosed (ND) or chronic ITP patients. Sample size = 10 HC, 4 untreated newly diagnosed ITP, 4 chronic ITP, mean +/- SD.

Fig S1. Identification of human peripheral blood monocytes by flow cytometry. Monocytes were identified by hierarchical gating based CD4 intermediate expression, CD14 high and HLADR high. Monocyte subsets were identified as classical (C-MCs; $CD14^{++}CD16^{-}$), intermediate (I-MCs; $CD14^{++}CD16^{+}$) and non-classical (NC-MCs; $CD14^{+}CD16^{++}$) monocytes.

Fig S2. Peripheral blood monocyte and platelet counts in ITP patients treated with TPO-RA. **(A)** Monocyte counts and **(B)** platelet counts patients with chronic ITP who had been treated with TPO-RA (n= 9). Dashed line represents the normal laboratory ranges for each measure.

Fig S3. Viability of *in-vitro* monocytes following 24 hour culture. Monocytes were cultured without treatment (non-treated; NT), or with 1μM dexamethasone (dex) or 100ng/ml lipopolysaccharide (LPS) for 24 hours. Cell viability was determined by flow cytometry**.**

Table I: Patient demographics

Ex-vivo phenotyping		
Demographic	**Newly diagnosed (n=11)**	**Chronic ITP (n=9)**
Age (yrs); median (range)	37 (22-80)	72 (37-81)
Female; no. (%)	4 (37)	3 (30)
Treatment when sample taken – no. (%)		
Treatment naïve*	11 (100)	n/a
GC*	11 (100)	n/a
TPO mimetic	n/a	9 (64)
In-vitro phenotyping		
Demographic	**Newly diagnosed (n=4)**	**Chronic ITP (n=4)**
Age (yrs); median (range)	36 (22-74)	72 (35-86)
Female; no. (%)	0 (0)	1 (25)
Treatment when sample taken – no. (%)		
Treatment naïve*	4 (100)	n/a
GC*	n/a	n/a
TPO mimetic	n/a	1 (25)
MMF	n/a	2 (50)
no treatment	4(100)	1(25)

*11 untreated, newly diagnosed patients had a matched post-glucocorticoids (GC) samples taken on average 13 days after initiation of therapy (range 8-26 days).

Table SI: *Ex-vivo* monocyte phenotyping panel

Antigen	Fluorochrome	mAb clone	Source
CD4	FITC	OKT4	Biolegend
CD14	BV785	M5E2	Biolegend
CD16	BV650	3G8	Biolegend
HLADR	BV711	L243	Biolegend
CD64	BV510	10.1	Biolegend
CD80	APC	2D10	Biolegend
CD86	AlexaFluor700	FUN-1	BD Biosciences
CD163	PE-Dazzle594	GHI/61	Biolegend
CD206	PE	15-Feb	Biolegend
CCR2	PerCP-Cy5.5	K036C2	Biolegend
CCR5	BV421	J418F1	Biolegend
CX3CR1	PE-Cy7	2A9-1	Biolegend

Table SII: Mean fluorescence intensity of monocyte subset cell surface markers as determined by *ex-vivo* flow cytometry of ITP patients prior and following glucocorticoid treatment *in-vivo*

Marker	HC C-MC	HC I-MC	HC NC-MC	ITP pre C-MC	ITP pre I-MC	ITP pre NC-MC	ITP post C-MC	ITP post I-MC	ITP post NC-MC
CD14	4567 ± 1570	5794 ± 1582	1271 ± 288.1	4077 ± 2008	5331 ± 2415	941.1 ± 242.8	2708 ± 701.2	4027 ± 1086	992 ± 237.5
CD16	2001 ± 288.5	281.3 ± 47.81	43602 ± 7485	353 ± 339.4	3812 ± 3476	55253 ± 21694	551.2 ± 614.1	2514 ± 1686	59261 ± 19889
CD64	5111 ± 2909	5420 ± 3594	2732 ±2605	2862 ± 1078	3308 ± 1241	2000 ± 1127	3031 ± 948.9	3056 ± 921.5	1130 ± 441.5
CD80	89.5 ± 34.15	-19.5 ± 39.69	-1194 ± 227.8	38.13 ± 62.2	-33.35 ± 68.13	-706.2 ± 645.6	91.38 ± 88.76	-44.86 ± 36.37	-1157 ± 508.4
CD86	153.5 ± 94.08	109.1 ± 154.3	225 ± 208.1	67.74 ± 196.1	-64.18 ± 402.2	-171.6 ± 531.1	284.6 ± 101.2	237 ± 144.8	296.4 ± 103.5
CD163	3650 ± 2455	4784 ± 3126	769.4 ± 424.4	5144 ± 2152	9088 ± 3708	3552 ± 2645	4900 ± 2407	6096 ± 3042	828.7 ± 492.1
CD206	96.61 ± 111	107.6 ± 137.3	180.7 ± 188.4	400.1 ± 364	641.1 ± 655.4	1244 ± 1339	68.41 ± 179.2	61.13 ± 204.1	146.7 ± 218.2
CCR2	5709 ± 5483	4489 ± 4414	197.7 ± 248.5	5320 ± 5132	4749 ± 4529	265.9 ± 380	756 ± 888.6	1244 ± 1372	911.2 ± 1176
CCR5	1438 ± 1294	2141 ± 1661	2026 ± 2398	1737 ± 1704	3119 ± 3214	2778 ± 2878	756 ± 888.6	1244 ± 1372	911.2 ± 1176
CX3CR1	1238 ± 637.5	2420 ± 1230	5031 ± 2998	698 ± 687.6	1168 ± 981.5	1953 ± 1493	1502 ± 596.3	2331 ± 953.3	4478 ± 1776
HLADR	1184 ± 535.1	2588 ± 1048	3951 ± 2197	552.1 ± 334.8	1825 ± 1374	3133 ± 2321	907.5 ± 425.7	2340 ± 1587	2259 ± 1283

Data values represents average mean fluorescence intensity (MFI) ± standard deviation. HC: healthy control; ITP pre: untreated newly diagnosed patients with ITP; ITP post: ITP patient following two weeks of prednisolone treatment; C-MC: classical monocyte; I-MC: intermediate monocyte; NC-MC: non-classical monocyte

Table SIII: Mean fluorescence intensity of monocyte subset cell surface markers as determined by *ex-vivo* flow cytometry of ITP patients receiving TPO-RA treatment *in-vivo*

Marker	HC C-MC	HC I-MC	HC NC-MC	ITP/TPO-RA C-MC	ITP/TPO-RA I-MC	ITP/TPO-RA NC-MC
CD14	3932 ± 1430	5204 ± 1511	1088 ± 251.9	5843 ± 1832	7003 ± 2171	1267 ± 302.5
CD16	1796 ± 289.9	267.2 ± 58.37	41179 ± 6756	2501 ± 401	275.7 ± 52.53	45632 ± 8797
CD163	4109 ± 635.7	5282 ± 700.7	906.3 ± 226.6	3468 ± 681.8	4448 ± 1054	667.4 ± 313.4
CD206	-34.42 ± 21.85	-63.57 ± 18.54	29.09 ± 27.08	82.61 ± 234.2	62.56 ± 240.1	126.4 ± 236.7

Data values represents average mean fluorescence intensity (MFI) ± standard deviation. HC: healthy control; ITP/TPO-RA: patients with chronic ITP treated with TPO-RA; C-MC: classical monocyte; I-MC: intermediate monocyte; NC-MC: non-classical monocyte

Table SIV: Monocyte subset cell surface marker expression following 24 hr *in-vitro* treatment as determined by flow cytometry

Marker	HC - NT C-MC	HC - NT I-MC	HC - dex C-MC	HC - dex I-MC	HC - LPS C-MC	HC - LPS I-MC
CD16 (%)	-	30.62 ± 25.44	-	74.2 ± 20.81	-	28.41 ± 17.82
CD64 (MFI)	422.3 ± 87.48	436 ± 208	330 ± 144.1	502.4 ± 295.5	495.5 ± 111.5	699.3 ± 379.6
CD80 (MFI)	273 ± 152.6	531.3 ± 400.9	291 ± 130.9	474 ± 169.7	681.5 ± 252.5	1217 ± 497.9
CD163 (MFI)	115 ± 155.1	215.1 ± 551	1648 ± 2030	4610 ± 3394	95.32 ± 255.4	438.4 ± 1082
CD206 (MFI)	1018 ± 440.9	1069 ± 424	920.4 ± 556.7	1788 ± 697.7	925.3 ± 371.3	1037 ± 307.7

Marker	ND ITP - NT C-MC	ND ITP - NT I-MC	ND ITP - dex C-MC	ND ITP - dex I-MC
CD16 (%)	-	6.693 ± 5.884	-	56.7 ± 19.48
CD163 (MFI)	21.23 ± 51.84	96.02 ± 100.3	773.5 ± 297.3	2274 ± 564.4
CD206 (MFI)	967 ± 640.1	1256 ± 702.6	1289 ± 536.6	1667 ± 594.6

Marker	chronic ITP - NT C-MC	chronic ITP - NT I-MC	chronic ITP - dex C-MC	chronic ITP - dex I-MC
CD16 (%)	-	17.59 ± 19.9	-	68.98 ± 18.85
CD163 (MFI)	12.47 ± 34.9	61.89 ± 58.32	837 ± 488.2	2475 ± 1049
CD206 (MFI)	967 ± 640.1	1256 ± 702.6	1289 ± 536.6	1667 ± 594.6

Data values represents either average percentage (%) or average mean fluorescence intensity (MFI) ± standard deviation. HC: healthy control; ND ITP: untreated, newly diagnosed patients with ITP; C-MC: classical monocyte; I-MC: intermediate monocyte; NT: non-treated; dex: dexamethasone; LPS: lipopolysaccharide

Fig 1.

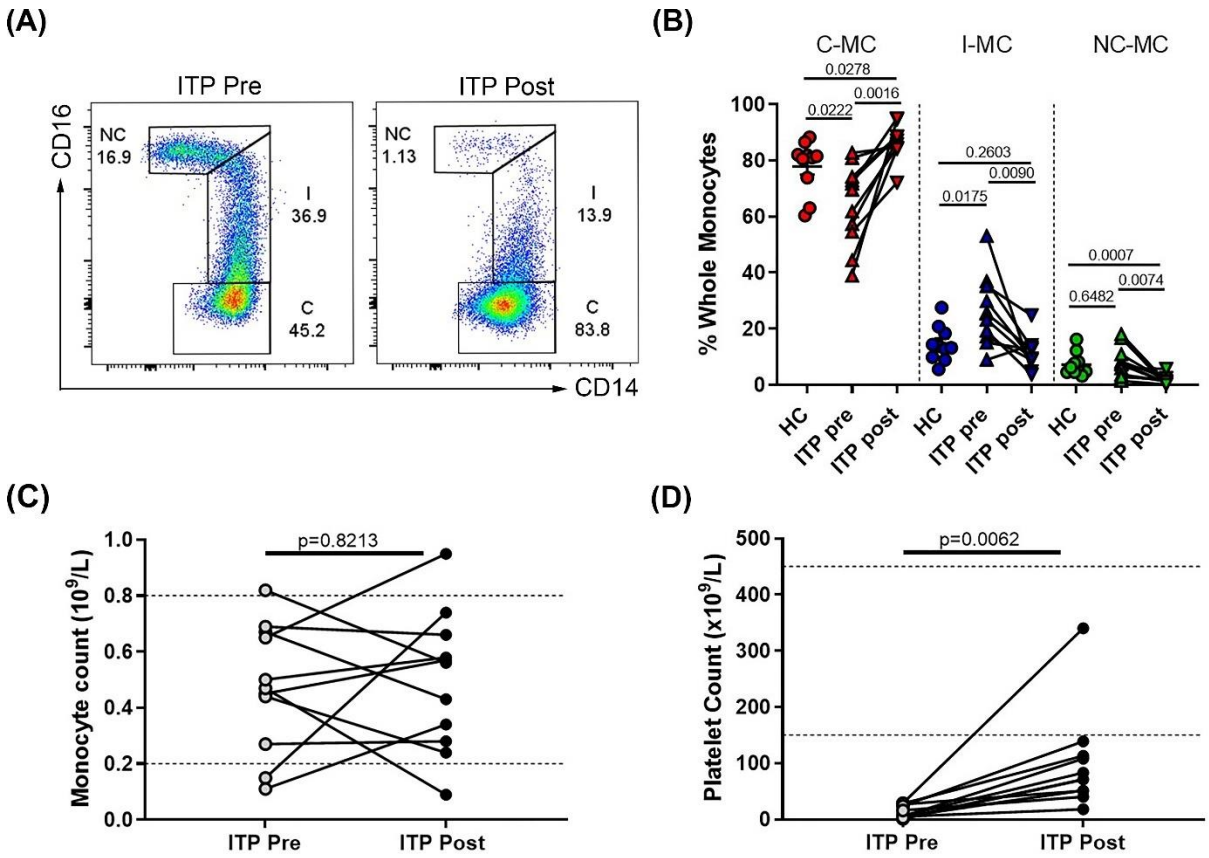

(A)
ITP Pre
ITP Post
CD16
CD14
NC
16.9
I
36.9
C
45.2
NC
1.13
I
13.9
C
83.8
(B)
C-MC
I-MC
NC-MC
% Whole Monocytes
0.0278
0.0222
0.0016
0.2603
0.0090
0.0175
0.0007
0.0074
0.6482
HC
ITP pre
ITP post
(C)
Monocyte count (10^9/L)
p=0.8213
ITP Pre
ITP Post
(D)
Platelet Count (x10^9/L)
p=0.0062
ITP Pre
ITP Post

Fig 2.

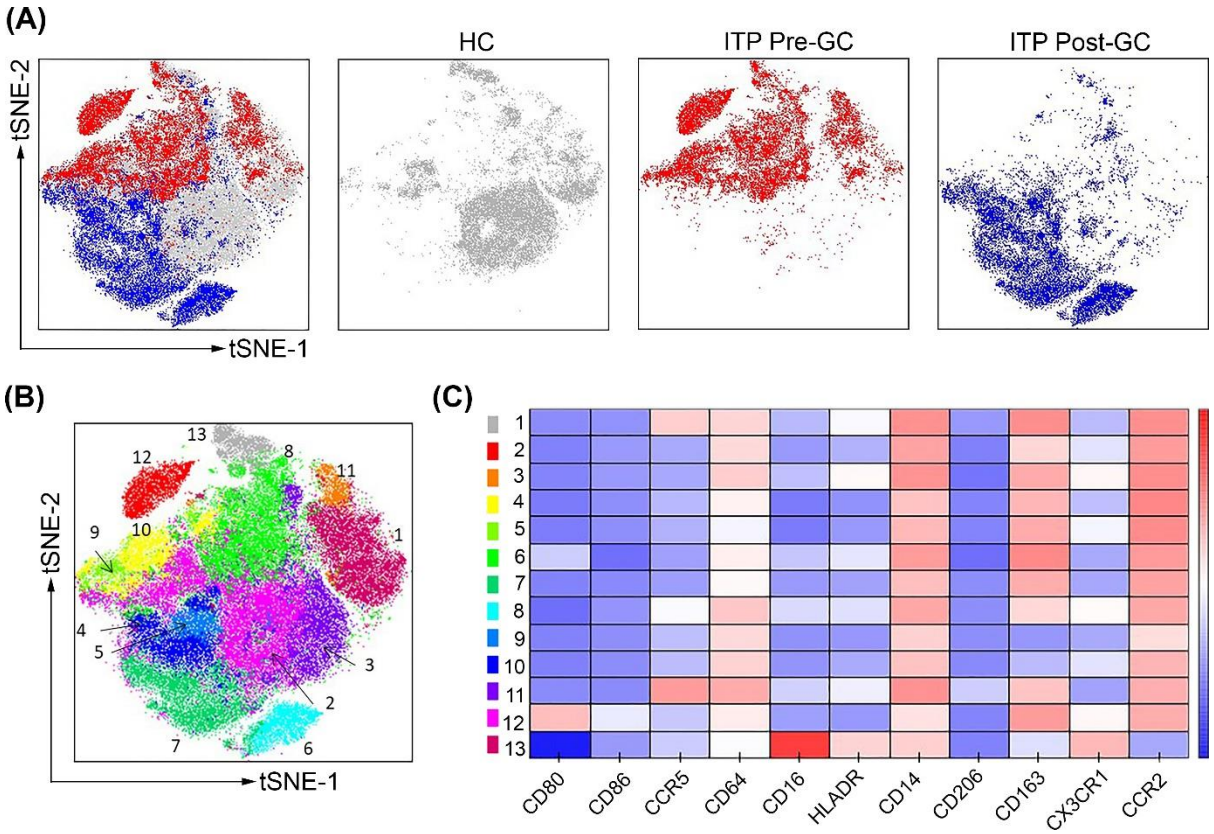
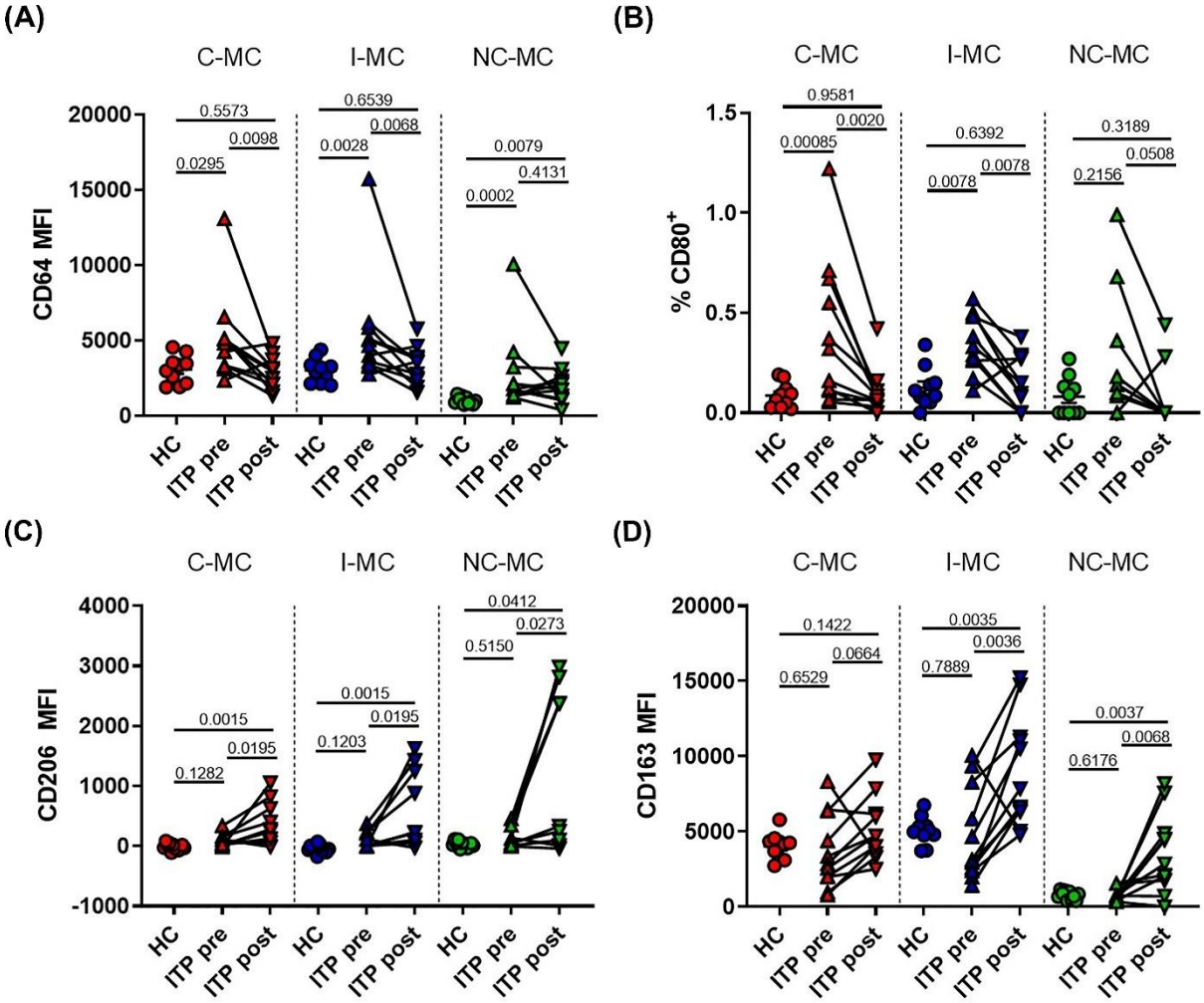

(A)
HC
ITP Pre-GC
ITP Post-GC
tSNE-2
tSNE-1
(B)
tSNE-2
tSNE-1
1
2
3
4
5
6
7
8
9
10
11
12
13
(C)
1
2
3
4
5
6
7
8
9
10
11
12
13
CD80
CD86
CCR5
CD64
CD16
HLADR
CD14
CD206
CD163
CX3CR1
CCR2

Fig 3.


(A)
C-MC
I-MC
NC-MC
CD64 MFI
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15000
10000
5000
0
0.5573
0.0098
0.0295
0.6539
0.0068
0.0028
0.0079
0.4131
0.0002
HC
ITP pre
ITP post
(B)
% CD80+
1.5
1.0
0.5
0.0
0.9581
0.0020
0.00085
0.6392
0.0078
0.0078
0.3189
0.0508
0.2156
(C)
CD206 MFI
4000
3000
2000
1000
0
-1000
0.0015
0.0195
0.1282
0.0015
0.0195
0.1203
0.0412
0.0273
0.5150
(D)
CD163 MFI
20000
15000
10000
5000
0
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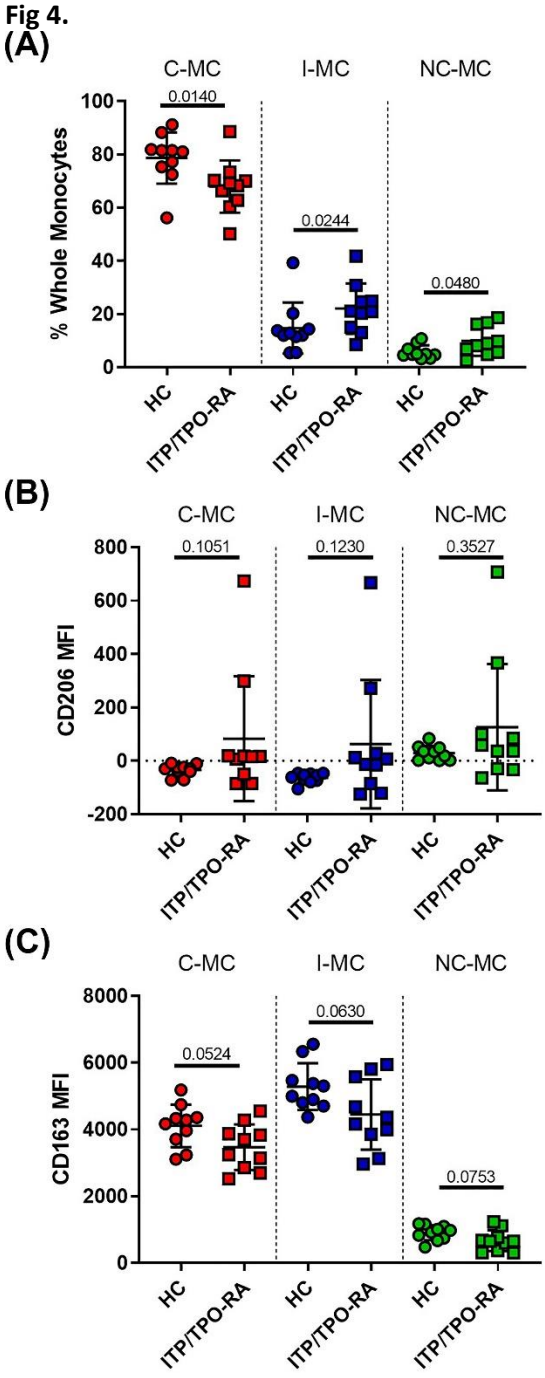
0.0037
0.0068
0.6176

Fig 4.


(A)
C-MC
I-MC
NC-MC
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0.0244
0.0480
% Whole Monocytes
100
80
60
40
20
0
HC
ITP/TPO-RA
(B)
C-MC
I-MC
NC-MC
0.1051
0.1230
0.3527
CD206 MFI
800
600
400
200
0
-200
HC
ITP/TPO-RA
(C)
C-MC
I-MC
NC-MC
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4000
2000
0
HC
ITP/TPO-RA

Fig 5.

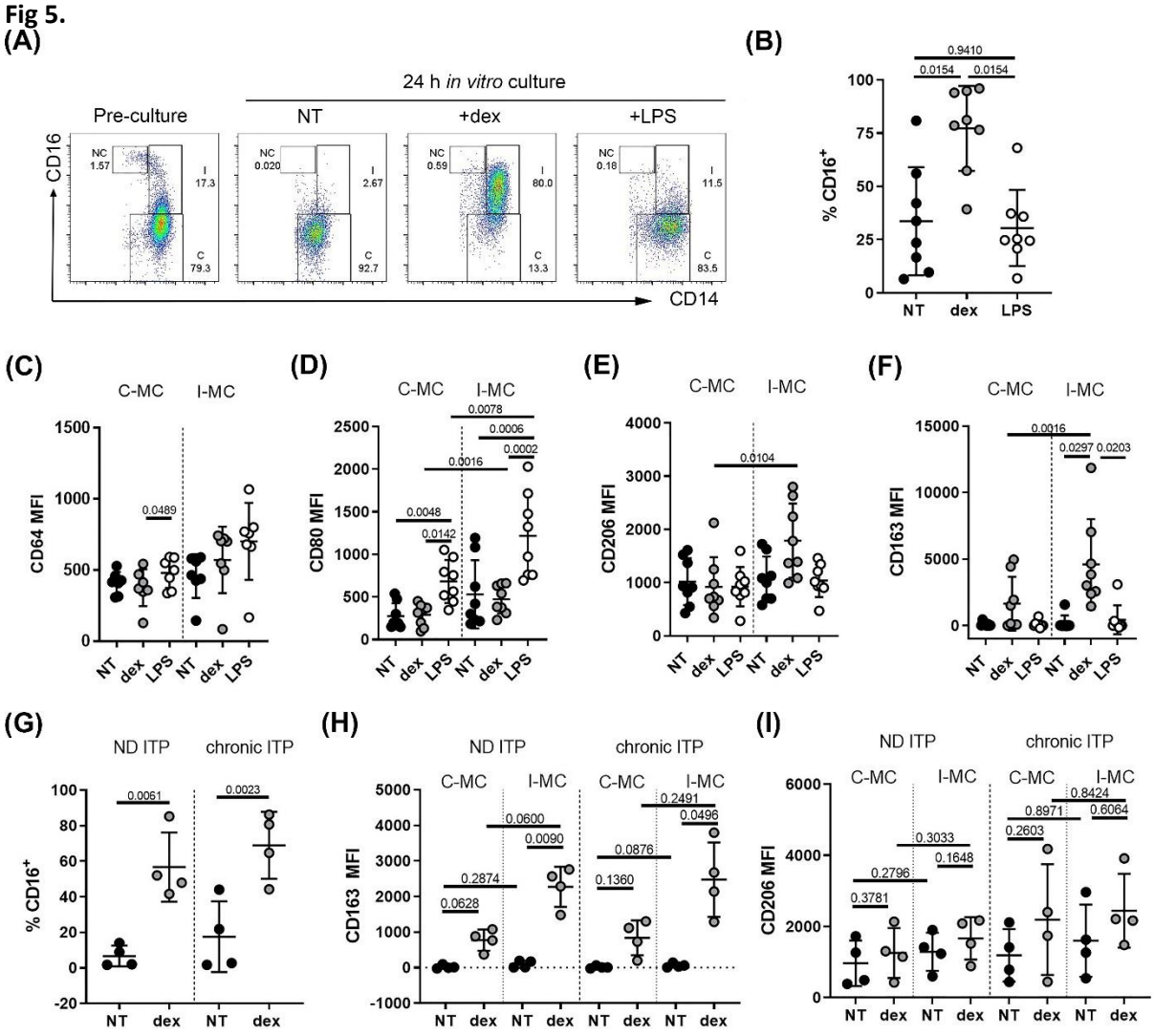
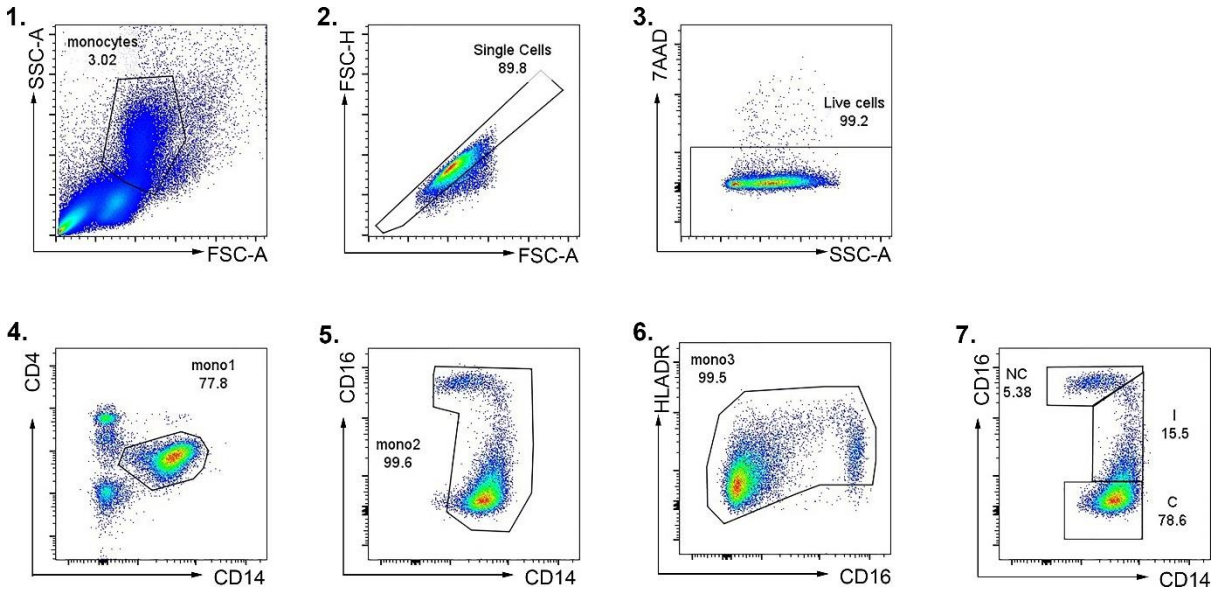

(A)
24 h *in vitro* culture
Pre-culture
NT
+dex
+LPS
CD16
CD14
(B)
% CD16+
NT
dex
LPS
(C)
CD64 MFI
C-MC
I-MC
(D)
CD80 MFI
(E)
CD206 MFI
(F)
CD163 MFI
(G)
ND ITP
chronic ITP
% CD16+
(H)
CD163 MFI
(I)
CD206 MFI

Fig S1.


1.
SSC-A
monocytes
3.02
FSC-A
2.
FSC-H
Single Cells
89.8
FSC-A
3.
7AAD
Live cells
99.2
SSC-A
4.
CD4
mono1
77.8
CD14
5.
CD16
mono2
99.6
CD14
6.
HLADR
mono3
99.5
CD16
7.
CD16
NC
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I
15.5
C
78.6
CD14

Fig S2.

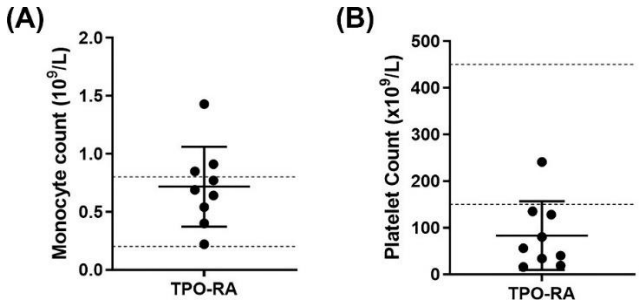
(A)
Monocyte count (10^9/L)
2.0
1.5
1.0
0.5
0.0
TPO-RA
(B)
Platelet Count (x10^9/L)
500
400
300
200
100
0
TPO-RA

Fig S3.