bjh research paper

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

Emily L. Williams,[1] Madeleine L. Stimpson,[1] Philippa J. P. Lait,[1] Lauren P. Schewitz-Bowers,[1] Lauren V. Jones,[1] Ashwin D. Dhanda,[2,3] Richard W. J. Lee[1,4,5] and Charlotte A. Bradbury[1,4]

[1]Translational Health Sciences, Bristol Medical School, University of Bristol, Bristol, [2]Faculty of Health, Peninsula Institute of Health Research, University of Plymouth, Plymouth, [3]South West Liver Unit, Derriford Hospital, University Hospitals Plymouth NHS Trust, Plymouth, [4]University Hospitals Bristol NHS Foundation Trust, Bristol, and [5]Moorfields Eye Hospital NHS Foundation Trust, London, UK

Received 14 July 2020; accepted for publication 17 September 2020

Correspondence: Emily L. Williams, University of Bristol, Biomedical Sciences Building, Bristol, BS8 1TD, UK.
E-mail: emily.williams@bristol.ac.uk

Richard W.J. Lee and Charlotte A. Bradbury contributed equally.

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 interleukin 17A and interferon gamma. The role of monocytes (MCs) 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 MCs. Using multiparameter flow cytometry analysis, we demonstrate the expansion of intermediate MCs ($CD14^{++}CD16^{+}$) in untreated patients with newly diagnosed ITP, with these cells displaying a pro-inflammatory phenotype, characterised by enhanced expression of CD64 and CD80. After 2 weeks of prednisolone treatment (1 mg/kg daily), the proportion of intermediate MCs reduced, with enhanced expression of the anti-inflammatory markers CD206 and CD163. Healthy control MCs were distinctly different than MCs from patients with ITP before and after 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 MCs in ITP pathogenesis and clinical response to GC therapy.

Keywords: autoimmunity, glucocorticoids, steroids, immune thrombocytopenia, monocyte subsets.

Immune thrombocytopenia (ITP) is a rare (2–3/100 000) autoimmune condition characterised by a low platelet count ($<100 \times 10^{9}$/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 GC 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 interleukin 17A (IL-17A) and interferon gamma (IFN-γ) producing $CD4^{+}$ T cells, as well as lower numbers of circulating forkhead box P3 $(FOXP3)^{+}$ regulatory T cells (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 MCs to the development of ITP is less well understood than

doi: 10.1111/bjh.17205

classical antibody and T cell-mediated autoimmunity. It is nonetheless likely that MCs shape these adaptive immune drivers of ITP, as well as the T-cell response to treatment.

Human MCs are classified into three subsets based on their cell surface expression of CD14 [lipopolysaccharide (LPS) co-receptor] and CD16 [activatory Fc gamma receptor III (FcγRIII)] into classical (C-MCs; $CD14^{++}CD16^{-}$), intermediate (I-MCs; $CD14^{++}CD16^{+}$) and non-classical (NC-MCs; $CD14^{+}CD16^{++}$) MCs.[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 patients with ITP have been shown to specifically promote the expansion of IFN-$\gamma^{+}CD4^{+}$ 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 (HCs) demonstrating reduced priming of naïve T cells and reduced pro-inflammatory $CD4^{+}$ T-cell polarisation, while also promoting secretion of IL-10 by Tregs, implying that I-MCs under homeostatic conditions attenuate $CD4^{+}$ T-cell activity.[17]

GCs, such as prednisolone or dexamethasone (dex), aim to suppress aberrant immune responses,[3,4] and while 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. In the present study, to further elucidate the potential role of MCs in disease pathogenesis and resolution following GC treatment, we examined the phenotype of the MC subsets before and after *in vivo* GC treatment in untreated patients with newly diagnosed ITP.

Patients and methods

Participants

Regulatory approval was granted in accordance with the NHS Health Research Authority (HRA) at the University Hospitals Bristol NHS Foundation Trust, UK. Untreated patients with newly diagnosed ITP, aged ≥16 years, had peripheral blood samples taken at the point of diagnosis before treatment and after an average of 2 weeks prednisolone (1 mg/kg daily) treatment, in accordance with international consensus guidelines.[3,4] Additional patients with varying levels of disease chronicity who had received TPO-RA therapy, were also recruited (HRA ref: 15/LO/2088). 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.

MC and platelet count

Peripheral blood was collected from patients by sterile venepuncture into ethylenediaminetetraacetic (EDTA) containing tubes. MC 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, Chicago, IL, USA).™ Human Monocyte Enrichment Cocktail (Stem Cell Technologies UK, Cambridge, UK) according to the manufacturer's instructions.

MC culture

Isolated MCs were cultured in 24-well low-adherent plates (Costar®, Corning, NY, USA) with 1 μmol/l dex or 100 ng/ml LPS (*Escherichia coli* O55:B5) (both Sigma-Aldrich, St. Louis, MO, USA) at a density of 1×10^{6} cells/ml in complete Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% (v/v) fetal calf serum (FCS), L-glutamine, and penicillin/streptomycin (Gibco, Paisley, SC, UK) for 24 h at 37°C in a 5% humidified CO_2 incubator. Cells were harvested by incubation on ice for 15 min.

Phenotyping by flow cytometry

PBMCs were assessed using a panel of fluorescent-conjugated antibodies outlined in Table SI. Cultured MCs 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, San Jose, CA, USA). 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 MCs [identified by live, singlets expressing CD4, CD14 and human

leucocyte antigen-DR isotype (HLA-DR)] 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, side scatter-area (SSC-A), forward scatter (FSC)-A, FSC-height (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 (GraphPad Software Inc., La Jolla, CA, USA). 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 patients with newly diagnosed ITP

The patients with ITP demographics are outlined in Table I. PBMCs from 11 untreated patients with newly diagnosed ITP, presenting with a platelet count of $<30 \times 10^9$/l, were examined by multiparameter flow cytometry to determine the MC subset phenotype before and after GC treatment [median (range) of 13 (8–26) days]. MCs were gated from isolated PBMCs based on $CD4^{int}$, CD14 and $HLA\text{-}DR^{hi}$ as described by Abeles *et al.*[23] and shown in Figure S1. Based on CD14 and CD16 expression (Fig 1A), untreated patients with newly diagnosed ITP had an expansion of I-MCs compared with HC [median (range) age 32 (23–52) years, all female], with a concurrent reduction of the C-MC. The I-MC and NC-MC populations were reduced after 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 before or after GC therapy (Fig 1C). There was a significant increase in platelet count after GC therapy (Fig 1D).

Table I. Patients' demographics.

Demographic	Newly diagnosed (n = 11)	Chronic ITP (n = 9)
Ex vivo phenotyping		
Age, years, median (range)	37 (22–80)	72 (37–81)
Female, *n/N*	4/11	3/9
Treatment when sample taken, *n/N*		
Treatment naïve*	11/11	n/a
GC*	11/11	n/a
TPO mimetic	n/a	9/9
Demographic	**Newly diagnosed (n = 4)**	**Chronic ITP (n = 4)**
In vitro phenotyping		
Age, years, median (range)	36 (22–74)	72 (35–86)
Female, *n/N*	0/4	1/4
Treatment when sample taken, *n/N*		
Treatment naïve*	4/4	n/a
GC*	n/a	n/a
TPO-RA	n/a	1/4
MMF	n/a	2/4
No treatment	4/4	1/4

GC, glucocorticoid, ITP, immune thrombocytopenia; MMF, mycophenolate mofetil; TPO-RA, thrombopoietin receptor agonists.
*11 untreated, newly diagnosed patients had a matched post-glucocorticoids (GCs) sample taken on average 13 days after initiation of therapy (range 8–26 days).

MCs from patients with newly diagnosed ITP before and after GC treatment have distinct phenotypes

The phenotypic differences between whole MCs from HCs and patients with ITP before (ITP pre-GC) and after GC treatment (ITP post-GC) based on multiparameter flow cytometry were visualised by generating a t-SNE 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 while some phenotypes were shared between these cohorts, some cellular clusters were unique. For example, cluster 12 is only present in untreated patients with newly diagnosed ITP, whereas cluster 6 is only observed in patients with ITP after GC therapy (Fig 2B,C). Detailed examination of these phenotypes in the MC subsets from untreated patients with newly diagnosed ITP 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 HCs. These pro-inflammatory markers were reduced after GC therapy on the C-MCs and I-MCs, but not on NC-MCs (Fig 3A,B respectively). CD206 and CD163 have previously been shown to be upregulated *in vitro* and *in vivo* after GC treatment.[21] In the ITP cohort, CD206 was upregulated on all MC subsets (Fig 3C), with CD163 being specifically upregulated on both I-MCs and NC-MCs, but not C-MCs (Fig 3D) following *in vivo* GC treatment. These data suggest that MCs from newly diagnosed ITP have a pro-inflammatory phenotype and that this switches to an anti-inflammatory phenotype after treatment, with the nature of this shift varying between MC subsets.

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

To demonstrate that the observed phenotypic changes were specifically due to GC therapy and not due to disease resolution, the PBMC phenotype of patients with chronic ITP receiving TPO-RA treatment (n = 9) was investigated. The platelet counts for the chronic ITP patient cohort was below

the normal range, although higher than the counts from the untreated newly diagnosed cohort (Fig 1D; Figure S2A). However, MC counts were within normal parameters (Fig S2B). Consistent with the untreated patients with newly diagnosed ITP, there was an increase in the proportion of I-MCs and NC-MCs in patients with ITP treated with TPO-RA with a decrease in C-MCs compared with the concurrently analysed HC cohort [median (range) age 27 (22–44) years, all female] (Fig 4A). These post-TPO-RA I-MCs did not express increased CD163 or CD206 compared with the HCs (Fig 4B,C respectively and Table SIII), suggesting that expression of these markers on I-MCs is specific to *in vivo* GC treatment.

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

To corroborate the *ex vivo* MC phenotype during active inflammation after diagnosis or after GC therapy, MCs from HCs [median (range) age 32 (23–58) years, all female] were treated *in vitro* with either LPS (to mimic inflammation) or dex (a synthetic GC) for 24 h under tissue culture conditions. Flow cytometry analysis of *in vitro* cultured MCs revealed that, unlike directly *ex vivo* where three MC subsets can be detected, there are only two subsets identified: C-MC and I-MC (Fig 5A). Therefore, further studies were focussed on only these two MC subsets. Despite previous reports that GC treatment increases MC viability,[20,21] in the present study there was good viability amongst all groups and therefore GC treatment did not augment MC survival (Figure 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 after *in vivo* GC treatment outlined in Fig 1, but is consistent with our previously reported *in vitro* data in HCs.[17]

There was no significant increase in CD64 mean fluorescent intensity (MFI) after LPS stimulation compared with untreated MCs (Fig 5C). However, there was a significant

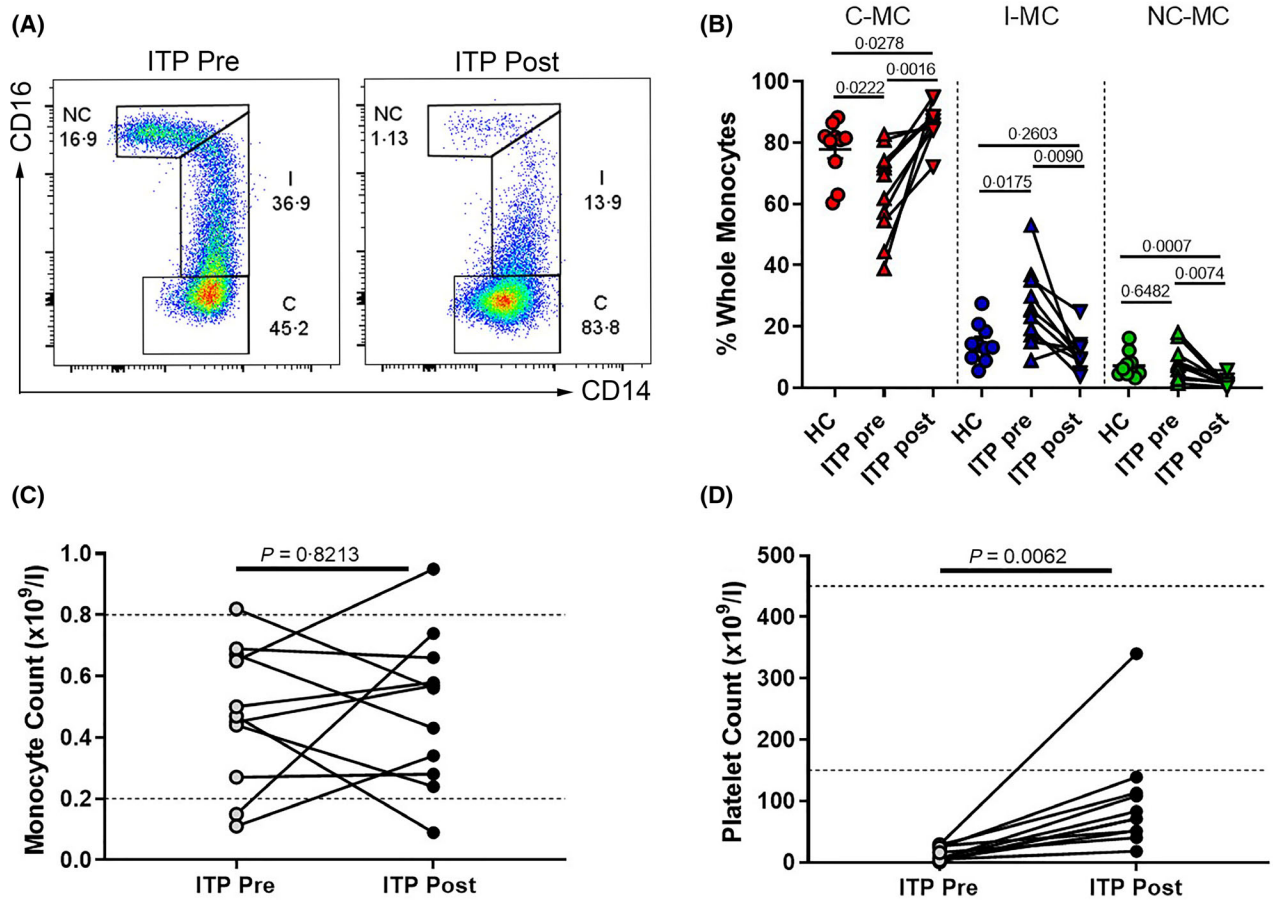

Fig 1. Intermediate monocytes (MCs) are expanded in untreated patients with newly diagnosed immune thrombocytopenia (ITP). (A) Representative plots showing the expression of CD14 (LPS receptor) and CD16 (Fc-γ receptor) on MC subsets (C, classical; I, intermediate and NC, non-classical) from peripheral blood MCs isolated from the same untreated patients with ITP (ITP pre; left) and after 2 weeks of glucocorticoid (GC) treatment (ITP post; right). (B) Cumulative data showing the percentage of each MC subset in healthy controls (HCs), patients with ITP before and after an average of 2 weeks treatment with 1 mg/kg/daily prednisolone. (C) Peripheral MC counts and (D) platelet counts from the same untreated patients with ITP and after on average, 2 weeks of GC treatment. Dashed line represents the normal laboratory ranges for each measure. Sample size = 11 patients with ITP and 10 HCs, mean ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

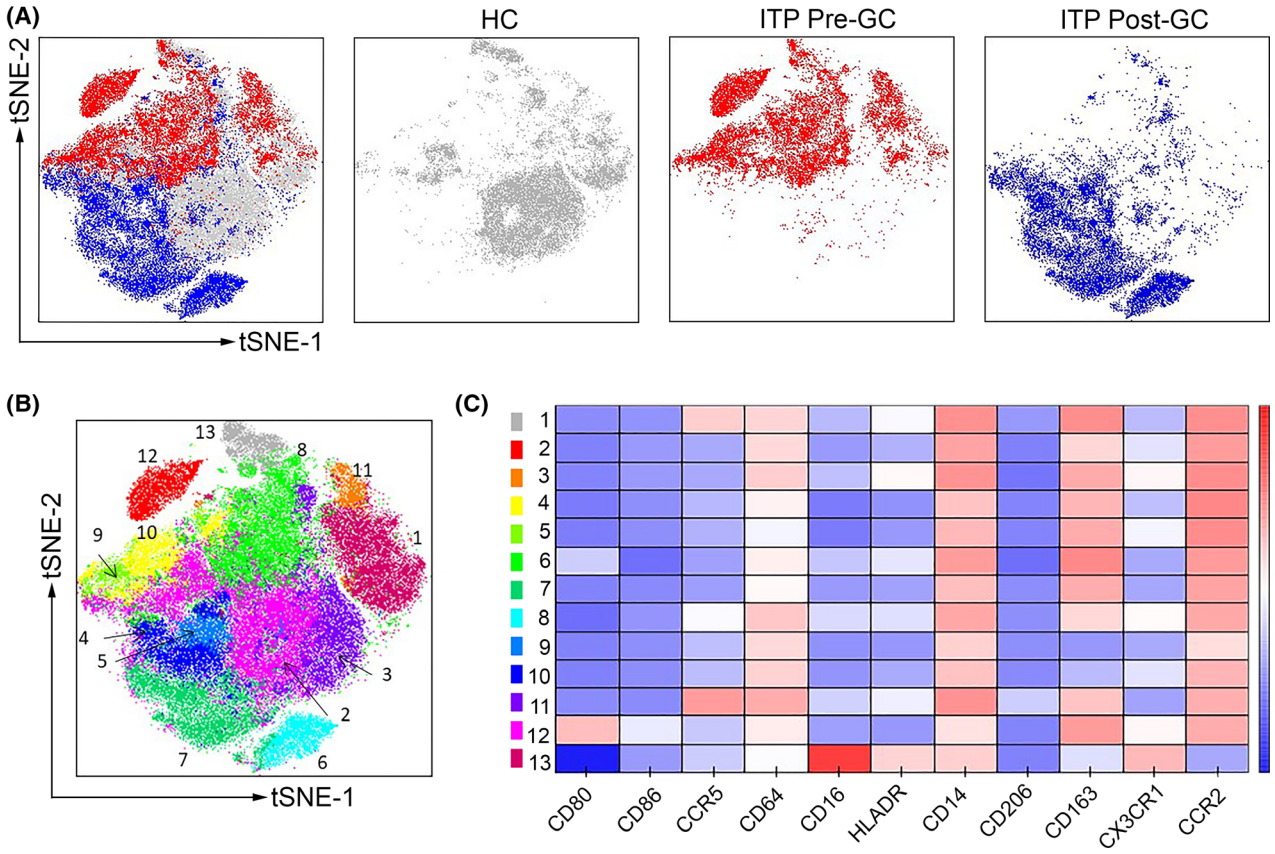

Fig 2. *Ex vivo* t-distributed stochastic neighbour embedding (t-SNE) analysis of monocytes (MCs) before and after glucocorticoid (GC) treatment in patients with untreated immune thrombocytopenia (ITP). (A) t-SNE analysis of healthy controls (HCs), untreated patients with newly diagnosed ITP (ITP pre-GC) and paired samples from patients with ITP after GC treatment (ITP post-GC). MCs 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 patients with ITP and 10 HCs. [Colour figure can be viewed at wileyonlinelibrary.com]

increase in expression of CD80 on both C-MCs and I-MCs after treatment with the inflammatory stimulus LPS compared with both NT- 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 was not significantly upregulated after dex treatment (Fig 5E). However, after dex treatment, I-MCs did have greater CD206 expression than the C-MCs in the same culture (Fig 5E). After dex treatment, the I-MCs had significantly upregulated CD163 compared with NT- and LPS-treated MCs (Fig 5F). In addition, I-MCs upregulated CD163 to a greater extent after dex treatment than C-MCs, the latter of which showed 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 while both subsets partly mimic *ex vivo* phenotypic changes observed in the untreated ITP cohort, the I-MCs are most changed after incubation with either dex or LPS treatment. Furthermore, MCs isolated from patients with newly diagnosed and chronic ITP and treated for 24 h with dex, showed similar expansion of $CD16^{+}$ I-MCs (Fig 5G). I-MCs significantly upregulated CD163 compared with C-MCs (Fig 5H); neither MC population significantly upregulated CD206 (Fig 5I). This suggests that MCs from patients with ITP 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 of $<30 \times 10^9$/l) has avoided confounding factors such as heterogeneous ITP treatments (current and previous), variable disease severity and chronicity. Consistent with previous reports, we observed an expansion of the I-MC population in patients with active ITP, which returns to a normal proportion of the peripheral MCs following successful treatment.[24] In addition, we demonstrate that MCs differ in their cell surface phenotype in untreated patients with newly diagnosed ITP before and after GC treatment, and in both cases, compared to HC samples. However, this does contrast with other autoimmune diseases, where I-MC expansion is seen after *in vivo* GC

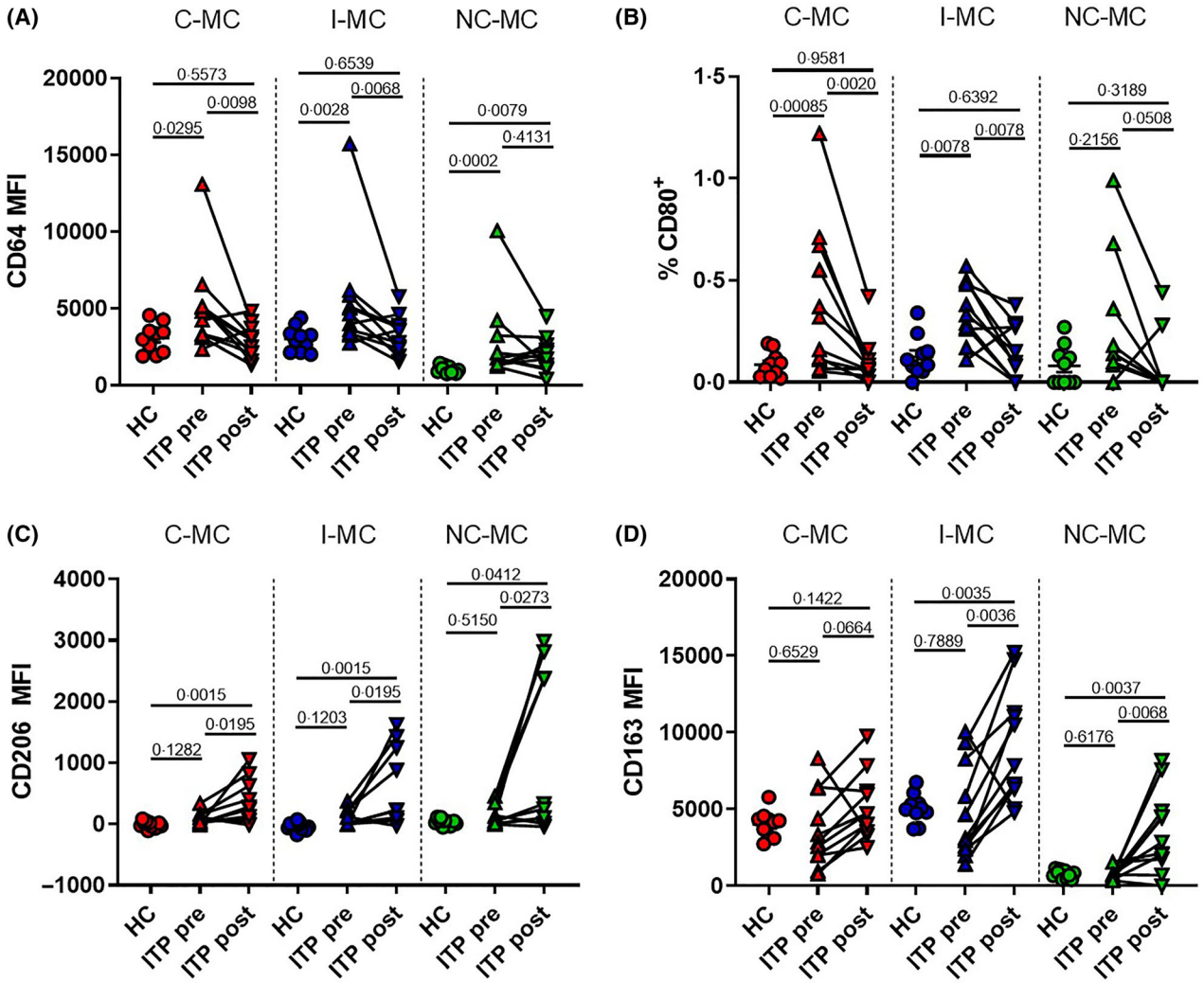

Fig 3. Monocyte (MC) subset cell surface expression before and after glucocorticoid treatment in untreated patients with immune thrombocytopenia (ITP). Cumulative data showing (A) CD64 mean fluorescent intensity (MFI), (B) percentage CD80, (C) CD206 MFI and (D) CD163 MFI of each MC subset in healthy controls (HCs), patients with ITP before (ITP pre) and after (ITP post) 2 weeks treatment with prednisolone. Sample size = 11 patients with ITP and 10 HCs, mean ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

treatment,[17] and suggests that other factors such as different disease-specific autoimmune context may influence MC phenotype before and after exposure to GCs. Variable disease duration and severity, or co-administration of other therapies may also play a role. Recently, in a cohort of untreated patients with ITP, Monzón Manzano *et al.*[25] found an increased proportion of C-MCs and decreased proportion of NC-MCs, but in contrast to our present results there was no change in the percentage of I-MCs. However, the untreated patients in the Monzón Manzano *et al.*[25] study had ITP for a minimum of 6 months, some for >12 months, with average platelet counts of ~100 × 10^9/l (hence not requiring treatment). This contrasts with our present patients with newly diagnosed (acute) untreated ITP with platelet counts of <30 × 10^9/l and a clinical need for prompt treatment. Therefore, it is possible that the differences between these cohorts in terms of disease chronicity, stability and severity of thrombocytopenia, explains the differing observations in the MC-subset proportions. Concordant with our observations, previous work by Liu *et al.*[26] demonstrated a reduction of CD64 (FcγRI) and consequentially a reduction of opsonised bead phagocytosis, following high-dose *in vivo* dex treatment. As expression of Fc receptors contributes to ITP pathogenesis,[27] this suggests that reduction of FcγRs after GC therapy may help treat ITP by reducing antibody-mediated platelet phagocytosis.

MCs are the precursors of macrophages, the vast diversity of which has been described as the 'M1/M2 paradigm'. M1-like macrophages are characterised by, but not limited to, expression of CD64 and CD80, with these M1 myeloid cells being pro-inflammatory and driving type I inflammatory responses in adaptive immune effector cells. In contrast, M2

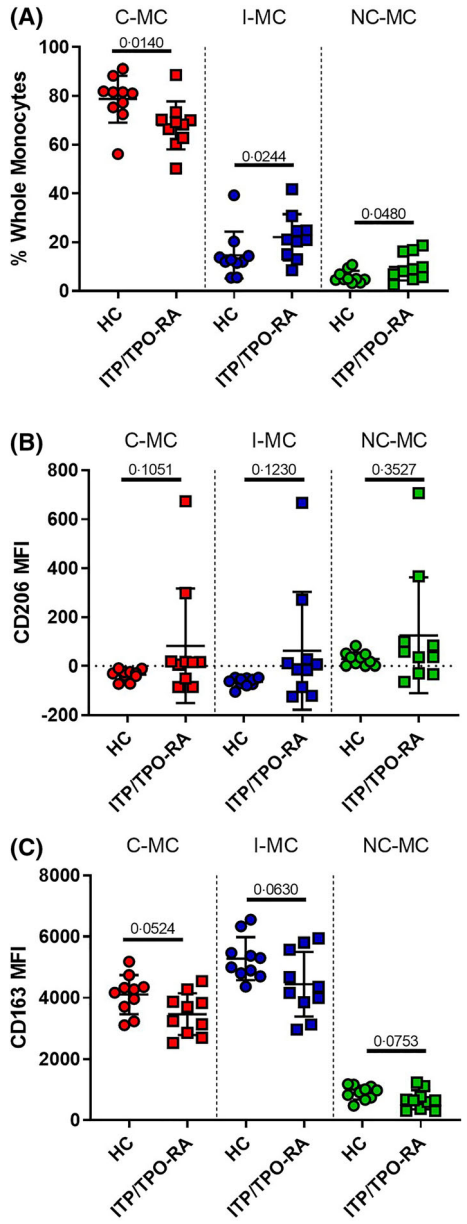

Fig 4. Monocytes (MCs) from patients with chronic immune thrombocytopenia (ITP) receiving thrombopoietin receptor agonist (TPO-RA) treatment do not express CD163 and CD206. Cumulative data showing (A) percentage of MC subsets, (B) CD206 and (C) CD163 mean fluorescent intensity (MFI) expression by each MC subset in healthy controls (HCs, $n = 10$), or patients with chronic ITP that had received TPO-RA (ITP/TPO-RA; $n = 9$), mean ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

macrophages, often expressing CD206 and CD163, are wound-healing or anti-inflammatory cells.[28] Our present study examined circulating MCs, which can share characteristics with these M1-like or pro-inflammatory cells and conversely M2-like or anti-inflammatory cells. Specifically, we showed that prior to treatment, all MC subsets showed a pro-inflammatory, activated phenotype, and this switched to an anti-inflammatory (or more M2-like) phenotype after *in vivo* GC therapy. This suggests that systemic administration of GCs, rather than MCs returning to a homeostatic phenotype, skews MCs to be anti-inflammatory. The present study clarifies that all MCs, in particular I-MCs, have the ability to be either pro- or anti-inflammatory depending on the context in which they are activated, shedding light on the dichotomous role of MCs in both health and disease.

As the *ex vivo* anti-inflammatory phenotype (namely CD206 and CD163 upregulation) is not observed with other therapeutic interventions such as TPO-RAs, we can conclude that the upregulation of these molecules is due to GC-specific effects on MCs. This is further supported by previous reports of CD163 and CD206 upregulation in MCs treated with GCs *in vitro*,[21,29] and as the *in vitro* upregulation of CD163 has been shown in the present study in both patients with newly diagnosed and chronic ITP, this phenotypic response to GCs is apparently retained regardless of disease stage. With regard to the upregulation of CD206 expression after *in vitro* dex treatment, our present findings were on the margins of significance (Fig 5E; $P = 0{\cdot}0515$ compared with HCs), and given that CD206 regulation by GCs has previously been examined in murine models or at the genomic level,[21,29,30] further investigation into the kinetics of CD206 protein expression after *in vitro* GC treatment in human MCs is warranted. Consistent with our present findings, Monzón Manzano *et al.*[25] also demonstrated an increase in I-MCs after TPO-RA treatment, but in contrast to our present findings they did not observe an increase in the proportion of NC-MCs or decrease in C-MCs compared with HCs (this may again simply reflect differences in our patient cohorts and treatment criteria). Nonetheless, there is a consistent increase in I-MCs across these studies, which may reflect ongoing ITP disease activity, as following successful treatment of ITP, MC subsets have previously been found to return to normal proportions.[24,25] Alternatively, increase in I-MCs may be an indirect TPO-RA effect, as TPO-RAs have been associated with other immune changes in B , T and Treg cells, and altered production of anti-platelet autoantibodies.[31–33]

Under steady state and during acute inflammation, C-MCs are released from the bone marrow and differentiate first into I-MCs and secondly into NC-MCs.[34] C-MCs are relatively short-lived (~1 day) and 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 present 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, after 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-

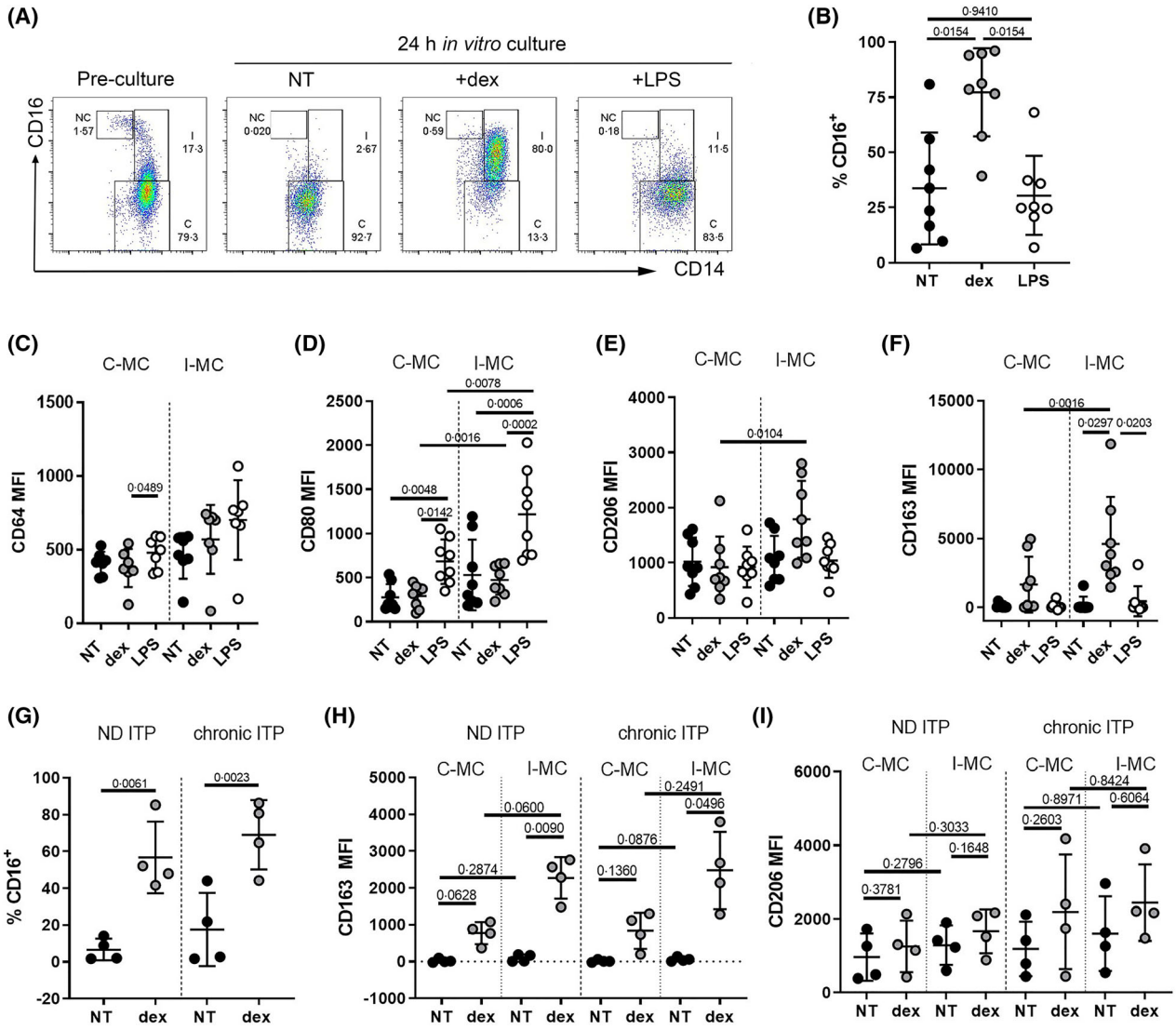

Fig 5. *In vitro* induction of CD80 after LPS and CD163 after glucocorticoid treatment. (A) Flow cytometry examples of CD14 and CD16 expressions on monocyte (MC) subsets before and after 24-h culture with either non-treatment (NT), 1 μmol/l dexamethasone (dex) or 100 ng/ml lipopolysaccharide (LPS). Cumulative data for (B) the percentage of CD16-expressing I-MCs after treatment, (C) CD64 MFI, (D) CD80 MFI (E) CD206 MFI and (F) CD163 MFI of C-MC and I-MC following 24-h treatment *in vitro* of HC peripheral blood MCs. (G) Percentage of CD16-expressing I-MCs after treatment, (H) CD163 MFI and (I) CD206 MFI on C-MC and I-MC after 24-h *in vitro*treatment of MCs from patients with newly diagnosed (ND) or chronic immune thrombocytopenia (ITP). Sample size = 8 HCs, 4 untreated newly diagnosed ITP, 4 chronic ITP, mean ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

MCs and NC-MCs, have been shown to persist in the periphery longer than C-MCs (~4·3 and ~7·4 days respectively) it is most likely that over the 2-week treatment period the pro-inflammatory MCs (observed before treatment) are being replaced by fresh bone marrow egressing C-MCs which, due to the systemic GC therapy, differentiate into the anti-inflammatory MCs seen post-GC therapy. Previous studies support this mechanism, demonstrating expansion of a CD14, CD16 and CD163 population *in vitro* after GC treatment, with CD163 expression being linked exclusively to GC treatment.[17,21,35,36] However, our present observed reduction in the percentage of circulating $CD16^+$ MCs after GC treatment *in vivo* has also been reported in multiple sclerosis[37] and in HCs.[38] This suggests that there are distinct differences with regard to CD16 expression after 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* after 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, while *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 patients with newly diagnosed and chronic ITP 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 patients with ITP 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 patients with ITP.[40] Alongside this, it has been shown that M1-like MC-derived macrophages (MDMs) generated from circulating MCs from patients with ITP 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 the present study upon diagnosis, could contribute to enhanced $CD4^+$ T-cell activity. In contrast, M2-MDMs from patients with ITP differentiated in the presence of dex for 7 days, similar to the post-GC MC phenotype observed in our present study, had decreased CD80 and increased CD163 expression and were able to suppress $CD4^+$ T-cell proliferation and cytokine production,[41] suggesting that GC-derived MCs are able to suppress $CD4^+$ T-cell responses. Combined with this, patients with newly diagnosed ITP treated with high-dose dex have enhanced circulating myeloid-derived suppressor cells ($CD11b^+CD33^+HLA\text{-}DR^-$) and M2-like macrophages ($CD68^+CD163^+$).[42] These data suggest that not only are the circulating peripheral MCs in patients with ITP 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.

Acknowledgements

We thank Andrew Herman and Lorena Sueiro Ballesteros for their flow cytometry support. We also thank the Bristol Eye Hospital Clinical Research Unit for their support in collecting HC peripheral blood samples. This research was funded by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Author Contributions

Emily L. Williams, Madeleine L. Stimpson, Lauren P. Schewitz-Bowers, Philippa J. P. Lait and Lauren V. Jones performed the research and analysed results. Emily L. Williams, Ashwin D. Dhanda, Richard W. J. Lee and Charlotte A. Bradbury designed the research. Emily L. Williams wrote the paper; all authors reviewed and approved the final manuscript.

Conflict of Interest

The authors have no conflicts of interest to disclose.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Identification of human peripheral blood monocytes by flow cytometry.

Fig S2. Peripheral blood monocyte and platelet counts in patients with ITP treated with TPO-RA.

Fig S3. Viability of *in vitro* monocytes following 24-h culture.

Table SI. *Ex vivo* monocyte phenotyping panel.

Table SII. Mean fluorescence intensity of monocyte subset cell surface markers as determined by *ex vivo* flow cytometry of patients with ITP before and after glucocorticoid treatment *in vivo*.

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

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

References

1. Hill QA, Newland AC. Fatigue in immune thrombocytopenia. *Br J Haematol*. 2015;**170**:141–9.
2. Neunert C, Arnold DM. Severe bleeding events in adults and children with primary immune thrombocytopenia: a systematic review: reply. *J Thromb Haemost*. 2015;**13**:1522–3.
3. Provan D, Arnold DM, Bussel JB, Chong BH, Cooper N, Gernsheimer T, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv*. 2019;**3**:3780–817.
4. Neunert C, Terrell DR, Arnold DM, Buchanan G, Cines DB, Cooper N, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia. *Blood Adv*. 2019;**3**:3829–66.
5. Ji X, Zhang L, Peng J, Hou M. T cell immune abnormalities in immune thrombocytopenia. *J Hematol Oncol*. 2014;**7**:72.
6. Cines DB, Cuker A, Semple JW. Pathogenesis of immune thrombocytopenia. *Presse Med*. 2014;**43**:e49–59.
7. McKenzie CG, Guo L, Freedman J, Semple JW. Cellular immune dysfunction in immune thrombocytopenia (ITP). *Br J Haematol*. 2013;**163**:10–23.
8. Zufferey A, Kapur R, Semple JW. Pathogenesis and therapeutic mechanisms in immune thrombocytopenia (ITP). *J Clin Med*. 2017;**6**:16.
9. Ji L, Zhan Y, Hua F, Li F, Zou S, Wang W, et al. The ratio of Treg/Th17 cells correlates with the disease activity of primary immune thrombocytopenia. *PLoS One*. 2012;**7**:e50909.
10. Ogawara H, Handa H, Morita K, Hayakawa M, Kojima J, Amagai H, et al. High Th1/Th2 ratio in patients with chronic idiopathic thrombocytopenic purpura. *Eur J Haematol*. 2003;**71**:283–8.
11. van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med*. 1968;**128**:415–35.

12. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010;**116**:e74–80.
13. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. *Immunol Res*. 2012;**53**:41–57.
14. Dhanda AD, Williams EL, Yates E, Lait PJ, Schewitz-Bowers LP, Hegazy D, et al. Intermediate monocytes in acute alcoholic hepatitis are functionally activated and induce IL-17 expression in CD4(+) T cells. *J Immunol*. 2019;**203**:3190–8.
15. Evans HG, Suddason T, Jackson I, Taams LS, Lord GM. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. *Proc Natl Acad Sci USA*. 2007;**104**:17034–9.
16. Zhong H, Bao W, Li X, Miller A, Seery C, Haq N, et al. CD16+ monocytes control T-cell subset development in immune thrombocytopenia. *Blood*. 2012;**120**:3326–35.
17. Liu B, Dhanda A, Hirani S, Williams EL, Sen HN, Martinez Estrada F, et al. CD14++CD16+ monocytes are enriched by glucocorticoid treatment and are functionally attenuated in driving effector T cell responses. *J Immunol*. 2015;**194**:5150–60.
18. Li J, Wang Z, Hu S, Zhao X, Cao L. Correction of abnormal T cell subsets by high-dose dexamethasone in patients with chronic idiopathic thrombocytopenic purpura. *Immunol Lett*. 2013;**154**:42–8.
19. Hogger P, Dreier J, Droste A, Buck F, Sorg C. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J Immunol*. 1998;**161**:1883–90.
20. Barczyk K, Ehrchen J, Tenbrock K, Ahlmann M, Kneidl J, Viemann D, et al. Glucocorticoids promote survival of anti-inflammatory macrophages via stimulation of adenosine receptor A3. *Blood*. 2010;**116**:446–55.
21. Ehrchen J, Steinmuller L, Barczyk K, Tenbrock K, Nacken W, Eisenacher M, et al. Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. *Blood*. 2007;**109**:1265–74.
22. Tsianakas A, Varga G, Barczyk K, Bode G, Nippe N, Kran N, et al. Induction of an anti-inflammatory human monocyte subtype is a unique property of glucocorticoids, but can be modified by IL-6 and IL-10. *Immunobiology*. 2012;**217**:329–35.
23. Abeles RD, McPhail MJ, Sowter D, Antoniades CG, Vergis N, Vijay GK, et al. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure. *Cytometry A*. 2012;**81**:823–34.
24. Yang Y, Zhang X, Zhang D, Li H, Ma L, Xuan M, et al. Abnormal distribution and function of monocyte subsets in patients with primary immune thrombocytopenia. *Clin Appl Thromb Hemost*. 2017;**23**:786–92.
25. Monzon Manzano E, Alvarez Roman MT, Justo Sanz R, Fernandez Bello I, Hernandez D, Martin Salces M, et al. Platelet and immune characteristics of immune thrombocytopaenia patients non-responsive to therapy reveal severe immune dysregulation. *Br J Haematol*. 2020;**189**:943–53.
26. Liu XG, Ma SH, Sun JZ, Ren J, Shi Y, Sun L, et al. High-dose dexamethasone shifts the balance of stimulatory and inhibitory Fcgamma receptors on monocytes in patients with primary immune thrombocytopenia. *Blood*. 2011;**117**:2061–9.
27. Norris PA, Segel GB, Burack WR, Sachs UJ, Lissenberg-Thunnissen SN, Vidarsson G, et al. FcγRI and FcγRIII on splenic macrophages mediate phagocytosis of anti-glycoprotein IIb/IIIa autoantibody-opsonized platelets in immune thrombocytopenia. *Haematologica*. 2020 [Online ahead of print]. https://doi.org/10.3324/haematol.2020.248385
28. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014;**6**:13.
29. Shepherd VL, Konish MG, Stahl P. Dexamethasone increases expression of mannose receptors and decreases extracellular lysosomal enzyme accumulation in macrophages. *J Biol Chem*. 1985;**260**:160–4.
30. Meers GK, Bohnenberger H, Reichardt HM, Luhder F, Reichardt SD. Impaired resolution of DSS-induced colitis in mice lacking the glucocorticoid receptor in myeloid cells. *PLoS One*. 2018;**13**:e0190846.
31. Bao W, Bussel JB, Heck S, He W, Karpoff M, Boulad N, et al. Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents. *Blood*. 2010;**116**:4639–45.
32. Li X, Zhong H, Bao W, Boulad N, Evangelista J, Haider MA, et al. Defective regulatory B-cell compartment in patients with immune thrombocytopenia. *Blood*. 2012;**120**:3318–25.
33. Kapur R, Aslam R, Speck ER, Rebetz JM, Semple JW. Thrombopoietin receptor agonist (TPO-RA) treatment raises platelet counts and reduces anti-platelet antibody levels in mice with immune thrombocytopenia (ITP). *Platelets*. 2020;**31**:399–402.
34. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J Exp Med*. 2017;**214**:1913–23.
35. Heideveld E, Hampton-O'Neil LA, Cross SJ, van Alphen FP, van den Biggelaar M, Toye AM, et al. Glucocorticoids induce differentiation of monocytes towards macrophages that share functional and phenotypical aspects with erythroblastic island macrophages. *Haematologica*. 2018;**103**:395–405.
36. Vakili S, Fischer T, Rappaport J. M2 differentiation of MonoMac-1 cell line induced by M-CSF and glucocorticoid pathways. *J Cell Physiol*. 2020;**235**:7383–91.
37. Fingerle-Rowson G, Angstwurm M, Andreesen R, Ziegler-Heitbrock HW. Selective depletion of CD14+ CD16+ monocytes by glucocorticoid therapy. *Clin Exp Immunol*. 1998;**112**:501–6.
38. Dayyani F, Belge KU, Frankenberger M, Mack M, Berki T, Ziegler-Heitbrock L. Mechanism of glucocorticoid-induced depletion of human CD14+CD16+ monocytes. *J Leukoc Biol*. 2003;**74**:33–9.
39. Evans HG, Gullick NJ, Kelly S, Pitzalis C, Lord GM, Kirkham BW, et al. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proc Natl Acad Sci USA*. 2009;**106**:6232–7.
40. Okamoto N, Homma M, Kawaguchi Y, Kabasawa N, Uto Y, Hattori N, et al. Increased expression of interleukin-17 is associated with macrophages in chronic immune thrombocytopenia. *Int J Clin Exp Pathol*. 2018;**11**:2419–29.
41. Feng Q, Xu M, Yu YY, Hou Y, Mi X, Sun YX, et al. High-dose dexamethasone or all-trans-retinoic acid restores the balance of macrophages towards M2 in immune thrombocytopenia. *J Thromb Haemost*. 2017;**15**:1845–58.
42. Shao X, Wu B, Cheng L, Li F, Zhan Y, Liu C, et al. Distinct alterations of CD68(+)CD163(+) M2-like macrophages and myeloid-derived suppressor cells in newly diagnosed primary immune thrombocytopenia with or without CR after high-dose dexamethasone treatment. *J Transl Med*. 2018;**16**:48.

