Dear editor,

We thank you for your advice, and we would like to re-submit our revised manuscript entitled “Different Phenotypes of Monocytes in Patients with New-Onset Mild Acute Pancreatitis” （ESPS Manuscript NO: 30400）for your further consideration as a basic research article for publication in *World Journal of Gastroenterology*.

We have clarified several issues in the revision and believe that we have addressed all of the concerns raised by the reviewers. The major changes in the revision have been marked in red. Please see our point-by-point responses below. In addition, we have carefully checked every sentence in the revision to eliminate/reduce any potential syntax error and this manuscript has been proofread by two native English biologists from *Medjaden*, a professional publication service company. We think that this manuscript is easily understood in terms of a scientific story and its language writing.

If I can be of any assistance regarding the process of this manuscript, please feel free to contact me. I look forward to hearing from you soon.

Sincerely,

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Responses to the Editor

Thank you for your advice. We have carefully revised the manuscript, according to the guidelines of your journal. In addition, we have carefully checked every sentence in the revision to eliminate potential syntax errors.

Please provide language certificate letter by professional English language editing companies (Classification of manuscript language quality evaluation is B).

Response: This manuscript has been proofread by two native English biologists from Medjaden, a professional publication service company. Please see the language certificate from *Medjaden Bioscience Limited* (attached).

Please provide the fixed line number.

Response: We have provided the fixed line numbers in the manuscript (Page 2, line 53).

Audio core tip:

In order to attract readers to read your full-text article, we request that the author make an audio file describing your final core tip, it is necessary for final acceptance. Please refer to Instruction to authors on our website or attached Format for detailed information.

Response: We have made an audio file describing our final core tip (attached).

Responses to the reviewers

Reviewed by 03558529,

This is a good study with detailed and well-performed phenotyping of monocyte populations which shows that changes to these populations can be detected early on in MAP pathogenesis, correlate well to clinical parameters (CRP) and may be a useful tool in diagnosis. The combination of markers used is unusual however the authors have found some interesting differences in

monocyte subtypes which could have implications for MAP screening. Although monocytes and macrophages are already known to be important role in the pathophysiology of MAP, the use of new-onset patients in this study makes the findings particularly novel and significant.

Comment 1. My main concern is the confusion in the manuscript between monocytes and macrophages. The use of M1-macrophage and M2-macrophage to describe the cells analysed in this study is misleading and should be changed. The terms monocyte and macrophage are used interchangeably to describe the cells which is incorrect in this instance. The cells in this study (monocytes) are described as being M1-like or M2-like which is a property of macrophages, not monocytes. I presume this is why many of markers used for the study are actually typical for analysis of macrophages rather than monocytes. In any case I think it would be more appropriate to refer to the cells in this study as pro-inflammatory and classical monocytes. Lines 115 to 117: 'Macrophages and monocytes are heterogeneous cell populations. Under an inflammatory condition, blood monocytes can maturate into macrophages, which are further activated.' It would be more accurate to state that monocytes are circulating blood cells which differentiate into macrophages when they enter the tissue. A lot of the introduction discusses M1 and M2 macrophages and their properties. However the present study investigates monocytes, not macrophages. Although monocytes can give rise to macrophages, they are not the same cell type, they have different cell markers and different activation states and properties. M1 and M2 polarization are properties of macrophages, not monocytes. It should be made clear in the text the difference between the two. For example line 143 'In this study, we characterized the numbers of different subsets of macrophages 'this is incorrect. Further discussion of monocyte subsets and markers should be given in the introduction, rather than macrophage subsets. I think that the use of M1 and M2 to describe the cells analysed in this study is misleading and

should be minimized.

Response: We understand his/her constructive comments. We realized the cells we studied were monocytes, and we have changed the term in the revision. I want to explain one question here. Monocytes and macrophages belong to the monocyte–macrophage lineage and are considered cell populations that may be adapted and respond to a wide variety of signs in their environment. It is well known that macrophages can be classified into two subpopulations, classically activated (M1) and alternatively activated/ (M2). In response to your concerns, we had searched some literatures. We found that the M1 and M2 classification, initially proposed for macrophages, can be extended to human peripheral blood monocytes [**Medeiros LT**, Peraçoli JC, Bannwart-Castro CF, Romão M, Weel IC, Golim MA, de Oliveira LG, Kurokawa CS, Medeiros Borges VT, Peraçoli MT. Monocytes from pregnant women with pre-eclampsia are polarized to a M1 phenotype. Am J Reprod Immunol. 2014; 72: 5-13] [**Babu S**, Kumaraswami V, Nutman TB. Alternatively activated and immunoregulatory monocytes in human filarial infections. J Infect Dis. 2009; 199: 1827-37]. The expression of both M1 and M2 markers is detected in circulating peripheral blood mononuclear cells [**Satoh N**, Shimatsu A, Himeno A, Sasaki Y, Yamakage H, Yamada K, Suganami T, Ogawa Y. Unbalanced M1/M2 phenotype of peripheral blood monocytes in obese diabetic patients: effect of pioglitazone. Diabetes Care. 2010; 33: e7]. Accordingly, we preferred to use M1 and M2 monocytes in the revision. In addition, we have rephrased the sentence ‘Macrophages and monocytes are heterogeneous cell populations. Under an inflammatory condition, blood monocytes can maturate into macrophages, which are further activated’ into “Monocytes are circulating while blood cells, which differentiate into macrophages when they enter the tissue” (Page 6, line 144-145).

Comment 2. A reasoning for the flow cytometry markers selected and gating strategy should be given. Monocytes are primarily distinguished by CD14 and

CD16 as classical (CD14(++)CD16(-)), intermediate (CD14(++)CD16(+)) and nonclassical/pro-inflammatory (CD14(+)CD16(++)) monocytes. Why was CD16 omitted from this study? Why was CD163 used instead?

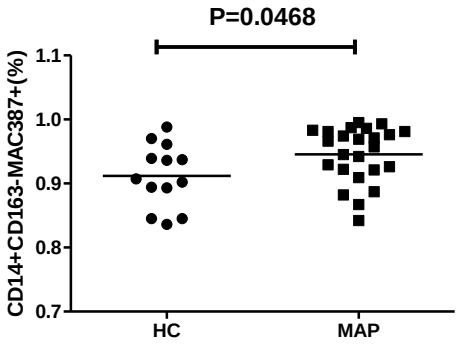
Response: We appreciate his/her advice. We have added the reasons for the flow cytometry markers selected and gating strategy (Page6, line 154-158; Page7, line 162-178). Monocytes can be classified into 3 subsets by CD14 and CD16. However, the 3 subsets of monocytes were not the cells we wanted to analyze. We analyzed the monocytes from another point of view, and we wanted to know which polarization status of peripheral blood monocytes presented in the MAP patients. That is why we choose CD163 instead of CD16.

Comment 3. It would be good to include the ratio between CD14+CD163- and CD14+CD163+ monocytes in Figure 1. The ratio between inflammatory vs non-inflammatory monocytes is as important as changes to overall number because they can balance each others actions.

Response: We appreciate his/her advice. We now include the ratio between CD14+CD163- and CD14+CD163+ monocytes in Figure 1 (Page 30).

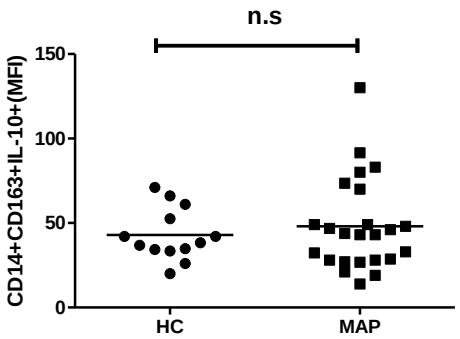
Comment 4. Figure 2. What is the percentage of CD14+CD163- cells which are positive for MAC387? Are there more CD14+CD163-MAC387+ cells in the MAP patients simply because there are more CD14+CD163- cells in the MAP patients? Or is a greater percentage of the CD14+CD163- expressing MAC387 in the MAP patients?

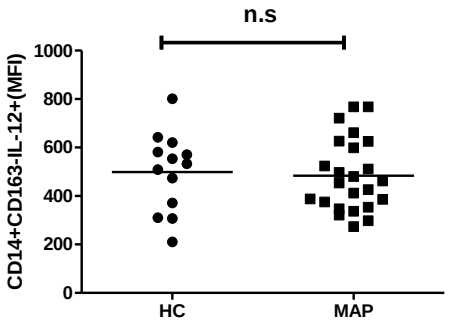
Response: Thank you for your comments. We compared the percentages of CD14+CD163-MAC387+ monocytes between the MAP patients and HC, and found higher percentages of the CD14+CD163-MAC387+ in the MAP patients (see figure below).

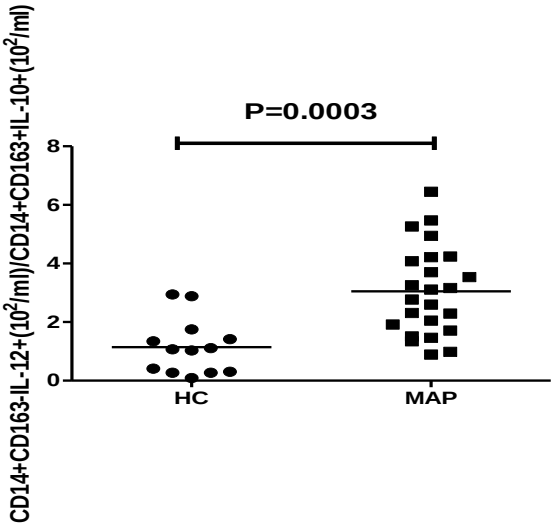


Comment 5. Figure 4. I would like to see the mean fluorescence intensity of IL-10 and IL-12 in the various monocyte populations rather than the percentage positive. Here also the ratio between IL-10-positive and IL-12-positive would be informative.

Response: We appreciate his/her advice. We compared the mean fluorescence intensity of anti-IL-10 and anti-IL-12 in the monocytes, and we found no significant difference between the MAP patients and the HC. We also analyzed the ratio between the numbers of IL-12-positive and IL-10-positive cells, and found the ratios of the numbers of CD14+CD163-IL-12+ to CD14+CD163+IL-10+ monocytes in the MAP patients were significantly higher than that in the HC (see figures below).







Comment 6. Figure 4. Were the monocytes expressing detectable levels of IL-10 or IL-12 in the absence of in vitro stimulation with LPS/PMA/ionomycin? This would be more relevant.

Response: We understood his/her comments. In our study, we detected low frequency of IL-10+ or IL-12+ monocytes from the MAP patients and controls following stimulated with LPS, PMA and ionomycin. Although we completely agree with his/her comments that detection of endogenously activated monocytes are more clinical relevant, we did not analyze the frequency of IL-10+ or IL-12+ monocytes without in vitro stimulation. We recognized that IL-10 or IL-12 secreted by monocytes without stimulation can be detected by ELISA. Indeed, a previous study has shown that higher levels of IL-12, but lower levels of IL-10 in the cultured monocytes without stimulation were detected from pregnant women with pre-eclampsia, as compared with that from the healthy pregnant women [**Medeiros LT**, Peraçoli JC, Bannwart-Castro CF, Romão M, Weel IC, Golim MA, de Oliveira LG, Kurokawa CS, Medeiros Borges VT, Peraçoli MT. Monocytes from pregnant women with pre-eclampsia are polarized to a M1 phenotype. Am J Reprod Immunol. 2014; 72: 5-13]. We are interested in further investigating it in the future studies.

Reviewed by 00069137,

The paper is interesting, well designed, and the idea behind the work is original. The paper is well written. I have some major comments and other minor comments.

Comment 1. The authors mix terminology when discussing monocytes and macrophages. The cells studied appear to be monocytes. (PBMC).

Response: We appreciate your constructive comments. We have changed to monocytes in the revision.

Comment 2. The sample is quite small. While the matching seems adequate, the usual ratio of cases to controls is either 1:1 or even 1:2.

Response: We understood his/her concern. We recognized the limitation of a relatively smaller sample size in the discussion section. Our hospital is relatively smaller one with limited MAP patients hospitalized so that we had difficult to recruit more patients in a short time period. Before the project began, we had estimated the minimum sample size by calculation formula:

Two groups of independent sample mean comparison:

$n_1=Cn_2$

$n_2= [(Z_{\alpha/2}+Z_{\beta})\ s/\ \delta\ ]^2(1+C)/C$

In this study: inspection level α=0.05, power of test 1-β=0.9,

Sstandard deviation s=358 (from preliminary experiment)

Expectation difference value δ =440(plasma amylase, U/L)

Sample size proportion C=2

So n_{1min}=20, n_{2min}=10

Although the sample size was relatively smaller this proof in principle study indicated imbalance of different subsets of peripheral monocytes may contribute to the pathogenesis of MAP and some measures may be valuable for evaluating the severity of MAP. Accordingly, the available findings are

clinically relevant and important for clinical practice in management of MAP patients.

Comment 3. Were patients stratified according to pancreatitis etiology? would differences be expected from biliary, alcoholic, or triglyceride induced AP?

Response: This is an interesting question. We did stratify the patients according to the etiology, including alcohol, hypertriglyceridemia, and cholelithiasis. However, we found no difference in the monocytes subsets in the MAP patients induced by these 3 factors.

Comment 4. The discussion of interleukin levels as biomarkers seems out of context. The discussion of how IL levels could be related to monocyte subpopulations is interesting. However, there are other sources of IL-10 or IL-12 besides monocytes.

Response: Thank you. Production of cytokines is an important function of monocytes, and pro-inflammatory and anti-inflammatory responses are mainly performed by cytokines. The function of cytokines in a certain extent reflects the function of the cells. That was why we discussed the interleukin in this manuscript. We will further investigate the functions of some monocyte subpopulations by cell sorting and cell culture in the following experiment and we will discuss the relationship between the IL levels and the monocyte subpopulations at that time.

Comment 5. At what period in the evolution of pancreatitis were samples drawn?

Response: We collected the fasting venous blood samples within 72 hours after upper abdominal pain occurred (Page 8, line 217-218).

Comment 6. At times in the discussion results are repeated textually instead of discussed in relation to the relevant literature.

Response: We have revised the discussion section by repeating the results and expending the discussion of similarity and difference of our findings with other reported in the revision (Page14, line 362-363; Page15, line 389-392).

Comment 7. It would be interesting to study to what extent monocyte subpopulations change in relation to pancreatitis or inflammation in general. A group with inflammation from another source would be helpful. This would give a pathophysiologic link more plausability and specificity.

Response: Our previous study has found significant changes in the numbers of peripheral blood different subsets of monocytes in patients with tuberculous pleural effusion (TPE) (Reference 13). We found increased numbers of peripheral blood CD14+CD163-, CD14+CD163-IL-12+, but decreased numbers of CD14+CD163+CD115+ cells in TPE patients, as compared with the healthy controls. However, we did not observed any significant difference in the numbers of CD14+CD163+IL-10+ monocytes between the TPE patients and controls. The different results from our current and previous studies may stem from different diseases.

Comment 8. It should be noted that a cause-effect association is difficult to establish. Are monocyte population changes a marker of inflammation (more likely)? or do they participate in pathogenesis (or repair??)?.

Response: Yes, the monocytes participate in both acute and chronic inflammation, and also contribute to the pathogenesis of infection and chronic inflammatory disease. When infections caused by various microbes occur, the pro-inflammatory monocytes are recruited to inflamed tissues, produce pro-inflammatory cytokines, participate in clearance of pathogens and dead cells. The patrolling monocytes are also recruited to sites of inflammation and contribute to wound healing [**Ingersoll MA**, Platt AM, Potteaux S, Randolph GJ. Monocyte trafficking in acute and chronic inflammation. Trends

Immunol. 2011; 32: 470-7.] [**Shi C**, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Rev Immunol. 2011; 11:762-74.]

Reviewed by 00456978,

It reads as a fairly good manuscript and my comments are limited to some style, language and typos. page3 line75 - "were correlated": change to "correlated"; page5 line122-123 - "help to tissue repair, but promote tumor growth and metastasis": I guess it would be more correct to say "help immunoregulation and tissue repair but that may promote ...". Firstly, the phrase as it is creates an impression that all M2 macrophages are tumor-associated. Secondly, keeping in mind the "Colourwheel of the macrophage activation" (for example from http://www.macrophages.com/macrophage-review), it is important to mention the regulatory function of M2 macrophages; page 5 line 132 - "MAC387+ monocytes/macrophages are recently recruited into the tumor...": perhaps it is better to refer to those as "recently infiltrating monocytes/macrophages" (as in Ref13), since they may have other functions in addition to association with tumors; same for p.9 l.244; p.6 l.159 - "no a history" -> "no history"; p.8 l.230 - "in the patients" -> "in the MAP patients"; p.11 l.309 - "the numbers of of peripheral blood different subsets of macrophages" -> "the number of of different subsets of peripheral blood macrophages". The study is generally well written, and in my opinion only few minor corrections need to be done.

Response: We appreciate his/her advice. Now, we have changed most sentences accordingly and carefully checked every sentence in the revision to (Page 4, line 108; Page 6, line 150-151; Page 7, line 166-167; Page 11, line 283-284; Page 8, line 194; Page 10, line 271; Page 13, line 351-352)

Reviewed by 00947129,

Zhang et al. investigated the numbers of different subsets of monocytes and

their associations with clinical markers of patients with mild acute pancreatitis (MAP). Overall, this is a nice study; however, there are some points that need attention:

Comment 1. The terms monocytes and macrophages are used interchangeably. However, these cells are not the same.

Response: We appreciate his/her advice. We have changed to monocytes in the revision.

Comment 2. The authors are investigating Map patients. Therefore, I do not quite understand one of their main conclusions: CD14+CD163+CD115+ macrophages (monocytes!) may be a biomarker for evaluating the severity of MAP. The severity of MAP by definition is mild. Even if a patient has higher CRP levels, the disease severity remains mild. The really interesting thing would be to include patients with moderate or severe disease.

Response: In this study, we found that the numbers of peripheral blood CD14+CD163+CD115+ monocytes were correlated significantly with the plasma CRP levels and the APACHE II scores in the MAP patients. It is well known that plasma CRP level (>150mg/L) and APACHE II score are two important measures to evaluate the severity of acute pancreatitis (including MAP, moderate acute pancreatitis and severe acute pancreatitis), so we concluded the number of peripheral blood CD14+CD163+CD115+ monocytes may be a biomarker for evaluating the severity of acute pancreatitis. Although MAP patients have a relatively low severity of AP they have different degrees of AP, displayed varying clinical symptoms and outcomes. Hence, it is important to evaluate the severity of MAP in patients. The goal of the current study was to evaluate the numbers of different subsets of monocytes in the MAP patients with early stage of AP so that we only studied MAP patients, but not MMP and SAP patients. It is ideal to including AP patients with different severities, such as MAP, moderate AP and SAP. However, our hospital is a relatively smaller one with limited numbers of moderate and severe AP

patients who meet the criteria for our study and we are interesting to further examining the numbers of different subsets of monocytes in moderate and severe AP patients in the future.

Comment 3. The method of sampling needs to be described in more detail. How much blood was taken, in what type of tubes, from where? The time of sampling is also critical as MAP resolves quickly.

Response: We appreciate his/her advice. Now, we have specified the method for collecting venous blood samples in the revision (Page 8, line 216-218).

Comment 4. Also, more data is needed concerning MAP patient characteristics. What was the etiology, body mass index and length of hospital stay in these patients?

Response: We appreciate his/her advice. Now, we have provided these data in Table 1 of the revision (Page10, line 264-266; Table 1).

Comment 5. Figure legends are considered as stand-alone. The experimental protocol and abbreviations need to be defined here as well. I'm not an expert in flow cytometry, so it was rather difficult for me to understand what the numbers mean in the SSC diagrams (e.g. 33.8 in top right panel of Fig. 1A).

Response: We appreciate his/her advice. Now, we have specified the abbreviations in the legends of the revision.

Comment 6. Note you are measuring amylase and lipase "activities".

Response: We appreciate his/her advice. Now, we have specified to measure the levels of plasma amylase and lipase "activities" (Page 4, line 96; Page8, line 209).

Comment 7. Superscripts and subscripts are missing in the manuscript (e.g. 106 cells in line #190, CO2 in line #193, in Table 1).

Response: We appreciate his/her advice. We have carefully checked the superscripts and subscripts in the manuscript, and revised them in the revision (Page 9, line 227 and 230; Table 1).

Comment 8. There are some sentences that need rephrasing (e.g. in line #308, activation degrees are associated the severity; in line 324, MAC387+ macrophages are recently recruited macrophages; in line 332, a positive feedback loop to strength pro-inflammatory responses).

Response: We appreciate his/her advice. We have rephrased these sentences in the manuscript (Page 13, line 351; Page 14, line 368; Page 14, line 376).

Comment 9. Abbreviations should be defined at first use (e.g. CBA). Lipase is abbreviated as LPS in Table 1, but LPS is also defined as lipopolysaccharide on page 7. This is a bit confusing.

Response: We are sorry for the confusion. Now, we have carefully checked all the abbreviations and specified them when they occurred first in the revision. We use lipopolysaccharide instead of LPS on page 9, line 228.