nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: The multiple myeloma micro-environment is defined by an inflammatory stromal cell landscape
Corresponding author name(s): Pieter Sonneveld, Tom Cupedo

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-RS30248A
Message: 15th Aug 2020

Dear Tom,

Thank you for providing a point-by-point response to your Resource manuscript entitled "An integrated definition of the immune and stromal micro-environment in multiple myeloma reveals stromal fibroblast-centric bone marrow inflammation". As noted in my previous correspondence that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the current manuscript for publication, but would be very interested in considering a revised version along the lines proposed in your revision plan.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

Specifically, please include the following:
All referees (Q1, Q2, Q3)
-Add new in vitro experiments to use age-matched BM stromal cells from control patients (ie undergoing hip replacement therapy).
-Move the UMAP clustering analysis from individual patients in Supp. Fig. 2e to main figure.
Q1, Q2 - Measure TNF and IL-1b concentrations from the multiple myeloma patient BM plasma.
Q1, Q3 - Increase the number of multiple myeloma patients and controls for the BM scRNA-seq analysis.
Q2 - Perform more in-depth computational bioinformatic analysis of the scRNA-seq datasets.
Q2 - Compare the BM immune cell compartments (scRNA-seq on CD38+ and CD38- cells) between controls and multiple myeloma patients; please also add scRNA-seq datasets of

the MM tumor cells.

We overrule the request from Q1 to include a cohort of MGUS patients - this request would require a new IRB and would likely not add to the existing study.

When you revise your manuscript, please take into account all reviewer and editor comments, and please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Resource format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
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Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Kind regards,

Laurie

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this study the authors identified a myeloma-specific stromal fibroblast population, with enhanced inflammatory signature, which spatially co-localized with tumor cells and support tumor cell survival. Moreover, this cell population is resistant to the conventional anti-tumor therapy.
The study is original and novel. However, despite the excellent work performed and the quality of data and presentation, there are several critical aspects to be elucidated. Statistic is adequate. The study is descriptive and lack of functional studies to validate their results and hypothesis. Moreover the number of the patients and the type of controls analyzed is too limited and non adequate and should be improved to support the

conclusions of the work. References are appropriate. The summary and abstract are appropriate and clear.
I suggest a series of major points to be addressed:
- The number of controls and patients is very limited (2 controls vs 10 MM patients). Moreover the controls used are patients underwent to cardiac surgery thus should not be consider as healthy controls. I suggest to increase the number of both MM patients and controls and to include a cohort of MGUS subjects
- The description of the methods and the results can be improved. In the methods are not reported the number and the characteristics of the patients. Fig.1 is built on data obtained from merged MM samples. Are the two clusters, F1 and F2, differentially distributed among the patients? Fig 3d-f does not show the results on Adult controls, indeed the authors report data on fetal bone marrow stromal cells, which are not age matched.
- Authors declare in the methods that Bone marrow sample were used within 72 hours after collection. I suspect that after 72 hours the immunophenotype of the cells can be altered. How did the authors store the samples and compare samples with a large different time of collection? They have to demonstrate that immunophynotype was not changed across the 72 hours.
- Authors did not find a clear distinction between mesenchymal stromal cells and fibroblast. I think that the have to clarify this issue. Although MSC and fibroblast may overlap the immune-phenotype, MSC are able to differentiate into osteoblast, chondrocyte and adipocytes whereas fibroblasts do not. I think that authors have to show this to define cells as fibroblasts.
- The paper is merely descriptive and it lacks of a functional demonstration of ISF pro-survival effects on MM cells. In vitro experiments with isolated ISF, co-coltured in the presence of MM cells, should be performed in order to check MM cell viability.
- Have the authors investigated potential differences between cluster F1 and F2? Can they really be considered as one homogeneous cell population?
- Have the authors normalized the results for the tumor load of each patient? I suggest to do it
- The authors highlight a crucial role for TNF-alpha and IL-1beta in the activation of stromal cells. I suggest to check BM plasma levels of these two cytokines in the cohort of patients and controls and to correlate to the % of ISF found in each patient.
- Line 22-24 page 11: the number of MRDneg is 4/12 while 8/12 patients are MRDpos, according to Suppl. Table 4. Please check.
- Fig. 6: did the authors check the % of inflammatory fibroblast populations in patients after treatment?
- Suppl Table 1: PH1 has 0% of plasma cells in BM, did the authors observe any differences compared with the other patients?

Reviewer #2:
Remarks to the Author:
Nature Immunology
Manuscript NI-RS30248A

The de Jong study, "An integrated definition of the immune and stromal micro-environment in multiple myeloma reveals stromal fibroblast-centric bone marrow inflammation" describes the hematopoietic and non-hematopoietic BM microenvironment in untreated multiple myeloma (MM) patients and non-MM controls using single cell RNA sequencing (scRNAseq). The scRNAseq data was used to define the cellular composition of

the MM environment with specific focus on the fibroblast and immune cell compartments. They also had access to paired samples from patients 28 days post induction therapy which they examine by bulk RNAseq on sorted fibroblasts. A second set of control non-MM samples were used as comparators for the bulk RNAseq analysis.

Figure 1-5: The majority of the paper focuses on an interesting potential feed forward inflammatory loop wherein immune cells from the MM environment, particularly lymphocytes and myeloid cells, drive the activation of fibroblasts via TNFa and IL1b. The result is the emergence of two populations of activated fibroblasts found which they refer to as F1 and F2 (Figure 1). Additional non-hematopoietic cells that appear to emerge in MM (compared with the non-MM controls) include SELP+ SEC, CDH5+ EC and osteolineage cells (OLC) however the authors do not further characterize these cells. The authors then show that F1 and F2 clusters of fibroblasts express a range of pro-inflammatory genes including chemokines, cytokines and activation markers.

Figure 2 suggests that CXCL12+CD44+ fibroblasts are enriched in MM whereas in non-MM controls the fibroblasts appear to express CXCL12 but little CD44. In the MM samples the CXCL12+CD44+ fibroblasts are localized in proximity to MM tumor cells.

Figure 3 shows that fibroblasts or stromal cells from different sources can respond in vitro to TNFa and IL1b however the responses are qualitatively and quantitatively different. The significance of this figure is unclear as the authors compared myeloma fibroblasts with fetal bone marrow derived stromal cells and adipose-derived stromal cells. The more relevant comparison would have been the 'control' bone marrow fibroblasts used in Figure 1d (and throughout the paper) instead of the other comparators. Separately the authors would need to provide evidence that fetal bone marrow derived stromal cells and adipose-derived stromal cells are fibroblasts or some other stromal subtype.

Figures 4 and 5 utilize scRNAseq data from immune cells in MM samples to ascertain which populations express TNFa and IL1b. They show that IL1b is primarily expressed by myeloid cells whereas TNFa is expressed by multiple lymphocyte populations and myeloid cells. Two major omissions from the paper are 1) a comparison of the immune compartments between controls and MM as shown in Figure 1 for non-immune cells (how does the tumor affect the immune cell compartment?); 2) the composition and inflammatory profile of the myeloma tumor cells. While immune cells are most likely expressing pro-inflammatory cytokines that influence local fibroblasts in MM the tumor cells themselves may be an equally important source of factors that activate the fibroblasts.

Figure 6 has the potential to be very interesting and informative. However, in its current form the data content is somewhat perplexing. The authors have collected bone marrow samples from the same patients 28 days after induction therapy and assessed the presence/absence of tumor cells. Most patients (7/11) exhibited tumor cell depletion whereas 4/11 patients remained tumor cell positive. Bulk RNAseq was then performed on fibroblasts sorted from bone marrow samples from these patients at the time of diagnosis and 28 days after induction therapy. This analysis showed no change in proinflammatory gene expression between diagnosis and post therapy. The authors concluded that fibroblasts maintain an 'activated' phenotype in the bone marrow after tumor cells are depleted. They also extrapolated from this finding to suggest that maintenance of an inflammatory fibroblast state may underlie relapse. While the fibroblast compartment may remain 'activated' in the absence of MM tumor cells, which would be an interesting and

unexpected finding, the authors have not examined the possibility that immune cells also maintain an activated/pro-inflammatory state following post-induction therapy. In addition, it remains possible that the fibroblasts have changed following tumor cell debulking but those changes are not captured with RNAseq analysis on candidate genes.

Overall, the authors have generated interesting gene expression data from valuable patient samples however available computational tools, analysis and thus insights were not maximized. The limited utilization of the samples and data and lack of experimental data to establish causality or functional relevance of the proposed hypotheses leave many important questions unanswered and dampen enthusiasm. The paper may be useful as a resource however a deeper dive on the computational analysis of these important patient samples would be necessary to glean more detailed and high impact insights for the myeloma field.

Additional points:

Figure 1a and p5: "clustering was driven by cellular identity, rather than patient characteristics": A separate analysis or UMAP colored by patient ID would be needed to make this point.

Figure 1d. The finding of MM-specific fibroblast subsets is exciting. Are the genes in 1d selected as some of the top genes differentially expressed in F1,F2 vs F3-6? Although the authors select the inflammation pathway to interrogate, guided by their MM versus normal BM data, it would be prudent to show in an unsupervised manner the top genes or pathways that distinguish F1 and F2 from the rest of the fibroblasts or from each other to understand differences and similarities. In addition, pseudotime trajectory analysis may enable developmental relationships between the various clusters in control v. MM samples. That is, are F1 and F2 related to one another and from which clusters in the control samples do they arise?

Figure 2. The authors should confirm whether or not ISF proximity to MM cells is restricted to F1 and F2 or applies to all fibroblasts in the MM environment.

Figure 3a. Expression of TNF-RI, TNF-RII and IL1R is not distinguishing ISFs from clusters F3-6 as the authors also acknowledge and in addition they are also expressed in normal BM (Figure 3a) at similar levels. Normal fibroblasts also respond to IL1 and TNFa treatment and acquire an ISF phenotype. Why do the authors propose that only F1 and F2 represent ISFs?

P10. "In sum, these analyses identify three specific bone marrow immune subsets able to interact with myeloma ISFs". Throughout the text, the authors discuss these findings with generic references to the immune cell subset in focus. It would be helpful to precisely reference the immune cell subset being discussed in the results section (as they do in the discussion. For example, CD14+ myeloid cells, CD38+CX3CR1+ NK cells and cytotoxic GZMB+ effector T cells).

With the exception of CX3CR1+ NK, CXCR1/2 expression seems to be restricted to only a handful of cytotoxic T cells and CD14+ myeloid cells making these links relatively weak. On the contrary, CD34- myeloid cells, which are admittedly less abundant, more uniformly express CXCR1/2 and IL1. Overall, although the expression of TNF and IL1 from many immune cells seems robust and may nurture MM cells, few of these cells appear to

express CXCR1/2 in the MM microenvironment. This potential discrepancy should be addressed.

Figure 5d. While CXCR4 is uniformly expressed in CD38+ cells the authors should clarify why they selected this marker.

Figure 6c,d,e. These subfigures are redundant. 6e is sufficient. Figure 6e appears to be missing an MRD neg sample from the CXCL8 plot.

Figure 6. The authors show that there are no differences in the stromal compartment before or after induction therapy with regards to these markers. It would be important to show whether other fibroblast markers or pathways are different in these paired samples. Why did the authors use bulk RNAseq rather than single cell RNAseq for the post-induction therapy samples? This would have enabled a high resolution and powerful comparison of immune and fibroblast/stromal composition following treatment. Without this data, or at the least an unbiased genome wide analysis of the bulk RNAseq data, it is difficult to conclude that fibroblasts have not changed in the post-induction therapy samples.

Reviewer #3:
Remarks to the Author:
The local tumor microenvironment has a major impact on tumor progression and resistance.
This clearly is the case in patients with multiple myeloma – there is a documented intensive interaction between the bone marrow microenvironment and the tumor cells - inducing MM cell proliferation and resistance.
The authors shed light on the tumor cell promoting interaction between myeloma cells and the microenvironment using scRNAseq. Overall, the manuscript is very interesting, well written, and the figures are illustrative.
This is an interesting report and clearly novel information with significant impact on the understanding of the pathogenesis of MM and future treatment strategies.
By generation of paired single cell transcriptomic datasets of the bone marrow immune and stromal microenvironments in patients with multiple myeloma the authors identified myeloma-specific inflammatory stromal fibroblasts, spatially co-localized with tumor cells inducing tumor cell survival and immune modulation. Myeloma stromal signatures were recapitulated in non-myeloma fibroblasts through stimulation with inflammatory cytokines. Additional analyses of the bone marrow of myeloma patients provide evidence for an immune cell-driven feed-forward loop of fibroblast activation. Follow-up analyses of myeloma patients revealed that even a successful anti-tumor therapy inducing MRD negativity was unable to revert tumor-supportive inflammation, predicting a role for fibroblast activation in disease persistence and relapse.

Major Comment:
Comparing 10 MM samples to 2 controls is barely adequate –5 controls should be provided. In this context, samples from control came from the sternum whereas samples from MM patients were derived from the iliac crest, - this might have an impact on the MM cells and the local microenvironment. In addition, the donors were patients undergoing cardiothoracic surgery with potential other comorbidities that might have an impact on the bone marrow microenvironment. I am wondering if this is an issue for interpreting the results. Thus, more and more appropriate controls should be included.

Minor:
Fig 1A: it is beautiful, but please show plot for all 10 patients and controls individually – if not in the manuscript, then at least in Suppl.
Fig 2: difficult to interpret – how many fibroblasts per MM cells? If the BM is packed with MM co-localization by chance is anticipated, right?

In order to shorten the introduction, consider to eliminate line 14-19.

No issue about the references and credit to previous work.

Author Rebuttal to Initial comments

Response to reviewers for NI-RS30248A **De Jong et al.**

We would like to thank the reviewers for their constructive criticism on our resource study "An integrated definition of the immune and stromal micro-environment in multiple myeloma reveals mesenchymal stromal cell-centric bone marrow inflammation". In response to the remarks from the reviewers we have substantially amended our study which now includes an increased number of patients and controls for mesenchymal stromal cells analyses, as well as an alternative source of control cells. We have also added single cell analyses of the tumor cells from the same patients, and single cell analyses of immune cells from control patients. In addition, we provide new data on inflammatory proteins in bone marrow supernatants of newly diagnosed patients, patients after treatment and controls. Together with extended bioinformatical analyses, including predictions of cellular interactions, we feel that our study has significantly improved. All comments by the individual reviewers are addressed in a point-to point fashion below.

Reviewer #1

In this study the authors identified a myeloma-specific stromal fibroblast population, with enhanced inflammatory signature, which spatially co-localized with tumor cells and support tumor cell survival. Moreover, this cell population is resistant to the conventional anti-tumor therapy.
The study is original and novel. However, despite the excellent work performed and the quality of data and presentation, there are several critical aspects to be elucidated. Statistic is adequate.
The study is descriptive and lack of functional studies to validate their results and hypothesis.
Moreover the number of the patients and the type of controls analyzed is too limited and non-adequate and should be improved to support the conclusions of the work.
References are appropriate. The summary and abstract are appropriate and clear.

I suggest a series of major points to be addressed:

- The number of controls and patients is very limited (2 controls vs 10 MM patients). Moreover the controls used are patients underwent to cardiac surgery thus should not be consider as healthy controls. I suggest to increase the number of both MM patients and controls and to include a cohort of MGUS subjects

In response to these comments by the reviewer we have increased the numbers of patients and controls in our analyses. We added 5 additional control patients, two of which are sternal aspirates as in the original submission, and 3 are femoral aspirates from patients undergoing hip-replacement surgery (see Supplementary tables 2 and 3 for patient characteristics). Results from both types of control patients were similar, and both showed the same differences in comparison to MM patients. We also added 3 additional newly diagnosed MM patients, bringing the total comparison to 13 MM patients and 7 non-cancer controls. Controls and MM patient niche datasets were age matched, as detailed in supplemental table 1
We agree with the reviewer that the control patients should not be denoted as healthy controls, and we now refer to these patients as either non-cancer controls or control patients throughout the manuscript

We share the reviewer's enthusiasm for analyzing stromal cells in MGUS patients. However, we feel that these types of analyses go beyond the scope of our current study. Since only a small fraction of MGUS patients will develop SMM, and only a percentage of those will develop MM, a clinically annotated cohort of patients with follow up of many years would be needed to exclude patients who never develop MM (or to compare those with the once that do progress). Together with the fact that we are limited to cell-rich samples due to the low percentage of stromal cells (0.002%), this would require a cohort that we at present do not have access to (if it exists). We are currently building such

a cohort through longitudinal follow-up of MGUS patients, but including sufficient patients with prolonged follow-up will take years.

- The description of the methods and the results can be improved. In the methods are not reported the number and the characteristics of the patients.

All patient characteristics were reported in supplementary tables, and we apologize for not making this sufficiently clear. We have extended the patient info in the supplemental tables (new supplemental table 1) and rephrased the text referencing these tables to better clarify this point.

Fig.1 is built on data obtained from merged MM samples. Are the two clusters, F1 and F2, differentially distributed among the patients?

UMAP renderings of individual patients were part of the original supplemental figures. As multiple reviewers remarked on this, we have moved this panel to Figure 1F to make the point that F1 and F2 (now referred to as MSC1 and MSC2) are present in all MM patients, and absent or strongly reduced in control patients. We have also added the enumeration of the MSC clusters per patient as Supplementary figure 2h.

Fig 3d-f does not show the results on Adult controls, indeed the authors report data on fetal bone marrow stromal cells, which are not age matched.

We agree with the reviewer that the fetal stromal cells were not the most optimal control. Remarks along these lines were made by multiple reviewers. We have now redone these experiments with mesenchymal stromal cells cultured from age-matched control patients undergoing hip-replacement surgery. The results and conclusions from these new experiments remained unaltered. The panels depicting fetal stromal cells and ADSC have been replaced with these new data in figure 3.

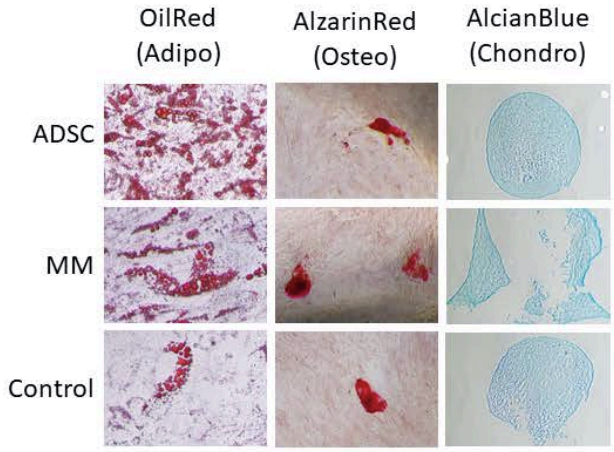
- Authors declare in the methods that Bone marrow sample were used within 72 hours after collection. I suspect that after 72 hours the immunophenotype of the cells can be altered. How did the authors store the samples and compare samples with a large different time of collection? They have to demonstrate that immunophenotype was not changed across the 72 hours.

The reviewer raises an important point. The 72 hrs stated in our manuscript was the maximal time allowed in the clinical protocol, yet all samples used in our study were frozen down within 24 hrs of sample collection, with the exception of 3 samples (PT1 and PT2 in scRNAseq and R7 in bulk seq) that took 48 hrs. We have updated the text in the methods section to reflect this fact.
To control for possible alterations in the immune-phenotype of the mesenchymal stromal cells, we initially performed single cell sequencing on a freshly isolated sample immediately following aspiration in our hospital, without a freeze-thaw cycle. We have now included this sample as supplemental figures 2K-L to show that the inflammatory phenotype is also found in these freshly prepared cells. Of note, all control patient samples were also left for 24hrs and subsequently viably frozen before isolation in order to avoid introducing any bias.

- Authors did not find a clear distinction between mesenchymal stromal cells and fibroblast. I think that they have to clarify this issue. Although MSC and fibroblast may overlap the immune-

phenotype, MSC are able to differentiate into osteoblast, chondrocyte and adipocytes whereas fibroblasts do not. I think that authors have to show this to define cells as fibroblasts.

We agree with the reviewer that we cannot definitively differentiate between fibroblasts and mesenchymal stromal cells in our analyses. The numbers of primary stromal cells that we can isolate are too low to allow for direct assessment of multilineage potential. This means that cells need to be propagated for multiple passages before we can assess their potential. When we do this, we see multilineage differentiation in the cultured cells from both MM patients and non-myeloma controls, similar to ADSCs (positive control) (Reviewer figure 1, below). Since we are unable to determine whether this potential is also present in cells directly ex-vivo, we agree with the reviewer that we currently do not have sufficient data to define the cells as fibroblasts. We therefore have altered the denomination of the cells to inflammatory mesenchymal stromal cells (iMSC).



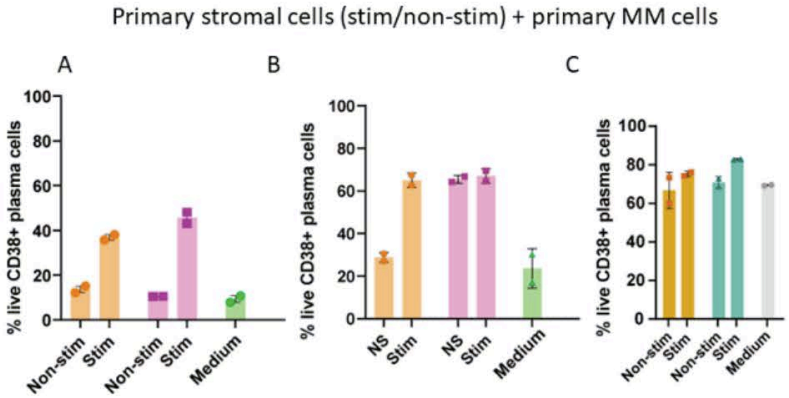
Reviewer Figure 1. *Trilineage potential of in vitro expanded bone marrow stromal cells. In vitro expanded MM stromal cells and control patient bone marrow cells were first expanded in vitro, subsequently cultured in differentiation medium and stained with the indicated dyes to reveal presence of Adipogenic, Osteogenic and Chondrogenic potential.*

- The paper is merely descriptive and it lacks of a functional demonstration of ISF pro-survival effects on MM cells. In vitro experiments with isolated ISF, co-coltured in the presence of MM cells, should be performed in order to check MM cell viability.

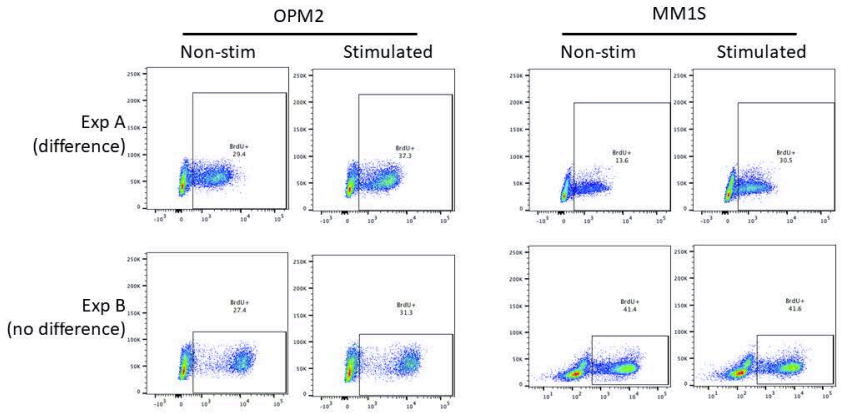
We agree with the reviewer on the added value of performing these cultures. In the past 6 months we have put every effort into setting up a system to test the pro-MM effects of inflammatory stromal cells, but have not been able to develop a system that provides a consistent outcome.

First, the number of ISF (iMSC) that we can isolate from fresh samples is too low for direct co-culture. This means that we need to expand cells in culture. We have done this by isolating the total adherent fractions of BM aspirates from MM patients and controls and propagating these cells for several passages. Subsequently cells were stimulated overnight with either TNFa or TNFa + IL1B to induce an ISF-like state as described in figure 3. These stimulated or non-stimulated cells were co-cultured with freshly isolated or thawed primary MM cells and viability of the tumor cells was assessed after overnight culture, 2 and 6 days of culture.

This resulted in an assay that in a few instances showed promising results, with non-stimulated stromal cells inducing survival similar to normal medium and stimulated stromal cells enhancing survival (Reviewer figure 2A). Unfortunately, in other experiments unstimulated stromal cells were equally capable of enhancing survival (Reviewer figure 2B). Finally, in some experiments MM cells survived regardless of the presence or absence of stromal cells (Reviewer Figure 2C). Also, when using the second batch of MM cells from the same patients, experiments did not yield the same results, all indicating the significant variation inherent to the plasma cells as well as possibly the stromal cells.



In an effort to increase reproducibility we subsequently invested in using human MM cell lines in a similar set-up, except using stromal cell-conditioned medium instead of co-cultures in an attempt to exclude confounding effects of cell-contact dependent signals inherent to stromal presence, yet independent of stimulation. Still, in these experiments the inherent variation in stromal cells appeared to be too high. Shown below are two representative experiments with two cell lines (OPM2 and MM1S) in combination with stimulated or non-stimulated conditioned medium. Since these cell lines do not need stromal support for survival, we used BrdU incorporation as a readout for enhanced proliferation. As can be seen in these panels, clear enhancement of proliferation could be seen in some experiments (Exp A) while this was completely absent from repeat experiments using the same set-up Exp B).



We are convinced from these experiments that there is currently no system that will allow the reproducible direct evaluation of the pro-MM effect of inflammatory stromal cells in vitro. This likely is due to donor-to-donor variations in primary MM cells as well as variations in primary stromal cells (either donor-derived or induced by passaging). We are putting effort in identifying stromal cell involvement in murine MM models, as we feel that this will be the only way to definitively proof this point. In the absence of these data, we have altered the text throughout, avoiding definitive statements claiming the generation of a pro-tumor microenvironment.

- Have the authors investigated potential differences between cluster F1 and F2? Can they really be considered as one homogeneous cell population?

We have added new data to clarify. The point that we are making is that there are relatively small differences in gene transcription between F1 and F2 (now denoted as MSC1 and MSC2). The top 50 differentially expressed genes are depicted in Supplemental table 6, in which the small logFold changes are apparent. The main differences are in the levels of inflammatory transcripts. We have added figure 2F and supplemental figure 2C to illustrate. Increased levels of transcription could suggest an intermediate stage of activation, which is supported by Fig 2G and the new pseudotime trajectory analysis using Monocle (Fig 3G).

- Have the authors normalized the results for the tumor load of each patient? I suggest to do it

We have correlated the percentage of tumor cells with the percentage of inflammatory stromal cells, which gave a correlation of 0.49. These data are now shown in supplemental figure 3J.

- The authors highlight a crucial role for TNF-alpha and IL-1beta in the activation of stromal cells. I suggest to check BM plasma levels of these two cytokines in the cohort of patients and controls and to correlate to the % of ISF found in each patient.

This is an interesting point. We have routinely collected bone marrow plasma during sample preparation and have now used these to analyze protein levels of TNFa, IL-1ß and several other inflammatory cytokines using multiplex bead assays and ELISA. Unfortunately, we were unable to reliably detect TNFa levels in the supernatants, which might be due to complexation of TNF with soluble TNFR, or because levels in the plasma are too low for detection. We did detect IL-1ß, with elevated levels in both newly-diagnosed MM patients as well as after induction therapy, and these data are now included in Figure 3D and 6J.
In addition, we detect elevated levels of IL-6, CXCL8, CCL2, and IL-18 protein in newly-diagnosed patients (Figure 1G) as well as post-induction therapy (Figure 6J). We do not wish to clain IL1b or TNF as the only possible mechanisms for ISF induction, and have added text (page 16, line 17-26, and model in supplemental figure 6) to highlight alternative options such as pattern recognition receptors.

- Line 22-24 page 11: the number of MRDneg is 4/12 while 8/12 patients are MRDpos, according to Suppl. Table 4. Please check.

This has been corrected.

- Fig. 6: did the authors check the % of inflammatory fibroblast populations in patients after treatment?

We fully agree with the reviewer that this is an interesting question. At this point in time, the absence of samples with sufficient cells to isolate the non-hematopoietic niche and perform scRNA-seq has made it impossible for us to perform such analysis.

- Suppl Table 1: PH1 has 0% of plasma cells in BM, did the authors observe any differences compared with the other patients?

We thank he reviewer for pointing this out. The percentages in the original supplemental table 1 were based on the assessment of bone marrow smears performed by a pathologist as part of routine diagnostic work-up. However, for all patients MM plasma cells were collected in the lab, and plasma cell percentages were independently determined using flow cytometry. The 0% plasma cells were clearly an error (as we are now including the sequencing of the plasma cells from this patient). In order to prevent similar discrepancies, we are now showing the percentage of plasma cells as determined by flow cytometry, which we believe is a more consistent manner of plasma cell enumeration for experimental purposes.

Response to reviewers for NI-RS30248A **De Jong et al.**

Reviewer #2

The de Jong study, "An integrated definition of the immune and stromal micro-environment in multiple myeloma reveals stromal fibroblast-centric bone marrow inflammation" describes the hematopoietic and non-hematopoietic BM microenvironment in untreated multiple myeloma (MM) patients and non-MM controls using single cell RNA sequencing (scRNAseq). The scRNAseq data was used to define the cellular composition of the MM environment with specific focus on the fibroblast and immune cell compartments. They also had access to paired samples from patients 28 days post induction therapy which they examine by bulk RNAseq on sorted fibroblasts. A second set of control non-MM samples were used as comparators for the bulk RNAseq analysis.

Figure 1-5: The majority of the paper focuses on an interesting potential feed forward inflammatory loop wherein immune cells from the MM environment, particularly lymphocytes and myeloid cells, drive the activation of fibroblasts via TNFa and IL1b. The result is the emergence of two populations of activated fibroblasts found which they refer to as F1 and F2 (Figure 1). Additional non-hematopoietic cells that appear to emerge in MM (compared with the non-MM controls) include SELP+ SEC, CDH5+ EC and osteolineage cells (OLC) however the authors do not further characterize these cells. The authors then show that F1 and F2 clusters of fibroblasts express a range of pro-inflammatory genes including chemokines, cytokines and activation markers.

Figure 2 suggests that CXCL12+CD44+ fibroblasts are enriched in MM whereas in non-MM controls the fibroblasts appear to express CXCL12 but little CD44. In the MM samples the CXCL12+CD44+ fibroblasts are localized in proximity to MM tumor cells.

Figure 3 shows that fibroblasts or stromal cells from different sources can respond in vitro to TNFa and IL1b however the responses are qualitatively and quantitatively different. The significance of this figure is unclear as the authors compared myeloma fibroblasts with fetal bone marrow derived stromal cells and adipose-derived stromal cells. The more relevant comparison would have been the 'control' bone marrow fibroblasts used in Figure 1d (and throughout the paper) instead of the other comparators.

We agree with the reviewer that primary mesenchymal stromal cells isolated from control patients are a better representation of the non-myeloma bone marrow. We have isolated and grown stromal cells from femoral heads after hip-replacement surgery, and repeated the stimulation experiments on these samples with similar results. The panels showing ADSC and fetal stromal cells have been replaced with this more relevant control (Figure 3).

Separately the authors would need to provide evidence that fetal bone marrow derived stromal cells and adipose-derived stromal cells are fibroblasts or some other stromal subtype.
ADSC are typically used as prototypic MSC cells with multilineage potential. Also, in light of the reviewer's previous remark, the ADSC, as well as the fetal-derived cells have been removed from the manuscript and replaced with the more appropriate age-matched bone marrow-derived stromal cells from non-cancer control patients.

Figures 4 and 5 utilize scRNAseq data from immune cells in MM samples to ascertain which populations express TNFa and IL1b. They show that IL1b is primarily expressed by myeloid cells whereas TNFa is expressed by multiple lymphocyte populations and myeloid cells.
Two major omissions from the paper are

1) a comparison of the immune compartments between controls and MM as shown in Figure 1 for non-immune cells (how does the tumor affect the immune cell compartment?)

We agree with the reviewer on the importance of this comparison. Therefore, we have performed scRNAseq on CD38+ and CD38- immune cells of 5 non-cancer control patients. These data are now presented in figures 4, 5 and supplemental figure 3. From these analyses it became clear that there is a specific increase in a CD8+ T cell population characterized by an IFN-response signature, in CD8+ Tscm and GZMK+CX3CR1- NK cells when comparing myeloma to controls (Figure 4D and G). Of these three populations, both CD8+ T cell populations also showed an increase in percentage of TNF transcribing cells (Figure 5A and C).

2) the composition and inflammatory profile of the myeloma tumor cells. While immune cells are most likely expressing pro-inflammatory cytokines that influence local fibroblasts in MM the tumor cells themselves may be an equally important source of factors that activate the fibroblasts.

We have added the scRNAseq data of isolated CD38+ multiple myeloma cells of the 13 MM patients for which we also present the immune and stromal data (Figure 5 and Supplemental figure 4), which shows no transcription of TNF and IL1B. Also, qPCR reveals a virtual absence of TNF and IL1B transcription in plasma cells of MM patients. Novel analyses using the CellphoneDB algorithm to predict putative cellular interactions based on transcription of receptor-ligand components indicated a possible specific interaction between a cluster of proliferating MM cells (P7) and the inflammatory stromal cell clusters (MSC1). This data is now presented in figure 5G-i.

Figure 6 has the potential to be very interesting and informative. However, in its current form the data content is somewhat perplexing. The authors have collected bone marrow samples from the same patients 28 days after induction therapy and assessed the presence/absence of tumor cells. Most patients (7/11) exhibited tumor cell depletion whereas 4/11 patients remained tumor cell positive. Bulk RNAseq was then performed on fibroblasts sorted from bone marrow samples from these patients at the time of diagnosis and 28 days after induction therapy. This analysis showed no change in proinflammatory gene expression between diagnosis and post therapy. The authors concluded that fibroblasts maintain an 'activated' phenotype in the bone marrow after tumor cells are depleted. They also extrapolated from this finding to suggest that maintenance of an inflammatory fibroblast state may underlie relapse. While the fibroblast compartment may remain 'activated' in the absence of MM tumor cells, which would be an interesting and unexpected finding, the authors have not examined the possibility that immune cells also maintain an activated/pro-inflammatory state following post-induction therapy. In addition, it remains possible that the fibroblasts have changed following tumor cell debulking but those changes are not captured with RNAseq analysis on candidate genes. In addition, it remains possible that the fibroblasts have changed following tumor cell debulking but those changes are not captured with RNAseq analysis on candidate genes

To clarify, and we have altered the text to better emphasize this point, the paired bone marrow samples were taken after the 4th course of induction therapy. Each course lasts 28 days, meaning that samples are taken after approximately 4 months of therapy.
We have now included new panels in Figure 6 to clarify the fact that the inflammatory transcriptome in mesenchymal stromal cells is unaltered after 4 months of treatment and that transcriptional changes between diagnosis and post-induction therapy are minimal (Figure 6F-G). Supplemental figure 5D-F show values of individual stromal cell-related genes to emphasize that these also remain similar.

We are in full agreement with the reviewer that residual immune cell activation could be a plausible explanation for the persistent inflammatory stromal cell transcriptome. We have altered the text on page 17, line 8-10, to highlight this fact. Unfortunately, we do not have the immune cell transcriptomes at the post-induction treatment time points. To strengthen the point that general bone marrow inflammation persists after induction therapy we have added panel 6J, which shows that in bone marrow plasma from these patients increased levels of IL-6 and CXCL8 are still detected post induction therapy. The same goes for IL-1β and IL-18 which are not transcribed by stromal cells and is another indication of broader bone marrow inflammation.

Overall, the authors have generated interesting gene expression data from valuable patient samples however available computational tools, analysis and thus insights were not maximized. The limited utilization of the samples and data and lack of experimental data to establish causality or functional relevance of the proposed hypotheses leave many important questions unanswered and dampen enthusiasm. The paper may be useful as a resource however a deeper dive on the computational analysis of these important patient samples would be necessary to glean more detailed and high impact insights for the myeloma field.

We have substantially increased our bioinformatical analyses of the datasets presented in our study. Foremost, the novel analyses now presented in Figure 5, using the CellphoneDB algorithm significantly add to our understanding of the possible biological role of the inflammatory stromal cells. Based on predicted receptor ligand interactions, inflammatory stromal cells have an additional mechanism of attracting tumor cells that is absent from other stromal cells: CCL2-CCR10. Moreover, a specific cluster of proliferative myeloma cells was identified as having the potential to be attracted to inflammatory stromal cells via CCL2/CCR2 interactions (Figure 5G-i), warranting future investigation into the interactions between stromal cells and proliferative subsets of tumor cells. Using CellphoneDB to study stromal – myeloid cell interactions a set of immune modulatory interactions specific for the inflammatory stromal clusters were identified. These include stromal cell transcription of ANXA1, VEGFA, C3, IL-6 and TNFRSF12A (Figure 5K). All of these proteins, when interacting with the transcribed counter-receptor on myeloid cell subsets can affect myeloid cell function, and add up to a pro-tumor microenvironment. Together these analyses generate and support novel hypothesis on the role of the stromal bone marrow microenvironment in Multiple Myeloma, that will be of value to the myeloma research community and can serve as a starting point for novel research efforts.
The full CellphoneDB analysis with all putative interactions of all the clusters from the 4 datasets is available for downloading from Github, allowing easy exploration.

Additional points:

Figure 1a and p5: "clustering was driven by cellular identity, rather than patient characteristics": A separate analysis or UMAP colored by patient ID would be needed to make this point.

Patient-specific UMAP renderings were generated to make this statement, and these were shown in the original supplemental figure 2. As the referencing of these UMAPs in the main text was insufficiently clear to multiple reviewers we have now included these as panel 1F in the new figure 1.

Figure 1d. The finding of MM-specific fibroblast subsets is exciting. Are the genes in 1d selected as some of the top genes differentially expressed in F1,F2 vs F3-6? Although the authors select the inflammation pathway to interrogate, guided by their MM versus normal BM data, it would be

prudent to show in an unsupervised manner the top genes or pathways that distinguish F1 and F2 from the rest of the fibroblasts or from each other to understand differences and similarities.

The inflammatory genes were chosen from the top-differentially expressed genes, in conjunction with the GSEA pathway analyses. We have now added these data to the supplemental information. Supplemental table 4 shows the top 50 differential genes in MSC1/2 (F1/2) versus MSC3-5. Supplementary table 5 shows the GESE pathway analysis on MSC1/2 versus MSC3-5

In addition, pseudotime trajectory analysis may enable developmental relationships between the various clusters in control v. MM samples. That is, are F1 and F2 related to one another and from which clusters in the control samples do they arise?

We agree with the reviewer that this is an important point. Our STRING network analyses (Fig3A) and stimulation experiments (Figure 3E-F) suggest that stimulation of mesenchymal stromal cells is sufficient to induce the inflammatory transcriptome. This is supported by the fact that in general inflammatory genes are higher expressed in F1 (MSC1) compared to F2 (MSC2) (Figure 3G). We used Monocle3 to perform pseudotime analysis. With a pseudotime start point in MSC5, trajectories run from MSC2 to MSC1, again supporting a model of increasing activation states (Figure 3H). MSC2 was predicted to be derived from MSC3.

Figure 2. The authors should confirm whether or not ISF proximity to MM cells is restricted to F1 and F2 or applies to all fibroblasts in the MM environment.

Our analysis provides no evidence for a preferential interaction between myeloma cells (or T cells) and F1 or F2. In fact, from our analysis and the images in Figure 2 it is clear that many MM cells are not engaging with CD44-expressing stromal cells. Even though the histology will likely only visualize the CD44hi stromal cells (a fraction of total F1/F2) this still indicates that interactions with F1/F2 are not mandatory for MM cells. This fits with our new CellphoneDB analyses which show a predicted preferential interaction of F1/F2 with the CCR2/CCR10 transcribing MM cell subset.
To make this point clearer, we have altered the text describing figure 2, and only conclude from this figure that CD44+ stromal cells co-localize with MM cells (a prerequisite for interaction) (page 7, lines 21-22 and 24-25). Similarly, we added images of CD44+ stromal cells interacting with CD3+ T cells. Again, a prerequisite for immune modulation, but no evidence of preferential interaction.

Figure 3a. Expression of TNF-RI, TNF-RII and IL1R is not distinguishing ISFs from clusters F3-6 as the authors also acknowledge and in addition they are also expressed in normal BM (Figure 3a) at similar levels. Normal fibroblasts also respond to IL1 and TNFa treatment and acquire an ISF phenotype. Why do the authors propose that only F1 and F2 represent ISFs?

We propose that ISF are an activated form of local tissue stromal cells, induced after inflammatory signaling. Most likely, ISF (F1/F2; MSC1/MSC2) derive from MSC3, as indicated earlier by pseudotime analysis. Our hypothesis would be that many normal stromal cells have the capacity to be activated and to take on an ISF phenotype. Why this only happens in a with a select number of cells in what seems to be a localized manner is of great interest and will be subject of novel research efforts.

P10. "In sum, these analyses identify three specific bone marrow immune subsets able to interact with myeloma ISFs". Throughout the text, the authors discuss these findings with generic references

to the immune cell subset in focus. It would be helpful to precisely reference the immune cell subset being discussed in the results section (as they do in the discussion. For example, CD14+ myeloid cells, CD38+CX3CR1+ NK cells and cytotoxic GZMB+ effector T cells).

We agree with the reviewer and have updated text and references throughout the manuscript.

With the exception of CX3CR1+ NK, CXCR1/2 expression seems to be restricted to only a handful of cytotoxic T cells and CD14+ myeloid cells making these links relatively weak. On the contrary, CD34- myeloid cells, which are admittedly less abundant, more uniformly express CXCR1/2 and IL1. Overall, although the expression of TNF and IL1 from many immune cells seems robust and may nurture MM cells, few of these cells appear to express CXCR1/2 in the MM microenvironment. This potential discrepancy should be addressed.

The reviewer is correct in her/his comment. We have performed additional experiments on plasma cells that revealed a discrepancy between transcription of CXCR1/2 as found by scRNAseq, and levels detected by flow cytometry. Transcription significantly underestimated the number of cells expressing CXCR1/2 surface protein. We have therefore decided to remove the CXCR1/2 emphasis from the manuscript, and focus instead on cell populations increased in myeloma versus control, and the TNF/IL1B transcription of these subsets. This revealed increased percentages and TNF transcription in IFN-responsive CD8+ T cells and CD8+ Tscm cells (Figure 5). Based on our CellPhoneDB receptor-ligand interaction data, we now include a much more detailed analyses of the myeloid compartment and the potential interactions these cells might have with the inflammatory stromal cells (Figure 5)

Figure 5d. While CXCR4 is uniformly expressed in CD38+ cells the authors should clarify why they selected this marker.

We apologize for not making this point sufficiently clear. CXCR4 was shown since previous literature on NK cells in MM made a distinction between CXCR4+ and CX3CR1+ NK cells. We have now removed this emphasize

Figure 6c,d,e. These subfigures are redundant. 6e is sufficient. Figure 6e appears to be missing an MRD neg sample from the CXCL8 plot.

We have updated figure 6 and removed the redundant plots as suggested by the reviewer.

Figure 6. The authors show that there are no differences in the stromal compartment before or after induction therapy with regards to these markers. It would be important to show whether other fibroblast markers or pathways are different in these paired samples.
The data presented are distilled from full-transcriptome deep RNA-sequencing. Other fibroblast markers were unaltered in these analyses we are now presenting these data in supplemental figure 5. The individual genes are listed in supplemental tables 10 and 11.

Why did the authors use bulk RNAseq rather than single cell RNAseq for the post-induction therapy samples?
This was due to the low cell numbers in the paired aspirates of post-induction treatment (less aspirate volume was taken). Stromal cells are scarce in bone marrow aspirates (0.002%), thus cell-

rich samples are required to perform robust scRNA seq analyses. With the limited cell numbers, we were only able to sort stromal cells and perform RNA sequencing after linear amplification of the RNA.

This would have enabled a high resolution and powerful comparison of immune and fibroblast/stromal composition following treatment. Without this data, or at the least an unbiased genome wide analysis of the bulk RNAseq data, it is difficult to conclude that fibroblasts have not changed in the post-induction therapy samples.
Our analyses are genome-wide transcriptional analyses on highly purified stromal cells. We have extended Figure 6 and added the unsupervised analyses to show all differences in the myeloma bone marrow stroma comparing newly-diagnosed to post induction chemotherapy, and myeloma to controls (Figure 6, Supplemental figure5 and Supplemental tables 10 and 11).

Response to reviewers for NI-RS30248A **De Jong et al.**

Reviewer #3

(Remarks to the Author)
The local tumor microenvironment has a major impact on tumor progression and resistance. This clearly is the case in patients with multiple myeloma – there is a documented intensive interaction between the bone marrow microenvironment and the tumor cells - inducing MM cell proliferation and resistance.
The authors shed light on the tumor cell promoting interaction between myeloma cells and the microenvironment using scRNAseq. Overall, the manuscript is very interesting, well written, and the figures are illustrative.
This is an interesting report and clearly novel information with significant impact on the understanding of the pathogenesis of MM and future treatment strategies.
By generation of paired single cell transcriptomic datasets of the bone marrow immune and stromal microenvironments in patients with multiple myeloma the authors identified myeloma-specific inflammatory stromal fibroblasts, spatially co-localized with tumor cells inducing tumor cell survival and immune modulation. Myeloma stromal signatures were recapitulated in non-myeloma fibroblasts through stimulation with inflammatory cytokines. Additional analyses of the bone marrow of myeloma patients provide evidence for an immune cell-driven feed-forward loop of fibroblast activation. Follow-up analyses of myeloma patients revealed that even a successful anti-tumor therapy inducing MRD negativity was unable to revert tumor-supportive inflammation, predicting a role for fibroblast activation in disease persistence and relapse.

Major Comment:
Comparing 10 MM samples to 2 controls is barely adequate –5 controls should be provided. In this context, samples from control came from the sternum whereas samples from MM patients were derived from the iliac crest, - this might have an impact on the MM cells and the local microenvironment. In addition, the donors were patients undergoing cardiothoracic surgery with potential other comorbidities that might have an impact on the bone marrow microenvironment. I am wondering if this is an issue for interpreting the results. Thus, more and more appropriate controls should be included.

We thank the reviewer for raising these important points, with which we can only agree. We have increased the number of MM patients to 13 and the number of controls to 7. Of the 5 additional control samples, 3 were taken from femoral heads after hip replacement surgery. Average age of both cohorts was 61 years (Range MM: 54-64; range controls: 27-75; see supplemental table 1 for characteristics of all patients split by experiment). The control patient cells from both sternal and femoral aspirates show similar results, and both are similarly different from MM. These findings, together with the clear increase in CD44+ cells in histological samples from MM iliac crest biopsies compared to control patient ileac crest biopsies (Figure 2) make us confident that location is not a factor driving stromal inflammation. For all experiments the age and gender distribution of the patient samples used in now presented in supplemental table 1. The underlying reason for the cardiac surgery or the hip-replacement surgery are now presented in supplementary table 3. We have actively excluded patients with a known history of inflammatory disease.

Minor:
Fig 1A: it is beautiful, but please show plot for all 10 patients and controls individually – if not in the manuscript, then at least in Suppl.

We apologize for the fact that this was not clear. The UMAP renderings for individual patients were in the original supplemental figure 2E, but referencing to this figure was not obvious enough. We

now include the patient specific renderings for the stromal cells in Figure 1F, for the immune cells in supplemental figure 3 and for the plasma cells in supplemental figure 4.

Fig 2: difficult to interpret – how many fibroblasts per MM cells? If the BM is packed with MM co-localization by chance is anticipated, right?

The reviewer is right in this regard. As mentioned also in reply to reviewer 2, our data does not provide any evidence for a preferential co-localization of MM cells and CD44+ stromal cells. in fact, many MM cells can be seen not interacting with CD44+ stromal cells, and we have added text to specifically detail this fact (page 7, line 21-22 and line 24-25). While this could be partly due to the fact that histology will likely only visualize CD44hi cells (a fraction of the inflammatory stroma), we favor the hypothesis that inflammatory stromal cells are interacting specifically with a subset of (possibly proliferating) MM cells. This is based on the predicted interactions between MSC1 and MM subset P7 in the new Figure 5, using the CellphoneDB algorithm. From the histology in figure 2 we only conclude that CD44+ stromal cells are interacting with MM cells and with T cells, a prerequisite for a biologically meaningful role in MM cell pathology or immune modulation. The mechanistic underpinnings of such interactions require novel lines of research.

In order to shorten the introduction, consider to eliminate line 14-19.

The introduction has been edited to accommodate the novel data in this revised resource.

Decision Letter, first revision:

Subject: Decision on Nature Immunology submission NI-RS30248B

Message: 13th Feb 2021

Dear Tom,

Thank you for providing your point-by-point response/rebuttal to the referees' comments on your revised Resource manuscript entitled, "An integrated definition of the immune and stromal micro-environment in Multiple Myeloma reveals mesenchymal stromal cell-centric bone marrow inflammation". As noted previously, we are very interested in the possibility of publishing your study in Nature Immunology. Accordingly, please revise your manuscript as indicated in your response and outlined below:

Q1
Add cytokine data (IL-1b, IL-6, IL-18, CCL2 and CXCL8).
Compare cytokine expression/abundance differences between MM patients who exhibit either low frequencies of CD138+ plasma cells and those with higher frequencies of CD138+ cells.

Q2
Clarify their bioinformatics dataset, including the number cells in the UMAP analyses and cutoffs used in differential gene expression.
Plot the DEG dataset in Supp Table 6 as a volcano plot and clarify DEGs in Fig. 6.
Add histology and flow cytometry for CD44 expression for BM MSCs.
Perform trajectory analysis of control MSCs.
Perform flow cytometry to analyze CD8+ Tscm and NK cells for MM patients and controls.
Add discussion of other stromal cell types.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Resource format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Please include a revised version of any required reporting checklist. It will be available to referees to aid in their evaluation of the manuscript goes back for peer review. They are available here:

Reporting summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please use the link below to submit your revised manuscript and related files:
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We hope to receive your revised manuscript within four weeks. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Kind regards,

Laurie

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The Authors responded to the majority of the issues of this reviewer
My concerns of this version are:
In the first version of the paper there was declared that the samples were process within 72hours but in the actual version the authors write 24 for most of the samples or 48 hours for some samples. Moreover the authors in the first version declared that the cell population that sustain the inflammation was fibroblast but now they state that these cells are mesenchymal stromal cells.
The authors did not prove the functional validation of the pro-inflammatory cytokines in the survival of the malignant clone.
They did not perform the correlation between plasma levels of cytokines (Il-1b, Il-6, CCl2, Il18, Cxcl8) and % iMSC as requested
As requested in the previous round of review, there are 2 samples with a very low % of CD138+ cells (0,9% (in the first version was 0%) and 1,2%) in these samples the authors have highlighted any difference with the samples with higher CD138+ %?

Reviewer #2:
Remarks to the Author:
The revised de Jong study, "An integrated definition of the immune and stromal micro-environment in multiple myeloma reveals stromal fibroblast-centric bone marrow inflammation" has improved substantially since the initial submission. The paper still lacks functional data to support any of the findings from the single cell RNA sequencing and unfortunately the MSC subtypes (from control and MM subjects) have not been adequately characterized (based on the RNAseq data) or validated (by flow or CyTOF). To strengthen the paper and bring it to the level for a full research article in Nature Immunology the authors should address the following remaining points.

1) The description of bioinformatic analysis of the data is insufficient. More details are needed throughout the paper. The figure legends and results section lack essential details related to all bioinformatic analysis (most of the paper). The number of cells in each UMAP should be added to the plot and clarified in the results and/or legends. What cut-off was used to include/exclude expressed genes?
2) The data in Figure 3e and 3f are potentially interesting. Were the analyses performed on the same day? The data should be plotted with the same y-axis scale in order to compare. Patient variability in Figure 3e is concerning.
3) Giving each of the clusters/subsets less arbitrary names would help the reader to

understand the relevance of the findings. I would urge the authors to name each cluster/subset based on a relevant marker, either one that is differentially expressed or one that is related to its predicted function.
4) The differences between MSC1 and MSC2 should be better highlighted in the results and main figures. For example, the DEGs in Supplemental Table 10 can/should be plotted as a volcano plot or heatmap. For all tables the expression levels of each gene should be included and the cut-offs should be clearly stated upfront. The number of DEGs (that reach statistical significance and expression level cut-offs) should be added to Figure 6c. The dots for each gene should be changed to black for visualization.
5) In the absence of any functional data, can the subsets of MSC be validated by flow?
6) The differences (or lack thereof) between PH and PT samples should be provided as separate UMAPs in Figure 1. Figure 1f suggests that MSC1/2 are more prominent/consistent in PH1-6 compared with PT1-7. Can the authors elaborate and clarify?
7) The authors have completely ignored the OLC, EC and SEC that emerge in the MM samples though these cells could be key contributors to disease and persistence after treatment. Can the authors at least elaborate on (acknowledge) these cells and the potential differences between control and MM?
8) Can the authors perform trajectory analysis for the MSCs in control subjects to better understand their predicted developmental relationships and discuss the findings?
9) Can the authors validate the increase in CD8 Tscm and GZMK+ NK cells by flow?
10) In the abstract the authors suggest that CD8+ effector T cells and CD8+ Tscm are sources of cytokines that activate the bone marrow stroma. Can the authors provide experimental evidence that the infiltrating (TNF-producing?) CD8 T cells can activate control MSCs (MSC4/5) to generate the MSC1/2 phenotype? This would greatly strengthen the paper by providing functional support of the in silico findings. Without these data the paper may be better suited for a 'resource' format.
11) The DEGs in Supplementary Table 11 should be shown in Figure 6 as a heatmap and discussed. The authors claim that the number of DEGs in Figure 6g is small but the way the graphic is made hides the differences. The number of DEGs (that reach statistical significance and expression level cut-offs) should be added to Figure 6g. The dots for each gene should be changed to black for visualization.
12) The model cartoon overstates the findings from the paper given no functional analysis between cell types was provided. Further, the cartoon oversimplified the data generated in the scRNAseq analysis. The authors have shown two subtypes of MSC in MM patient samples yet the cartoon shows only one type of iMSC. Finally, the relationships between MSCs in control samples and MM samples was ignored. The cartoon could be amended to reflect the concrete novel findings of this manuscript or otherwise omitted.

Reviewer #3:
Remarks to the Author:
The authors have answered adequately to all questions and comments, originality and significance for the community is from high level, therefore, I suggest to accept the manuscript for publication.

Author Rebuttal, first revision:

Reviewer #1

The Authors responded to the majority of the issues of this reviewer
My concerns of this version are:

In the first version of the paper there was declared that the samples were process within 72hours but in the actual version the authors write 24 for most of the samples or 48 hours for some samples.

We thank the reviewer for the opportunity to clarify this issue. The alterations in the revised version were made in response to remarks from this reviewer on the original submission, as explained in our 1st point-by-point-reply.
The 72 hrs stated in our manuscript was the maximal time allowed in the clinical protocol, yet all samples used in our study were frozen down within 24 hrs of sample collection, with the exception of 3 samples (PT1 and PT2 in scRNAseq and R7 in bulk seq) that took 48 hrs. We have updated the text in the methods section to reflect this fact.
To control for possible alterations in the immune-phenotype of the mesenchymal stromal cells, we initially performed single cell sequencing on a freshly isolated sample immediately following aspiration in our hospital, without a freeze-thaw cycle. We have now included this sample as supplemental figures 2K-L to show that the inflammatory phenotype is also found in these freshly prepared cells. Of note, all control patient samples were also left for 24hrs and subsequently viably frozen before isolation in order to avoid introducing any bias.

Moreover the authors in the first version declared that the cell population that sustain the inflammation was fibroblast but now they state that these cells are mesenchymal stromal cells.

We thank the reviewer for the opportunity to clarify this issue. The alterations in the revised version were made in response to remarks from this reviewer on the original submission, as explained in our 1st point-by-point-reply.
The numbers of primary stromal cells that we can isolate are too low to allow for direct assessment of multilineage potential. This means that cells need to be propagated for multiple passages before we can assess their potential. When we do this, we see multilineage differentiation in the cultured cells from both MM patients and non-myeloma controls, similar to ADSCs (positive control) (as shown in Reviewer figure 1, original reply). Since we are unable to determine whether this potential is also present in cells directly ex-vivo, we agree with the reviewer that we currently do not have sufficient data to define the cells as fibroblasts. We therefore have altered the denomination of the cells to inflammatory mesenchymal stromal cells (iMSC).

The authors did not prove the functional validation of the pro-inflammatory cytokines in the survival of the malignant clone.

The functional consequences of the bone marrow inflammation, and the increased levels of IL-18 and various chemokines that we see are indeed not addressed in our study. Moreover, we are careful not to suggest direct effects of the inflammation on the malignant cells, but to rather highlight the immune-modulatory potential of these cytokines. In general, bone marrow inflammation is increasingly recognized as a multifactorial driver of tumorigenesis in several hematological malignancies, impacting on biological processes as diverse as DNA repair, ROS production, mitochondrial stress, immune cell differentiation and malignant cell proliferation. Defining the role of bone marrow inflammation in multiple myeloma is a long-term project that we

are undertaking in the lab, but which we feel goes well beyond the scope of the current Resource study.

They did not perform the correlation between plasma levels of cytokines (Il-1b, Il-6, CCl2, Il18, Cxcl8) and % iMSC as requested

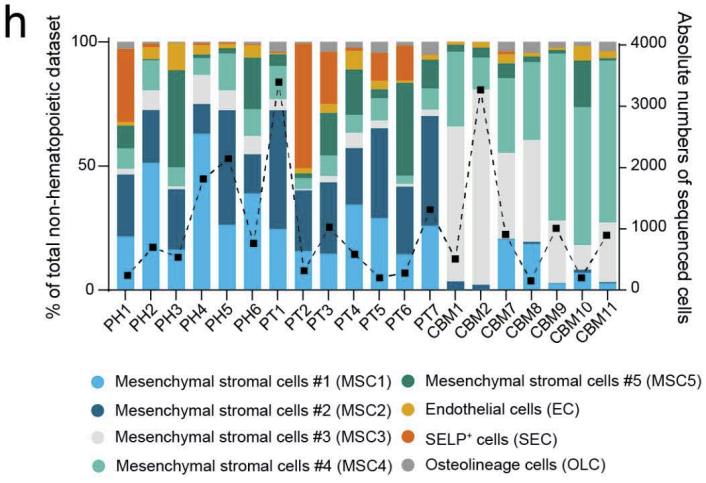
We apologize to the reviewer for not including the correlations of IL1b and TNF with percentage of iMSC as requested originally. We had performed the analysis for IL-1b and no correlation was found. TNF unfortunately was undetectable. These results should have been included in our revised manuscript and the omission was unfortunate and a mistake on our part.
For the additional cytokines the reviewer is referring to in this second review (IL6, CCL2, IL18 and CXCL8) we have now performed the correlations and added these in Supplemental Fig 3C. None of the cytokines show a significant correlation to percentage of iMSC.

As requested in the previous round of review, there are 2 samples with a very low % of CD138+ cells (0,9% (in the first version was 0%) and 1,2%) in these samples the authors have highlighted any difference with the samples with higher CD138+ %?

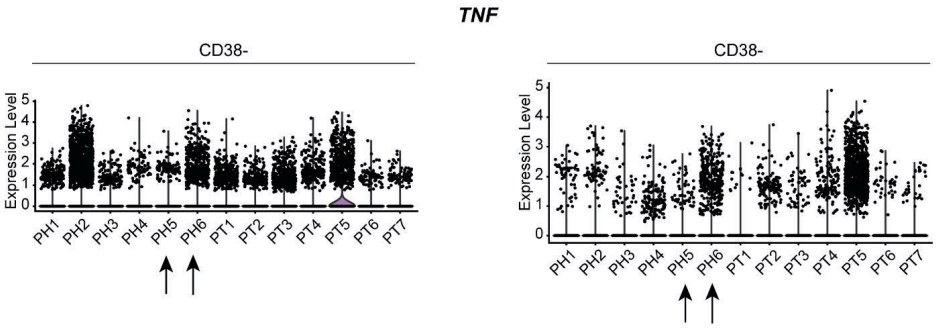
In our initial response to this comment, we had focused on explaining why the apparent absence of MM cells (0%) was incorrect. We have now evaluated the two patients with lowest MM cell numbers to determine any deviations from the rest of the dataset. The patients in question are PH5 (1.2%) and PH6 (0.9%).

Both patients are hyperdiploid IgG Myeloma's at R-ISS stage I. M-protein levels are within the normal range: 2.84 g/dL (PH5) and 2.04 g/dL (PH6), average M-protein is 2 g/dL with range of 0.22-3.85 g/dL. Overall survival is 359d (PH5) and 389d (PH6). Average in this cohort is 437d, with range of 28-752d.

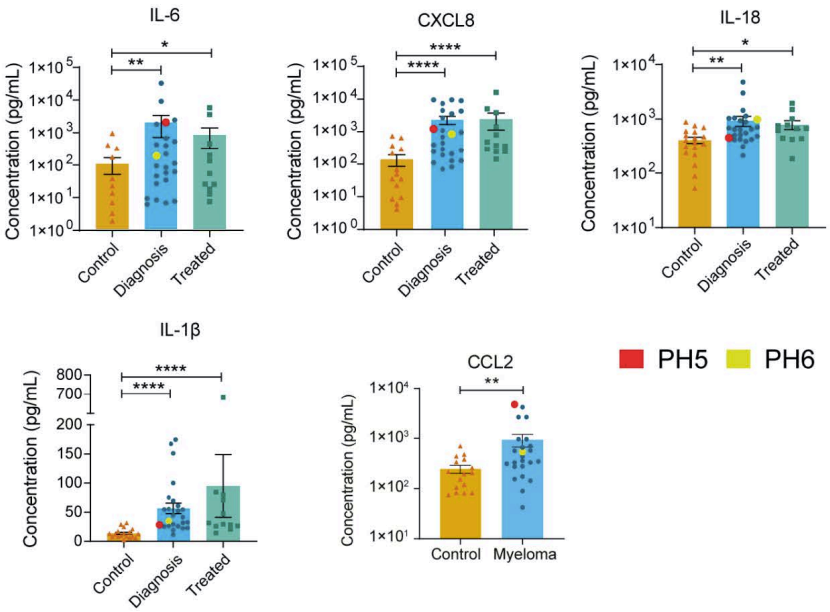
Percentage of iMSCs in the bone marrow is similar to that of other patients, as depicted in supplemental figure 2H (PH5 and PH6).



Expression levels of TNF in hematopoietic cells are also comparable to other patients as shown below:



As are the protein levels of inflammatory proteins:



With all the information we currently have we cannot identify any differential features defining these two patients.

Reviewer #2

The revised de Jong study, "An integrated definition of the immune and stromal micro-environment in multiple myeloma reveals stromal fibroblast-centric bone marrow inflammation" has improved substantially since the initial submission. The paper still lacks functional data to support any of the findings from the single cell RNA sequencing and unfortunately the MSC subtypes (from control and MM subjects) have not been adequately characterized (based on the RNAseq data) or validated (by flow or CyTOF). To strengthen the paper and bring it to the level for a full research article in Nature Immunology the authors should address the following remaining points.

We thank the reviewer for the positive feedback. Confusion appears to have arisen on the nature of our manuscript. The reviewer is assessing our study as a full research paper, not as the Resource study that it is. While we have indeed tried to present the resource data as much as possible along hypothesis-driven narratives to increase readability and broaden the interest and scope, the core of our study still is the unique paired datasets of non-hematopoietic niche, tumor microenvironment and tumor cells from 13 newly diagnosed multiple myeloma patients, compared to non-cancer controls.

1) The description of bioinformatic analysis of the data is insufficient. More details are needed throughout the paper. The figure legends and results section lack essential details related to all bioinformatic analysis (most of the paper). The number of cells in each UMAP should be added to the plot and clarified in the results and/or legends. What cut-off was used to include/exclude expressed genes?

We apologize for the apparent unclarities. We have added the number of cells in each UMAP. For the single cell RNAseq data and for the Bulk seq data no gene expression cut-offs were used, and all significantly altered genes were included in the analyses. We have added Log2Fold change cut-offs and significance cut-offs to all tables and legends, as well as to the volcano plots.

The methods section has been updated to better report the methodology. All the code that was used to generate the data in our study is available online via GitHub, as referenced in the manuscript.

2) The data in Figure 3e and 3f are potentially interesting. Were the analyses performed on the same day? The data should be plotted with the same y-axis scale in order to compare.

Figure 3e and f (stimulations of various MM and control patient stromal cells) were performed on multiple days, and with multiple donors. We therefore were purposefully not directly comparing the two situations, and to only conclude that similar genes are induced in myeloma cells and controls. We have adjusted the y-axis to allow easier comparison.

Patient variability in Figure 3e is concerning.

As stated in response to the previous remark, these studies aim to make the point that the genes found in the single cell analyses are induced by stimulation. Multiple myeloma is a heterogeneous disease and variability was therefore expected. Moreover, the in vitro expansion of cells prior to the experiment is also a likely contributor to this variability. This notwithstanding, this figure depicts a clear increase in inflammatory transcripts in all patients in response to TNF or IL-1b.

3) Giving each of the clusters/subsets less arbitrary names would help the reader to understand the relevance of the findings. I would urge the authors to name each cluster/subset based on a relevant marker, either one that is differentially expressed or one that is related to its predicted function.

We share the reviewer's preference for clarity and understanding, but on this issue we respectfully disagree. Renaming the MSC clusters with gene names would imply functional significance of such a gene in the absence of supporting data.
MSC clusters 3-5 (present in MM and controls) are composed of cells that are largely homogeneous in gene expression. The differentially expressed genes are not unique to a single cluster, but rather expressed to higher levels/frequencies of cells in specific communities leading to the algorithm forcing cells into separate clusters. Whether these remaining cluster (MSC3-5) also represent functionally different populations of cells has yet to be determined experimentally.

To highlight the unique inflammatory nature of MSC1 and MSC2 we refer to these as iMSC (inflammatory MSC) in the manuscript.

4) The differences between MSC1 and MSC2 should be better highlighted in the results and main figures. For example, the DEGs in Supplemental Table 10 can/should be plotted as a volcano plot or heatmap.

We have now presented the 100 most differential genes that distinguishing MSC1 from MSC 2 in a heatmap in the new figure 3G, as well as in Supplementary table 7.

For all tables the expression levels of each gene should be included and the cut-offs should be clearly stated upfront. The number of DEGs (that reach statistical significance and expression level cut-offs) should be added to Figure 6c. The dots for each gene should be changed to black for visualization.

We have altered all tables and figures as per reviewer's suggestion. Tables now depict the average expression level per gene and the Log2Fold change and significance cut-offs. No cut-offs for expression levels were used.

5) In the absence of any functional data, can the subsets of MSC be validated by flow?

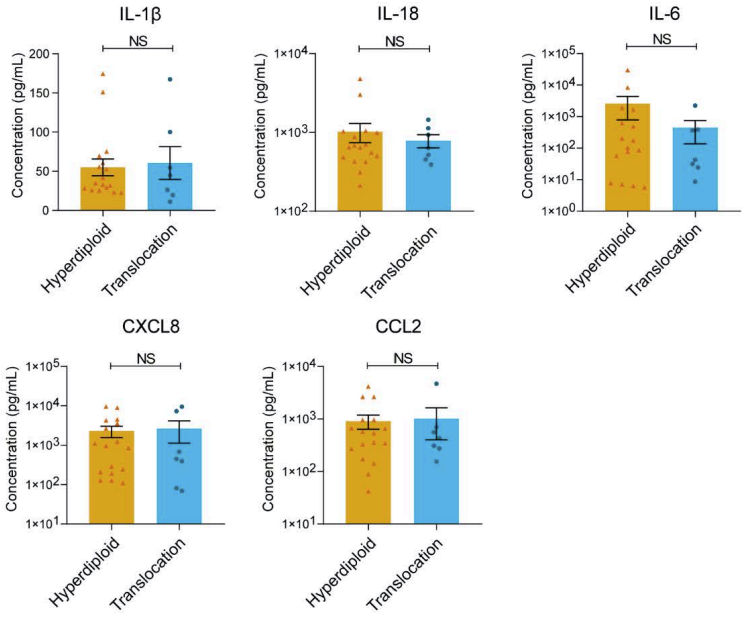
We thank the reviewer for this remark. We have extensively tried to identify surface markers allowing flow-cytometric detection of the MSC clusters. This has proven unsuccessful so far, mainly due to the fact that very few unique markers exist, and clusters are mainly driven by variations in expression levels and percentage of positive cells.
At this point the only suitable marker for the inflammatory MSC (iMSC) that can be used in flow cytometry is CD44. However, this is not a unique marker for iMSC, but rather enriched on iMSC compared to other MSC (Figure 1E). In figure 2 we showed that CD44 can be used to identify iMSC in histology. We have analyzed ex-vivo bone marrow MSC for CD44 expression by flow cytometry, and can now show that the percentage of CD44+ MSC is significantly higher in MM patients compared to control patients. We have added these data to the manuscript as new figure 2A. In this way the increase in CD44+ iMSC has now been validated both by histology (figure 2B-D) and by flow cytometry (Figure 2A).

6) The differences (or lack thereof) between PH and PT samples should be provided as separate

UMAPs in Figure 1. Figure 1f suggests that MSC1/2 are more prominent/consistent in PH1-6 compared with PT1-7. Can the authors elaborate and clarify?

The reviewer raised an interesting point. We have generated the separate UMPAs and present these now as Figure 1 G-H). Quantification of the different clusters suggests minor differences between hyperdiploid and translocated patients, especially in the ratio of MSC1 and MSC2. However, variation is high, as visualized in supplemental figure 2H, and no significant differences are identified. In addition to the extra panels, we have added accompanying text on page 7, lines 6-10.

Comparing inflammatory protein expression in Bone marrow plasma also did not show differences between hyperdiploid and translocated patients as shown below for the reviewer.



7) The authors have completely ignored the OLC, EC and SEC that emerge in the MM samples though these cells could be key contributors to disease and persistence after treatment. Can the authors at least elaborate on (acknowledge) these cells and the potential differences between control and MM?

We can only agree with the reviewer's remark. We have focused our investigations on the MSC populations, driven by the clear differences between MM patients and control patients, and the fact that MSC represent the majority of cells in our analyses. This notwithstanding, OLC, EC and SELP+ cells are all potential MM niche populations. EC and SELP+ cells are both increased in MM patients (Figure S2G). We have added text (page 6, lines 2-7) to acknowledge this fact. The nature of the SEC cells is still unknown, yet their differential nature is potentially an interesting finding for follow-up investigation. We have also added the cluster-defining markers of the SELP+ cluster in supplementary table 4 to allow scrutiny of this population by the research community.

8) Can the authors perform trajectory analysis for the MSCs in control subjects to better understand their predicted developmental relationships and discuss the findings?

We have performed the trajectory analyses on the control patient only and are now showing these data as Supplemental figure 3D. The results are largely in line with the trajectories in the MM patients. There appear to be a few more trajectories in the control dataset, but the relevance of that prediction is at this point unclear.

9) Can the authors validate the increase in CD8 Tscm and GZMK+ NK cells by flow?

We have used a new cohort of newly-diagnosed MM patients (15) and control patients (5) to validate these populations by flow cytometry (Figure 4H and gating in new supplemental Figure 4). Tscm were defined by expression of CD8, CD3, CD38, CD45RA, CD27 and CD95. Tscm were also increased in MM patients by this flow cytometric analyses.
$CD56^{bright}$ NK cells were defined by absence of CD3 and expression of CD56 (bright). In addition, as CD27 was one of the defining markers of the $CD56^{bright}$ cluster in the transcriptomic analyses (Suppl Fig 5D), we analyzed this separate as well. Both total $CD56^{bright}$ and $CD56^{bright}$ $CD27^{+}$ NK cells were increased in MM patients compared to controls. We confirmed the GZMK transcription by both CD56bright populations by qPCR on sorted cells (Supplemental Fig 5E)

10) In the abstract the authors suggest that CD8+ effector T cells and CD8+ Tscm are sources of cytokines that activate the bone marrow stroma. Can the authors provide experimental evidence that the infiltrating (TNF-producing?) CD8 T cells can activate control MSCs (MSC4/5) to generate the MSC1/2 phenotype? This would greatly strengthen the paper by providing functional support of the in silico findings. Without these data the paper may be better suited for a 'resource' format.
While we share the interest of the reviewer for further delineating the mechanistic underpinnings of immune cell – stromal cell interactions in MM, we feel that the experiments suggested by the reviewer go beyond the Resource nature of our manuscript, and would better fit a Research manuscript, as the reviewer also is suggesting.

11) The DEGs in Supplementary Table 11 should be shown in Figure 6 as a heatmap and discussed. The authors claim that the number of DEGs in Figure 6g is small but the way the graphic is made hides the differences. The number of DEGs (that reach statistical significance and expression level cut-offs) should be added to Figure 6g. The dots for each gene should be changed to black for visualization.

It was not our intention to hide any data in figure 6G, and we have changed the figure to present the data more clearly, indicating number of DEG and cut-offs. The initial figure had no expression level cut-offs and included all DEG.
In response to the concerns raised by this reviewer we reanalyzed this dataset which led to the identification of a previously unnoticed error in the data processing. A few samples had been wrongly labelled as either diagnosis or post-induction therapy. We have corrected this in the novel version of Figure 6 and supplemental table 13. This correction has two minor consequences:

1. The number of DEG (without expression level cut-off) increased from 51 to 158 in the new analyses
2. In the new analyses we see a small but significant overrepresentation of T cell-related genes, as depicted in supplemental figure 7D and supplemental table 13

There is still no difference in any of the MSC-related genes as shown in supplemental figure 7E-F and no difference in the inflammatory transcriptome as shown in figure 6G-H.

We have added a heatmap showing the top differential DEG as new supplemental Fig 7G.

12) The model cartoon overstates the findings from the paper given no functional analysis between cell types was provided. Further, the cartoon oversimplified the data generated in the scRNAseq analysis. The authors have shown two subtypes of MSC in MM patient samples yet the cartoon shows only one type of iMSC. Finally, the relationships between MSCs in control samples and MM samples was ignored. The cartoon could be amended to reflect the concrete novel findings of this manuscript or otherwise omitted.
We thank the reviewer for these comments. We have altered the model cartoon to focus more on our findings. The text has also been altered (page 17, line 1-10, and figure legend) to clearly state the hypothetical nature of the model. We feel that including the hypothetical model is of value as it provides a possible direction for future experimental validation or falsification.

Reviewer #3

(Remarks to the Author)
The authors have answered adequately to all questions and comments, originality and significance for the community is from high level, therefore, I suggest to accept the manuscript for publication.

We thank the reviewer for the constructive feedback that helped to improve our study.

Decision Letter, second revision:

Subject: Your manuscript, NI-RS30248C

Message: Our ref: NI-RS30248C

29th Mar 2021

Dear Dr. Cupedo,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "An integrated definition of the immune and stromal micro-environment in Multiple Myeloma reveals mesenchymal stromal cell-centric bone marrow inflammation" (NI-RS30248C). Please carefully follow the step-by-step instructions provided in the personalised checklist attached, to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments and please make sure to upload your checklist.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "An integrated definition of the immune and stromal micro-environment in Multiple Myeloma reveals mesenchymal stromal cell-centric bone marrow inflammation". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Immunology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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If you have any further questions, please feel free to contact me.

Best regards,

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Fax: 212 696 9752
E-mail: immunology@us.nature.com

On behalf of

Laurie A. Dempsey, Ph.D.
Senior Editor
Nature Immunology

l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Reviewer #1:
Remarks to the Author:
Authors responded to the queries of this reviewer.

Reviewer #2:
Remarks to the Author:
The authors have adequately addressed all of my concerns as they pertain to this manuscript to be published as a resource paper. I commend the authors for making every effort to address all reviewer concerns and for bringing this informative work and elegant paper to forefront.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-RS30248D

Message: In reply please quote: NI-RS30248D

Dear Tom,

I am delighted to accept your Resource manuscript entitled "The multiple myeloma micro-environment is defined by an inflammatory stromal cell landscape" for publication in an upcoming issue of Nature Immunology.

The manuscript will now be copy-edited and prepared for the printer. Please check your calendar: if you will be unavailable to check the galley for some portion of the next month, we need the contact information of whom will be making corrections in your stead. When you receive your galleys, please examine them carefully to ensure that we have not inadvertently altered the sense of your text.

Acceptance is conditional on the data in the manuscript not being published elsewhere, or announced in the print or electronic media, until the embargo/publication date. These restrictions are not intended to deter you from presenting your data at academic meetings and conferences, but any enquiries from the media about papers not yet scheduled for publication should be referred to us.

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Your paper will be published online soon after we receive your corrections and will appear in print in the next available issue. Content is published online weekly on Mondays and Thursdays, and the embargo is set at 16:00 London time (GMT)/11:00 am US Eastern time (EST) on the day of publication. Now is the time to inform your Public Relations or Press Office about your paper, as they might be interested in promoting its publication. This will allow them time to prepare an accurate and satisfactory press release. Include your manuscript tracking number (NI-RS30248D) and the name of the journal, which they will need when they contact our office.

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Kind regards,

Laurie

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