Cell Genomics Transparent Peer Review Record

Cystatin C is glucocorticoid-responsive, directs recruitment of Trem2+ macrophages and predicts failure of cancer immunotherapy

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Summary

Initial submission:	Received : 6/13/2023
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First round of review:	Number of reviewers: 3 Revision invited : 11/6/2023 Revision received : 3/23/2023
Second round of review:	Number of reviewers: 2 Accepted : 5/30/2023
Data freely available:	Yes
Code freely available:	Yes

This transparent peer review record is not systematically proofread, type-set, or edited. Special characters, formatting, and equations may fail to render properly. Standard procedural text within the editor's letters has been deleted for the sake of brevity, but all official correspondence specific to the manuscript has been preserved.

CellPress

Referees' reports, first round of review

Reviewer #1: The manuscript by Kleeman et al. reports that cystatin C is glucocorticoid-responsive, directing recruitment of Trem2+ macrophages and predicts failure of cancer immunotherapy. Importantly, in this manuscript the authors show for the first time a direct link between GC signaling, CyC and Trem2+ macrophages. Thus the manuscript represents a significant contribution into the field, is well written and easy to follow, while the experimental design is well done and the conclusions are supported by the experimental evidences. Here are small problems to be fixed:
- General to all the Figures: the fonts are mostly too small for the Journal format;
- The reference to Fig. 1a is missed within the text. Appropriate text describing this scheme should be added into the main text;
- The reference to Fig. 5e is also missed within the text;
- The reference to Fig. 6d is also missed within the text.

Reviewer #2: In the paper by Kleeman et. al., the authors address the role of glucocorticoids with the induction of cystatin C, a secreted cysteine protease. They used the UK Biobank GWAS data to identify the genetic link of the latent CyC, and then associated Cyc production following GR engagement with myeloid cells across multiple tissues. Cystatin production PGS was associated with reduced lifespan in the UK biobank, which was correlated with elevated metabolic syndrome, hypertension, obesity, and cardiovascular disease. Patients with Cyc also predicted increased mortality risk to many cancers. Mechanistically, GR-mediated Cyc induction has previously been shown in rodent models. However, interestingly Cyc induction in myeloid cells was only apparent in cells during diseased settings, and not following GR activation. Using Cyc-deficient tumors, the authors went on to find reduced Trem2+ macrophages which have been previously associated with tumor progression. They then show that Cyc levels could be used to predict patient responses to caner immunotherapy with anti-PD1 antibodies. Overall, the authors conclude that CyC is an effector for glucocorticoid signaling during inflamed settings (including metabolic disease, cardiovascular disease, kidney failure, and cancer) that promotes Trem2+ macrophage recruitment and overall immunosuppression.

Data in this manuscript is presented clearly and in a rigorous manner. The authors have accumulated a remarkable quantity of data to communicate an exciting story. Many observations from this study apply to a myriad of disease settings, so appears to be broadly relevant to a wide range of researchers. I have no major requests for revisions.

Minor Comments:
-In the KPC tumor model, it is unclear whether the Trem2+ macrophages derive from infiltrating monocytes or tissue resident macrophage origins. In addition, loss of Trem2+ macrophages may also result from cell death or down-regulation of Trem2 expression. These should likely be discussed.
-Some figures are difficult to read and need larger fonts. An example is figure 3E
-Supplemental Figures need legend titles.

Reviewer #3: Comments enter in this field will be shared with the author; your identity will remain anonymous.

The article by Kleeman et al seeks to establish a causal relationship between Cystatin C levels and resistance to cancer immunotherapy using genomic study approaches combined with mathematical modeling. The authors also include some wet-lab mechanistic studies. The paper is a tremendous amount of information, data and effort. The approach is based on a lot of assumptions that may or may not be biologically relevant (assume the deviation from correlation with eGFR-creatine is all due to cellular production). Nonetheless, we recognize that defining assumptions is the nature of mathematical modeling. The paper's strength is in the sample size, the inclusion of a validation cohort and assessing the approach in genetically diverse backgrounds.
However, the author's reasoning and logical flow from one point to the next creates a barrier for the reader and presents the largest challenge for this manuscript. The authors lead the reader from one assumption to the next, each one being based on the validity of the previous remark/assumption/conclusion. Biology rarely fits a simple algebraic equation (CyC= renal plus cell production). Furthermore, the authors jump from one concept or model to the next as if they are "randomly selecting" data that supports their reasoning. Therefore the authors need to alter the manuscript language to reflect their bias in logic and make the transitions "softer" and more indicative of their statements as hypotheses. The manuscript is an interesting interpretation of a lot of data/analyses, worthy of publication. The rationale for why certain models and approaches are chosen needs to be clearer. Also including models that may not have supported the rationale should be included in the discussion.
The manuscript exams the role of CST3 gene (CyC) and it's correlation to cancer immunotherapy. This is not a novel concept and has been shown in many manuscripts- these references and concepts should be included. The authors use genomic data to devise a score to show association, however the association is post-treatment which limits clinical utility. The authors also try to link a mechanism of increased production by macrophages through activation by GC in a diseased state. The data support this as a possible mechanism contributing to a complex biological system. The conclusions need to incorporate the concept that this is complex system and this pathway may be important contributor. Many of the conclusions are overstated:
"Moreover, the peak in CyC-residual appears to be delayed by approximately 18 hours compared to the expected peak in plasma cortisol, consistent with our in vitro experiments. Taken together, these findings demonstrate that CyC production is directly induced by GCs, and that CyC has a reduced diurnal amplitude and offset periodicity compared to plasma cortisol. "
""On the basis of these results, we hypothesized that monocyte-like THP-1 cells would have high basal CST3 gene expression and secretion of CyC without GC-inducibility, and we confirmed this by RT-PCR (Figure 4f) and ELISA (Figure 4g) "
The data do not support an exclusive role of this specific monocyte population (myeloid and other macrophage lineages showed stronger correlation) and figures 4f and 4g do not support the conclusion well either.
Statements that their signature is superior to predicting people with disease is overstated.
Reviewer specific comments:
1) Overall many figures suffer from very small scale and difficult to read
2) Better explanation of PGS decile scale in Figure 1f.
3) Correlations of residual CyC as a marker for increased mortality are confusing. Is this just another (better?) marker of excessive inflammation and how does this relate to your downstream story? Figure 2D-E: Results contradictory between TCGA and UKB validation cohorts for many of the cancers so what can we take from this?
4) Figure 3a: where is rs2749527 in UKB group?
5) Supplemental Figure 3b: the trans correlation for CST3 does not hold in another European cohort and is not mentioned in the text. Explanation is required
6) Explain better the purpose of Trans-eQTL analysis analysis in visceral fat? Is there any association in other tissues - should be stated.
7) Figure S3e- this approach is a stretch to supplement plasma bilirubin for cortisol and make this conclusion. This concept can be added to discussion but should be removed from results.
8) I don't understand the significance of this: "In support of this, we identified circadian rhythmicity from cosinor regression of spleen CST3 gene expression against time of death, which was attenuated compared to the canonical GC target FKBP5 (amplitude = 0.060 versus 0.24, Figure S4a)."
9) Figure 4f and 4g need a control - high relative to what? The authors use human data from data

sets to support their conclusions and then use only cell lines for experimental support. Validation should be done with human primary cells (they are easy to get) - could use human B cells or T cells as control.
10) The in vitro Dexamthasome dose seems rather high? Is 100nM relevant? The vivo 20 mg/kg = 20 mg/kg*70 kg = 1400 mg (COVID dose is 6 mg daily). This is unreasonably high.
11) Why does Cyc not go up in total mouse after dexamethasone treatment when you showed in vitro you get upregulation and Cyc is GR responsive?
12) "Altogether, these findings indicate that CyC production is relatively constant in health, and in health does not significantly increase in response to GC agonism, explaining the validated utility of CyC as a marker of renal function in patients without acute illness." This connection is not sound logic.
13) "As GR is expressed in macrophages but not in monocytes61, we hypothesized that, while monocytes have high constitutive basal CyC production, macrophages would secrete CyC in response to GC signaling" Another example of seemingly random hypotheses - why would monocytes have high consititutive CyC without a GR receptor - what is controlling that? The literature does not support this simplistic statement in fact it has been shown that Monocytes have more transcriptional alterations following GC signaling than human macrophages. This manuscript relies on in vitro experiments with cell lines that have been shown to not behave similarly to macrophages. Primary cells should be investigated. GC comparison of CyC production (RNA and protein) for constitutive monocyte, macrophage and in presence of dexamethasone should be shown in primary cells (in vitro differentiated macrophages from blood monocytes). The authors later recognize that GC signaling is required but not sufficient for CST3 expression (although this is not shown with knockouts/etc). GC targets are ubiquitous and so difficult to distinguish.
14) "The findings that CyC is primarily expressed by myeloid cells and that GC responsive CyC secretion is predominantly mediated by macrophages have the potential to explain our finding" - This has not been shown in a direct comparison of CyC levels, and even in the CST3 expression data the DCs are just as high. You have shown that myeloid cells are the largest producers in the blood. Your data does not support the exclusive effect of macrophages.
15) Do we need a direct correlation of CyC protein vs CST3 expression? We keep seeing Cy residual and other "substitute readouts." The real correlation should be measured in primary cells.
16) Minor: Figure 4h does not show melanoma CST3 expression. Wrong reference in text. Also grammar errors in same paragraph.
17) The use of cell lines should be validated in primary cells, e.g. in Figure 5, treatment to THP-1. Also the data for 18 hr is so variable that this is not a plausible conclusion. It would be nice to see a correlation of the transcription data to the CyC protein data in these analyses per sample. There are numerous samples in the protein assay (Fig 5B) but only 3 in the transcription data. The qPCR data suggests an issue with reproducibility.
18) The authors fail to recognize other work related to cancer and CST3 expression, which has shown significant expression by various tumors, and by protein and not only RNA. Others have reported differential prognosis based on CyC. Others have also shown the CyC is GC-responsive and may relate to the differences in cancer outcomes. The authors should address these data in light of their work.
19) In addition to showing CyC in murine cancer models the authors could refer to lots of work in human cancers that have shown this increase in CyC in plasma of cancer patients. Overstated conclusions - figure 5h - needs an in part by the tumor (they do not know if the in vivo elevations are also mediated by inflammatory effects).
20) The CST3-/- is a nice experiment. Why pancreatic cancer - rationale needs improvement. The language needs correction: "While isogenic sgScrambled and CST3-/- Mm1 clones had equivalent doubling times in vitro (sgScrambled: 23.3 hours, 95% CI 21.6-25.4; CST3-/-): 24.0 hours, 95% CI 22.4-25.8, Figure S6c)"
21) Also the rationale for the immune related component is difficult to follow - what does the cysteine proteases and role of GCs have to do with this- the point is the rationale is difficult to follow. The rationale for including anti-PD-L1 in the bi-flank model is not clear. The data look the same as without the PD-L1. The untreated control in this model should be included. This is not a CBI responsive tumor. The experiment in an immunodeficient mouse would be a better experiment for immune-related contribution.
22) Minor: The tumor volume scales should be the same for all graphs
23) Minor: Fig 6e: nomenclature is CD not Cd
24) Minor: Fig 6c y-axis should actually by %Ki67+ tumor cells? PI is undefined and vague
25) Minor: Endpoint tumor weights at endpoint (language)

26) Reference to Figure S7c is incorrect.
27) Comment: Trem2 might regulate trafficking of other cells into the tumor - but your data did not show differences in the other cell populations?
28) How can you call the CD8+ population effector T cells? What gene expression is showing this? It looks similar to CD4 - "effector" implies function which is not included in your data.
29) Switching to the COVID model from the cancer immunotherapy is a bit of whiplash. Need better explanation for the TREM2 definition of cluster 0. Also this data needs ICU patients not treated with Dexamethasone as a control.
30) Alternate hypothesis is CyC plays a role in survival of the cells (alters metabolic condition of the tumor?)
31) Figure S8 - why is this data supplemental? A) need better explanations for reader of the C clusters. Why BCC for analyses? This is an example of changing models without clear rationale. The macrophages CST3 expression showed no effect although your work has focused on the CyC production from this population. The production by the MOs was an overstated conclusion as being the most relevant as the other populations (DCs) were not really studied.
32) The association of CyC and poor prognosis in many cancers is not new data. How does a complicated PGS score, based on post-treatment response help as a biomarker, since the pre-treatment levels did not correlate? The odds ratio being a positive or negative benefit in figure 6h is confusing and should be more clear (odds of more PFS or worse?). The data does not hold for individual cancers.
33) Methods
- Define FACS buffer
- Medias should contain concentrations of all ingredients (how much glutamine?)
34) Discussion: Well-written. The authors present their arguments and model. However many statements do not recognize the biological complexity of GC-induced immunosuppression, CyC secretion and Cancer immunosuppressive mechanisms. The tone of the discussion should reflect their pathway as one possible mechanism of many. Some statements are not helpful nor necessary (e.g. suggesting CyC plays a role in immune privilege). The portion on POTENTIAL mechanisms is nice and indicates this is a possibility and is the authors hand-waving at this point.

Authors’ response to the first round of review

Reviewer comments
We would like to thank all three reviewers for their insightful and constructive comments. We have addressed all of the comments in detail and a better manuscript has emerged as a consequence. We provide a detailed point-by-point response in red for each reviewer below.

Reviewer #1
The manuscript by Kleeman et al. reports that cystatin C is glucocorticoid-responsive, directing recruitment of Trem2+ macrophages and predicts failure of cancer immunotherapy. Importantly, in this manuscript the authors show for the first time a direct link between GC signaling, CyC and Trem2+ macrophages. Thus the manuscript represents a significant contribution into the field, is well written and easy to follow, while the experimental design is well done and the conclusions are supported by the experimental evidences.

We thank the reviewer for the precise and insightful summary of our work and for the positive feedback on our research.

Here are small problems to be fixed:
- General to all the Figures: the fonts are mostly too small for the Journal format;

We agree and have modified the formatting.

- The reference to Fig. 1a is missed within the text. Appropriate text describing this scheme should be added into the main text;

We have updated the relevant reference (line 131).

- The reference to Fig. 5e is also missed within the text;

We have updated the relevant reference (line 412).

- The reference to Fig. 6d is also missed within the text.

We have updated the relevant reference (line 501).

Reviewer #2
In the paper by Kleeman et. al., the authors address the role of glucocorticoids with the induction of cystatin C, a secreted cysteine protease. They used the UK Biobank GWAS data to identify the genetic link of the latent CyC, and then associated Cyc production following GR engagement with myeloid cells across multiple tissues. Cystatin production PGS was associated with reduced lifespan in the UK biobank, which was correlated with elevated metabolic syndrome, hypertension, obesity, and cardiovascular disease. Patients with Cyc also predicted increased mortality risk to many cancers. Mechanistically, GR-mediated Cyc induction has previously been shown in rodent models. However, interestingly Cyc induction in myeloid cells was only apparent in cells during diseased settings, and not following GR activation. Using Cyc-deficient tumors, the authors went on to find reduced Trem2+ macrophages which have been previously associated with tumor progression. They then show that Cyc levels could be used to predict patient responses to caner immunotherapy with anti-PD1 antibodies. Overall, the authors conclude that CyC is an effector for glucocorticoid signaling during inflamed settings (including metabolic disease, cardiovascular disease, kidney failure, and cancer) that promotes Trem2+ macrophage recruitment and overall immunosuppression.

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We thank the reviewer for the systematic, comprehensive, and insightful summary of our work and for the positive feedback on our research.

Minor Comments:
-In the KPC tumor model, it is unclear whether the Trem2+ macrophages derive from infiltrating monocytes or tissue resident macrophage origins. In addition, loss of Trem2+ macrophages may also result from cell death or down-regulation of Trem2 expression. These should likely be discussed.

We are grateful for the reviewer raising this important question. We are unable to comment precisely on the origin of the Trem2+ macrophages in this tumor model without performing specific lineage tracing experiments that are beyond the scope of this present study. We have referenced this question in the modified discussion (lines 661-665). We note that there is significant uncertainty in the field regarding the mechanism by which macrophages acquire a Trem2+/Apoe+ differentiation program. We agree that while it is biologically plausible that CyC could drive recruitment of Trem2+ macrophages, we do not have any definitive evidence to prove this, and it is equally possible that CyC is necessary for survival of Trem2+ macrophages or maintenance of Trem2 expression. In response to the reviewer, we have modified the discussion to reflect this uncertainty (lines 665-679)

-Some figures are difficult to read and need larger fonts. An example is figure 3E

We agree with this point, which was also raised by reviewer #1, and have modified the formatting.

-Supplemental Figures need legend titles.

We apologize for the accidental omission and legend titles have now been added.

Reviewer #3
The article by Kleeman et al seeks to establish a causal relationship between Cystatin C levels and resistance to cancer immunotherapy using genomic study approaches combined with mathematical modeling. The authors also include some wet-lab mechanistic studies. The paper is a tremendous amount of information, data and effort. The approach is based on a lot of assumptions that may or may not be biologically relevant (assume the deviation from correlation with eGFR-creatine is all due to cellular production). Nonetheless, we recognize that defining assumptions is the nature of mathematical modeling. The paper's strength is in the sample size, the inclusion of a validation cohort and assessing the approach in genetically diverse backgrounds.

However, the author's reasoning and logical flow from one point to the next creates a barrier for the reader and presents the largest challenge for this manuscript. The authors lead the reader from one assumption to the next, each one being based on the validity of the previous remark/assumption/conclusion. Biology rarely fits a simple algebraic equation (CyC= renal plus cell production). Furthermore, the authors jump from one concept or model to the next as if they are "randomly selecting" data that supports their reasoning. Therefore the authors need to alter the manuscript language to reflect their bias in logic and make the transitions "softer" and more indicative of their statements as hypotheses. The manuscript is an interesting interpretation of a lot of data/analyses, worthy of publication. The rationale for why certain models and approaches are chosen needs to be clearer. Also including models that may not have supported the rationale should be included in the discussion.

The manuscript exams the role of CST3 gene (CyC) and it's correlation to cancer immunotherapy. This is not a novel concept and has been shown in many manuscripts- these references and concepts should be included. The authors use genomic data to devise a score to show association, however the association is post-treatment which limits clinical utility. The authors also try to link a mechanism of increased production by macrophages through activation by GC in a diseased state. The data support this as a possible mechanism contributing to a complex biological system. The conclusions need to incorporate the concept that this is complex system and this pathway may be important contributor. Many of the conclusions are overstated:

"Moreover, the peak in CyC-residual appears to be delayed by approximately 18 hours compared to the expected peak in plasma cortisol, consistent with our in vitro experiments. Taken together, these findings demonstrate that CyC production is directly induced by GCs, and that CyC has a reduced diurnal amplitude and offset periodicity compared to plasma cortisol. "

"On the basis of these results, we hypothesized that monocyte-like THP-1 cells would have high basal CST3 gene expression and secretion of CyC without GC-inducibility, and we confirmed this by RT-PCR (Figure 4f) and ELISA (Figure 4g) "

The data do not support an exclusive role of this specific monocyte population (myeloid and other macrophage lineages showed stronger correlation) and figures 4f and 4g do not support the conclusion well either.

Statements that their signature is superior to predicting people with disease is overstated.

We thank the reviewer for the constructive comments provided and appreciate their positive feedback on the multiple strengths of the manuscript. We are grateful that they have spent considerable time on identifying aspects that if were to be addressed would improve the manuscript and strengthen its conclusions.
Suggested adjustments to the language, results, and conclusions have been included in the revised manuscript. The suggested additional experiments have been performed and their results have helped refine and validate our hypotheses and conclusions. During the revision process we have performed three specific requested sets of experiments. Firstly, we repeated the dexamethasone treatment time course in macrophage-like THP-1 cells (Figure 5a-b) to confirm the reproducibility of the CyC induction findings. Secondly, we obtained human primary monocytes which were subsequently differentiated into macrophages (M1 and M2) and dendritic cells (immature and mature) using established strategies. This allowed us to examine the effect of 18-hour dexamethasone treatment on the extracellular concentration of CyC in each primary cell type, altogether confirming the role of macrophages in glucocorticoid-inducible expression of CyC (Figure S4c-g). Thirdly, we obtained Rag1 knock-out mice and inoculated sgScrambled and CST3-/- tumors. Growth curves were similar between the two groups (Figure S6g), confirming the relevance of the adaptive immune system in the growth defect phenotype we observed in CST3-/- tumors.

1) Overall many figures suffer from very small scale and difficult to read.

We have modified the formatting in concordance with all reviewer requests.

2) Better explanation of PGS decile scale in Figure 1f.

The figure legend has been updated accordingly.

3) Correlations of residual CyC as a marker for increased mortality are confusing. Is this just another (better?) marker of excessive inflammation and how does this relate to your downstream story? Figure 2D-E: Results contradictory between TCGA and UKB validation cohorts for many of the cancers so what can we take from this?

We are grateful for the reviewer making us aware that the text around cystatin C residual is insufficiently clear – we have made modifications to update this. We do not view any of the metrics of cystatin C discussed in the manuscript to be a marker of excessive inflammation. We posit that cystatin C is regulated by a multitude of factors, of which inflammation can be one example.

We introduced the concept of 'cystatin C residual' primarily as an approach to validate our polygenic score; we have now included a more comprehensive introduction of this concept and explanation in the main text (lines 173-176) and the discussion (lines 590-595). As cystatin C production is a latent (unmeasured trait), it is necessary to use a surrogate measure to validate the score. By calculating the deviation between cystatin C and creatinine in UK Biobank, we are able to estimate renal function-independent variation in cystatin C. This is an exploratory approach and our analyses are not predicated on it. With regard to the variation between TCGA and UKB, we note that for a limited number of cancer types there are apparently contradictory effect sizes for CyC-production PGS, although it is unlikely that these would meet a significance threshold. We would argue that there are significant differences in patient selection between UK Biobank and TCGA – for example, almost all patients in TCGA received surgical resection. Furthermore, we propose that this potential discordance between cancer types would support cystatin C modulating the host response to the tumor, rather than modulating the tumor itself. This would be consistent with our overall hypothesis that cystatin C might also regulate host anti-tumor

immunity.

4) Figure 3a: where is rs2749527 in UKB group?

Figure has been annotated accordingly.

5) Supplemental Figure 3b: the trans correlation for CST3 does not hold in another European cohort and is not mentioned in the text. Explanation is required.

This is clarified in a later part of the current text, where we demonstrate that a potential explanation for this discordance relates to the macrophage content in the visceral adipose tissue of patients selected on the basis of cardiovascular disease in the STARNET cohort. We have modified the figure legend of Figure S3b to clarify this point.

6) Explain better the purpose of Trans-eQTL analysis analysis in visceral fat? Is there any association in other tissues - should be stated.

In the absence of large-scale macrophage specific eQTL datasets, we focused on a tissue which can have a very high macrophage content (visceral adipose fat). We have amended the text to make this rationale clearer.

7) Figure S3e- this approach is a stretch to supplement plasma bilirubin for cortisol and make this conclusion. This concept can be added to discussion but should be removed from results.
We agree with the reviewer's comment and have removed the concept from the text and figures.

8) I don't understand the significance of this: "In support of this, we identified circadian rhythmicity from cosinor regression of spleen CST3 gene expression against time of death, which was attenuated compared to the canonical GC target FKBP5 (amplitude = 0.060 versus 0.24, Figure S4a)."

We are proposing from our in vitro data that the transcriptional and post-translational regulation of cystatin C might differ from canonical glucocorticoid receptor targets such as FKBP5. For example, we notice relatively delayed RNA expression and secretion of cystatin C (>18 hours after GR stimulation). This may be reflective of mechanisms that limit variations in GC-induced GC expression. The cosinor regression gives an indication of the amplitude of variation in CST3 and FKBP5 during the 24-hour cycle. We have updated the Methods to provide additional clarity (lines 1625-1626). The dynamics of cystatin C expression and secretion was further elucidated as part of the experiment suggested by reviewer 3 in specific comment 15 (discussed below).

9) Figure 4f and 4g need a control - high relative to what? The authors use human data from data sets to support their conclusions and then use only cell lines for experimental support. Validation should be done with human primary cells (they are easy to get) - could use human B cells or T cells as control.

We are grateful for the authors drawing our attention to this point that was unclear in the text. The adjective 'high' was included erroneously here and has been removed.

With regard to primary cell culture experiments – we recognize the potential limitations of experimentation within the THP-1 system and have performed a set of experiments using primary human monocyte cells. Primary cells were differentiated into M1 macrophage, M2 macrophage and matured dendritic cell subsets using established methodologies. We treated primary cells with 100nM dexamethasone for 18 hours and measured supernatant protein expression of cystatin C (normalized to cellular protein content) using the established assays. These experiments identified significantly elevated

extracellular CyC in M2 macrophage (p=0.05, Figure S4d), increased CyC in M1 macrophages (p=0.07, Figure S4c) and unchanged CyC in dendritic cells (p>0.39, Figure S4e-f). In addition to the new figure panels, the main text (lines 375-386) has been updated with these data.

10) The in vitro Dexamthasome dose seems rather high? Is 100nM relevant? The vivo 20 mg/kg = 20 mg/kg*70 kg = 1400 mg (COVID dose is 6 mg daily). This is unreasonably high.

With regard to in vitro dosing, we here used the same dose as used by the ENCODE consortium's glucocorticoid receptor experiments. We chose to adopt this dose so that our results were directly comparable to their publicly available results. With regard to in vivo dosing, there is precedent in the literature for this dexamethasone dosing1, and we were able to confirm that this dose consistently repressed endogenous production of corticosterone, thereby validating on-target effects. Nevertheless, we accept the point of the reviewer and have acknowledged the high dexamethasone dosing in the manuscript with appropriate adjectives (line 355).

11) Why does Cyc not go up in total mouse after dexamethasone treatment when you showed in vitro you get upregulation and Cyc is GR responsive?

This point was discussed in first manuscript draft and has been emphasized in the revised manuscript in recognition of the reviewer's question – on the basis that we do not see GC-inducible CyC expression in monocytes. As such, in a healthy mouse, with low tissue recruitment of macrophages, the absence of elevations in serum CyC upon dexamethasone administration is in keeping with the other results of the manuscript.

12) "Altogether, these findings indicate that CyC production is relatively constant in health, and in health does not significantly increase in response to GC agonism, explaining the validated utility of CyC as a marker of renal function in patients without acute illness." This connection is not sound logic.

We thank the reviewer for drawing our attention to this point. We apologize for not making our logic clearer in the text. We had omitted to include a reference at this point in the text to the CKD-EPI marker study2 (now added, line 362). In this paper the authors collate multiple studies where renal function was directly measured alongside cystatin C and/or creatinine. The authors report that cystatin C is a highly accurate marker of renal function using an equation they derive. However, they note in the methods that the performance of cystatin C is reduced if they included studies of renal transplant patients: "We excluded studies involving transplant recipients because our preliminary analyses showed large variations among these studies in the relationship between serum cystatin C levels and measured GFR." Our findings as well as theirs support a model where: in healthy people and healthy mice, cystatin C production is fairly constant and so levels are very closely coupled to underlying glomerular filtration rate, making it a good marker of renal function in these patients. However, in the context of disease states, recruitment of macrophages into inflamed tissues would result in glucocorticoid-induced cystatin C production, thereby inducing variability in cystatin C production and uncoupling plasma cystatin C levels from renal function. This can explain the findings of the CKD-EPI authors as renal transplant patients are routinely treated with exogenous glucocorticoids. We hope that the reviewer agrees that the text is now improved in clarity.

13) "As GR is expressed in macrophages but not in monocytes61, we hypothesized that, while monocytes have high constitutive basal CyC production, macrophages would secrete CyC in response to GC signaling"

Another example of seemingly random hypotheses - why would monocytes have high consititutive CyC without a GR receptor - what is controlling that? The literature does not support this simplistic statement

in fact it has been shown that Monocytes have more transcriptional alterations following GC signaling than human macrophages. This manuscript relies on in vitro experiments with cell lines that have been shown to not behave similarly to macrophages. Primary cells should be investigated. GC comparison of CyC production (RNA and protein) for constitutive monocyte, macrophage and in presence of dexamethasone should be shown in primary cells (in vitro differentiated macrophages from blood monocytes). The authors later recognize that GC signaling is required but not sufficient for CST3 expression (although this is not shown with knockouts/etc). GC targets are ubiquitous and so difficult to distinguish.

A) With regard to expression of glucocorticoid receptors in macrophages versus monocytes, we have based some of our thinking on this related manuscript (also cited in the text [line 367])3. Here, the authors demonstrate that monocyte-to-macrophage differentiation substantially enhances sensitivity to glucocorticoids, with comparatively few GC-inducible genes in monocytes (n=529) compared to macrophages (n=4,222). They provide a possible explanation for this by showing that monocyte-to-macrophage differentiation is associated with marked increases in RNA and protein expression of glucocorticoid receptor. They replicated this finding in primary cells (mouse-derived). Consistent with this, using estimates of enhancer-gene pair activity, we identify increased activity of the distal CST3 enhancer in macrophage-like versus monocyte-like THP-1 cells (Figure S4h-i). We are not aware of any direct contradictory studies at this time but would, of course, review any references suggested by the reviewer.

B) As discussed above, we are grateful for the reviewer's suggestion that we extend our findings from the THP-1 experimental system into primary human cells. We have performed a series of dexamethasone induction experiments (discussed above, [lines 375-386]) in primary human monocytes, macrophages and dendritic cells (Figure S4c-f). In light of the significant cost of repeated acquisition of primary human monocytes which cannot proliferate in vitro, we have prioritized cell harvesting at the 18-hour timepoint where we would expect to only detect elevations in extracellular CyC levels (cf. CST3 gene expression), as shown in Figure 5a-b.

14) "The findings that CyC is primarily expressed by myeloid cells and that GC responsive CyC secretion is predominantly mediated by macrophages have the potential to explain our finding" - This has not been shown in a direct comparison of CyC levels, and even in the CST3 expression data the DCs are just as high.

You have shown that myeloid cells are the largest producers in the blood. Your data does not support the exclusive effect of macrophages.

We are very grateful for drawing our attention to the potential importance of dendritic cells in GC-inducible CyC secretion. As discussed above, we have generated primary human dendritic cells by established differentiation protocols and measured the effect of dexamethasone on extracellular cystatin C (Figure S4f-g). We did not find GC-induced induction of CyC in dendritic cells (cf. macrophages), supporting biological importance of macrophages in our proposed model of CyC function.

15) Do we need a direct correlation of CyC protein vs CST3 expression? We keep seeing Cy residual and other "substitute readouts." The real correlation should be measured in primary cells.

We are very grateful to the reviewer for raising this important point about the direct correlation between CST3 gene expression and extracellular CyC protein in macrophages in the context of glucocorticoid receptor agonism. As shown in Figure 5a-b, there is a time lag between increased RNA expression of CST3 (significantly elevated by 6 hours) and increased extracellular CyC protein (significantly elevated by 18 hours). This is consistent with the expected time delays between transcription, translation and

secretion. As such, computing the direct correlation between CST3 gene expression and extracellular CyC protein at a single timepoint is unlikely to be informative. We do, however, agree that primary cell experiments are a meaningful addition to the narrative of the manuscript and have included these updated data in Figure S4c-g. Furthermore, we have modified the first paragraph of the discussion to directly reference the use of "substitute readouts" of CyC production used in this manuscript (lines 590-595) to address the concern raised by the reviewer.

16) Minor: Figure 4h does not show melanoma CST3 expression. Wrong reference in text. Also grammar errors in same paragraph.

We thank the reviewer for raising these points and have amended the text.

17) The use of cell lines should be validated in primary cells, e.g. in Figure 5, treatment to THP-1. Also the data for 18 hr is so variable that this is not a plausible conclusion. It would be nice to see a correlation of the transcription data to the CyC protein data in these analyses per sample. There are numerous samples in the protein assay (Fig 5B) but only 3 in the transcription data. The qPCR data suggests an issue with reproducibility.

As discussed above, we have completed experiments in primary cells that support glucocorticoid-mediated induction of CyC expression in macrophages. With regard to Figure 5a-b: previously we did not perform measurements of RNA and extracellular protein from the same samples (i.e. from the same well of a 6-well plate) and as a result, the number of biological replicates differed. To confirm reproducibility, we have repeated all macrophage-like THP-1 experiments used to generate Figure 5a-b and replicated our original findings. To ensure direct comparison of protein and RNA measurements, we adopted a modified experimental protocol allowing us to isolate RNA (for measurement of CST3 expression by qPCR), cellular protein fraction (for normalization of extracellular CyC) and cell supernatant (for measurement of extracellular CyC by ELISA) from each well, with 6 biological replicates per timepoint.

18) The authors fail to recognize other work related to cancer and CST3 expression, which has shown significant expression by various tumors, and by protein and not only RNA. Others have reported differential prognosis based on CyC. Others have also shown the CyC is GC-responsive and may relate to the differences in cancer outcomes. The authors should address these data in light of their work.

While it was our intention to reference the wider literature that has reported an association between cystatin C plasma levels and cancer outcomes, as well as the link between GC and cystatin C levels (paragraph 2 of introduction), we recognize that we could have been more comprehensive in our referencing. The manuscript has been updated with greater context from the background literature (references 15, 18, 19, 20, 21, 22, 23, 24, 25, 26). We would be grateful if the reviewer could advise on potential further references after reviewing our current additions.

19) In addition to showing CyC in murine cancer models the authors could refer to lots of work in human cancers that have shown this increase in CyC in plasma of cancer patients. Overstated conclusions - figure 5h - needs an in part by the tumor (they do not know if the in vivo elevations are also mediated by inflammatory effects).

With regard to the first point: we have updated the text to reflect the wider body of work in this space (lines 440-441) With regard to the second point: the text has been adjusted (line 467).

20) The CST3-/- is a nice experiment. Why pancreatic cancer - rationale needs improvement. The language needs correction: "While isogenic sgScrambled and CST3-/- Mm1 clones had equivalent

doubling times in vitro (sgScrambled: 23.3 hours, 95% CI 21.6-25.4; CST3-/-): 24.0 hours, 95% CI 22.4-25.8, Figure S6c)"

With regard to the first point: we and others have found the KPC-derived Mm1 model to be highly reproducible. It recapitulates the low immunogenicity and lack of immunotherapy responsiveness of human pancreatic cancer. We have modified the results (lines 542-543) and methods to reflect this (lines 1285-1287). We consider the Mm1 tumor model an example of an epithelial tumor model that has applicability to solid tumors, especially tumors with limited responsiveness to immunotherapy, as our hypothesis relates to cystatin C as a mechanism for immunotherapy resistance.

With regard to the second point: the text was updated accordingly.

21) Also the rationale for the immune related component is difficult to follow - what does the cysteine proteases and role of GCs have to do with this- the point is the rationale is difficult to follow. The rationale for including anti-PD-L1 in the bi-flank model is not clear. The data look the same as without the PD-L1. The untreated control in this model should be included. This is not a CBI responsive tumor. The experiment in an immunodeficient mouse would be a better experiment for immune-related contribution.

We acknowledge the request of the reviewer to provide a clearer rationale. In light of the well-established role of cystatin C-inhibited proteins such as cathepsins in immunity, and specifically anti-tumor immunity, we hypothesized that cystatin C could play a direct modifier of anti-tumor immunity. In support of this, glucocorticoids are known to profoundly modulate anti-tumor immunity, through mechanisms that are not fully characterized. As cystatin C is a glucocorticoid response gene, it can be viewed as a putative effector of glucocorticoid-induced immune suppression.

Anti-PD-L1 administration does not enable an immune reaction, instead it accentuates the existing immune reaction. It requires the migration of T cells into the tumor. As we were ultimately interested in the therapeutic relevance of cystatin C, we designed the experiment in this way, particularly in the context of other studies that have used a similar approach. In support of a subtle but detectable effect on tumor growth in the absence of cystatin C, induction of anti-PD-L1 antibody treatment significantly altered the tumor size ratio between paired scramble and CyC-knockout tumors (see Figure A below). This would suggest that, in the context of cystatin C knockout, PD-L1 can have a moderate effect that is detectable in this system.

As additional verification that suppressed tumor growth in CST3-/- tumors is at least partly related to immune control, we have experimented on immunodeficient mice. Ten 8-week-old C57BL/6J Rag1-null (defined by the absence of mature B and T cells, JAX #002216) mice were injected with either sgScrambled (n=5) or CST3-/- (n=5) cells with 200,000 cells injected subcutaneously in right flank. Tumor volumes were estimated longitudinally by caliper measurements, and tumors were weighed on mice sacrifice (day 41).

We found that CST3-/- tumor growth was much more heterogenous in Rag1-null (range at day 33: 121-1372 mm3) versus wild-type mice (range at day 33: 480-1960 mm3) and did not identify significant differences in tumor volumes at any timepoint ($p>0.05$, Figure S6g).

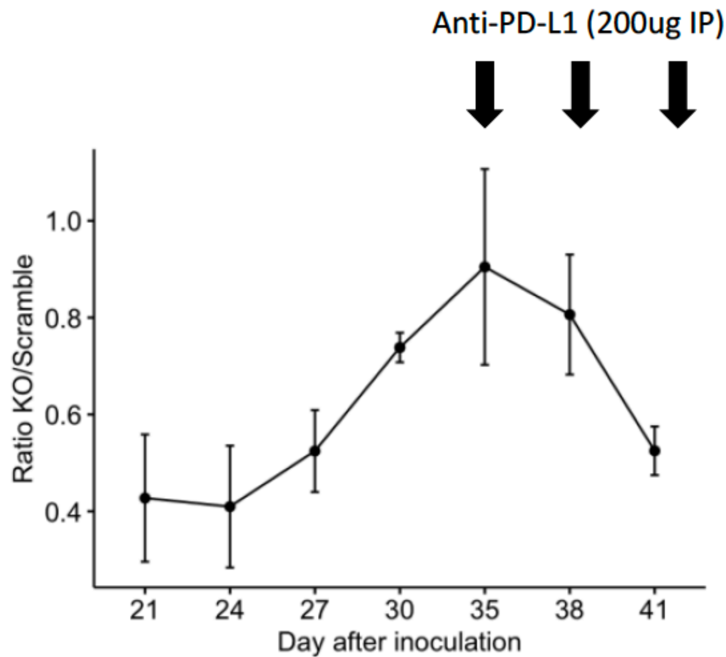

Figure A: Ratio of tumor volume in bi-flank CyC knockout (KO) and sgScrambled (Scramble) tumors following tumor inoculation. Dates of intraperitoneal anti-PD-L1 injections are annotated.

22) Minor: The tumor volume scales should be the same for all graphs.
The figures were updated accordingly.

23) Minor: Fig 6e: nomenclature is CD not Cd

We amended the labels accordingly.

24) Minor: Fig 6c y-axis should actually by %Ki67+ tumor cells? PI is undefined and vague.

Yes this is correct- the figure has been updated accordingly.

25) Minor: Endpoint tumor weights at endpoint (language).

The text has been updated accordingly.

26) Reference to Figure S7c is incorrect.

The text has been updated accordingly.

27) Comment: Trem2 might regulate trafficking of other cells into the tumor - but your data did not show differences in the other cell populations?

Yes, we would agree with this assertion. This would suggest that Trem2+ macrophages would directly modulate existing cells within the tumor microenvironment cf. modulating recruitment. There is emerging evidence that Trem2+ cells directly modify T cell function.

28) How can you call the CD8+ population effector T cells? What gene expression is showing this? It looks similar to CD4 - "effector" implies function which is not included in your data.

Agreed – this was an overstatement on our part, these cells were relabeled Cd8+ T cells.

29) Switching to the COVID model from the cancer immunotherapy is a bit of whiplash. Need better explanation for the TREM2 definition of cluster 0. Also this data needs ICU patients not treated with Dexamethasone as a control.

We accept that the COVID setting has the potential to create some complexity in the narrative. We note that COVID offers unique biological insights as, at different timepoints, patients have been treated with and without dexamethasone, and there is a significant availability of single-cell RNA sequencing of PBMCs. This provides an opportunity to test the effect of exogenous glucocorticoids in PBMCs in relatively matched patient populations. We are grateful for the comment around identification of the TREM2+ cluster – we have updated the manuscript to make this more apparent (lines 528-529). Briefly, TREM2 expression is poorly detected in human PBMC dataset (for reasons unclear, but reported by other groups). As such, we reconstructed expression using an established approach (gene-weighted density estimation), implemented in the Nebulosa package. This demonstrated that expression was broadly limited to a single pre-defined cluster (cluster 0). Many thanks for drawing our attention to the importance of the non-dexamethasone-treated control group, which has been added in Figure S7f and text (lines 531-532).

30) Alternate hypothesis is CyC plays a role in survival of the cells (alters metabolic condition of the tumor?)

We acknowledge that this potential effect warrants consideration. The doubling time in vitro (Figure S6c) and growth profiles (Figure S6g-h) in immunodeficient mice are unaffected by cystatin C genotype would argue against cystatin C having a strong effect on cellular survival.

31) Figure S8 - why is this data supplemental? A) need better explanations for reader of the C clusters. Why BCC for analyses? This is an example of changing models without clear rationale. The macrophages CST3 expression showed no effect although your work has focused on the CyC production from this population. The production by the MOs was an overstated conclusion as being the most relevant as the other populations (DCs) were not really studied.

With regard to the changes in dendritic cells, we fully accept the reviewer's comments. We have investigated the dynamics of GC-induced cystatin C secretion in primary human dendritic cells (Figure S4ef), and did not identify evidence of GC inducibility. We also note that CST3 gene expression was increased in macrophages in Figure S8, albeit not meeting a significance threshold.

With regard to the choice of BCC data: this is a unique dataset where single-cell RNA sequencing was performed before and after treatment with immunotherapy. Unfortunately, data sets like this are not yet available for other tumors. They are particularly useful resources to understand dynamic resistance mechanisms to immunotherapy, in this case whether cystatin C plays a role in these mechanisms. While this is a different epithelial tumor, the fundamental mechanisms of cystatin C-mediated immune suppression may still apply. This data were included in the supplementary material as they reflect publicly

available data, and as they are not necessary to substantiate the primary conclusions from the paper.

32) The association of CyC and poor prognosis in many cancers is not new data. How does a complicated PGS score, based on post-treatment response help as a biomarker, since the pre-treatment levels did not correlate? The odds ratio being a positive or negative benefit in figure 6h is confusing and should be more clear (odds of more PFS or worse?). The data does not hold for individual cancers.

The cystatin C production polygenic score is derived by structural equation modeling using eGFR-cystatin C and eGFR-creatinine summary statistics that were calculated across the unselected European participants in UK Biobank (irrespective of whether they have cancer or any treatment – more than 90% do not a recorded cancer diagnosis). We then calculated the polygenic score statistic for each participant in our independent collated cohort of patients treated with immunotherapy, and found a positive associated between the UKB-derived polygenic score and adverse outcomes. As such, we would argue that the PGS is not based on post-treatment response, but is a germline predisposition to cystatin C production that ought to be applicable across European populations, irrespective of treatment. With regard to the point about the wider literature on associations between plasma CyC levels and cancer outcomes, we have updated the manuscript introduction to better reflect the context of our work (line 92). We hope that the reviewer agrees that the CyC-production PGS has advantages over plasma-based metrics of CyC as it substantially reduces the effect of potential confounders as discussed in the first paragraph of the discussion. We are grateful for the comment around the clarity of Figure 6h and we have modified the figure accordingly. With regard to the variability across individual cancers, the sample size for melanoma and urothelial tumors is substantially larger than all other tumors examined (Table S2a), and it is difficult to interpret the results of the comparatively small cohort of renal cell carcinoma patients.

33) Methods
- Define FACS buffer

This have been updated in the text, FACS buffer refers to 2% FBS in PBS.

- Medias should contain concentrations of all ingredients (how much glutamine?)

This has been updated in the text, with particular reference to glutamine.

34) Discussion: Well-written. The authors present their arguments and model. However many statements do not recognize the biological complexity of GC-induced immunosuppression, CyC secretion and Cancer immunosuppressive mechanisms. The tone of the discussion should reflect their pathway as one possible mechanism of many. Some statements are not helpful nor necessary (e.g. suggesting CyC plays a role in immune privilege). The portion on POTENTIAL mechanisms is nice and indicates this is a possibility and is the authors hand-waving at this point.

We are grateful for the reviewer's constructive comments on the discussion and we have amended the text accordingly.

References

1. Gong, H. et al. Glucocorticoids antagonize estrogens by glucocorticoid receptor-mediated activation of estrogen sulfotransferase. Cancer Res. 68, 7386–7393 (2008).
2. Inker, L. A. et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. N. Engl. J. Med. 367, 20–29 (2012).
3. Diaz-Jimenez, D., Petrillo, M. G., Busada, J. T., Hermoso, M. A. & Cidlowski, J. A. Glucocorticoids mobilize macrophages by transcriptionally up-regulating the exopeptidase DPP4. J. Biol. Chem. (2020) doi:10.1074/jbc.RA119.010894.

Referees' report, second round of review

Reviewer #1: I appreciate the authors work making improvements on the manuscript

Reviewer #3: The authors have thoroughly addressed all of my original concerns.

Authors' response to the second round of review

None