The role of icIL-1RA in keratinocyte senescence and development of the senescence-associated secretory phenotype

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MS TITLE: The role of icIL-1RA type 1 in oral keratinocyte senescence and the development of the senescence associated secretory phenotype

AUTHORS: Sven E Niklander, Hannah L Crane, Lav Darda, Daniel W Lambert, and Keith D Hunter
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. In particular, two of the reviewers took issue with the model shown in Figure 8. They suggest that you either need to perform experiments to demonstrate the role of icIL-1RA in IL-6 and IL-8 regulation or you might consider performing additional experiments or revise your model and statements regarding this mechanism of regulation. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper by Niklander et al. aims to understand the role of the IL-1 signaling antagonist IL-1RA in regulating the SASP in keratinocyte senescence. The role of the SASP in keratinocytes and carcinogenesis of this cell type has not been fully explored, and this study provides some insights into the underlying mechanism. The authors provide evidence in cells lines and patient samples that IL-1RA is downregulated as cells progress from normal to malignant and show in vitro that these changes correlate with SASP expression/secretion. Overall, this is an interesting study that sheds new light on IL-1 antagonists in the SASP. Some of the conclusions are overstated and the statements related to how this leads to carcinogenesis need to be tempered.

Comments for the author

Major comments

1. The authors frame the message of the paper that IL-1RA loss and subsequent SASP upregulation is important in carcinogenesis of keratinocytes. However, they have no data to show this, other than correlation between IL-1RA expression and disease status (normal vs. malignant). Statements related to how these data give insights into the tumorigenesis process need to be tempered. For instance, in the intro (last paragraph), the author say they used this model “in order to understand how its downregulation contributes to the transformation towards HNC.” The authors do not provide any data towards this goal. Careful rewording of these statements is recommended. I would also recommend removing Fig 8B as no evidence of these processes are provided within the manuscript.

2. The results clearly show that knockdown of IL-1RA correlates with p65 phosphorylation and SASP expression/secretion, but there is only correlative data. This needs to be discussed. Moreover, conclusions such as “icIL-1RA1 modulates IL-6 and IL-8 levels by altering the activation of the NF-kB pathway…” need to be tempered as there is no direct evidence to demonstrate this.

3. Similar to comment #2, the authors state that “icIL-1RA has a role in the onset of senescence… by regulating IL-6 and IL-8 levels…”; however, this is not clearly shown. Either the authors should use neutralizing antibodies to definitively demonstrate that the onset of senescence in these cells is due to the SASP or they need to temper this conclusion. Many other senescent stimuli that have not been investigated herein may be contributing to the senescence phenotype.

4. It would be helpful for the reader to discuss why only IL-1B treatment seems to affect IL-1 signaling in these cells.

5. It is not clear why inhibition of cGAS does not affect IL1B or IL8 (Fig. 6).

Minor comments

1. It is hard to read the axes of all graphs.

2. In Fig 1D, sIL-1RN is clearly down (especially in OSCC), but the authors state there are no significant differences.

3. Fig 1E- it’s not clear from the figure what the red IF image represents. Need to label “IL-1RA”.

4. B-gal needs to be quantified in Fig S4C and S3A.

Reviewer 2

Advance summary and potential significance to field

In the manuscript Niklander et al, describe that IL-1RA expression is lost during oral carcinogenesis at the protein and RNA level, while IL-1R1 expression increases. Furthermore, they identify cytoplasmic IL-1RA (icIL-1RA) loss as a regulator of the SASP (IL-8 and IL-6) via ROCK inhibition. Thus, they conclude icIL-1RA loss in early oral carcinogenesis induces a premature activation of senescence and therefore SASP which promotes carcinogenesis. Overall, the manuscript presents interesting data with insightful findings. However, this is dampened by the difficulty in following the logic along the manuscript and the lack of consistency between the use of the different cell types. For example, if the authors aim is to find differences between NOK, OD and OSCC in relation to IL-1RA1 and induction of senescence, all the different groups (NOK, OD and OSCC) should be plotted together to determine a statistical significance between groups.

Comments for the author

Here are some comments that I believe should be addressed by the authors prior to publication:

Fig 1B. Not sure how the immortal cells lines fit in as immortalising them seems to affect IL1RA1 expression levels. Why are there 2 bands in the WB for IL1RA? Can it be post-translationally modified? How where the cells immortalised?

Fig 1C-1d. The authors state that there are no differences between intracellular and secreted IL-1RA but there are clear differences, particularly between normal and OSCC. They cannot base their conclusions based on statistics as two of the groups (normal and OSCC) have only 2 samples. Also, is the secreted form only regulated at the transcriptional level? Could post-translational modifications not be important to whether this is secreted or not? (Fig1B seems to show it undergoes post-translational modifications). An ELISA assay for the secreted isoform would be more informative.

Fig 1E. Normal NOK should be included to confirm IL-1RA expression levels. Labelling for what it is stained for is missing.

Fig 1F. is redundant as it's already show in Fig 1E

Fig 1G. It is interesting to see 'islets' and small clusters of cells staining positive for IL-1RA in OSCC. Do the authors have any rational as to why this might be? As they are later looking into senescence, have they looked into additional markers for senescence in these samples? E.g. Ki-67 or others?

Fig S1C-F should include NOK as a control.

Figure 2. What are the IL-1R expression levels in normal, OD and OSCC in human tissue samples? Do they correlated inversely with IL-1RA? Are there also difference in IL-1R expression between mortal and immortal cell lines?

Figure 3. The levels of total IL1RA should be shown to demonstrate specificity of the icIL-1RA KD.

Figure S3. The induction of beta-Gal is not convincing. There are very few cells with light blue staining. Also, the authors should explain better the rational of using ROCK inhibitors and how this treatment influences IL1RA expression, if it does. Are the cells not already immortalised? The change of comparisons between immortalised and mortal cells lines is very confusion and should be simplified.

Maybe figure 4B could go into supplementary?

Figure 4. In my opinion, the conclusions should be made by comparing the empty plasmid and the icIL-1RA1. Thus, only IL-8 is release in B16 when stimulated with IL-1b. Do the authors have any insights into the mechanisms of icIL-1RA1 in these cell types? Is NF-kB also implicated?

In figure 5 the authors find that IL-1RA decreases during senescence. However, in Figure 1, they see downregulation of IL-1RA during malignant progression. Are the authors conclusions that senescence stimulates tumor progression?

Fig 5 and S4. What does the % represent? % of senescence based on what? It is a bit confusion for the reader so I would remove it and just indicate passage number.

Fig 5. The authors state that OD release more SASP than NOK. However, in figure 3 they show no differences in IL8, IL6 release between OD and NOKs.

Fig 6. Ideally, both D6 and D25 should be assessed to reach a conclusion

Figure 6H. Total p65 should be shown. The authors state a dose-dependent increase in p16. Could the authors elaborate as to whether this is something expected and why? Quantification of this increase should also be shown.

To confirm a role for cGAS further inhibitors and RNAi targeting the cGAS/STING pathway should be used, e.g. KD of STING or cGAS.

Figure 7. Altogether figure 7 seems to be repetitive or complementary of Fig 3.

Fig 7C. Population doublings should be represented through different passages and not form a single time-point. This way you can see whether the proliferative arrest is over time and not at one specific time point.

Fig 7E. To confirm a role for NFkB functional assays blocking/RNAi targeting NFkB should be used.

Reviewer 3

Advance summary and potential significance to field

This is an interesting and well perfomed study documenting that levels of the IL-1R receptor antagonist icIL-RA1 decrease early during dysplastic transformation of oral keratinocytes. The authors show that knock down of icIL-RA1 results in elevation of IL-6 and IL-8 secretion and earlier onset of replicative senescence in dysplastic oral keratinocytes and that overexpression of icil-RA1 inhibits responses to IL-1b. This indicates that the loss of icIL-RA during disease progression may have functional consequences to disease development and that IL-1 signalling may be an early disease prevention target.

Comments for the author

The authors present a model in Figure 8 indicating that loss of icIL-RA may accentuate IL-1a responsiveness resulting in increased IL-6 and IL-8 expression and therefore an increase in senescence. It is intriguing that the B16 and D20 cells did not respond to recombinant IL-1a treatment but did to IL-1b treatment. To mechanistically verify their model it would be good for the authors to validate the role of IL-1a, IL-1b and IL-6 and IL-8 by knocking down expression of these cytokines and examining their effects on acquisition of a senescent phenotype with and without manipulation of icIL-RA levels.

Minor points

add scale bars to Figure 1G explain the colours in Figure 4B Quantify changes in CCF during senescence It would be good to show positive controls for detection of iCIL-RA variants by PCR It would be good to show a positive control for recombinant IL-1a activity

First revision

Author response to reviewers' comments

Reviewer 1

Advance Summary and Potential Significance to Field:

The paper by Niklander et al. aims to understand the role of the IL-1 signalling antagonist IL-1RA in regulating the SASP in keratinocyte senescence. The role of the SASP in keratinocytes and carcinogenesis of this cell type has not been fully explored, and this study provides some insights into the underlying mechanism. The authors provide evidence in cells lines and patient samples that IL-1RA is downregulated as cells progress from normal to malignant and show in vitro that these changes correlate with SASP expression/secretion.

Overall, this is an interesting study that sheds new light on IL-1 antagonists in the SASP. Some of the conclusions are overstated and the statements related to how this leads to carcinogenesis need to be tempered.

Reviewer 1 Comments for the Author:

Major comments

1. The authors frame the message of the paper that IL-1RA loss and subsequent SASP upregulation is important in carcinogenesis of keratinocytes. However, they have no data to show this, other than correlation between IL-1RA expression and disease status (normal vs. malignant). Statements related to how these data give insights into the tumorigenesis process need to be tempered. For instance, in the intro (last paragraph), the author say they used this model "in order to understand how its downregulation contributes to the transformation towards HNC." The authors do not provide any data towards this goal. Careful rewording of these statements is recommended. I would also recommend removing Fig 8B as no evidence of these processes are provided within the manuscript.

R: Thank you very much for your feedback. We agree that this is not directly assessed in this paper, but the overall evidence from the cell culture model in HNSCC is supportive of this; in part the progressive changes in the transcriptome which were presented in Hunter et al, 2006. In order to address this, we have reworded the last paragraph of the introduction to: "As IL-1RA downregulation is progressively observed through OD to OSCC, we examined the contribution of IL-1RA to the development of senescence and a pro-tumourigenic SASP (which has not been characterized in oral keratinocytes) which may be an important component of the malignant transformation process".
As recommended, figure 8b was removed from the manuscript.

2. The results clearly show that knockdown of IL-1RA correlates with p65 phosphorylation and SASP expression/secretion, but there is only correlative data. This needs to be discussed. Moreover, conclusions such as "icIL-1RA1 modulates IL-6 and IL-8 levels by altering the activation of the NF-kB pathway…" need to be tempered as there is no direct evidence to demonstrate this.

R: We agree and have added the following statement to the discussion related to these data, in order to temper the strength of the comment: "Although this needs further validation as these are observations from correlative data".

As recommended, we modified the mentioned statement to: "These data suggest that alterations in icIL-1RA1 expression result in changes in IL6 and Il8 levels which may be mediated by alterations in the activity of the NF-kB pathway".

3. Similar to comment #2, the authors state that "icIL-1RA has a role in the onset of senescence… by regulating IL-6 and IL-8 levels…"; however, this is not clearly shown. Either the

authors should use neutralizing antibodies to definitively demonstrate that the onset of senescence in these cells is due to the SASP or they need to temper this conclusion. Many other senescent stimuli that have not been investigated herein may be contributing to the senescence phenotype.

R: We modified that conclusion to: “Altogether, these findings suggest that in oral keratinocytes, icIL-1RA1 might be involved in both the onset of senescence and also in the development of the SASP, which are parallel but separate processes”

4. It would be helpful for the reader to discuss why only IL-1B treatment seems to affect IL-1 signaling in these cells.

R: The following paragraph was added to the discussion: “Interestingly, only treatment with recombinant IL-1¤ and not recombinant IL-1¤ had an effect on IL-6 and IL-8 production by the D20 and B16 oral dysplastic and oral cancer cell lines. A possible explanation for this is that those cells lines already exhibited high levels of endogenous IL-1¤ (Fig 2D),thus adding more IL-1¤ had no effect, whereas IL-1¤ expression by oral cancer cell lines has been reported to be very low or undetectable (Al-Sahaf et al., 2019) (doi: 10.1002/ijc.31852)”.

5. It is not clear why inhibition of cGAS does not affect IL1B or IL8 (Fig. 6).

R: According to a recent paper (Yang et al., 2017) (https://doi.org/10.1073/pnas.1705499114), there are differences in the effect of cGAS ablation on the SASP depending on the method used to induce senescence. In that paper, the effect on IL8 in IR-induced senescence isn’t huge in fibroblasts (and it’s different to MEF). This suggests that the effects on cGAS ablation might dependent on the method employed to induce senescence (we used replicative exhaustion) and on the cell type (we worked with NOKs).

Minor comments

1. It is hard to read the axes of all graphs.

R: The resolution of the figures has been improved to address this issue.

2. In Fig 1D, sIL-1RN is clearly down (especially in OSCC), but the authors state there are no significant differences.

R: We agree that in Figure 1D sIL-1RA levels do appear to decrease in OD and OSCC cells lines compared to NOKs, but this is not statistically significant (p value of 0.3377 and of 0.1272 respectively). This can be explained by giving more detail on how the data is presented. The OD dysplasia group has 2 mortal oral dysplasias, with similar levels of sIL-1RA to mortal NOKs (Fig 1 J), and 2 immortal OD which have low levels very similar to iNOKs (Fig 1 J). iNOKs have not been included in Figure 1D. sIL-1RA mRNA expression also decreased in immortal cells compared to mortal (see fig 1j) thus as there are no any immortal NOKs in figure 1D, it looks like a decrease (which, nevertheless, was not significant). We don’t think it would be appropriate to add the iNOKs to this figure, as this would generate confusion, but it is important to have both mortal and immortal ODs, as in vivo oral dysplasias are a mixture of mortal and immortal cells.

To avoid confusion to the reader, we added the following statement to result and discussion section respectively:

“Despite expression levels of sIL-1RN seemed to decrease in OD and OSCC cell lines compared to NOKs, no significant differences were observed (Fig 1D)”.

“Despite expression levels of sIL-1RN seeming to decrease in OD and OSCC cell lines compared to NOKs, there were no statistically significant differences between the groups. This is explained by

the fact that the OD group is composed of a mixture of both immortal and mortal cell lines, and it is worth noting that sIL-1RN mRNA expression did decrease in immortal cells compared to mortal cells. No any immortal NOKs were included in the NOK group, this has resulted in no statistically significant result overall."

3. Fig 1E- it's not clear from the figure what the red IF image represents. Need to label "IL-1RA".

R: We added the missing label

4. B-gal needs to be quantified in Fig S4C and S3A.

R: B-gal staining has been quantified in both images. Also, a positive control has been added to Fig S3 A and B.

Reviewer 2
Advance Summary and Potential Significance to Field:
In the manuscript Niklander et al, describe that IL-1RA expression is lost during oral carcinogenesis at the protein and RNA level, while IL-1R1 expression increases. Furthermore, they identify cytoplasmic IL-1RA (icIL-1RA) loss as a regulator of the SASP (IL-8 and IL-6) via ROCK inhibition. Thus, they conclude icIL-1RA loss in early oral carcinogenesis induces a premature activation of senescence and therefore SASP which promotes carcinogenesis. Overall, the manuscript presents interesting data with insightful findings. However, this is dampened by the difficulty in following the logic along the manuscript and the lack of consistency between the use of the different cell types. For example, if the authors aim is to find differences between NOK, OD and OSCC in relation to IL-1RA1 and induction of senescence, all the different groups (NOK, OD and OSCC) should be plotted together to determine a statistical significance between groups.

R: Thank you very much for your comments and feedback. When we showed *icIL-1RN* expression we combined the different cell types in different groups (normal, dysplastic and OSCC), as we wanted to show how icIL-1RN expression was lost during oral carcinogenesis, and there are two relevant points to assess: how IL1RA expression varies with clinical type of sample and how it varies with replicative fate. These, we feel are linked (a point which we have made in earlier papers), but are worthwhile showing separately in this context. If we combined all of them in one grouping we will not be able to show the nuance of the decrease of IL-1RA during oral carcinogenesis

Reviewer 2 Comments for the Author:
Here are some comments that I believe should be addressed by the authors prior to publication:

- Fig 1B. Not sure how the immortal cells lines fit in as immortalising them seems to affect IL1RA1 expression levels. Why are there 2 bands in the WB for IL1RA? Can it be post-translationally modified? How where the cells immortalised?

R: The immortal oral dysplasias cell lines (D19 and D20) are spontaneous immortalized cell lines and have been extensively characterised in the papers from McGregor et al. in 1997 and 2002 (https://cancerres.aacrjournals.org/content/57/18/3886.long,https://cancerres.aacrjournals.org/content/62/16/4757.long). This is important as the majority of oral dysplasias and OSCC are a mixture of mortal and immortal cells – and much more so the case in precancerous lesions(Leung et al., 2017)(doi: 10.1111/his.13260). We also used immortal NOKs which helped to confirm our suspicious that immortalization affects the expression of IL-1RA. The NOKs were experimentally immortalized by knocking down p16 and overexpressing hTERT. The details of that process can be found in the original papers (which were added to the material and methods section) "The following cell lines/cell cultures used in this study have been described previously: FNB6, D6, D25, B16, B22, D19, D20 (McGregor et al., 1997, McGregor et al., 2002) (https://cancerres.aacrjournals.org/content/57/18/3886.long,https://cancerres.aacrjournals.org/content/62/16/4757.long), OKF4 (Rheinwald et al., 2002)(doi: 10.1128/mcb.22.14.5157-5172.2002), OKF6 (Natarajan et al., 2006) (doi: 10.2353/ajpath.2006.051027)."

We do not know exactly what the second band represents, but it be another transcript variant of icIL-1RA. None of the commercially available antibodies are specific for the different variants of IL- 1RA; thus we could not assess this.

- Fig 1C-1d. The authors state that there are no differences between intracellular and secreted IL-1RA but there are clear differences, particularly between normal and OSCC. They cannot base their conclusions based on statistics as two of the groups (normal and OSCC) have only 2 samples. Also, is the secreted form only regulated at the transcriptional level? Could post-translational modifications not be important to whether this is secreted or not? (Fig1B seems to show it undergoes post-translational modifications). An ELISA assay for the secreted isoform would be more informative.

R: We agree that in Figure 1D sIL-1RA levels do appear to decrease in OD and OSCC cells lines compared to NOKs, but this is not statistically significant (p value of 0.3377 and of 0.1272 respectively). This can be explained by giving more detail on how the data is presented. The OD dysplasia group has 2 mortal oral dysplasias, with similar levels of sIL-1RA to mortal NOKs (Fig 1 J), and 2 immortal OD which have low levels very similar to iNOKs (Fig 1 J). iNOKs have not been included in Figure 1D. sIL-1RA mRNA expression also decreased in immortal cells compared to mortal (see fig 1j) thus as there are no any immortal NOKs in figure 1D, it looks like a decrease (which, nevertheless, was not significant). We don't think it would be appropriate to add the iNOKs to this figure, as this would generate confusion, but it is important to have both mortal and immortal ODs, as in vivo oral dysplasias are a mixture of mortal and immortal cells.

To avoid confusion to the reader, we added the following statement to result and discussion section respectively:

"Despite expression levels of sIL-1RN seemed to decrease in OD and OSCC cell lines compared to NOKs, no significant differences were observed (Fig 1D)".

"Despite expression levels of sIL-1RN seeming to decrease in OD and OSCC cell lines compared to NOKs, there were no statistically significant differences between the groups. This is explained by the fact that the OD group is composed of a mixture of both immortal and mortal cell lines, and it is worth noting that sIL-1RN mRNA expression did decrease in immortal cells compared to mortal cells. No any immortal NOKs were included in the NOK group, this has resulted in no statistically significant result overall."

The intracellular variant is transcribed by alternate splicing of the same gene as sIL-1RA and has at the 5'end, within the exon coding the leader sequence, a replacement of 85bp by a different sequence of 130 bp. Also, the intracellular variant lacks a leader peptide, therefore cannot be secreted. In keratinocytes, icIL-1Ra is regulated at the transcriptional level by IL-1a, as deleting the promoter region led to nearly complete loss of promoter activity (La and Fischer, 2001).

sIL-1RN transcripts were considerably lower than *icIL-1RN* transcripts in oral keratinocytes (although we recognize that that direct comparison is not reliable due to possible differences in primer affinity). Also, all the data available on IL1RN expression on keratinocytes points to the direction that sIL-1RA is not expressed or expressed at very low levels by keratinocytes (Phillips et al., 1995) (doi: 10.1111/j.1365-2249.1995.tb02295.x) and that icIL-1RA corresponds to the main variant (La and Fischer, 2001, Garat and Arend, 2003) (doi: 10.4049/jimmunol.166.10.6149, doi: 10.1016/s1043- 4666(03)00182-0).

- Fig 1E. Normal NOK should be included to confirm IL-1RA expression levels. Labelling for what it is stained for is missing.

R: The FNB6 cell line is a well-documented normal oral keratinocyte cell line that has been immortalized through p16 knock down and overexpression of hTERT and is widely used in research as a control for normal oral keratinocytes (McGregor et al., 2002). Despite the FNB6 cells have been immortalized, we showed that they still retained IL-1RA expression, thus we used them as positive controls of IL-1RA expression.

We added the missing label of figure 1 E.

- Fig 1F. is redundant as it's already show in Fig 1E

R: Fig 1F represents a high-resolution image at a higher magnification of IL-1RA expression in FNB6 cells, which gives more detail of IL-1RA localization in the different cell compartments, therefore we believe is complementary to Fig 1E.

- Fig 1G. It is interesting to see 'islets' and small clusters of cells staining positive for IL-1RA in OSCC. Do the authors have any rational as to why this might be? As they are later looking into senescence, have they looked into additional markers for senescence in these samples? E.g. Ki-67 or others?

R: Oral dysplasias and oral squamous cell carcinomas are a mixture of mortal and immortal cells (Hunter et al., 2006) (doi: 10.1158/0008-5472.CAN-06-0186). As we showed that IL-1RA expression is lost once cells achieved immortality, we believe that focal expression of IL-1RA in some clusters of OSCC cells probably represents the presence of mortal cells, in which expression of IL-1RA is still retained.

It is well documented that Ki-67 expression increases in oral dysplasias and OSCCs compared to normal oral mucosa, thus we did no explore this and we have also show this recently (Leung et al., 2017)(doi: 10.1111/his.13260). We agree that it would be interesting to assess the expression of senescence markers in these samples, but this was beyond the aim of this study. In a recent paper from our group (Leung et al., 2017), we explored the expression of different senescent markers in normal mucosa, oral dysplasias and OSCC patient tissues samples. We found an increase in p16 expression in severe ODs, but this was likely to be associated with HPV infection. We did also find an increase in p53 and ¤H2AX expression with increasing grades of dysplasia, but these are not specific senescent markers, thus do not necessarily reflect an increase in senescent cells.

- Fig S1C-F should include NOK as a control.

R: The aim of the experiments represented in Fig S1C-F was to show whether IL-1a or IL-1b was able to induce IL-1RN expression in cells in which IL1RN expression was lost. Thus, we do not think that adding a control would add much as does not modifies what we intended to show.

- Figure 2. What are the IL-1R expression levels in normal, OD and OSCC in human tissue samples? Do they correlated inversely with IL-1RA? Are there also difference in IL-1R expression between mortal and immortal cell lines?

R: We did not analyse IL-1R1 in patient tissue samples. Nevertheless, a recent paper (Sun et al., 2015)(doi: 10.1371/journal.pone.0132677. eCollection 2015) found IL-1R1 to be expressed in 40 of 41 human tongue squamous cell carcinomas. In that paper, the authors showed that IL-1b and IL- 1R1 promoted cancer growth and metastasis by up-regulating CXCR4, which could be reversed by inhibiting IL-1R1 with IL-1RA. This is consistent with the decrease in IL-1RA expression in OSCC we and others have reported and suggests an inverse correlation between IL-1R1 and IL-1Ra in OSCC.

We compared IL1R1 expression between mortal and immortal cell lines as suggested. In both immortal normal and immortal dysplastic keratinocytes, there was a significant decrease in IL1R1 transcripts when compared to their mortal counterpart. That data has been added to figure 2B. Same as with immortal cell lines, mortal dysplastic oral keratinocytes presented with higher transcripts levels of IL1R1 than mortal oral keratinocytes. That data has been added to Figure 2A. The following statement was added to the result section: "IL-1R1 transcript levels were significantly up-regulated in immortal and mortal OD compared to normal and immortal NOKs (Fig 2A). Same as with IL1RN, IL-1R1 expression decreases in immortal cells compared to their mortal counterpart (Fig 2B)".

- Figure 3. The levels of total IL1RA should be shown to demonstrate specificity of the icIL-1RA KD.

R: We apologize for the confusion. The immunoblots from Fig3A- B are showing total IL-1RA levels, but the label was missing. We added the correct label.

- Figure S3. The induction of beta-Gal is not convincing. There are very few cells with light blue staining. Also, the authors should explain better the rational of using ROCK inhibitors and how this treatment influences IL1RA expression, if it does. Are the cells not already immortalised? The change of comparisons between immortalised and mortal cells lines is very confusion and should be simplified.

R: In Figure S3 we wanted to show that after removing Y-27632 the cells did not senesce immediately, thus it was expected not to find many blue cells when staining for beta galactosidase activity. We have quantified Beta gal expression and added a positive control to Fig S3.

Primary oral keratinocytes are very difficult cells to work with as they are very sensitive to any stress and have a short life spam (after 8-10 passages they senesce). We had many issues to generate stable IL1RA KD cells, as the cells usually did not grow after transfection, selection, etc… One of the reasons of that was induction of premature senescence (among many others). To overcome that issue, we treated the cells with Y-27632. Our preliminary data shows that rock inhibition has no or minor effect on IL1RN expression. We have a paper recently accepted in FEBS OpenBio where we show that immortalization with Y-27632 is reversible. Oral keratinocytes chemically immortalized with Y-27632 stop growing few passages after Y-27632 is removed from culture conditions, which is accompanied with an increase in p16 expression and a beta-galactosidase staining > 90%, consistent with a senescent growth arrest (see https://doi.org/10.1002/2211-5463.13012)

- Maybe figure 4B could go into supplementary?

R: We agree, but there is no fit for Fig 4B in any of the supplementary figures (and we do not think that can go as a sup figure by itself). We believe that figure 4B is important to support the experiments shown in Fig 4, thus we have left it there.

- Figure 4. In my opinion, the conclusions should be made by comparing the empty plasmid and the icIL-1RA1. Thus, only IL-8 is release in B16 when stimulated with IL-1b. Do the authors have any insights into the mechanisms of icIL-1RA1 in these cell types? Is NF-kB also implicated?

R: We modified the conclusion from Fig 4 as recommended and made the comparison only with the empty plasmid group. Now it reads: “In B16 icIL-1RA1 transfected cells, a decrease in IL-8 secretion (but no Il-6) was observed, but only when cells were stimulated with IL-1β. No changes in D20 icIL- 1RA1 transfected cells were observed (Fig 4G-J)”.

As we did not find a major effect on cell behaviour after re-expressing icIL-1RA1 in immortal cells in which expression has been lost, we did not explore this further. We believe icIL-1RA1 does not act as a tumour suppressor and that its expression is lost during immortalization, but we do not have a better insight about the mechanisms of icIL-1RA1 in these cells. This will be the focus of ongoing research in this cell model.

- In figure 5 the authors find that IL-1RA decreases during senescence. However, in Figure 1, they see downregulation of IL-1RA during malignant progression. Are the authors conclusions that senescence stimulates tumor progression?

R: We cannot conclude that from our data. We showed that IL-1RA expression is lost during the oral carcinogenesis process, that IL-1RA expression is lost during the acquisition of immortality and that IL-1RA expression is decreased during replicative senescence, which we showed is important for the development of the SASP. The SASP can have oncogenic actions, and this has been shown in many cancers (breast, skin, gastric, among others), but the effects of the SASP is

tissue specific and depends on many different factors. We do believe that the SASP from senescent oral keratinocytes (normal or dysplastic) has the potential to stimulate tumour progression, but this has to be demonstrated. The hypothesis arising from the data is that loss of expression of icIL1RA allow for the development of the SASP in parallel with the development of replicative senescence – we have shown that the two are closely linked, but not necessarily synonymous processes. The loss is maintained in the small % of cells which pass through crisis and become immortal (thus no IL1RA in spontaneously immortal cells, but it may be retained in experimentally immortalised cells). Thus the association with low IL1RA in SCC. The mechanism whereby Il1RA expression is lost (seemingly irreversibly) is not known and is the focus of ongoing work.

- Fig 5 and S4. What does the % represent? % of senescence based on what? It is a bit confusion for the reader so I would remove it and just indicate passage number.

R: The % represents % of cells stained positive for Beta galactosidase. The following was added to each of the figure legends of those figures: “Senescence % is based on the % of cells stained positive for SA-□-GAL”.

- Fig 5. The authors state that OD release more SASP than NOK. However, in figure 3 they show no differences in IL8, IL6 release between OD and NOKs

R: We combined the data from the two NOKs and two ODs cell cultures and compared IL-6 and IL-8 secretion, which showed that in general, senescent OD cells express higher levels of IL-6 and IL-8 than NOKs. We are aware that this is a comparison of a small cohort. It is just an observation form our data that be found important to point out. These data were added to Fig S4H.

- Fig6. Ideally, both D6 and D25 should be assessed to reach a conclusion. To confirm a role for cGAS further inhibitors and RNAi targeting the cGAS/STING pathway should be used, e.g. KD of STING or cGAS.

R: The experiments represented in Fig 6 are a proof of principle prompted by the observation of cytoplasmic chromatin fragments observed in senescent oral keratinocytes, which has not been described before. We agree that to validate this further we need to treat a larger panel of cell lines and perform other experiments, as the ones proposed. We believe is beyond the scope of this paper to explore this further, as our intention was to report these initial findings. These finding will be followed up in our ongoing investigations.

- Figure 6H. Total p65 should be shown. The authors state a dose-dependent increase in p16. Could the authors elaborate as to whether this is something expected and why? Quantification of this increase should also be shown.

R: Whilst it would be ideal to have total p65 data, in Figure 6H we used beta actin as a loading control, and equal quantities of protein per lane are observed. We do not attribute the decrease in p65 phosphorylation to a sample loading problem.

We quantified p16 expression and found no significant increase. Thus, we removed the following statements from the results and discussion section respectively: “A dose dependent increase of p16Ink4a protein was also noted (Fig 7H)” and “p16 protein expression rose in a dose dependent manner. This was unexpected, as removal of cGAS has been shown to prevent the onset of senescence and decrease p16Ink4a expression (Gluck et al., 2017) (doi: 10.1038/ncb3586). One possible explanation is that in this experiment cGAS was inhibited in already senescent cells, where p16Ink4a regulation might be different”

- Figure 7. Altogether figure 7 seems to be repetitive or complementary of Fig 3.

R: Figure 7 corroborates data shown in figure 3 and shows the effects of icIL-1RA1 KD during senescence. Although some data seems to be repetitive (increase in IL-6, IL-8 and NF-KB activation after KD of icIL-1RA1), we believe is essential for the manuscript, as provides a comparison between proliferative and senescent WT and KD cells.

- Fig 7C. Population doublings should be represented through different passages and not form a single time-point. This way you can see whether the proliferative arrest is over time and not at one specific time point.

R: We agree with that comment, but the cells senesced very fast after removing Y-27632 from culture conditions (after one passage for KD cells and two passages for WT cells). Nevertheless, we showed that the cessation of proliferation was accompanied by an increase in p16 expression and in SA-¤-GAL activity, indicative of a senescent state. We have added two graphs showing PPDs through the different passages to Fig 7c.

- Fig 7E. To confirm a role for NFkB functional assays blocking/RNAi targeting NFkB should be used.

R: We agree with this suggestion, but it is not possible at present to do any of this work due to restricted access to laboratories. We tempered down all statements related to the possible relation between icIL-1RA1 and NFkB (please see response to point 2 of reviewer 1) and will pursue this in future work.

Reviewer 3
Advance Summary and Potential Significance to Field:
This is an interesting and well performed study documenting that levels of the IL-1R receptor antagonist icIL-RA1 decrease early during dysplastic transformation of oral keratinocytes. The authors show that knock down of icIL-RA1 results in elevation of IL-6 and IL-8 secretion and earlier onset of replicative senescence in dysplastic oral keratinocytes and that overexpression of icil-RA1 inhibits responses to IL-1b. This indicates that the loss of icIL-RA during disease progression may have functional consequences to disease development and that IL-1 signalling may be an early disease prevention target.

Reviewer 3 Comments for the Author:
The authors present a model in Figure 8 indicating that loss of icIL-RA may accentuate IL-1a responsiveness resulting in increased IL-6 and IL-8 expression and therefore an increase in senescence. It is intriguing that the B16 and D20 cells did not respond to recombinant IL-1a treatment but did to IL-1b treatment. To mechanistically verify their model it would be good for the authors to validate the role of IL-1a, IL-1b and IL-6 and IL-8 by knocking down expression of these cytokines and examining their effects on acquisition of a senescent phenotype with and without manipulation of icIL-RA levels.

R: Thank you very much for your feedback. In relation to the lack of response of the B16 and D20 cell lines to IL-1a, we added the following to the discussion: “Interestingly, only treatment with recombinant L-1¤ and not recombinant IL-1¤ had an effect on IL-6 and IL-8 production by the D20 and B16 oral dysplastic and oral cancer cell lines. A possible explanation for this is that those cells lines already exhibited high levels of endogenous IL-1¤, thus adding more IL-1¤ had no effect, whereas IL-1¤ expression by oral cancer cell lines has been reported to be very low or undetectable (Al-Sahaf et al., 2019) (doi: 10.1002/ijc.31852).”

We agree that the proposed experiments would help to verify our model, but we believe is beyond the scope of this paper (which is already at the figure limit), thus we decided to remove our model proposal (figure 8b). Thus, testing this model will form the basis of our future work, as we currently have no access to laboratories.

Minor points
- add scale bars to Figure 1G

R: We added the magnification used to take the microphotographs to figure 1G legend “Images were taken using a 10X and 20X objective (F).

- explain the colours in Figure 4B

R: The following was added to the figure legend of figure 4B: “Blue: nuclei stained with DAPI. Green cells: positive transfected cells with a vector encoding for GFP.”

- Quantify changes in CCF during senescence

R: Quantification of CCF was done and added to figure S5C

- It would be good to show positive controls for detection of iCIL-RA variants by PCR

R: Keratinocytes are known to express icIL-1RA (La et al., 2001, Garat and Arend, 2003) (doi: 10.4049/jimmunol.166.10.6149, doi: 10.1016/s1043-4666(03)00182-0) and we corroborated that with previous experiments using oral keratinocytes, thus we used the NOKs or FNB6 cells as positive controls (as we showed that in FNB6 cells icIL-1RA expression was similar compared to NOKs, despite being immortal).

- It would be good to show a positive control for recombinant IL-1a activity

R: We added a positive control for recombinant IL-1a activity (Fig S1H). We added the following to the manuscript: “Recombinant IL-1α significantly increased icIL-1RN mRNA levels in FNB6 cells, confirming also that the recombinant protein was functional (Fig S1H)”.

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Second decision letter

MS ID#: JOCES/2020/252080

MS TITLE: The role of icIL-1RA type 1 in oral keratinocyte senescence and the development of the senescence associated secretory phenotype

AUTHORS: Sven E Niklander, Hannah L Crane, Lav Darda, Daniel W Lambert, and Keith D Hunter
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The paper by Niklander et al. aims to understand the role of the IL-1 signalling antagonist IL-1RA in regulating the SASP in keratinocyte senescence. The role of the SASP in keratinocytes and carcinogenesis of this cell type has not been fully explored, and this study provides some insights into the underlying mechanism. The authors provide evidence in cells lines and patient samples that IL-1RA is downregulated as cells progress from normal to malignant and show in vitro that these changes correlate with SASP expression/secretion.

Comments for the author

The authors have adequately addressed my concerns.

Reviewer 2

Advance summary and potential significance to field

This is an interesting study which would advance our knowledge on the role of IL-1RA expression during oral carcinogenesis. However, I find the study preliminary and the conclusions still not supported by the data presented.

Comments for the author

I find the new data presented by the authors still do not support their conclusion and therefore cannot recommend the manuscript is accepted for publication.