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A Novel Strategy for Evasion of NK Cell Immunity by Tumours Expressing Core2 O-glycans

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 25 February 2011

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office. Having received consistent comments from two expert scientists, I am able to reach a decision on your paper to facilitate efficient proceedings. As obvious from their reports, both referees are in principle supportive of the proposed novel tumor escape mechanism involving the NKG2D-NKG2DL system. Still, both these scientists request significant further experimentation to emphasize the importance of the system under study compared to alternative activated receptors/lectins. Additional efforts should therefore improve mechanistic details as well as physiological significance. As the comments are very explicit, there is no need to repeat them here in any detail. I do however urge you to focus your attention on the essential concerns to overcome these major critiques before returning a revised version of your paper for external re-assessment.

Be also reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision entirely depends on the content within the last version of your manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours truly,
Editor
The EMBO Journal
REFEREE REPORTS:

Referee #1:

In this manuscript, Tsuboi and colleagues identify a novel mechanism of tumor-mediated NK cell evasion that is mediated by galectin-3 interaction with polylactosamine-enriched core2 O-glycan present on the NKG2D ligand MICA.

This study provides evidence of a novel tumor escape mechanism that compromises the NKG2D-NKG2DL system. In addition, these findings represent an interesting contribution to the understanding of how galectins (particularly galectin-3) participate in tumor-immune escape. Overall, the manuscript is interesting, novel and carefully done. However, more compelling evidence about the mechanisms underlying the described effects and the pathophysiologic relevance of endogenous galectin-3-MICA-NKG2D interactions are necessary for its publication in EMBO J.

One major concern is that, as presented, this work appears to be biased toward an involvement of O-glycans, galectin-3, MICA and NKG2D in the suppressive effects on NK cells without a significant rational framework. As tumor-NK cells involve many other activating receptors (NCRs) and polylactosamine bind other many galectins, the authors should justify their selection of the NKG2D-NKG2DL system over many other systems operating in NK cell effector function and their decision to study galectin-3 among 15 members of the galectin family which also bind polylactosamine and exert immunomodulatory effects.

Specific comments:

Major concerns:

1. Authors did not provide a working hypothesis and a clear objective for this work. After reading the Introduction, one gets the impression that most of this work arises as a consequence of a fortuitous finding (“In the present study, we discover that...”).

2. As NK cells express a wide array of activating receptors, it is not clear why the authors have directly focused on the NKG2D-MICA system as a major target of O-glycan modulation and galectin inhibition without exploring other NK cell activating receptors. To further develop this story in an unbiased manner the authors should present blocking experiments on at least some other receptors that are critical for NK cell function.

3. As NKG2D recognizes many different ligands, it is also hard to understand why the authors focused their research on MICA without considering the possibility of a participation of MICB or the ULBPs. Was the role of other NKG2DL addressed? Examination of the role of other NKG2D ligands is needed to report in an unbiased fashion the role of galectin-3 in NK-tumor cell interactions.

4. Authors postulate that binding of galectin-3 to polylactosamine-enriched O-glycans on MICA represents a novel tumor escape mechanism. Although interesting, this hypothesis is still speculative in light of the results presented. Authors have not performed in vivo experiments (tumor growth) in galectin-3- and/or NKG2D knockout mice. These experiments are critical to validate the role of galectin-3 or NKG2D in the mechanism reported.

5. The effect of MICA-bound galectin-3 on NKG2D-mediated NK cell response was addressed in a very preliminary fashion as only secretion of granzyme B was assessed. Authors should investigate NK cell effector functions in more detail (at least regulation of cytotoxicity and IFN- secretion should be included in this study).

6. Closely related to the previous issue is the fact that MICA can be released into the culture media or into the serum of tumor-bearing patients (as authors mentioned in the Discussion). This has been demonstrated to be a tumor immune escape mechanism. As galectin-3 is secreted by different cell types and binds to O-glycans present on MICA ectodomain, is it possible that galectin-3 could bind to soluble MICA. Was this possibility addressed? If this is the case, what would be the consequence
of this binding on NK cell mediated antitumor responses? If soluble MICA has been claimed to contribute to NKG2D down-regulation, binding of galectin-3 to soluble MICA would prevent such down-regulation and would likely contribute to counteract this tumor escape mechanism.

7. Data about NK cell activation shown in Figure 6 must include a control of NK cells cultured in wells coated with an isotype-matched irrelevant human mAb or chimeric protein since as performed, there might be stimulatory effects mediated through CD16 engagement on the human NK cells by the human Fc of the chimeric protein bound to the plates.

Other points:

1. In the in vivo experiments shown in Suppl Figure 4 with beige mice and NK-cell depleted animals, it is sort of hard to understand that tumors grew in the presence of a xenogeneic immune system only devoid of NK cells. The authors should provide an explanation for this. Also, the authors did not mention the number of animals per group used in these experiments. Also, in the Materials and Methods section neither the origin of these animals nor the number of cells injected into each group were mentioned.

2. There are some sentences that appear to be incomplete (bottom of pages 15 and 18).

3. Figure 1A and Suppl Figure 2A show partially overlapping information. I would suggest presenting only one that combines the information of both.

4. How is the killing of bladder tumors by resting NK cells? Authors only used NK cells activated for 3 days with IL-2.

5. There are some spelling errors such as "lignd" instead of "ligand". Please, revise the manuscript.

6. There are no quality controls for the treatment with tunicamycin. The authors should include a control that this drug worked efficiently to prevent N-glycosylation?

7. Data in Suppl Figure 6 should be accompanied by flow cytometry staining to get a better idea on the expression of galectin-3 and MICA on cell lines.

8. It would be interesting to identify the cellular source of galectin-3. Is this lectin derived from tumor themselves or from the tumor microenvironment (including galectin-3+ macrophages, dendritic cells or endothelial cells?).

9. Data presented in Suppl Figure 7 give no clue about "affinity" (as mentioned on page 13). To support such statement, experiments with Biacore or an equivalent technology should be performed. Moreover, this figure has another weak point since less binding of the MICA-Ig chimeric constructs could also be interpreted as weaker affinity of the anti-MICA detection mAb for MICAC2-Ig than for MICA-Ig. To overcome this technical flaw, the authors should perform a competition assay using biotinylated MICA-IgG and investigate which chimeric molecule (MICA-IgG or MICAC2-IgG) displaces or inhibits more extensively the binding of biotinylated MICA-IgG to NKG2D-Fc.

10. Data presented in Suppl Figure 8 are not convincing since authors are staining for NKG2D using the 1D11 mAb (which is a mAb suitable for blocking/masking experiments). Therefore, less binding should not necessarily be interpreted as down-regulation. Moreover, authors should provide specific fluorescence index (SFI) values and mean {plus minus} SEM and a statistical analysis in order to support their conclusions and relevance of the findings.

11. How is the cell surface expression of MICA and galectin-3 on tumor cells from patients that are C2GnT(pos) or C2GnT(neg)? This is a simple flow cytometry or IHC experiment that would further support authors' conclusions.

12. In the discussion, authors describe tumor immune escape mechanisms that involve MICA and NKG2D. Authors should include the reference of Salih HR and coworkers (J Immunol 2002;169: 4098) as this is a seminal paper which describes MICA shedding as potential tumor escape mechanism. Also, Fuertes and colleagues (J Immunol 2008;180: 4606) described another tumor
immune escape mechanism involving MICA-NKG2D that could be cited. The authors might also wish to update some of the references on tumor-immune escape mechanisms or galectins.

13. Legend to Figure 4 does not mention the use of PNGase as in the text. Please, clarify this issue.

14. Band presented in Figure 3 upper panel is over-exposed or overloaded.

15. In Figure 4, it is desirable to include a control which does not express MICA nor galectin-3.

In summary, the findings presented in this manuscript are novel, interesting and carefully done but more compelling evidence on the mechanisms and physiologic relevance of this effect is still needed to support the conclusions raised by the authors and to receive an enthusiastic endorsement for the broad audience of EMBO Journal.

Referee #2:

This study by Tsuboi et al. starts with the observation that expression of the O-glycan branching enzyme core2 b-1,6-N-acetylglucosaminyltransferase (C2GnT) in tumor cells correlates with poor survival of patients with bladder cancer. In order to elucidate the underlying molecular mechanisms the authors take advantage of two human bladder tumor cell lines (KK-47 and YTS) expressing low (KK-47) or high levels of C2GnT (YTS-1). By using these parental cell lines together with derivatives that overexpress C2GnT (KK-47-C2) or are silenced for C2GnT expression (YTSCKD) they find that C2GnT overexpression strongly promotes tumor development in (T cell deficient) nude mice, whereas cells expressing C2GnT at low levels barely form tumors. The latter effect is in part due to NK cell activity, because low C2GnT-expressing cells form tumors in NK cell-depleted or in NK-dysfunctional (beige) mice. Also, in vitro cytotoxicity assays revealed an increased susceptibility of tumors with low C2GnT expression for cytolysis by human (!) NK cells and here increased susceptibility appeared predominantly due to ligation of the activating NK receptor NKG2D.

In the following biochemical experiments, Tsubo and colleagues provide evidence that the NKG2D ligand MICA expressed by KK47 or YTS is O-glycosylated by C2GnT allowing for binding of galectin-3. In a last series of experiments authors reach the conclusion that masking of MICA modified by C2GnT with galactin-3 impairs NKG2D binding and thereby efficient NK-mediated cytolysis of C2GnT expressing tumor cells.

Overall this is an impressive and comprehensive study linking poor survival of cancer patients to post-translational modification of a particular ligand of an activating NK cell receptor. Most of the data are convincing except for the last and most critical part on functional consequences of MICA modification for NKG2D-mediated NK cell recognition.

MAJOR

Hypothesis that O-glycosylation of MICA interferes with NKG2D binding and NK recognition requires more experimental support.

Authors should:
- Compare binding of soluble NKG2D (e.g. NKG2D-Fc) to KK-47, KK-47-C2, YTS, YTSCKD in flow cytometry (including staining for MICA/B (BAMO1, BAMO3) and proper controls (irrelevant Fc, isotype). This experiment should be also done after pretreatment of cells with endo-b-galactosidase, if possible with viable cells.
- Compare binding of MICA-Fc and MICAC2-Fc (produced in KK-47, KK-47-C2) to human and mouse NK cells in flow cytometry.
- Repeat experiment shown in Figure 6A using BAMO3 (instead of BAMO1; BAMO1 has previously been shown to block MICA-NKG2D interaction (see Friese et al., 2003) and therefore data shown in Fig. 6A raise concerns)
- Repeat experiment Fig. 2C/D with NKL cell line as effector cells and blockade of NKG2D (anti-NKG2D) as well as of MICA.

Tumor formation assays in beige mice or mice depleted of NK cells suggest C2GnT-dependent NK recognition of KK47 and YTS bladder tumor cell lines. However, in vitro cytotoxicity assays were performed with human NK cells and NK cells from mouse and man profoundly differ in the receptor...
repertoire determining NK cell activation. Therefore in vitro cytotoxicity assays should also be performed with mouse NK cells and NKG2D-dependent recognition should be documented by inclusion of an appropriate anti-NKG2D antibody and an anti-MICA antibody.

Antibodies BAMO1 and BAMO3 recognize both MICA and MICB, but authors only refer to MICA in text and figures.

MINOR
Figure 2A and Suppl. Fig. S4A are identical (except for bar colors that are reversed). Repeated inclusion of this single data set is meaningful for comparison, but should explicitly stated by the authors (e.g. Fig. legend S4A) to avoid concerns of scientific misconduct. Alternatively, it is recommended to incorporate Fig.S4A-C into Fig.2, because these represent essential experiments.

Suppl. Fig. S4A-C should use same scale for y-axis to allow for proper comparison.

Authors must mention in text (Results section) and figure legends that human NK cells were used for in vitro cytotoxicity assays. Likewise antibodies used for immunoblotting should be mentioned in the legends.

No mention in methods section about immunoblotting procedure and antibodies used, e.g. for detection of MICA.

Point-by-point responses to the referees’ comments

We thank the referees for their thorough reading of our manuscript and numbers of valuable comments. We address each specific comment below.

Referee #1

In this manuscript, Tsuboi and colleagues identify a novel mechanism of tumor-mediated NK cell evasion that is mediated by galectin-3 interaction with polylactosamine-enriched core2 O-glycan present on the NKG2D ligand MICA.

This study provides evidence of a novel tumor escape mechanism that compromises the NKG2D-NKG2DL system. In addition, these findings represent an interesting contribution to the understanding of how galectins (particularly galectin-3) participate in tumor-immune escape. Overall, the manuscript is interesting, novel and carefully done. However, more compelling evidence about the mechanisms underlying the described effects and the pathophysiologic relevance of endogenous galectin-3-MICA-NKG2D interactions are necessary for its publication in EMBO J.

One major concern is that, as presented, this work appears to be biased toward an involvement of O-glycans, galectin-3, MICA and NKG2D in the suppressive effects on NK cells without a significant rational framework. As tumor-NK cells involve many other activating receptors (NCRs) and polylactosamine bind other many galectins, the authors should justify their selection of the NKG2D-NKG2DL system over many other systems operating in NK cell effector function and their decision to study galectin-3 among 15 members of the galectin family which also bind polylactosamine and exert immunomodulatory effects.

We have added several new results and paragraphs and explained why we focused on the NKG2D-MICA system in this study by responding to Referee #1’s concern No.2 and No.3 below.

Page 20, Line 1.
We have added a new paragraph to "Discussion" to explain how we decided to study galectin-3 among 15 members of galectin family.

Specific comments:
Major concerns:

1. **Authors did not provide a working hypothesis and a clear objective for this work. After reading the Introduction, one gets the impression that most of this work arises as a consequence of a fortuitous finding ("In the present study, we discover that...").**

   As Referee #1 suggested, we have revised our manuscript to mention our major objective and working hypothesis more explicitly than our original manuscript.

   Page 4, line 4.
   We have added a new sentence clearly mentioning our objective in "Introduction".

   Page 6, line 13. Page 8, line 19.
   We have added several new sentences clearly mentioning our working hypotheses based on our extensive clinical survey in "Results".

2. **As NK cells express a wide array of activating receptors, it is not clear why the authors have directly focused on the NKG2D-MICA system as a major target of O-glycan modulation and galectin inhibition without exploring other NK cell activating receptors. To further develop this story in an unbiased manner the authors should present blocking experiments on at least some other receptors that are critical for NK cell function.**

   New Supplementary Figure S4.
   We performed a series of blocking experiments on NK activating receptors other than NKG2D as Referee #1 suggested. NKG2C (as another C-type lectin receptor) and NKp46, NKp30 and NKp44 (as the Ig-super family activating receptors) were blocked with their blocking antibodies, and then measured NK-mediated cytotoxicity against the bladder tumour cells. The most effective inhibition was observed when NKG2D was blocked (New Supplementary Figure S4), suggesting that NKG2D plays the most critical role in NK cell killing of the bladder tumour cells.

   Page 8, line 23.
   We have added several new sentences to explain the new results of the blocking experiments and clarified why we focused on NKG2D in our study.

3. **As NKG2D recognizes many different ligands, it is also hard to understand why the authors focused their research on MICA without considering the possibility of a participation of MICB or the ULBPs. Was the role of other NKG2D ligands addressed? Examination of the role of other NKG2D ligands is needed to report in an unbiased fashion the role of galectin-3 in NK-tumor cell interactions.**

   New Supplementary Figure S5.
   To address the issues raised by Referee #1, we performed several additional experiments. We made a comparison of the expression levels among the five NKG2D ligands in the bladder tumour cells by RT-PCR and performed the blocking experiments on MICA/B and ULBP-2. MICA expression was the highest in all five ligands and anti-MICA/B efficiently inhibited NK cell killing of the bladder tumour cells, suggesting that MICA is a critical ligand for NK cell killing of the bladder tumour cells.

   Page 9, line 4.
   We have added several new sentences to explain the new results and clarified why we focused on the NKG2D-MICA interaction in our study.

4. **Authors postulate that binding of galectin-3 to polylactosamine-enriched O-glycans on MICA represents a novel tumor escape mechanism. Although interesting, this hypothesis is still speculative in light of the results presented. Authors have not performed in vivo experiments (tumor growth) in galectin-3- and/or NKG2D knockout mice. These experiments are critical to validate the role of galectin-3 or NKG2D in the mechanism reported.**

   We agree with Referee #1 that in vivo experiments are required to validate the role of NKG2D and galectin-3 in the immunoevasion mechanism that we reported. We then did several additional
experiments. The experimental systems that we chose were different from the ones that Referee #1 suggested. The reason why we took another approach was detailed below.

We performed the following two series of experiments to show that NKG2D and galectin-3 play a major role in evasion from NK cell immunity in vivo.

In vivo role of NKG2D Guerra et al. demonstrated that NKG2D plays a critical role in tumour growth of prostate cancer and B cell lymphoma by using the transgenic models with NKG2D-deficient mouse background (Guerra et al., 2008). However, these transgenic-NKG2D-deficient models are not applicable to bladder tumours disseminating into circulation. To address the in vivo role of NKG2D in eliminating the bladder tumour cells after dissemination into circulation, we decided to use nude mice whose NKG2D was neutralised by an anti-mouse NKG2D antibody (CX5) for in vivo experiments. It has been reported that CX5 efficiently neutralise mouse NKG2D in vivo (Ogasawara et al., 2004).

New Figure 8A and 8B
We performed in vitro and in vivo experiments by using CX5. These new results suggest that NKG2D plays a major role in NK cell killing of C2GnT-non-expressing bladder tumours in vivo and that C2GnT expression renders cells resistant to NKG2D-mediated NK cell attack in vivo.

Page 15, Line 23.
We have added several new sentences to explain the new results (New Figure 8A and 8B).

In vivo role of tumour cell-surface-bound galectin-3 Galectin-3 is a multifunctional protein. Tumour-associated galectin-3 is implicated in a variety of biological functions including tumour cell adhesion, proliferation, differentiation, apoptosis and angiogenesis. Moreover, galectin-3 is found inside cells, extracellularly and in the circulation (Dumic et al., 2006). When we use galectin-3-knockout mice or galectin-3-knockdown tumour cells to examine the survival of the bladder tumour cells in circulation, it is expected that it will be difficult to interpret the results and evaluate the role of galectin-3 in vivo due to its multifunction and complex localization. We should focus on the bladder tumour cell-surface-bound galectin-3, since the immunoevasion which we reported in this study involves only cell-surface-bound galectin-3 (MICA-bound galectin-3).

New Figure 8C and 8D and New Supplementary Figure S15
We thus decided to use the bladder tumour cells whose cell-surface-bound galectin-3 was removed by endo- -galactosidase treatment for in vivo experiments. However, we were concerned that after the cleavage by endo- -galactosidase, cell-surface galectin-3 may be restored in a short time by de novo synthesis of poly-N-acetyllactosamine and secretion of galectin-3 from tumour cells. We first examined NK-mediated cytotoxicity against the cell-surface-galectin-3-removed KK-47-C2 cells. KK-47-C2 cells were resistant to NK cell attack, but the cell-surface-galectin-3-removed KK-47-C2 cells were efficiently killed by NK cells in vitro (New Figure 8C). We confirmed that little of the cell-surface-galectin-3 was restored during the experiments (New Supplementary Figure S15). We then used the cell-surface-galectin-3-removed KK-47-C2 cells for tumour formation assays. Although KK-47-C2 cells formed a number of foci, the cell-surface-galectin-3-removed KK-47-C2 cells were efficiently killed in mice and formed significantly less foci than KK-47-C2 cells (New Figure 8D). These new results indicate that most of galectin-3-removed KK-47-C2 cells were killed within several hours by NK cells in vivo, suggesting that tumour cell-surface-bound galectin-3 plays an important role in immunoevasion mechanism from NK cell immunity by C2GnT-expressing tumour cells in vivo.

Page 16, Line 19.
We have added several new sentences to explain the new results (New Figure 8C and 8D).

We appreciate the Referee #1’s suggestion that the results from the in vivo experiments using NKG2D and/or galectin-3 knockout mice will strongly validate the immunoevasion mechanism that we reported. We also believed that the use of the knockout mice would be a good way to do that. However, the existing transgenic-NKG2D-deficient mouse or galectin-3-deficient mouse systems are not applicable to examination of cell survival of the bladder tumour cells after dissemination into circulation. Another way to examine bladder tumours in mice is to induce experimental bladder tumours using N-butyl-N-(4-hydroxybutyl)nitrosamine (Bryan, 1977), but it would be difficult to obtain any information about the relationship between metastasis and glycosylation of bladder...
tumour cells with this system. We do hope to develop some new experimental systems using NKG2D and/or galectin-3 knockout mice and carry out the experiments to validate our immunoevasion mechanism in the future, but we thought that the in vivo experimental systems that we chose were useful as alternative systems at this point and also support our conclusion.

5. The effect of MICA-bound galectin-3 on NKG2D-mediated NK cell response was addressed in a very preliminary fashion as only secretion of granzyme B was assessed. Authors should investigate NK cell effector functions in more detail (at least regulation of cytotoxicity and IFN-γ secretion should be included in this study).

New Figure 7.
We examined the secretion of INF- and human NK cell-mediated cytotoxicity against the bladder tumour cells and analysed the effect of galectin-3 on those NK cell functions as suggested. We have obtained three new results and combined them with the result of granzyme B secretion to make a new figure (New Figure 7).

Page 14, line 21.
We have added a new paragraph to explain the new results (New Figure 7) and revised "Materials and Methods" accordingly.

6. Closely related to the previous issue is the fact that MICA can be released into the culture media or into the serum of tumor-bearing patients (as authors mentioned in the Discussion). This has been demonstrated to be a tumor immune escape mechanism. As galectin-3 is secreted by different cell types and binds to O-glycans present on MICA ectodomain, is it possible that galectin-3 could bind to soluble MICA. Was this possibility addressed? If this is the case, what would be the consequence of this binding on NK cell mediated antitumor responses? If soluble MICA has been claimed to contribute to NKG2D down-regulation, binding of galectin-3 to soluble MICA would prevent such down-regulation and would likely contribute to counteract this tumor escape mechanism.

We agree with Referee #1 that it is possible that galectin-3 binds to soluble MICAC2 shed by tumour cells through poly-N-acetyllactosamine. We tested the possibility by the following experiments.

New Figure 4K and 4L and New Figure 6L
Galectin-3 co-immunoprecipitated with MICAC2 (New Figure 4K and 4L). Binding of MICAC2-IgG to NKG2D-Fc was inhibited by galectin-3 in a dose-dependent manner (New Figure 6L). These results taken together suggest that galectin-3 binds to soluble MICAC2.

We also agree with the Referee #1’s suggestion that binding of galectin-3 to soluble MICAC2 would prevent down-regulation of NKG2D, thereby counteracting the tumour escape mechanism by soluble MICAC2.

New Supplementary Figure S17
To test this possibility, we examined the ability of MICAC2-IgG to down-regulate NKG2D in the presence of galectin-3. We have obtained a new result (New Supplementary Figure S17). MICAC2-IgG has the ability to down-regulate NKG2D even in the presence of galectin-3 (New Supplementary Figure S17C). Compared with the ability of MICA-IgG to down-regulate NKG2D in the presence of galectin-3, the NKG2D down-regulation by MICAC2-IgG was slightly reduced as Referee #1 predicted (New Supplementary Figure S17B and S17C). However, the difference was small and substantial amounts of NKG2D were still present at the NK cell surface after stimulation with either MICA-IgG or MICAC2-IgG in the presence of galectin-3. This suggests that the counteracting effect of galectin-3 binding on tumour escape mechanism by soluble MICAC2 is small.

Page 19, line 4.
We have added a new paragraph to explain the new results (New Supplementary Figure S17).

7. Data about NK cell activation shown in Figure 6 must include a control of NK cells
cultured in wells coated with an isotype-matched irrelevant human mAb or chimeric protein since as performed, there might be stimulatory effects mediated through CD16 engagement on the human NK cells by the human Fc of the chimeric protein bound to the plates.

New Figures 7 and 8.
We need to use a control-IgG chimera for NK activation and FACS staining experiments as Referee #1 suggested. We redid the whole NK activation and FACS staining experiments including the control experiments with the control-IgG chimeras. We used CD43-IgG chimera as a control, because CD43 (a T-cell marker) has no certain interacting partners and we used to use the chimera for several experiments (Sawada et al, 1994). We have shown the new results in New Figures 7 and 8.

In addition, we need to avoid the stimulation of NK cells through CD16 and binding of secondary antibody to Fc receptor on NK cells as Referee #1 pointed out. To do this, we pre-incubated NK cells with FcR Blocking Reagent (Mylteni Biotech). We accidentally omitted to write this procedure in our original manuscript. We are sorry for any confusion caused by this. We have added the description of this procedure to "Materials and Methods".

Other points:

1. **In the in vivo experiments shown in Suppl Figure 4 with beige mice and NK-cell depleted animals, it is sort of hard to understand that tumors grew in the presence of a xenogeneic immune system only devoid of NK cells. The authors should provide an explanation for this. Also, the authors did not mention the number of animals per group used in these experiments. Also, in the Materials and Methods section neither the origin of these animals nor the number of cells injected into each group were mentioned.**

In our original manuscript, we mentioned that we used beige mice and NK-depleted mice for tumour formation assay. The mice we actually used were SCID/beige and NK-depleted nude mice. The severe combined immunodeficiency (SCID)/beige mice were generated by backcrossing beige mutation onto C57BL/6-scid/scid strain (Christianson et al, 1996). SCID/beige and NK-depleted nude mice were used for in vivo tumour formation assay in order to study the role of NK cells in rejection of tumour cells by tumour immunosurveillance systems (Harding et al, 2010; Hartman et al, 2009; Ohyama et al, 2002; Ohyama et al, 1999). In our original manuscript, we accidentally wrote "beige" and "NK-depleted" for "SCID/beige" and "NK-depleted nude", respectively. We apologise for any confusion caused by this.

We have revised "Results" and "Materials and Methods" accordingly.

2. **There are some sentences that appear to be incomplete (bottom of pages 15 and 18).**

We have revised the sentences that Referee #1 pointed out.

3. **Figure 1A and Suppl Figure 2A show partially overlapping information. I would suggest presenting only one that combines the information of both.**

We have included the information of Supplementary Figure 2A (our original manuscript) in New Figure 1A (our revised manuscript) as suggested. We have revised the legends for New Figure 1A accordingly.

4. **How is the killing of bladder tumors by resting NK cells? Authors only used NK cells activated for 3 days with IL-2.**

Page 24, line 13.
We measured specific lysis of KK-47 cells by resting human and mouse NK cells. We have added the result to our revised manuscript.

5. **There are some spelling errors such as "lignd" instead of "ligand". Please, revise the manuscript.**
We have corrected the spelling errors.

6. **There are no quality controls for the treatment with tunicamycin. The authors should include a control that this drug worked efficiently to prevent N-glycosylation?**

New Supplementary Figure S7.

To confirm that tunicamycin efficiently worked to prevent N-glycosylation of the cell-surface proteins of the bladder tumours as requested, we presented a result from the positive control experiments that we previously did as New Supplementary Figure S7.

Page 10, line 10.
We have added a new sentence to "Result" to explain the new result.

7. **Data in Suppl Figure 6 should be accompanied by flow cytometry staining to get a better idea on the expression of galectin-3 and MICA on cell lines.**

New Figure 4.

To support that the bladder tumour cells express MICA or MICAC2 and that galectin-3 exists at the cell surface, we performed flow cytometric analyses as suggested. We stained the cells with anti-MICA (BAMO3) and anti-galectin-3 and analysed staining by flow cytometry. We have included the new results in New Figure 4.

Page 11, line 6.
We have added several new sentences to explain the new results (New Figure 4).

8. **It would be interesting to identify the cellular source of galectin-3. Is this lectin derived from tumor themselves or from the tumor microenvironment (including galectin-3+ macrophages, dendritic cells or endothelial cells)?**

We detected substantial amounts of galectin-3 in the total cell-surface proteins from the cultured bladder tumour cells (New Figures 4 and 5, New Supplementary Figures S9 and S11). These results indicate that at least the tumour cell itself is a source of galectin-3. However, it has been reported that patients with metastatic tumours have higher concentrations of circulating galectin-3 than those with localized tumours (Iurisci et al, 2000; Saussez et al, 2008; Vereecken et al, 2006), suggesting that tumour may not be a sole source of galectin-3. We think that tumour cell-surface bound galectin-3 is derived from both tumours themselves and tumour microenvironments.

9. **Data presented in Suppl Figure 7 give no clue about "affinity" (as mentioned on page 13). To support such statement, experiments with Biacore or an equivalent technology should be performed.**

New Supplementary Figure S14.

We agree with the concern raised by Referee #1 that ELISA is not suitable for the comparison of affinity in old Supplementary Figure 7 in our original manuscript. To make a comparison of the affinity for NKG2D-F between MICA-IgG and MICAC2-IgG by more precise methods, we analysed the MICA-NKG2D interaction using surface plasmon resonance-based biosensor (BIACORE3000) as suggested. We have obtained a sensor gram and added the new result to Supplementary Figure S7 in our previous manuscript to make New Supplementary Figure S14B and removed the saturation curves by ELISA.

Page 14, line 12.
We have added several new sentences to explain the new result (New Supplementary Figure S14).

Moreover, this figure has another weak point since less binding of the MICA-Ig chimeric constructs could also be interpreted as weaker affinity of the anti-MICA detection mAb for MICAC2-Ig than for MICA-Ig. To overcome this technical flaw, the authors should perform a competition assay using biotinylated MICA-IgG and investigate which chimeric molecule (MICA-IgG or MICAC2-IgG) displaces or inhibits more extensively the binding of biotinylated MICA-IgG to NKG2D-Fc.
New Figures 4 and 6L.
In our original manuscript, we used an anti-MICA/B (clone BAMO1) to analyse the expression levels of MICA/B molecules by ELISA. To check if the affinity of BAMO1 for MICA molecules is affected by C2GnT, we tested BAMO1 binding to cells by flow cytometry. BAMO1 binding to C2GnT-expressing cells was slightly but significantly reduced compared with C2GnT-non-expressing cells or cells with reduced C2GnT expression (New Supplementary Figure S13). This new result indicates that BAMO1 binding to MICA is affected by core2 O-glycosylation as Referee #1 suggested. The reason for this is that BAMO1 is a blocking antibody and that BAMO1 binds to 1+2 region of MICA (NKG2D binding site). We appreciate the referee #1’s pointing this out.

We then tested another MICA antibody, BAMO3 to analyse the expression levels of MICA molecules. There is no difference in BAMO3 binding to cells between C2GnT-expressing and C2GnT-non-expressing cells (New Figure 4B and 4G). This is because BAMO3 is specific to the 3 region of MICA which is independent of NKG2D binding site (Salih et al, 2003; Spreu et al, 2006). Therefore, we did flow cytometric analyses (New Figure 4) and redid ELISA (New Figure 6L) by using BAMO3.

We have added the following new results from the experiments using BAMO3 instead of BAMO1, New Figure 4A-4J, New Figure 6L and New Supplementary Figure S11.

We have revised "Results" and "Materials and Methods" accordingly.

10. Data presented in Suppl Figure 8 are not convincing since authors are staining for NKG2D using the ID11 mAb (which is a mAb suitable for blocking/masking experiments). Therefore, less binding should not necessarily be interpreted as down-regulation. Moreover, authors should provide specific fluorescence index (SFI) values and mean (plus minus) SEM and a statistical analysis in order to support their conclusions and relevance of the findings.

New Supplementary Figure S16
To detect the expression levels of NKG2D on the NK cell surface, we used another anti-NKG2D monoclonal antibody (clone ON72, Beckman Coulter) which is suitable for flow cytometry (Verneris et al Blood 2004) as suggested. And also, we did flow cytometric analysis on three independently prepared samples and presented all the results (experiment 1., 2. and 3.) (Supplementary Figure S16A-S16E). We then did the statistical analysis using those three results as requested and showed the result as a bar graph (Supplementary Figure S16F). We have added the new result (New Supplementary Figure S16) to show the expression levels of NKG2D on the NK cell surface.

Page 18, line 12.
We have added several sentences to "Discussion" to explain the new result (Supplementary Figure S16).

11. How is the cell surface expression of MICA and galectin-3 on tumor cells from patients that are C2GnT(pos) or C2GnT(neg)? This is a simple flow cytometry or IHC experiment that would further support authors' conclusions.

New Supplementary Figure S11
To confirm the cell-surface expression of MICA, MICAC2 and galectin-3 in bladder tumour cells from patients, we prepared primary culture from C2GnT-negative (P1) and C2GnT-positive (P2) tumour specimens and then stained the primary cultured cells with anti-MICA and anti-galectin-3. The results from the flow cytometric analyses were shown in New Supplementary Figure S11.

Page 13, line 1.
We have added several new sentences to explain the new result (New Supplementary Figure S11) and also a new paragraph to Supplementary Materials and Methods to explain the procedures of the preparation of primary cultures.

12. In the discussion, authors describe tumor immune escape mechanisms that involve MICA and NKG2D. Authors should include the reference of Salih HR and coworkers (J Immunol 2002;169: 4098) as this is a seminal paper which describes MICA shedding as potential tumor escape mechanism. Also, Fuertes and colleagues (J Immunol 2008;180: 4606) described another
tumor immune escape mechanism involving MICA-NKG2D that could be cited. The authors might also wish to update some of the references on tumor-immune escape mechanisms or galectins.

Page 18, line 4.
We have revised "Discussion" by including two literatures (Salih et al. (2002) and Fuertes et al. (2008)) as suggested. And also, we have updated the reference list by adding 5 new literatures.

13. Legend to Figure 4 does not mention the use of PNGase as in the text. Please, clarify this issue.

Page 36, line 27.
We have added the description about PNGase treatment to the legend for New Figure 5 (Old Figure 4) as requested.

14. Band presented in Figure 3 upper panel is over-exposed or overloaded.

New Figure 3B
We have rescanned another blot that is not over-exposed to make a new blot data and replaced the old over-exposed blot with the new blot (New Figure 3B) as suggested.

15. In Figure 4, it is desirable to include a control which does not express MICA nor galectin-3.

We agree with Referee #1 and we also thought that it was desirable to include any control cells which express neither MICA nor galectin-3. However, as far as we checked, all the tumor cells such as other bladder cancer cells, breast cancer cells and prostate cancer cells express both MICA/B and galectin-3 more or less. We decided to present these results (Old Figure 4, New Figures 4 and 5) without including the control cells not expressing MICA and galectin-3, since the most important information provided by these results is that galectin-3 binds MICAC2 from C2GnT-expressing tumor cells.

In summary, the findings presented in this manuscript are novel, interesting and carefully done but more compelling evidence on the mechanisms and physiologic relevance of this effect is still needed to support the conclusions raised by the authors and to receive an enthusiastic endorsement for the broad audience of EMBO Journal.

Referee #2

This study by Tsuboi et al. starts with the observation that expression of the O-glycan branching enzyme core2 b-1,6-N-acetylgalcosaminyltransferase (C2GnT) in tumor cells correlates with poor survival of patients with bladder cancer. In order to elucidate the underlying molecular mechanisms the authors take advantage of two human bladder tumor cell lines (KK-47 and YTS) expressing low (KK-47) or high levels of C2GnT (YTS-1). By using these parental cell lines together with derivatives that overexpress C2GnT (KK-47-C2) or are silenced for C2GnT expression (YTSC2KD) they find that C2GnT overexpression strongly promotes tumor development in (T cell deficient) nude mice, whereas cells expressing C2GnT at low levels barely form tumors. The latter effect is in part due to NK cell activity, because low C2GnT-expressing cells form tumors in NK cell-depleted or in NK-dysfunctional (beige) mice. Also, in vitro cytotoxicity assays revealed an increased susceptibility of tumors with low C2GnT expression for cytolysis by human (!) NK cells and here increased susceptibility appeared predominantly due to ligation of the activating NK receptor NKG2D. In the following biochemical experiments, Tsuboi and colleagues provide evidence that the NKG2D ligand MICA expressed by KK47 or YTS is O-glycosylated by C2GnT allowing for binding of galectin-3. In a last series of experiments authors reach the conclusion that masking of MICA modified by C2GnT with galactin-3 impairs NKG2D binding and thereby efficient NK-mediated cytolysis of C2GnT expressing tumor cells.

Overall this is an impressive and comprehensive study linking poor survival of cancer patients to post-translational modification of a particular ligand of an activating NK cell receptor. Most of the data are convincing except for the last and most critical part on functional consequences of MICA
modification for NKG2D-mediated NK cell recognition.

**MAJOR**

Hypothesis that O-glycosylation of MICA interferes with NKG2D binding and NK recognition requires more experimental support.

Authors should:

- Compare binding of soluble NKG2D (e.g. NKG2D-Fc) to KK-47, KK-47-C2, YTS, YTSC2KD in flow cytometry (including staining for MICA/B (BAMO1, BAMO3) and proper controls (irrelevant Fc, isotype). This experiment should be also done after pretreatment of cells with endo-b-galactosidase, if possible with viable cells.

New Figure 4, New Figure 6 and New Supplementary Figure S13

We performed a series of flow cytometric analysis on the bladder tumour cells as Referee #2 suggested. We analysed cell-surface expression of MICA using anti-MICA (BAMO3) (New Figure 4) and NKG2D-Fc chimera (New Figure 6) before and after endo-β-galactosidase treatment. We also performed FACS analysis on the bladder tumour cells using another anti-MICA antibody (BAMO1) (New Supplementary Figure S13) according to the Referee #2’s suggestion. The Referee #2 was concerned that BAMO1 binding to MICA may be affected depending on O-glycosylation. Actually this was the case. New Supplementary Figure S13 showed that binding of BAMO1 was slightly reduced by core2 O-glycans (New Supplementary Figure S13B and S13D). We thought that this was because BAMO1 binding site resides in the 1+2 region of MICA which is O-glycosylated (New Supplementary Figure S12). We then decided to use anti-MICA (BAMO3) for FACS analysis (New Figure 4). BAMO3 is specific to the 3 region of MICA which is independent of NKG2D binding site (Salih et al., 2003; Spreu et al., 2006), and there is no difference in BAMO3 binding to cells between C2GnT-expressing cells and C2GnT-non-expressing cells. We appreciate Referee #2’s pointing out the possible difference in reactivity to MICA between BAMO1 and BAMO3.


We have added several sentences to explain the new results from flow cytometric analyses (New Figures 4 and 6 and New Supplementary Figure S13) and revised "Results" and "Figure legends" accordingly.

*Compare binding of MICA-Fc and MICAC2-Fc (produced in KK-47, KK-47-C2) to human and mouse NK cells in flow cytometry.*

New Figure 6G-6K

We performed a series of flow cytometric analysis on both human and mouse NK cells using MICA-IgG and MICAC2-IgG as Referee #2 requested. We have added the new result as New Figure 6G-6K in our revised manuscript.

Page 14, line 6.

We have added several sentences to explain the new results from flow cytometric analyses (New Figure 6G-6K) and revised "Results" and "Figure legends" accordingly.

*Repeat experiment shown in Figure 6A using BAMO3 (instead of BAMO1; BAMO1 has previously been shown to block MICA-NKG2D interaction (see Friese et al., 2003) and therefore data shown in Fig. 6A raise concerns)*

New Figure 6L

We performed ELISA using BAMO3 as requested. We agree with Referee #2 that BAMO1 is not suitable for examination of the expression levels of MICA/B, because BAMO1 is a blocking antibody. And also, we made a comparison of the anti-MICA/B antibody reactivity to cells between BAMO1 and BAMO3. BAMO1 binding to C2GnT-expressing cells was slightly but significantly reduced compared with C2GnT-non-expressing cells (New Supplementary Figure S13) as described above. In contrast, there is no difference in BAMO3 binding to cells between C2GnT-expressing and C2GnT-non-expressing cells (New Figure 4B and 4G). Therefore, we redid the same ELISA experiment as old Figure 6A in our original manuscript and added the new result as New Figure 6L in our revised manuscript.

Page 14, line 17.
We have added several sentences to explain the new results (New Figure 6L) and revised "Materials and Methods" and "Figure legends" accordingly.

Repeat experiment Fig. 2C/D with NKL cell line as effector cells and blockade of NKG2D (anti-NKG2D) as well as of MICA.

New Supplementary Figure S6
We agree with Referee #2 that we need to confirm that C2GnT-expressing tumour cells are more resistant to NK cell attack than C2GnT-non-expressing cells (or cells with reduced C2GnT expression) by using a clonal NK cell line such as NKL cells. We used another clonal NK cell line, KHYG-1, since we were unable to obtain NKL cells. KHYG-1 cells are of NK cell origin, displaying NK cell cytotoxic activity and IL-2-dependent proliferation (Miah et al, 2008; Suck et al, 2006; Yagita et al, 2000). We purchased this cell line from Japan Collection Research Bioresources (JCRB) (Osaka, Japan). We performed a series of cytotoxic assay against bladder tumour cells using KHYG-1 cells as effector cells and obtained the new results (New Supplementary Figure S6) equivalent to the results using human NK cells (New Figure 2F).

Page 9, line 10.
We have added several sentences to explain the new results (New Supplementary Figure S6) using KHYG-1 cells.

Tumor formation assays in beige mice or mice depleted of NK cells suggest C2GnT-dependent NK recognition of KK47 and YTS bladder tumor cell lines. However, in vitro cytotoxicity assays were performed with human NK cells and NK cells from mouse and man profoundly differ in the receptor repertoire determining NK cell activation. Therefore in vitro cytotoxicity assays should also be performed with mouse NK cells and NKG2D-dependent recognition should be documented by inclusion of an appropriate anti-NKG2D antibody and an anti-MICA antibody.

We agree with Referee #2 that it is necessary to examine cytotoxicity against human bladder tumour cells using mouse NK cells.

New Figure 2E
We performed in vitro cytotoxicity assay using mouse NK cells. Similarly to the results with human NK cells, C2GnT-non-expressing bladder tumour cells were efficiently killed by mouse NK cells and C2GnT-expressing tumour cells were more resistant to NK cell attack than C2GnT-non-expressing tumour cells (New Figure 2E). This supports the result from our in vivo tumour formation (New Figure 2A-2D), consistent with the observation that MICA functions as a mouse NKG2D ligand in mice (Elsner et al, 2007; Friese et al, 2003).

Page 8, line 11.
We have added several new sentences to explain the new results (New Figure 2E).

Antibodies BAMO1 and BAMO3 recognize both MICA and MICB, but authors only refer to MICA in text and figures.

New Supplementary Figure S5 and New Supplementary Figure S8
We agree with Referee #2 that it is necessary to explain why we mainly referred to MICA. When we blocked MICA/B of KK-47-cells with BAMO1, NK-mediated killing was reduced to about 20% compared with control IgG (New Supplementary Figure S5B), indicating that MICA/B plays a major role as an NKG2D ligand. In addition to this, RT-PCR analysis revealed that the expression level of MICA is the highest of all five NKG2D ligands (New Supplementary Figure S5A). Based on these two new results, we decided to mainly refer to MICA rather than MICA/B. On the other hand, we have confirmed that MICB is also core2 O-glycosylated and carries poly-N-acetyllactosamine (New Supplementary Figure S8). This suggests that MICB also participates in the immunoevasion in the same way as MICA.

Page 9, line 5. Page 10, line 22.
We have added several new sentences to explain the new results (New Supplementary Figure S5 and S8).
MINOR

Figure 2A and Suppl. Fig. S4A are identical (except for bar colors that are reversed). Repeated inclusion of this single data set is meaningful for comparison, but should explicitly stated by the authors (e.g. Fig. legend S4A) to avoid concerns of scientific misconduct. Alternatively, it is recommended to incorporate Fig.S4A-C into Fig.2, because these represent essential experiments.

New Figure 2A-2D and New Figure 8D We appreciate Referee #2’s pointing this out and we apologise for the confusion caused by using the same figure. We have incorporated Supplementary Figure 4A-4C in our original manuscript into Figure 2 to make New Figure 2A-2D as suggested. In addition, we have presented New Figure 2A in New Figure 8 (as New Figure 8D, left panel) for the comparison purpose. We have explicitly stated the re-use of New Figure 2A in the legend for New Figure 8D.

Page 38, line 16. We have added several sentences to explain the new figures and revised "Figure legends" accordingly.

Suppl. Fig. S4A-C should use same scale for y-axis to allow for proper comparison. Authors must mention in text (Results section) and figure legends that human NK cells were used for in vitro cytotoxicity assays. Likewise antibodies used for immunoblotting should be mentioned in the legends.

No mention in methods section about immunoblotting procedure and antibodies used, e.g. for detection of MICA.

New Figure 2A-2D and New Supplementary Table SII We apologize for the confusion caused by using the different scales in our previous manuscript. We have changed the scale for y-axis as requested. And also, we have added a detail list of the antibodies that we used in this study (New Supplementary Table SII). We have revised "Results" and "Figure legends" to mention about NK cells and antibodies that we used in our experiments more clearly as requested.

Page 24, line 19. We have added a new paragraph in "Materials and Methods" in our revised manuscript to detail the procedure of Western blotting.

References


Saussez S, Lorfevre F, Lequeux T, Laurent G, Chantrain G, Vertongen F, Toubeau G, Decaestecker C, Kiss R (2008) The determination of the levels of circulating galectin-1 and -3 in HNSCC patients could be used to monitor tumor progression and/or responses to therapy. Oral Oncol 44: 86-93


Acceptance letter

I have already received final comments from two referees. As you can see, there seems a little
confusion related to figure-labeling that I kindly ask you to address sending a corrected text file before official acceptance to the paper.

The editorial office will be in touch soon related to necessary paper work.

I do like to take this opportunity to congratulate to this paper and remain with best regards.

Yours truly

Editor
The EMBO Journal

Ref#1:

The authors have adequately addressed all the comments I raised in my previous review.

This study now reports in an unbiased manner an important mechanism of tumor-immune escape mediated by MICA and galectin-3.

Ref#2:

All my concerns were adequately addressed.

Minor:
Figure 4 K,L: numbering of lanes discrepant between Figure and Legend

Figure S5A: GAPGH