Targeting MICA/B with cytotoxic therapeutic antibodies leads to tumor control [version 2; peer review: 2 approved]

Mathieu Bléry1, Manel Mrabet-Kraiem1, Ariane Morel1, Florence Lhospice1, Delphine Bregenon1, Cécile Bonnafous1, Laurent Gauthier1, Benjamin Rossi1, Romain Remark1, Stéphanie Cornen1, Nadia Anzeriz1, Nicolas Viaud1, Sylvia Trichard1, Sabrina Carpentier1, Alix Joulin-Giet1, Gwendoline Grondin1, Veronika Liptakova2, Younghoon Kim2, Laurent Daniel3, Aurélie Haffner3, Nicolas Macagno3, Laurent Pouyet4, Ivan Perrot1, Carine Paturel1, Yannis Morel1, Alexander Steinle2,5, François Romagné4, Emilie Narni-Mancinelli6, Eric Vivier1,3,6

1Innate Pharma, Marseille, France
2Institute for Molecular Medicine, Goethe-University Frankfurt am Main, Frankfurt am Main, Germany
3Assistance Publique des Hôpitaux de Marseille, Hôpital de la Timone, Marseille, France
4MI-mAbs, Aix Marseille University, Marseille, France
5Frankfurt Cancer Institute, Frankfurt am Main, Germany
6Aix Marseille University, CNRS, INSERM, CIML, Marseille, France

* Equal contributors

Abstract

Background: MICA and MICB are tightly regulated stress-induced proteins that trigger the immune system by binding to the activating receptor NKG2D on cytotoxic lymphocytes. MICA and MICB are highly polymorphic molecules with prevalent expression on several types of solid tumors and limited expression in normal/healthy tissues, making them attractive targets for therapeutic intervention.

Methods: We have generated a series of anti-MICA and MICB cross-reactive antibodies with the unique feature of binding to the most prevalent isoforms of both these molecules.

Results: The anti-MICA and MICB antibody MICAB1, a human IgG1 Fc-engineered monoclonal antibody (mAb), displayed potent antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) of MICA/B-expressing tumor cells in vitro. However, it showed insufficient efficiency against solid tumors in vivo, which prompted the development of antibody-drug conjugates (ADC). Indeed, optimal tumor control was achieved with MICAB1-ADC format in several solid tumor models, including patient-derived xenografts (PDX) and carcinogen-induced tumors in immunocompetent MICagen

Open Peer Review

Approval Status: ✔ ✔

version 2 (revision)
27 Oct 2021

version 1
13 Sep 2021

1. Jacques Zimmer, Luxembourg Institute of Health (LIH), Esch-sur-Alzette, Luxembourg
2. Roberta Castriconi, University of Genova, Genoa, Italy

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transgenic mice.

Conclusions: These data indicate that MICA and MICB are promising targets for cytotoxic immunotherapy.

Keywords
MICA, ADC, cancer immunotherapy

This article is included in the Medical Biotechnology gateway.

This article is included in the European Research Council (ERC) gateway.

This article is included in the Clinical Medicine gateway.

This article is included in the Immunological Disruptions and Interventions collection.

This article is included in the Cancer collection.
Corresponding author: Eric Vivier (vivier@ciml.univ-mrs.fr)

Author roles: Bléry M: Conceptualization, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing - Original Draft Preparation, Writing - Review & Editing; Mrabet-Kraiem M: Conceptualization, Investigation, Methodology, Project Administration, Resources, Validation, Visualization, Writing - Original Draft Preparation, Writing – Review & Editing; Morel A: Investigation, Supervision; Lhospice F: Investigation; Bregnon D: Investigation; Bonnafous C: Investigation, Supervision; Gauthier L: Investigation, Supervision; Rossi B: Investigation; Remark R: Investigation, Supervision; Cornen S: Investigation, Supervision; Anceriz N: Investigation, Supervision; Viaud N: Investigation; Trichard S: Investigation; Carpentier S: Formal Analysis; Joulin-Giet A: Investigation; Grondin G: Investigation; Liptakova V: Investigation; Kim Y: Investigation; Daniel L: Investigation, Supervision; Haffner A: Investigation, Supervision; Macagno N: Investigation, Supervision; Pouyet L: Investigation, Supervision; Perrot I: Formal Analysis; Paturel C: Investigation, Supervision; Morel Y: Conceptualization, Funding Acquisition, Supervision; Steimle A: Conceptualization, Resources, Supervision; Romagné F: Conceptualization, Funding Acquisition, Supervision; Narni-Mancinelli E: Visualization, Writing - Original Draft Preparation, Writing – Review & Editing; Vivier E: Conceptualization, Funding Acquisition, Methodology, Project Administration, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: M.M-K., A.M., D.B., C.B., B.R., R.R., C.P., S.C., N.A., N.V., S.T., A.J-G., G.G., Y.M., I.P., E.V are employees of Innate Pharma. A.S. had research contracts with Innate Pharma, and PDI Therapeutics, as well as consulting and stock ownership of PDI Therapeutics. The other authors have no competing financial interests to declare.

Grant information: The E.V. laboratory at CIML and Assistance-Publique des Hôpitaux de Marseille is financially supported by the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement Nos 694502 and 875102), the Agence Nationale de la Recherche including the PIONEER Project (ANR-17-RHUS-0007), MSDAVENIR, Innate Pharma and institutional grants to the CIML (INSERM, CNRS, and Aix-Marseille University) and to Marseille Immunopole. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Bléry M, Mrabet-Kraiem M, Morel A et al. Targeting MICA/B with cytotoxic therapeutic antibodies leads to tumor control [version 2; peer review: 2 approved] Open Research Europe 2021, 1:107 https://doi.org/10.12688/openreseurope.13314.2
First published: 13 Sep 2021, 1:107 https://doi.org/10.12688/openreseurope.13314.1
Introduction

Cancer is one of the most feared diseases of the 21st century, but immuno-oncology has revolutionized its treatment. Strategies based on the use of therapeutic monoclonal antibodies (mAbs) directed against immune checkpoint inhibitors1-3, or cytotoxic mAbs against specific tumor antigens have proved particularly effective4,5. Cytotoxic mAbs have been engineered to improve their therapeutic efficacy through the Fc-mediated immune effector function or by using antibody drug conjugates (ADCs). ADCs combine the specificity of a monoclonal antibody (mAb) with the toxicity of a cytotoxic payload6. ADCs allow a maximal anti-tumor efficacy via the specific targeting of the cytotoxic agent to tumor cells and minimize toxicity against healthy tissues6. An ADC is constituted by a mAb that is associated to a cytotoxic drug by a linker. The mAb must be as specific as possible for a cell surface tumor-associated antigen or overexpressed by tumor cells, in order to spare healthy tissue. Also, the targeted tumor antigen has to be internalized together with the ADC to deliver the cytotoxic agent inside the tumor cell. Another constraint of the ADC is the linker that bind the mAb to the cytotoxic agent. The linker can be cleavable or non-cleavable, meaning that the cytotoxic agent is released by a low pH or under reducing environment or require proteolytic degradation. In any cases, the release of the cytotoxic agent in the circulation must be avoided to prevent tissue damage. Conjugation of the linker to the mAb must not alter the binding specificity and affinity of the mAb. Finally, the cytotoxic agent coupled to the mAb can either disrupt microtubules or damage DNA, such as the pyrrolobenzodiazepines (PBD), both first and second class of ADC eventually inducing mechanisms causing cell death. Originally discovered in Streptomyces species, pyrrolobenzodiazepines (PBDs) are based on naturally occurring antitumour antibiotics that bind to the DNA minor groove. The dimerization of two PBD units allows to crosslink DNA by binding to the N2 position of guanine on opposing strands of DNA7. The rationally designed pyrrolobenzodiazepine (PBD) dimers emerged around ten years ago as a new class of drug for antibody-drug conjugates (ADC). Different PBD payloads including talirine and tesirine have been developed and evaluated clinically8. Talirine (SGD-1882) is highly hydrophobic with limited therapeutic index. All talirine-ADCs were subsequently discontinued because of high clinical toxicity9. Tesirine (SG3249) was designed to combine potent antitumor activity with desirable physicochemical properties such as favorable hydrophobicity and improved conjugation characteristics10,11. Recently Loncastuximab tesirine was approved under accelerated approval for the treatment of adult patients with relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy. Camidanlumab tesirine shows promising clinical trial results in the treatment of Hodgkin lymphoma. ADC design is very important to overcome toxicities and optimize therapeutic index. We developed site-specific technology based on bacterial transglutaminase (BTG) conjugation of derivative linker-drug to the glutamine at positions 295 and 297 thereby yielding homogeneous ADCs. As previously described these homogeneous ADCs display improved pharmacokinetics and better therapeutic indexes11. Classical dipeptide lysosomal-cleavable linker with high stability valine-alanine (VA) was selected12.

The number of approved ADCs has doubled in the last three years, reflecting the potential of this powerful tool to fight cancers6. Various tumor antigens have been targeted in this context. The most appropriate targets are accessible hematologicaltumor cell surface molecules (e.g. CD22, CD79b, BCMA, CD30, CD33), or highly expressed solid tumor antigens (e.g. Her-2, Nectin-4, Trop-2). Many targets have been explored in clinical development using the ADC approach and 11 of them have been approved. Targets with limited expression in normal tissues, which are well internalized and play a role in tumor biology (growth, metastasis or resistance), are potentially great assets for ensuring a positive efficacy/toxicity balance for this approach13.

Major histocompatibility complex (MHC) class I-related chain A and B polypeptides (MICA and MICB) are cell surface molecules with low levels of expression at steady state. However, they can be induced by cellular stresses, such as infections and tumor transformation14-20. The induction of their expression is tightly controlled by E2F, the DNA damage response and p5321-24. Together with members of the UL16-binding protein (ULBP) family, MICA and MICB act as ligands of the human NKG2D immunoreceptor, which is expressed on the surface of T cells (e.g. CD8+ T cells, γδ T cells) and natural killer (NK) cells25-27. Following ligand binding, NKG2D directly delivers activating signals promoting cytotoxicity and cytokine production28. MICA and MICB are frequently associated with epithelial tumors, induced by microbial infections, and is aberrantly expressed in certain autoimmune disease lesions29-32. MICA expression is also associated with hematological malignancies including leukemia and multiple myeloma30-32. MICA has a structure resembling the protein fold of MHC class I molecules, with an α1-α2 platform domain and a membrane-proximal Ig-like α3 domain33. Our objective was to develop an efficient therapeutic tool targeting MICA and MICB in oncological indications. We generated a group of cytotoxic mAbs targeting the products of the MICA and MICB alleles. The limited efficiency of these mAbs in models of solid tumors in vivo was the rationale to develop a novel ADC comprising the human anti-MICA/B Ab conjugated to highly potent (PBD) toxin with an intracellular cleavable linker using site-specific bacterial transglutaminase (BTG) technology yielding homogeneous ADCs. As previously described, these homogeneous ADCs display improved pharmacokinetics and better therapeutic indexes34. Preclinical efficacy and preliminary toxicity results validate MICA/B as an attractive therapeutic target for ADC approach in multiple solid tumors.
Results
MICA/B protein levels are low in healthy tissues and high at the tumor bed

We started by analyzing MICA/MICB protein levels in a large series of healthy tissues and tumors, as the quantification of MICA/MICB mRNA is not predictive of the amounts of the corresponding proteins expressed at the cell surface, due to the complex processes regulating these transcripts. We therefore developed a novel mAb directed against both MICA and MICB, named Mia4, which specifically stained the MICA and MICB proteins on formalin-fixed, paraffin-embedded tumor tissue sections (Figure 1a). Healthy tissues were barely stained with the anti-MICA/B Mia4 mAb, including organs with high levels of blood flow, exposure, and clearance: the skin, liver, lung and all vital organs, heart and spleen. Only limited expression was detected on the testis and ovary (Figure 1b). By contrast, tumor biopsy specimens from patients with head and neck squamous cell carcinoma (HNSCC), mesothelioma, ovarian, endometrial cancers, breast and melanoma were stained with the anti-MICA/B Mia4 mAb, revealing high levels of MICA/B expression in tumors (Figure 1c). Importantly, MICA/B expression was observed at the membrane of tumor cells, in addition to intracytoplasmic expression (Figure 1a and 1b). Indeed, 20 to 100% of biopsy specimens from patients with ovarian adenocarcinoma, breast cancer (estrogen receptor-positive (ER+) and -negative (ER-)), metastatic melanoma, head and neck (H&N), non-small cell lung cancer (NSCLC, squamous and adenocarcinoma), mesothelioma and urothelial cancer, scored with more than 50% of cells positive for MICA/B expression (Figure 1d). Breast cancer tumor biopsies were scored based on the percentage of tumor cells expressing MICA (Figure 1e). Important membranous MICA/MICB staining was observed in tumor biopsies at various scores (Figure 1f). These results confirm the identification of MICA/B as an interesting candidate tumor antigen.

MICAB1, a pan-allelic antibody, promotes in vitro antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP) and in vivo antitumor function

For the targeting of MICA and MICB in cancer patients, we generated anti-MICA and MICB antibodies, by immunizing mice with recombinant soluble human MICA-Fc recombinant protein or a range of human C1R cells negative for endogenous MICA and MICB expression and engineered to express MICA alleles (Table 1). MICAB1 was selected based on its binding capacity to the products of 28 different alleles of MICA with Luminex technology, indicating that its epitope binding site was not affected by MICA and MICB allele polymorphisms (Figure 2a).

We then evaluated the ability of MICAB1 with Fc optimization to promote peripheral blood mononuclear cell (PBMC)-mediated ADCC towards human C1R cells expressing the MICA*001 or MICB*002 allele. MICAB1 mediated strong ADCC by restim PBMCs (Figure 2b). Similarly, Fc-engineered MICAB1 induced primary human NK cell activation in cocultures of PBMCs with human C1R cells expressing different alleles MICA or MICB, as shown by the induction of the cell surface activation marker CD137, whereas Fc-silent MICAB1 did not (Figure 2c). Epidermal growth factor receptor (EGF-R), CD20 and MICA and MICB were expressed on the surface of A549 lung cancer cells and Raji B-cell lymphoma cells (Figure 2d, upper panels). MICAB1, targeting MICA and MICB, promoted an efficient tumor target cell lysis by PBMCs when compared to the anti-EGF-R mAb cetuximab and the anti-CD20 mAb rituximab, which are widely used in clinical practice (Figure 2d, lower panels). MICAB1 also promoted the phagocytosis of MICA-expressing C1R target cells by monocyte-derived macrophages in vitro (Figure 2e). Thus, MICAB1 efficiently promotes the ADCC and ADCP of MICA-expressing tumor target cells in vitro.

We then assessed the in vivo efficacy of MICAB1. We used a tumor model consisting of NOD-SCID mice bearing invasive MICA-expressing B-cell lymphomas, resulting from the intravenous (i.v.) injection of Raji-MICA*001 tumor cells (Figure 3a). These mice were simultaneously treated with a range of doses of MICAB1 Fc-engineered mAb. We found that MICAB1 Fc-engineered mAb treatment was effective over a range of doses, with 50% and 100% of mice surviving at doses of 0.5 and 5 mg mAb/kg of body weight, respectively, versus 30% of the mice treated with the control mAb at a dose of 5 mg/kg (Figure 3a). MICAB1 Fc-engineered mAb treatment also decreased the numbers of Raji-MICA*001 and MICA*008 tumor cells (exhibiting low and medium levels of surface MICA, respectively), recovered from the peritoneal cavity of NOD-SCID mice, relative to control mAb-treated mice (Figure 3b). In this model MICAB1 Fc-engineered mAb was more potent than commercial anti-MICA/B mAb BAMO3 at controlling tumor cells with low level of MICA (Figure 3b).

We then evaluated the therapeutic effect of MICAB1 mIgG2a mAb on tumor models based on subcutaneous (s.c.) injection of B16F10-MICA*001 in immunocompetent MICA-gen transgenic mice (Figure 3c and d). Beneficial effects of MICAB1 were moderate for the treatment of solid tumor models, compared to intravenous or intraperitoneal tumor models.

Generation and characterization of MICAB1 PBD1G-ADCs

We then aimed to improve the therapeutic effect of the MICAB1 mAb in solid tumors, using a MICAB1 Fc-silent antibody conjugated to potent cytotoxic payloads. We checked that MICA was internalized after binding to the MICAB1 mAb. The binding of the MICAB1 Fc-silent mAb coupled to a pH-sensitive dye on MICA-transfected Raji cells led to an increase in the intensity of the fluorescence signal during the first hour, consistent with rapid internalization (Figure 4a). For the payloads, we focused on the highly potent pyrrolobenzodiazepine family (PBD), to maximize the potential efficacy of MICAB1-ADCs against a broad range of tumors with various levels of MICA and MICB expression. The MICAB1 Fc-silent mAb was coupled to a first-generation PBD (PBD1G) with BTG technology, which has been reported to limit off-target toxicity, at a drug-antibody ratio (DAR) of 2, via a intracellular valine-alanine cleavable peptide linker (Figure 4b). In vivo, the MICAB1-ADCs had a strong effect in a xenogenic colon carcinoma model HCT-116, in nude mice, at doses as low as 0.05 mg/kg, administered once weekly for...
Figure 1. MICA and MICB expression are restricted to tumor tissues. (a) Two breast cancer slides were stained with Mia4 mAb developed by Innate Pharma or the isotype control (IC). MICA/MICB staining was positive only with Mia4 Ab compared to IC. Arrows indicate MICA/B-positive tumor-endothelium. Scale bars: 250 µm. (b–c) Representative immunohistochemical staining for MICA and MICB on 15 out of 32 tissues from three healthy donors (b) and on biopsy specimens from head and neck squamous cell carcinoma (HNSCC), mesothelioma, ovarian cancer, endometrial cancer, breast cancer and melanoma (c). Scale bars are indicated. (d) Percentage of patients with 50% of MICA/B-positive tumor cells on biopsy specimens from primary breast cancer estrogen receptor-positive (ER+) (n=20), breast cancer estrogen receptor-negative (ER-) (n=15), metastatic melanoma (n=72), HNSCC (n=82), squamous non-small-cell lung carcinoma (NSCLC) (n=24), mesothelioma (n=20), urothelial tumors (n=170), adenocarcinoma NSCLC (n=32), ovarian adenocarcinoma (n=73). (e–f) Different subtypes of breast cancer were scored based on proportion of MICA/MICB positive cells (score 0 (no MICA/MICB expression), score 1 (< 10%), score 2 (10% to 50%) score 3 (51 to 80%) and score 4 (>80%) tumor cells expressing MICA/MICB). (e) Patient proportions with different scores presented as histograms. (f) Two representative slides with scores 4 and 2. Strong cell surface MICA/MICB staining at different score. The black scale bars correspond to 50 and 100 µm.

We then tested the MICAB1-ADCs in patient-derived xenograft (PDX) models, which present the obvious advantage of originating from cancer patients, and hence may better reflect human tumor physiology and preserve the heterogeneity of the original tumors. We performed immunohistochemical staining (IHC) to assess MICA/B expression in a panel of 146 PDXs corresponding to different types of human cancer (breast, lung and colon cancer). The distribution of MICA expression in PDXs matched that observed in patients (Figure 1 and Table 2). MICAB1 PBD1G-ADCs had a curative effect in a PDX model of HER-2+ breast cancer, at doses as low as 0.05 mg/kg once weekly for eight weeks, with a complete remission rate of 100% on day 85 (Figure 4d). MICAB1 PBD1G-ADCs also increased the survival of C57BL/6 mice receiving injections of MICA-transfected B16F10 melanoma cells, and the cured mice developed long-term protective immunity when rechallenged with B16F10 MICA*001 (Figure 4e). Thus, MICAB1 PBD1G-ADCs were reactive against several xenogenic and syngeneic tumor models in vivo.

MICAB1 PBD2G ADCs inhibit tumor growth in vivo

The PBD1G-ADCs have raised safety issues in several clinical trials. We therefore coupled the MICAB1 mAb to a second-generation PBD (PBD2G), less toxic for the target cells and with a better therapeutic index.
The therapeutic effect of MICAB1 PBD2G-ADCs was then evaluated in vivo, in immunodeficient NOD-SCID mice into which H1703 lung carcinoma cells had been injected. MICAB1 PBD2G-ADC treatment was effective at a single low dose of 0.3 mg/kg, with one third of the mice displaying complete remission and a significant delay of apparent tumor growth, by ~40 to 60 days relative to the control mAb (Figure 5a). In a second xenogenic model, nude mice engrafted with HCT116 colon carcinoma were treated with MICAB1 PBD2G-ADCs at doses of 0.5, 1 and 2 mg/kg, which delayed apparent tumor growth, by ~20, 50 and 80 days, respectively, relative to the isotype control PBD2G conjugate (Figure 5b). We then assessed two breast cancer PDXs, HER-2<sup>high</sup> (HBCx-5) and HER-2<sup>low</sup> luminal (HBCx-34) with high and medium levels of MICA expression, respectively, in nude mice. Single injection of MICAB1 PDB2G-ADC at low dose of 0.5 mg/kg effectively controlled tumor growth in both breast cancer models with 100% of complete remission on day 85 (Figure 5c).

Although the transgenic MICAgen mouse represents a valuable model to study MICA in vivo<sup>34</sup>, IHC analyses of tissues from these mice revealed MICA expression in several organs, whereas no such expression was observed in single human organs (Table 3 and Figure 5d). Nevertheless, we established a cancer model in MICAgen mice by injecting a carcinoma, methylicholanthrene (MCA), which presents the advantage of reconstituting real carcinogenesis and the associated tumor microenvironment. Importantly, MCA-induced fibrosarcomas displayed cell-surface MICA expression (Figure 5e). The therapeutic effect of MICAB1 PBD2G-ADCs was then evaluated in mice bearing tumors induced by MCA injection (Figure 5f). MICAB1 PBD2G-ADC treatment displayed signs of activity even for a single dose of 0.5 mg/kg, relative to the control group treated with IC-PBD2G at the same dose. A single injection of 2 mg/kg MICAB1 PBD2G-ADC delayed tumor growth considerably by 20 days. Of note, no therapeutic effect was observed with the MICAB1 mAb in MCA-induced fibrosarcomas bearing MICAgen mice even at optimal doses of 10 mg/ml once weekly for three weeks (Figure 5g).

Altogether, these results showed that MICAB1-PBD2G-ADC are efficient to treat solid tumors with 100% of complete remission on day 85 in two PDXs treated mice and are well tolerated in MICAgen humanized mice.

**Discussion**

MICA and MICB are tightly regulated stress-induced proteins that trigger the immune system by binding to the activating receptor NKG2D on cytotoxic lymphocytes. Targeting the NKG2D-MICA/B axis is a very attractive immunotherapeutic approach. Conceptually different ways of targeting MICA are currently being investigated, including bispecific T-cell engagers B2-OKT3 (MICA, CD3), bispecific antibodies, such as JZC01 (MICA, VEGFR2)<sup>39</sup>, 2A9-MICA (MICA, BCMA)<sup>40</sup>, cytotoxic anti-α<sub>3</sub> domain-specific mAbs for inducing ADCC<sup>41</sup>,<sup>42</sup> and CAR-NK cells. Targeting MICA/B using an ADC approach is, to our knowledge, totally novel.

It was previously reported that MICA and MICB are predominantly expressed intracellularly in tumor and normal tissue using commercially available antibodies directed against MICA α1/2 or α3 domains<sup>36</sup>. In contrast, our results indicate that limited MICA expression was detected on healthy tissues in comparison with an important expression in tumors in large cohorts of patients, that includes both membranous and intracellular expression, as for other solid tumor antigens (Her-2, Nectin-4, TROP-2).

Using in vitro assays with fluorescent dye, we show here that MICAB1 induces a high level of MICA internalization, supporting the development of ADCs targeting MICA and MICB.
Figure 2. MICAB1, a pan-allelic antibody, mediates potent antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). (a) Luminex assay characterizing MICAB1 monoclonal antibody (mAb) binding to 28 different alleles of MICA. Results are presented as the median fluorescence intensity corrected for the background control. Representative data from two independent experiments are shown. (b) Peripheral blood mononuclear cells (PBMCs) from healthy donors were incubated with 51Cr-chromium-loaded C1R tumor cell line expressing MICA*001 or MICB*002 in the presence of a range of doses of MICAB1 Fc-engineered or isotype control mAb. After four hours, 51Cr chromium levels in the supernatants were determined. The data shown are representative of two independent experiments. Mean of percentage of lysis (n=3) and SD are shown. The percentage of lysis is significantly different between the MICAB1 Fc-engineered antibody and the isotype control for C1R MICA001 (p-value = 9.6e-08) and C1R MICB002 (p-value = 2.4e-09). The interaction between the antibody and its concentration is also significant in both experiments (p-values = 0.018 and 0.14, respectively). (c) C1R cells expressing different alleles of MICA were incubated with PBMCs from healthy donors and 10 µg/ml of MICAB1-Fc-engineered, MICAB1-Fc silent or isotype control Fc-engineered mAb for 24 hours. The data shown are the frequencies of natural killer (NK) cells expressing CD137 among total NK cells and are representative of three independent experiments (Friedman test p-value = 0.0007). Pairwise comparisons conclude that the percent of CD137+ NK cells obtained with MICAB1 Fc-engineered is significantly different from those obtained with all other antibodies (adjusted p-values =0.043). (d) PBMCs from healthy donors were incubated with 51Cr-chromium-loaded A549 cells naturally expressing MICA *001:12/*004 and MICB *002/*007 or Raji-MICA*001 in the presence of a range of doses of MICAB1 Fc-engineered or isotype control, cetuximab or rituximab. Upper panels: MFI of mAb binding at the cell surface (n=1) revealed with a secondary Ab. Lower panels: After four hours, 51Cr chromium levels were determined in the supernatants. The data shown are representative of two independent experiments. Mean of percentage of lysis (n=3) and SD are shown. Antibody, its concentration and the interaction between both have a significant effect on the percentage of lysis in A549 (p-value = 4.9e-05 for antibody and 0.00016 for interaction) and in Raji-MICA*001 (p-value = 9.7e-09 for antibody and 0.01 for interaction). Pairwise comparisons conclude to a significant difference between MICAB1 Fc-engineered and the isotype control (p-value=1.3e-05 in A549 and 1.7e-09 in Raji-MICA*001) or Rituximab (p-value = 0.03) but not with Cetuximab (p-value=0.14). (e) C1R cells expressing MICA*001 or MICA*008 were loaded with PKH67 and added to macrophages in the presence of a range of doses of MICAB1 Fc-engineered or isotype control mAb. The cells were incubated for two hours at 37°C. Macrophage intracellular fluorescence intensity is shown (in relative units) as a function of antibody concentration. Data of one representative donor are shown.

using intracellular cleavable linkers. We observed complete remission in most of the tumor models tested with the MICAB1-ADC approach. In addition, a strong vaccination effect was obtained with MICA-ADC in a syngeneic MICA-transfected melanoma model.

Our ADC technology includes an enzymatic conjugation method using BTG, facilitating the site-specific conjugation of the linker-toxin to the mAb, thereby making it possible to control the drug-antibody ratio. We coupled the MICAB1-Fc silent mAb to potent DNA binder payloads, to promote the killing of MICA/MICB-expressing tumor cells. ADCs carrying a new generation of PBDs are currently in clinical development. ADCT-402 (lincustaximab tesirine), an ADC against CD19 conjugated to a PBD-dimer toxin, has recently been approved for the treatment of diffuse large B-cell lymphoma and mantle cell lymphoma, on the basis of clinical results obtained in phase I and pivotal phase II clinical trials. As the therapeutic index is lower for these potent payloads, good targets must display tumor-restricted expression. In addition, our technology, based on site-specific conjugation with an Fc silent mAb, improves the therapeutic index of ADCs. The use of a genetically engineered mouse model expressing human MICA (MICAgen mice), in conjunction with carcinogen-induced tumors, further validates the presence of a therapeutic window for our ADCs, without major toxicity, even in the presence of low levels of MICA on normal tissues.

Like other cell surface tumor antigens (Her-2, Nectin-4, CD30), MICA is subject to proteolytic cleavage. The shedding of this protein generates soluble truncated MICA molecules including only the extracellular domain. Given the levels of soluble MICA and MICB found in the serum of cancer patients (~100 pg/mL), the concentrations of PBD2G-ADCs at doses currently used in clinical practice (~500 and 700 ng/mL), the efficacy of MICAB1 PBD2G-ADCs should not be affected by the presence of soluble MICA, but further investigations aiming at addressing this point will be needed.

Therefore, several approaches are proposed to harness anti-tumor immunity via the targeting of MICA/B. Our results are based on an array of in vivo models including MCA-induced fibrosarcoma in an immunocompetent ad hoc MICA-transgenic mouse model, and PDX. Importantly, efficacy of MICAB1-PBD2G at low dose was observed in two-breast cancer PDXs, including HBCx-34 which is resistant to another ADC targeting trastuzumab emtansine (KADCYLA), targeting the HER2 tumor antigen. Thus, MICAB1-PBD2G appears a promising therapeutic tool for further clinical development. The next steps for the clinical development of MICA ADCs will include the assessment of their safety profile in non-human primates.

Methods

Antibodies

Cat#: MICAB1, RRID:AB_2892111, Innate Pharma; Cat# MICAB1-mlgG2a, RRID:AB_2892224, Innate Pharma; Cat# MICAB1 Fc-silent RRID:AB_2892226; Innate Pharma; Cat# Mia4 mAb AB_2892227, Merk Serono; Cat# Erbitux, RRID: AB_2892606, ROCHE; Cat# Mabthera, AB_2892607 Innate Pharma; Cat# IC-mlgG2a RRID:AB_2892322, Innate Pharma; Cat# IC Fc-silent RRID:AB_2892233, Innate Pharma; Cat # Iso type control RRID:AB_2892234, Innate Pharma; Cat#MICAB1-PBD2G, AB_2892235, Innate Pharma; Cat#MICAB1-PBD1G, AB_2892236, Innate Pharma; Cat# IC-PBD2G AB_2892237, Innate Pharma; Cat#IC-PBD1G, AB_2892238, Innate Pharma; Cat#MICAB1-Cyp5, AB_2892239, Innate Pharma; Cat# IC-Cyp5 RRID:AB_2892241, Innate Pharma; Cat#MICAB1- APC, RRID:AB_2892242, Innate Pharma; Cat# IC-APC RRID:AB_2892243, BAMO monoclonal mouse anti-human alpha3 MICA/B antibody from Tubingen university.
**Figure 3.** MICAB1 Fc-engineered prevents tumor growth but shows moderate therapeutic effect. (a) Raji B-cell lymphoma cells expressing the MICA*001 allele were injected intravenously into NOD-SCID immunodeficient mice simultaneously treated once with a range of doses of MICAB1 (0.05, 0.5, 2.5 or 5 mg/kg, n=8 to 18, pool of two independent experiments) or isotype control monoclonal antibody (mAb) (2.5 or 5 mg/kg; n=17). Kaplan–Meier curves of mouse survival are shown. Pairwise comparison by Log-rank Mantel–Cox test was conducted between isotype control and MICAB1 0.05 mg/kg (p-value = ns), 0.5 mg/kg (p-value = 0.04), 2.5 mg/kg (p-value = 0.02) and 5 mg/kg (p-value = 0.009). There is a significant difference of survival between MICAB1 0.5 mg/kg and 5 mg/kg (p-value = 0.04). (b) Raji B-cell lymphoma cells expressing the MICA*001 or MICA*008 allele at low and medium levels were injected intraperitoneally into NOD-SCID immunodeficient mice simultaneously treated once with 0.5 mg/kg of body weight of MICAB1-Fc-engineered mAb or BAMO3 mAb or isotype control mAb. Tumor cell counts in peritoneal cavity lavage 24 hours later are shown. n=6-8 per group. Kruskal-Wallis test, p-value = 0.001. Tumor cell counts obtained with MICAB1 are significantly different from IC (p-value = 0.0017) but not from BAMO3 (p-value > 0.05). (c) B16-F10 MICA*001 were engrafted s.c. in MICAgen mice. Five days later, mice bearing tumors were randomized into two groups (n=10/group) and treated weekly for three weeks with 10 mg/kg of either MICAB1-mAbG2a or isotype control mAb. Tumor volumes per mouse over time are shown. The interaction antibody:time is significant (analysis of variance (ANOVA) on linear mixed-effects model (lmer) p-value = 0.0004), meaning that the tumor growth across time is different between antibodies. The pairwise difference of least squares mean for the factor antibody is statistically significant (p-value = 0.046). (d) Doubling time of tumor volumes from (c) are shown. Unpaired Wilcoxon test, p = 0.06.

### Cell lines

| Cell line   | Characteristics                        |
|-------------|----------------------------------------|
| A549        | Human lung cancer cells                |
| B16F10      | Mouse melanoma cells                   |
| C1R         | Human B lymphoblast                    |
| H1703       | Human lung carcinoma cells             |
| HBCx-34     | HER-2low luminal breast cancer patient-derived xenografts |
| HBCx-5      | HER-2high breast cancer patient-derived xenografts |
| HCT-116     | Human colon carcinoma cells            |
| MCA-induced tumors | Methylcholanthrene induced tumors   |
| Raji        | Human B-cell lymphoma cells            |

### Mice

Ethical approval for Innate Pharma study was obtained from CEEA-IPH (CNREEA registration code CEEA – 070). Project authorization using animals for scientific purposes was provided by the French Ministry of Higher Education, Research and Innovation under the number APAFIS#25600. Approval number for the use of animals at Innate Pharma used for scientific purposes is C 13 055 30. MI-mAbs Authorization (Apafis#6653- 2016072612069416 v6) was delivered by the French Minister of Enseignement supérieur, de la recherche et de l'innovation after approval by the Marseille Ethical Committee for Animal Experimentation (Comité National de Réflexion Ethique sur l’Expérimentation Animale no. 14). Mice were handled in accordance with national and European laws for laboratory animal welfare and experimentation (EEC Council Directive 2010/63/EU, September 2010). Xentech authorization to use animals in the CERFE facility was obtained by The Direction Départementale de la Protection des Populations, Ministère de l’Agriculture et de l’Alimentation, France “Direction of the Veterinarian Services, Ministry of Agriculture and Food, France” (agreement No. D-91-228-107). All PDXs experiments were performed in accordance with French legislation concerning the protection of laboratory animals and in accordance with a currently valid license for experiments on vertebrate animals, issued by the French Ministry of Higher Education, Research and Innovation (#14073).

C57BL/6J and nude mice were reared at Janvier Laboratories and Charles River laboratories. Transgenic mice with a 23.4 kb human genomic fragment (MICAgent mice), comprising the entire huMICA*007 allele and expressing MICA in a strictly controlled manner, as recently described

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**Cell line**

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- **HBCx-5**: HER-2high breast cancer patient-derived xenografts
- **HCT-116**: Human colon carcinoma cells
- **MCA-induced tumors**: Methylcholanthrene induced tumors
- **Raji**: Human B-cell lymphoma cells
**a** Raji wt

- Sytox Blue
- MICA1-Cyp5 at t=1H
- MICA1-Cyp5 at t=4H
- IC-Cyp5 at t=1H
- IC-Cyp5 at t=4H
- MICA1-Cyp5 overnight

**b**

PBD 1st Generation (SGD-1882) with val-ala linker

PBD 2nd Generation (SG-3199) with val-ala linker

**c**

Colon carcinoma (HCT116)

- Tumor volume (mm³)
- Days (post cell injection)
- IC-PBD1G 0.05 mg/kg x3
- MICA1-PBD1G 0.05 mg/kg x3

**d**

HER-2 Breast cancer PDX (HBCx-5)

- Tumor volume (mm³)
- Days post randomization
- IC-PBD1G 0.05 mg/kg x8
- MICA1-PBD1G 0.05 mg/kg x8

**e**

Melanoma (B16F10 MICA*001)

- Percentage survival
- Days (post cell injection)
- 0.1 mg/kg
- 0.25 mg/kg
- 0.25 mg/kg
- 0.5 mg/kg
- 0.5 mg/kg
- 0.1 mg/kg

- Treatment: Vehicle IC-PBD1G MICA1-PBD1G

- Mice rechallenged with B16F10 MICA*001 in one flank
- Cured mice rechallenged
- Naive mice with B16F10 MICA*001
- Cured mice not rechallenged
mice from Charles River Laboratories and used by Xentech for PDXs models. Mice were received in the SOPF experimental zone at least one week before the beginning of the experiments. All animal experiments were performed in accordance with the rules of the Innate Pharma ethics and animal welfare committees.

**Human primary cells**

Peripheral blood samples from healthy donors were obtained from the Etablissement Francais du Sang (EFS, Marseille) under a written consent obtained from each volunteer by the EFS (transfer agreement #AC-2019-3428). Tumors tissues from patients were obtained at the time of surgical resection under approved protocol and written informed consent from each patient. The protocol was approved by local ethics and human investigations committee (IDRCB #2017-A00778-45) and by the Assistance Publique des Hôpitaux de Marseille (AP-HM).

**IHC**

Formalin-fixed paraffin embedded blocks were sliced in 5 μm-thick sections and immunostainings performed on a Discovery Ultra or a Benchmark Ultra (Ventana). All samples except breast cancer tissues were stained on a Discovery Ultra automaton. After pre-treatment with cell conditioning 1, sections were incubated for 1 hour at 37°C with anti-MICA/B (clone Mia4, Innate Pharma) primary antibody or mouse IgG1 isotype control at 2 μg/mL (for staining on the Discovery Ultra) or at 6.6 μg/mL (for staining on the Benchmark Ultra). Then, signal amplification using the discovery Amp HQ kit (760-052) or the UltraView kit (760-500) was performed. After revelation with 3,3-diaminobenzidine, counterstaining with hematoxylin and bluing, sections were washed, dehydrated, cleared and coverslipped. Stained sections were finally scanned on a slide scanner (S60 Nanozoomer, Hamamatsu or a Pannoramic scan II, 3DHistech). Staining was interpreted and scored by trained pathologists that determined the MICA/B expression on the tumor cells. Samples with more than 1% of MICA/B positive tumor cell were considered MICA/B positive.

**Normal tissues used for IHC analysis**

Panel of multiple organs of normal tissue microarray (cat. n. FDA999k, BioMax Us) were stained with Mia4 as per the previously described IHC protocol. In total 32 types of normal human organs were analyzed including cerebrum,
Membranous staining on tubules. No staining on glomerulus.

Strong membranous and cytoplasmic staining in the spermatogonia in all the seminiferous ducts. Membranous and cytoplasmic staining in the epithelium of the bronchioles and on 5% of pneumocytes. Membranous and cytoplasmic staining in the mucosa.

Membranous staining on less than 1% of cells and background noise.

Weak membranous and cytoplasmic staining in the mucosa.

Membranous (and slightly cytoplasmic) staining on endometrial glands (epithelial cells). Staining also shown (n=13 or 14 mice per group). One experiment was performed. Interaction between antibody and time is significant (ANOVA on lmer p-value = 2.6e-14). Pairwise comparisons show significant differences between MICAB1-PBD2G and IC-PBD2G (p-value = 0.0006), shown (n=13 or 14 mice per group). One experiment was performed. Interaction between antibody and time < 0.05). (g) MICAgen mice were injected s.c. with MCA. Around 100 days later, mice bearing fibrosarcomas were randomized into five groups and treated once with vehicle or 0.5 or 2 mg/kg MICAB1 PBD2G-ADCs or IC-PBD2G or vehicle. Tumor volumes per mouse over time are shown (n=13 or 14 mice per group).)

Table 3. MICA and MICB protein expression in formalin-fixed, paraffin-embedded (FFPE) tissues from MICAgen mouse as determined by immunohistochemistry. This table summarizes MICA/MICB expression observed on epithelial cells in most of the MICAgen mice tissues using Mia4 mAb.

| IHC analysis of MICA expression on MICAgen mice | |
|-----------------------------------------------|-----------------------------------------------|
| Lymph node                                    | Membranous staining on less than 1% of cells and background noise. |
| Stomach                                       | Membranous and cytoplasmic staining in the mucosa. |
| Colon                                         | Weak membranous and cytoplasmic staining in the mucosa. |
| Prostate                                      | Membranous and cytoplasmic staining in the epithelium of the prostate glands. Membranous staining in 2% of the epithelial cells of the prostatic urethra. |
| Lung                                          | Membranous and cytoplasmic staining in the epithelium of the bronchioles and on 5% of pneumocytes. |
| Testis                                        | Strong membranous and cytoplasmic staining in the spermatogonia in all the seminiferous ducts. |
| Kidney                                        | Membranous staining on tubules. No staining on glomerulus. |
| Ovary                                         | Membranous and cytoplasmic staining on oocytes and in the stroma. |
| Fallopian tubes                               | Membranous and cytoplasmic staining on the epithelium of the fallopian tubes. |
| Uterus                                        | Membranous (and slightly cytoplasmic) staining on endometrial glands (epithelial cells). Staining also observed in the vascular endothelium. |
| Liver                                         | Membranous staining on hepatocytes and on the epithelium of some central veins. |

Membranous staining on cerebellum, adrenal gland, ovary, pancreas, parathyroid gland, hypophysis, testis, thyroid gland, breast, spleen, tonsil, thymus gland, bone marrow, lung, heart, esophagus, stomach, small intestine, colon, liver, salivary gland, kidney, prostate, uterus, uterine cervix, skeletal muscle, skin, peripheral nerve, mesothelium, eye and larynx, each type taken from three normal human individuals, single core per case.

Production and purification of antibodies
Antibodies were produced as previously described31.

The sequences encoding the light chain variable and heavy chain variable domains of the various antibodies were inserted into the SLX-192 expression vector in frame, it could be replaced with Gateway™ pDEST™26 Vector (Thermofisher...
Production of MICAB1 Fc-silent having N297S mutation
Antibodies were produced as previously described. Briefly, IPH43 Fc-silent was produced as chimeric antibodies bearing human IgG1 constant regions comprising an asparagine to serine substitution at Kabat heavy chain residue 297 (N297S), thereby eliminating native N297-linked glycosylation. The antibody comprised an acceptor glutamine at amino acid residue 295 (Kabat EU numbering) of their heavy chains, such that the reactive linker was conjugated to residue Q295 on each heavy chain of the antibodies (each antibody has two conjugated moieties; DAR=2), and lacking significant effector function.

Luminex assay
A LifeCodes LSA-MIC Luminex kit (Immucor, cat. n. 265300R) was used to determine the binding of MICAB1 and an isotypic control to the products of 28 different alleles of MICA, as recommended by the manufacturer and described elsewhere.

Binding of MICAB1 to cells expressing MICA
Cell staining was performed with the range of doses of MICAB1 or isotype control (IC) mAb indicated in the corresponding experiments, to monitor binding to MICA on cells. Various doses of antibody were incubated with the cells for 1 hour at 4°C. The cells were then washed and goat anti-human IgG (H+L)-PE secondary antibody (IM1626, BC) was added and the cells were incubated for a further 30 minutes at 4°C. The cells were washed and then acquired and analyzed on a FACS CANTO II (HTS) (BECTON DICKINSON (BD)) flow cytometer equipped with FACS Diva software, and analyzed with FlowJo X 10.0.7r2 software.

^51^Chromium release assay
Target cells were stained with 50 μCi of ^51^chromium (PERKIN ELMER, cat. no. NEZ030002MC) per million cells for 1 h at 37°C. 50 μL of antibody were loaded onto a 96-well plate, completed with 100 μL of ^51^chromium-loaded target cells and then 50 μL of NK or PBMC. The plates were then incubated for 4 h at 37°C, and 50 μL of supernatant was collected and transferred to a LumaPlate (Perkin Elmer, cat. no. 6006633). Plates were dried at 56°C and read with a TopCount (NXT™ Perkin Elmer apparatus. A control condition without antibody was used to assess basal target cell lysis by NK or PBMC. In addition, a control without antibody and without NK or PBMC was used to determined spontaneous ^51^chromium release from the target cells. A control with 2% Triton X-100 on target cells was used to determine the maximal level of ^51^chromium release from target cells. Experiments were performed in duplicate or triplicate, depending on the number of effector cells obtained.

ADCC assay
PBMCs were isolated from buffy coat samples (EFS: Etablissement Français du Sang, Marseille) by density gradient separation (Pancoll tube: PAN BIOTECH, cat. no. P04-60100). The PBMC preparation was then enriched in NK cells by negative selection with a human NK cell isolation kit and LS separation columns (MACS-Miltenyi, cat. no. 130-092-657) in accordance with the manufacturer’s instructions. PBMCs were used at and effectortarget (E:T) cell ratio of 100:1 or 200:1, whereas purified NK cells were used at an E:T ratio of 10:1. A range of antibody doses was added to the effector cells. ^51^Chromium-loaded target cells were added and classical chromium release assay performed.

ADCP assay
Monocytes were isolated with the human Kit CD14 microbeads (Miltenyi, cat. no. 130-050-201), resuspended in macrophage medium (complete medium: RPMI-1640 containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and 1x NEAA supplemented with 20 ng/mL M-CSF and 1x penicillin/streptomycin solution) and differentiated for seven to nine days, with medium renewal every two to three days. The day before the ADCP experiment, monocyte-derived macrophages were used to seed a flat-bottomed 96-well plate (100 000 macrophages/well) in complete RPMI medium, incubated overnight at 37°C and starved from FCS for 2 h in RPMI without additives. Antibodies were diluted and added to the plates containing macrophages. PKH67 (SIGMA, cat. no. PKH67GL-1KT)-labeled target cells were added to macrophages with an E:T ratio of 1:4 (400 000 target cells/well). The plate was centrifuged for 2 min at 300 x g and incubated for 2 h at 37°C. After incubation, the supernatants containing non-phagocytosed target cells in suspension were discarded, and trypan blue (diluted 1/2 in PBS, 50 μL/well) was added to the plate and incubated with the cells for 1 minute to quench extracellular fluorescence. The trypan blue was then removed and the plate was analyzed on an EnSpire® Multimode Plate Reader (Perkin Elmer) to quantify residual PKH67 fluorescence from cells subjected to phagocytosis, corresponding to macrophage intracellular fluorescence.
overnight at 37°C. Internalization was stopped by placing the plate on ice for 10 minutes. The cells were washed and resuspended in cold PBS containing the mortality marker Sytox blue (Thermo Fisher Scientific, cat. no. S34857) diluted 1/10000. Cells were acquired and analyzed on a FACS CANTO10 (BECTON DICKINSON (BD)) flow cytometer, with FACS Diva software.

Conjugation of MICAB1 to first- or second-generation (1G or 2G) pyrrolobenzodiazepines
Conjugations were performed as previously described\(^1\). PBD1G was purchased from Levena and PBD2G was provided by Spirogen. A two-step process based on use of click chemistry reactive groups was used in which a lysine-based linker with a reactive group is first bound to the acceptor glutamines of the antibodies, followed by reaction with a second compound that includes the PBD1G (purchased from Levena) or PBD2G (given from Spirogen) and a complementary reactive group. To obtain the intermediate antibody bound to a reactive linker, 5 mg/mL mutant mAb was incubated with 20 equivalents of a reactive lysine-based linker (NH2-PEG-N3) (Click chemistry tools, cat. n. AZ101) per site of coupling and 2 U/mL BTG (Zedira, cat. n. T153) overnight at 37°C in PBS. The mAb-reactive linker conjugate was purified by affinity chromatography on protA. In order to produce the final ADC comprising a pyrrolobenzodiazepine (PBD) dimer, the azide-functionalized antibody above (2 mg/mL in PBS/1,2-propanediol 50/50 v/v) was then incubated with 1.75 molar equivalent of DBCO-val-ala-PBD (DBCO-val-ala-PBD1G, purchased from Levena) or (DBCO-val-ala-PBD2G, given form SPIROGEN) or per site of coupling (the DBCO reacts with the azide). The mixture was incubated for 48-72h at RT with gentle agitation. Completion of the reaction was controlled by LC-ESI-MS (DAR >1.9). Excess of derivatized-PBD was removed by dialysis (MWCO=10 kDa), followed by purification by size exclusion chromatography (Superdex 200 10/300GL column, GEHealthcare). The final compounds were concentrated on Amicon 30K devices.

LC/MS analysis of ADCs
Entire antibody was analyzed for DAR determination as previously described\(^1\). ADC products were eluted on a PLRP-S polymeric reverse-phase column (2.1 x 50 mm, 5 μm, 4000 Å, Agilent) heated at 80°C, at a flow rate of 0.35 mL/min, using the following gradient: 0–1 min, 5% B; 1–4 min, 5–50% B; 4–5 min, 50% B; 5–6 min, 50–5% B; 6–7 min, 5% B (A, water + 0.1% formic acid; B, acetonitrile + 0.1% formic acid). Analytes were ionized by electrospray and detected by a micro-TOF QII mass spectrometer (Bruker) operating in positive TOF-MS mode. Raw data were analyzed with Data Analysis software (Bruker), and deconvolution was performed using MaxEnt1, which could be replaced with OA-Analysis Engine\(^5\).

In vivo experiments. Mice had specific and opportunistic pathogen free (SOPF) health status. Changes to litter were made every week for animals housed in a group. Animals housed in pairs were changed once every two weeks. The litter was composed of dusted poplar wood and the enrichment included a poplar brick as well as short cotton fibers. Mice were housed in ventilated cages with a maximum of five per cage of 500 cm\(^2\) on the ground. Animals were observed daily including weekends and holidays. Mice were housed in standardized temperature conditions (22°C ± 2°C) with relative humidity between 40 -75 % and had a 12-hour day and night daily lighting cycle. Both supply and exhaust air were HEPA-filtered and renewed 15-20 times per hour. Complete irradiated maintenance diet for rats, mice and hamsters and autoclaved tap water were available ad libitum in each cage. The acclimatization period before handling was a minimum of five days and the animals were handled only in dedicated rooms.

For the in vivo experiments in PDx models performed at Xentech, the animal care and housing were in accordance with French regulatory legislation concerning the protection of animals used for scientific purposes. Mice were housed in groups of a maximum of seven animals during the acclimation period and a maximum of six animals during the experimental phase. Mice were housed inside individually ventilated cages (IVC) of polysulfone (PSU) plastic (mm 213 W x 362 D x 185 H, Allentown, USA) with sterilized and dust-free bedding. Food and water were sterilized. Animals were housed under a light-dark cycle (14-hour circadian cycle of artificial light) and controlled room temperature and humidity. At request, the environmental conditions were monitored and the data were retained in the Central Animal House Archive. Drinking water was provided ad libitum. Each mouse was offered daily a complete pellet diet (150-SP-25, SAFÉ) throughout the study. The analytical certificate of animal food and water was retained at the CERFE premises. HBCx-5 and HBCx-34 breast tumor-bearing mice received estrogen diluted in drinking water. β-estradiol powder was suspended in 10% absolute ethanol and 90% distilled water to obtain the final concentration of 850 mg/L. This stock solution was added at 1/100 into the drinking water to obtain a final concentration of 8.5 mg/L. All animals were weighed before each experiment and identified by a unique pattern for ear punch numbering system. Each cage was identified by a paper tag indicating: cage number, mice strain and number, tumor code, date of experiment.

**In vivo MICAB1 efficacy in mouse tumor models**

**Disseminated Raji MICA*001 model.** 15x10\(^4\) Raji MICA*001 cells were injected intravenously (i.v.) into the tail vein in CB17 SCID females NOD (non-obese diabetic) SCID mice of 8-12 weeks old. The day of cell engraftment, different groups (n = 8–18 pool of two independent experiments) were treated with MICAB1-Fc-engineered Ab or IC. Mice were observed and weighed two to three times per week. All mice were used for this experiment without any exclusion. Mice were sacrificed by cervical dislocation when the weight loss was more than 20% or 15% (the day before weekend). End of protocol was at day 100. No adverse events were described. Group of animals for experiment 1: MICAB1 at 0.05 - 0.5 - 5 mg/kg (n=8) and IC at 5 mg/kg (n=8). Group of animals for experiment 2: MICAB1 at 0.5 – 2.5 mg/kg (n=10) and IC at 2.5 mg/kg (n=9).

**Disseminated Raji MICA*001 and Raji MICA*008 i.p. models.** 15x10\(^4\) Raji MICA*001 cells with low level of MICA expression or Raji MICA*008 with medium level of MICA expression were injected intraperitoneally (i.p.) into NOD-SCID
immunodeficient mice, which were then treated with single injection of i.v. 0.5 mg/kg MICAB1-Fc-engineered mAb or BAMO3 mAb or isotype control mAb (n= seven or eight mice per group). 24h after treatment, peritoneal cavity lavage (PCL) were performed to analyze cell number using microscope. The monitoring of mice weight was not performed. Six groups of animals: MICAB1 (one group for each tumor cell line), BAMO3 (one group for each tumor cell line), IC (one group for each tumor cell line) and eight mice per group were used. A total of 44 8-9 week-old NOD SCID female mice were used. All mice were used for this experiment without any exclusion. Stratified randomization according to the mice age was used. End of protocol was at day 1. No adverse events were described.

**Solid B16-F10 MICA*001 tumor model.** $3 \times 10^5$ B16F10-MICA*001 tumor cells mixed with Matrigel (Corning) (1: 1) were inoculated s.c. in MICAgen mice at 0 day. A total of 32 8-10-week-old MICAgen male or female mice were used. Stratified randomization was used when average tumor volume (TV) is $\sim116$ mm$^3$ at day 5. The day of randomization 20 mice with $25 \text{mm}^3 < TV < 116 \text{mm}^3$ were selected, 12 mice were excluded (four mice with $TV < 25 \text{mm}^3$, three mice $TV > 116 \text{mm}^3$, and five mice with malformed tumors). Two groups of mice (10 mice per group) were i.v. injected with $10 \text{mg/kg}$ of MICAB1-mIgG1a or IC-mIgG2a once a week for three weeks. Mice were monitored once to twice a week, the measured parameters including, TV and general health conditions. Tumor size was monitored with a digital caliper (Mitutoyo) and TVs were calculated as follows: $TV = (\text{length} \times \text{width} \times 3.14/6)$. Body weight was not monitored for this experiment. Mice were sacrificed by cervical dislocation when tumors reach $1800 \text{mm}^3$ or when necrosis of tumors. End of protocol was at day 28 and end of experiment at day 35. No adverse events were observed.

**In vivo MICAB1 ADC efficacy in mouse tumor models ADC-PBD1G efficacy**

**Xenogeneic model (HCT116)**

A total of 32 10-week-old NUDE NMRI female mice were used. $2 \times 10^6$ HCT-116 were mixed with Matrigel (BD Biosciences) and s.c. inoculated in the right flank of mice. After nine days, stratified randomization was used when average TV is $\sim 106 \text{mm}^3$. 12 mice were excluded ($n = 10 \text{TV} < 81 \text{mm}^3$, $n = 10 \text{TV} > 136 \text{mm}^3$, $n = 1$ malformed tumors) and 20 mice were selected ($81 \text{mm}^3 < TV < 136 \text{mm}^3$). Mice were randomized to obtain two homogeneous groups (n=10 mice per group) with a similar distribution of tumor sizes. Mice were treated with i.p. injection of MICAB1-PBD1G or IC-PBD1G at 0.05 mg/kg, once a week for three weeks. Injections were performed on anesthetized mice that were previously maintained for few minutes in a chamber containing 3.5% isoflurane. Mice were monitored once to two times a week, the measured parameters including TV and general health conditions. Tumor size was monitored with a digital caliper (Mitutoyo) and TV were calculated as above. No adverse events were described. Mice were sacrificed by cervical dislocation when tumors reach $2000 \text{mm}^3$ or when necrosis of tumors was observed. Weight monitoring was not performed for this experiment. End of protocol was at day 44.

**PDX model (HBCx-5)**

The anti-tumor activity of MICAB1-va-PBD1G was determined in (HBCx-5). A total of 46 female SHO mice (Crl:SHO-Prkdcscid Hhr) aged 6–9 weeks old were used. The protocol has been previously described\(^b\). Briefly, 20 mm$^3$ of tumor fragment from (tumors from donor mice, passage), was placed in the interscapular region, SC. Breast tumor-bearing mice received estrogen diluted in drinking water ($\beta$-estradiol, 8.5 mg/L), from the date of tumor implant to the date of inclusion (i.e. D0). Stratified randomization was used when average TV was $\sim130 \text{mm}^3$ at day 40. 20 mice were allocated, according to their TV, to give homogenous mean and median TV ($60 \text{mm}^3 < TV < 200 \text{mm}^3$) in each treatment arm and 26 mice were excluded with TV less than $60 \text{mm}^3$ or more than $200 \text{mm}^3$. Inclusion rate was 43%. Treatments were initiated 40 days post implantation of the tumor. A total of two groups were (10 mice per group) treated with IC-PBD1G (0.05 mg/kg qwk x 8 i.p.) or MICAB1-PBD1G (0.05 mg/kg qwk x 8 i.p.). All treatment doses were adjusted for body weight at time of dosing. In each experimental group, the mentioned dose was applied for all mice. Injected volume was around 100 μL. MICAB1-PBD1G or IC-PBD1G were i.p. administered on the enrolment day (i.e. D0). TV was evaluated by measuring perpendicular tumor diameters, with a caliper, twice a week during the whole experimental period. All animals were weighed at the same time as tumor size measurement. Relative body weight (RBW) loss was considered as an adverse effect of the treatment. Mice were observed every day for physical appearance, behavior and clinical changes. Mice were sacrificed by cervical dislocation when tumors reached $2000 \text{mm}^3$. Mice were sacrificed when necrosis of tumors was observed. No weight loss was reported. No adverse events were observed. The end of protocol was at 84 days.

**Syngeneic models (B16F10 MICA*001)**

A total of 90 eight-week-old C57BL6NRj female mice were used. $5 \times 10^5$ B16F10 MICA*001 was mixed with Matrigel (1:1) (BD Biosciences) and s.c. inoculated in the right flank of mice. Stratified randomization was used when average TV was $\sim150 \text{mm}^3$ at day 4. The day of randomization, 70 mice were selected to obtain seven homogeneous groups with similar tumor size distribution ($80 \text{mm}^3 < TV < 188 \text{mm}^3$) (10 mice per group) and 20 mice were excluded (four mice with $TV < 80 \text{mm}^3$ and 16 mice with $TV > 188 \text{mm}^3$). Mice were treated at day 4 with MICAB1-PBD1G or IC-PBD1G at doses of 0.1, 0.25, 0.5 mg/kg. 13 mice with complete tumor regression (from MICAB1-PBD1G treated groups) in addition to 10 new (naïve) C57Bl/6 females mice were used in three groups. Sample size was decided according to number of available mice in complete remission. Two groups with nine mice in complete remission and 10 naïve mice were re-challenged with s.c injection of $5 \times 10^5$ B16F10MICA*001 tumor cells. Four mice in complete remission were not re-challenged and were used as tumor-free mice controls. Mice were monitored two to three times a week, the measured parameters including mouse weight, TV and general health conditions. Tumor size was monitored with a digital caliper (Mitutoyo) and TV were calculated as above. All mice were fine, without weight loss. Mice were euthanized by cervical dislocation when TV reached
2000 mm$^3$ or tumor necrosis was observed. No adverse events were observed. The end of protocol was at 82 days.

**ADC-PBD2G efficacy**

**Xenogeneic model (H1703)**

A total of 33 eight-week-old NUDE NMRI female mice, were used. 7×10$^6$ H1703 was mixed with Matrigel (1:1) (BD Biosciences) and s.c. inoculated in the right flank of mice. After nine days, stratified randomization was used when average TV was ~91 mm$^3$.

The day of randomization, 27 mice was selected (55 mm$^3$<TV<141 mm$^3$) and six mice were excluded (four mice with TV<55 mm$^3$ and two mice with TV>141 mm$^3$). Mice were randomized to obtain three homogeneous groups (n=9 mice per group) with a similar distribution of tumor sizes. Mice were treated with single i.v. injection of MICAB2-PBD1G or IC-PBD2G at 0.3 mg/kg or vehicle. Injections were performed on anesthetized mice that were previously maintained for few minutes in a chamber containing 3.5% isoflurane. Mice were monitored once to two times a week, the measured parameters including TV, body weight and general health condition. Tumor size was monitored with a digital caliper (Mitutoyo) and TVs were calculated as above. No adverse events were described. Mice were sacrificed by cervical dislocation when tumors reached 1500 mm$^3$-3000 mm$^3$ or when necrosis of tumors was observed. No body weight loss was described for this experiment. The end of protocol was at day 131.

**Xenogeneic model (HCT116)**

A total of 70 eight-week-old NUDE NMRI female mice were used. 2×10$^6$ HCT116 was mixed with Matrigel (1:1) (BD Biosciences) and s.c. inoculated in the right flank of mice. After five days, stratified randomization was used when average TV was ~106 mm$^3$. The day of randomization, 63 mice were selected (55 mm$^3$<TV<171 mm$^3$). Seven mice were excluded (TV<55 mm$^3$). Mice were randomized to obtain seven homogeneous groups (n=9 mice per group, seven groups) with a similar distribution of tumor sizes. Mice were treated with single i.v. injection of MICAB2-PBD1G (at three indicated dose) or IC-PBD2G (at three indicated dose) or vehicle. Doses were 0.5 or 1 or 2 mg/kg. Injections were performed on anesthetized mice that were previously maintained for few minutes in a chamber containing 3.5% isoflurane. Mice were monitored once to two times a week, the measured parameters including TV, body weight and general health conditions. Tumor size was monitored with a digital caliper (Mitutoyo) and TVs were calculated as above. No adverse events were described excepted for one mouse from the MICAB1-PBD2G group. This mouse was sacrificed at day 47 because of oedema. Mice were sacrificed by cervical dislocation when tumors reached 1500 mm$^3$-2000 mm$^3$ or when necrosis of tumors was observed. No body weight loss was described for this experiment. The end of protocol was at day 83.

**PDXs models (HBCx-5 and HBCx-34)**

The anti-tumor activity of MICAB1-va-PBD2G was determined in the Her2+ breast cancer PDX model (HBCx-5) and ER+PR+HER2-breast cancer PDX model (HBCx-34).

**HBCx-5 model**

A total of 49 female nude mice aged 6-9 weeks old were used. The protocol has been previously described$^{36}$. Briefly, 20 mm$^3$ of tumor fragment from (tumors from donor mice, passage (n-1)) was placed in the interscapular region, SC. Breast tumor-bearing mice received estrogen diluted in drinking water (β-estradiol, 8.5mg/L), from the date of tumor implant to the end of the experiment. Stratified randomization was used when average TV was ~140 mm$^3$ at day 40. 20 mice were allocated according to their TV to give homogenous mean and median TV (75 mm$^3$<TV<220 mm$^3$) in each treatment arm and 29 mice were excluded with TV less than 75 mm$^3$ or more than 220 mm$^3$. Inclusion rate was 41%. Treatments were initiated 40 days post implantation of the tumor. Two groups (10 mice per group) were treated with a single i.v. injection of MICAB1-PBD2G (0.5 mg/kg) or vehicle. All treatment doses were adjusted for body weight at time of dosing. In each experimental group, the mentioned dose was applied for all mice. Injected volume was around 100 μL. MICAB1-PBD2G or vehicle were i.v. administered on the enrolment day (i.e. D0). TV was evaluated by measuring perpendicular tumor diameters, with a caliper, twice a week during the whole experimental period. All animals were weighed at the same time as tumor size measurement. RBW loss was considered as an adverse effect of the treatment. Mice were observed every day for physical appearance, behavior and clinical changes. Mice were sacrificed by cervical dislocation when tumors reached 2000 mm$^3$ or when necrosis of tumors was observed. No weight loss was reported. No adverse events were observed. The end of protocol was at 79 days.

**HBCx-34 model**

A total of 47 female nude mice (Hsd:Athymic Nude-Foxn1nu) aged 6-9 weeks old were used. The protocol has been previously described$^{36}$. Briefly, 20 mm$^3$ of tumor fragment from (tumors from donor mice, passage (n-1)) was placed in the interscapular region, SC. Breast tumor-bearing mice received estrogen diluted in drinking water (β-estradiol, 8.5mg/L), from the date of tumor implant to the date of inclusion (i.e. D0). Stratified randomization was used when average TV was ~140 mm$^3$ at day 38. 19 mice were allocated according to their TV to give homogenous mean and median TV (108 mm$^3$<TV<288 mm$^3$) in each treatment arm. 28 mice were excluded with TV less than 108 mm$^3$ or more than 288 mm$^3$. Inclusion rate was 41%. Treatments were initiated 38 days post implantation of the tumor. Mice were treated with i.v. single injection of MICAB1-PBD2G (0.5 mg/kg, 10 mice per group) or vehicle (nine mice per group). All treatment doses were adjusted for body weight at time of dosing. In each experimental group, the mentioned dose was applied for all mice. Injected volume was around 100 μL. MICAB1-PBD2G or vehicle were i.v. administered on the enrolment day (i.e. D0). TV was evaluated by measuring perpendicular tumor diameters, with a caliper, twice a week during the whole experimental period. All animals were weighed at the same time as tumor size measurement. RBW loss was considered an adverse effect of the treatment. Mice were observed every day for physical appearance, behavior and clinical changes. Mice were sacrificed by cervical dislocation when tumors reached 2000 mm$^3$ or when necrosis of tumors was observed. No weight loss was reported. No adverse events were observed. The end of protocol was at 79 days.
of tumors was observed. No weight loss was reported. No adverse events were observed. The end of protocol was at day 81.

MCA induced tumor models

**Efficacy of MICAB1-PBD2G on 3-MCA model**

A total of 90 7-14-week-old, transgenic male mice expressing human MICA*007 allele (MICAgan mice) were induced with 100 μg 3-MCA injected s.c. Mice were randomized into five groups (n=15 per group) when tumors reached ~150 mm³. 75 mice were selected (105 mm³ < TV < 288 mm³) and 15 mice were excluded with TV less than 105 mm³. Stratified randomization was used when average TV was ~150 mm³ to obtain homogeneous groups with similar tumor size distribution. Around 80-100 days after MCA treatment, mice were injected i.v. with MICAB1-PBD2G or IC-PBD2G at 0.5 mg/kg or 2 mg/kg or the vehicle (30 mM histidine, 200 mM sorbitol and 0.02% Tween 20 in PBS1X, pH=6) once on the day of randomization. Treatments were not blinded. Mice were observed and TV were evaluated by measuring perpendicular tumor diameters, with a caliper, one to twice a week during the whole experimental period. Body weight was not monitored for this experiment. Mice were sacrificed by cervical dislocation when tumors reached 1400 mm³ or when necrosis of tumors was observed. No adverse effects were observed. The end of protocol was at day 70 after first treatment.

**Comparison between MICAB1-PBD2G and MICAB1-mIgG2a**

The experimental design was the same as previously described. A total of 76 mice were used. Stratified randomization was used when average TV reached 120 mm³ to obtain homogeneous TV distribution. 52 mice were selected (120 mm³ < TV < 300 mm³) and 24 mice without tumor growth were excluded. Mice were randomized into four groups (n=13 per group), and injected i.v. once with MICAB1-PBD2G or IC-PBD2G at 2 mg/kg at 10mg/kg or three times with MICAB1-mIgG2a or IC-mIgG2a at 10 mg/kg per week. The first injection was on the day of randomization. Treatments were not blinded. Mice were observed and TV was evaluated by measuring perpendicular tumor diameters with a caliper and body weight was monitored once to twice a week during the whole experimental period. No weight loss was reported. No adverse effects were observed. Mice were sacrificed by cervical dislocation when tumors reached 1400 mm³ or when necrosis of tumors was observed. The end of protocol was at day 60 after first treatment.

Quantification and statistical analysis

Details on statistics used can be found in figure legends. All statistical analyses were performed using R version 4.0.3. The effects of the antibody and its concentration on the percentage of lysis, the MedFI and the phagocytosis are evaluated with a two-ways Anova : x~Antibody + Concentration+Antibody:Concentration

Data are shown with means and error bars showing the SD or median and range. Data from one representative experiment tested in simplicate are shown without SD or range.

Tumor growth models are analyzed with a linear mixed-effects model (lmer):

Volume ~ Antibody + Time + Antibody:Time + (1|Mouse.ID). Antibody, time and the interaction are fixed effects. Mouse.ID is the random effect. ANOVA was performed on this model to evaluate the significance of each fixed effect.

Pairwise differences of least squares mean for the factor antibody were computed. Confidence intervals and p-values were based on the t-distribution using degrees of freedom based on Kenward-Roger methods. The difference of percent of CD137+ NK cells between MICAB Fc-engineered and Isotype or Fc silent was evaluated by the Friedman rank sum test. The difference of cell counts between antibodies in the two Raji models (Raji *01, Raji*08) was evaluated by the Kruskal-Wallis rank sum test and pairwise comparisons are estimated by Wilcoxon rank sum exact tests. Kaplan Meier curves were used for survival representations and log-rank tests were applied to evaluate the difference of survival. Significance was assumed with *p < 0.05; **p ≤ 0.01; ***p < 0.001, ****p < 0.0001.

**Abreviations**

| Abreviations | Description |
|--------------|-------------|
| 2A9-MICA | Bispecific antibody consists of human MICA extracellular region and a single-chain antibody fragment (scFv) that targets BCMA |
| Ab | Antibody |
| ADC | Antibody-drug conjugates |
| ADCC | Antibody dependent cellular cytotoxicity |
| ADCP | Antibody-dependent cellular phagocytosis |
| B2-OKT3 | Bispecific T-cell engagers created in the tandem single-chain variable fragment BiTE format that targets MICA on tumor cells and CD3ε on human T cells |
| BCMA | B-cell maturation antigen |
| BiTE | Bispecific T cell engager |
| BTG | Bacterial transglutaminase |
| CAR-NK | Chimeric antigen receptor on natural killer cells |
Data availability

Underlying data

Figshare: Targeting MICA/B with cytotoxic therapeutic antibodies leads to tumor control. [https://doi.org/10.6084/m9.figshare.c.5549109.v1](https://doi.org/10.6084/m9.figshare.c.5549109.v1).

This project contains the following underlying data:

- **Fig. 1a** (NDPI files containing MICA/MICB specific positive staining with Mia4 Ab compared to IC in two breast cancer slides)
- **Fig. 1b** (NDPI files containing MICA/MICB staining on tissues slides from healthy donors)
- **Fig. 1c** (NDPI and mrxs files containing MICA/MICB staining on tumor slides from head and neck squamous cell carcinoma (HNSCC), mesothelioma, ovarian cancer, endometrial cancer, breast cancer and melanoma)
- **Fig. 1d** (XLSX file containing number of patients in different indication scored based on proportion of MICA/B positive cells)
- **Fig. 1e** (XLSX file containing number of patients with different subtypes of breast cancer scored based on proportion of MICA/MICB positive cells)
- **Fig. 1f** (MRXS files containing membranous MICA/MICB positive staining on two breast cancer slides at different scores)
- **Fig. 2a** (XLSX file containing BCM (background corrected mean fluorescence intensity) of MICAB1 at 10 μg/ml with different allele of MICA/B)
This project contains the following underlying data:

- XLSX file containing number of patients with different subtypes of breast cancer scored based on proportion of MICA/MICB positive cells

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgments

We would like to acknowledge Astra-Zeneca and Spirogen for providing toxins used in this study. These toxins were provided under a materials transfer agreement. We would like to acknowledge Tubingen University for BAMO-3 antibody.
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Open Peer Review

Current Peer Review Status:  

Version 2

Reviewer Report 15 November 2021
https://doi.org/10.21956/openreseurope.15342.r27925

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Jacques Zimmer
Department of Infection and Immunity, Luxembourg Institute of Health (LIH), Esch-sur-Alzette, Luxembourg

The authors satisfactorily addressed all concerns, and so I have no further comments to make.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: natural killer cells; major histocompatibility complex

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 October 2021
https://doi.org/10.21956/openreseurope.14384.r27610

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Roberta Castriconi
1 Department of Experimental Medicine (DIMES), University of Genova, Genoa, Italy
2 Department of Experimental Medicine (DIMES), University of Genova, Genoa, Italy

The authors have addressed a crucial and challenging issue in the context of mAb-mediated therapies against tumors, and represented by a general low efficacy of these approaches in solid
tumors. The study proposes a novel therapeutic strategy based on the targeting of MICA and MICB molecules with mAb conjugated with a second generation of pyrrolobenzodiazepine less toxic for the target cells (MICAB1-PBD2G). The study, based on an array of in vivo models of solid tumors, including MCA-induced fibrosarcoma in an immunocompetent MICA-transgenic mouse model, and PDX, shows that MICAB1-PBD2G is a very effective approach. All the experiments have been well conducted and well described with an appropriate statistical analysis. All the sections of the manuscript are well written and complete. Overall, the study represents an important step forward in optimizing the therapies of solid tumors.

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and does the work have academic merit?  
Yes

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Yes

Are all the source data underlying the results available to ensure full reproducibility?  
Yes

Are the conclusions drawn adequately supported by the results?  
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: NK cells, neuroblastoma, immunotherapies

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 27 September 2021

https://doi.org/10.21956/openreseurope.14384.r27601

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Jacques Zimmer

1 Department of Infection and Immunity, Luxembourg Institute of Health (LIH), Esch-sur-Alzette, Luxembourg
2
Department of Infection and Immunity, Luxembourg Institute of Health (LIH), Esch-sur-Alzette, Luxembourg

In this manuscript, Bléry et al. report on the generation and evaluation of MICA/B-targeting antibodies that are tested in vitro and in vivo in a large panel of cancer models. Overall, the efficiency of the antibody MICAB1 was not sufficient in vivo against a solid tumor (melanoma), and this was the reason why antibody-drug conjugates (ADC) were developed and used in cancer models, among them patient-derived xenografts and carcinogen-induced tumors. The ADC lead to tumor control in the various configurations tested.

The article is sound, well performed and timely, as the targeting of some of the ligands of the NK and T cell activating receptor NKG2D, namely MICA/MICB, have not yet been tested with the ADC method but could represent, as this work shows, an interesting addition to the immunotherapeutic arsenal against cancer, and particularly solid tumors.

Although this manuscript is globally very relevant and interesting, some items could be clarified or a bit modified:

1. The Introduction is very short, especially in the last paragraph, where some background information about the toxin PBD, the associated technology and the mechanism of action could be added.

2. There are many abbreviations throughout the article that are not all explained at first appearance; adding a table with all of them listed and explained (for example, ADC: antibody-drug conjugate, etc) would help.

3. Likewise, a table of the cell lines used (there are a lot) with their name, the species of origin, where they come from and what is their histology (for example: melanoma) and another table of the mouse models used would help.

4. The Mia4 antibody clearly distinguishes normal from cancerous tissues with a bright positive staining of the latter. Would it be possible to demonstrate the specificity of this antibody for MICA/MICB by including a reference antibody for comparison, such as the clone 6D4 or another one (for the IHC and/or for flow cytometry by staining MICA/MICB-transfected C1R cells with both antibodies)?

5. What was the rationale for the choice of C1R cells for the transfection experiments? The absence of endogenous MICA/MICB, the low expression of HLA class I, other?

6. When the MICAB1-Fc engineered antibody was used, was the isotype control in all cases also Fc-engineered? This is stated in one figure legend but not everywhere in the text.

7. Could the authors indicate one or two reference(s) for the use of CD137 as a NK cell-activation marker?

8. In figure 2b, ADCC induced by MICAB1-Fc engineered was compared with the isotype control. Was the "basic" level of NK cell cytotoxic activity (natural killing) evaluated in parallel?
In figure 2c, in the C1R-neo condition, 25% of the cells are CD137+, although there is no ligand for the antibody on this target cell line (in principle).

10. The *in vitro* experiments were often done only twice.

11. Figure 4: the color code in the right part of figure 4e is not optimal.

Minor points
1. There are some typos throughout the manuscript or sentences whose structure could be slightly modified.

2. Adverse events in the mouse experiments: was there a precise list that was checked? Body weight surveillance is considered frequently as a standard in invasive mouse experiments.

3. What were the "general health conditions" controlled. Here, a table might be more precise.

4. On page 20, what is the vehicle for the control groups of the *in vivo* models? The same than in the MCA model?

5. In the legend to figure 2, is the value $p=0.14$ considered as significant, or is this a typo?

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and does the work have academic merit?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** natural killer cells; major histocompatibility complex

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
Emilie Narni-Mancinelli

Minor points

1. There are some typos throughout the manuscript or sentences whose structure could be slightly modified.

2. Adverse events in the mouse experiments: was there a precise list that was checked? Body weight surveillance is considered frequently as a standard in invasive mouse experiments.

3. What were the "general health conditions" controlled. Here, a table might be more precise.

   **Author Response:** Limitations related to weight loss, appearance and behavior of the animals (prostration, prickly hair, isolation, breathing difficulties (dyspnea), paralysis, etc.) were the criteria used to monitor the general health conditions.

4. On page 20, what is the vehicle for the control groups of the in vivo models? The same than in the MCA model?

   **Author Response:** Yes the vehicle was composed of 30 mM histidine, 200 mM sorbitol and 0.02% Tween 20 in PBS1X, pH=6.

5. In the legend to figure 2, is the value p=0.14 considered as significant, or is this a typo?

   **Author Response:** No p=0.14 is not significant as mentioned in the legend « Pairwise comparisons conclude to a significant difference between MICAB1 Fc-engineered and the isotype control (p value=1.3e-05 in A549 and 1.7e-09 in Raji-MICA*001) or Rituximab (p-value = 0.03) but not with Cetuximab (p-value=0.14))

Major points:

1. The Introduction is very short, especially in the last paragraph, where some background information about the toxin PBD, the associated technology and the mechanism of action could be added.

   **Author Response:** The introduction has been revised accordingly.

2. There are many abbreviations throughout the article that are not all explained at first appearance; adding a table with all of them listed and explained (for example, ADC: antibody-drug conjugate, etc) would help.

   **Author Response:** A table has been added in the revised manuscript as follow:

   | Abbreviation | Definition |
   |--------------|------------|
   | 2A9-MICA Bispecific antibody | consists of human MICA extracellular region and a single-chain antibody fragment (scFv) that targets BCMA Ab Antibody ADC Antibody-drug conjugates ADCC Antibody dependent cellular cytotoxicity ADCP Antibody-dependent cellular phagocytosis B2-OKT3 Bispecific T-cell engagers created in the tandem single-chain variable fragment BiTE format that targets MICA on tumor cells and CD3ε on human T cells BCMA B-cell maturation antigen BiTE Bispecific T cell engager BTG Bacterial transglutaminase CAR-NK Chimeric antigen receptor on natural killer cells E2F E2 Factor protein EGF-R Epidermal growth factor receptor ER Estrogen receptor H&N Head and neck HBCx-34 Human breast cancer xenografts number 34 HBCx-5 Human breast cancer xenografts number 5 HER2 Human epidermal growth factor 2 HNSCC Head Neck squamous cell carcinoma IC Isotype control IHC Immunohistochemical staining JZC01 Bispecific antibody consisting of MICA and an
anti-VEGFR2 single chain antibody fragment mAb Monoclonal antibody MCA
Methylcholanthrene mg/kg Milligram per kilogram of mice MHC Major
histocompatibility complex MICA*001 or MICB*002 MHC class I chain-related protein
A allele 001 or B allele 002 MICA and MICB or MICA/B MHC class I chain-related protein
A and B MICagen MICA transgenic mouse NOD-SCID Non Obese Diabetic
Severe Combined ImmunoDeficiency OKT3 CD3ε binding single-chain variable
fragment (scFv) p53 Tumor protein 53 PBD Pyrrolobenzodiazepine PBD1G, SGD-1882
First-generation PBD PBD2G, SG-3249 Second-generation PBD PBMC Peripheral
blood mononuclear cell PDX Patient-derived xenografts TNBC Triple negative breast
cancer Trop-2 Tumor-associated calcium signal transducer 2 VEGFR2 Vascular
endothelial growth factor receptor 2

3. Likewise, a table of the cell lines used (there are a lot) with their name, the species of
origin, where they come from and what is their histology (for example: melanoma) and
another table of the mouse models used would help.

Author Response: A table has been added in the revised manuscript as follow:

| Cell line Characteristics | A549 Human lung cancer cells | B16F10 Mouse melanoma cells | C1R Human B lymphoblast | H1703 Human lung carcinoma cells | HBCx-34 HER-2low luminal breast cancer patient-derived xenografts | HBCx-5 HER-2high breast cancer patient-derived xenografts | HCT-116 Human colon carcinoma cells | MCA-induced tumors | Methylcholanthrene induced tumors | Raji Human B-cell lymphoma cells |
|---------------------------|-----------------------------|-----------------------------|------------------------|-------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|---------------------------------|---------------------------|---------------------------------|---------------------------------|

4. The Mia4 antibody clearly distinguishes normal from cancerous tissues with a bright
positive staining of the latter. Would it be possible to demonstrate the specificity of this
antibody for MICA/MICB by including a reference antibody for comparison, such as the
clone 6D4 or another one (for the IHC and/or for flow cytometry by staining MICA/MICB-
transfected C1R cells with both antibodies)?

Author Response: IHC was performed on RAJI (negative control) and C1R MICA*001
FFPE cell lines with the commercial clone BAMO1 or our proprietary clone (Mia4).
Both clones strongly stain C1R MICA*001 cells and not Raji cells. It is important to
note that we were not able to detect endogenous MICA/B expression on cell lines or
cancer tissues with the commercial clone BAMO1, highlighting the poor sensitivity of
this antibody for IHC application. To our knowledge, there is no other commercially
available anti-MICA/B for IHC application on FFPE samples.
What was the rationale for the choice of C1R cells for the transfection experiments? The absence of endogenous MICA/MICB, the low expression of HLA class I, other?

Author Response: C1R cells were selected based on both the absence of HLA class I and no detectable cell surface MICA/MICB. (STORKUS, W. et al. PNAS 1989, Groh, V. et al. Science 1998, Steinle, A. et al. Immunogenetics 2001)

When the MICAB1-Fc engineered antibody was used, was the isotype control in all cases also Fc-engineered? This is stated in one figure legend but not everywhere in the text.

Author Response: We confirm that when the MICAB1-Fc engineered antibody was used, isotype control was also Fc engineered in all cases as indicated in the Methods of the revised manuscript.

Could the authors indicate one or two reference(s) for the use of CD137 as a NK cell-activation marker?

Author Response: Raghvendra M Cancer Therapy (2017) (DOI: 10.1158/1078-
8. In figure 2b, ADCC induced by MICAB1-Fc engineered was compared with the isotype control. Was the "basic" level of NK cell cytotoxic activity (natural killing) evaluated in parallel?

**Author Response:** Basic level of NK cell cytotoxic activity is added in the figure below. This basal activity is similar to the one observed at the lowest mAb concentration.

**Competing Interests:** No competing interests were disclosed.