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Ciblage du CD56 comme option thérapeutique dans le carcinome à cellules de Merkel

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Résumé

Le carcinome à cellules de Merkel (CCM) est un cancer de la peau agressif. Bien que les inhibiteurs de point de contrôle immunitaire constituent une avancée majeure pour le traitement des patients avec un CCM métastatique, il reste nécessaire de développer de nouvelles options thérapeutiques pour les patients ne répondant pas à ces thérapies.

Le CD56 est un antigène membranaire présent à la surface des cellules tumorales du CCM et a été identifié comme une potentielle cible thérapeutique dans plusieurs cancers solides et hémopathies. Dans ce contexte, l'objectif de notre étude était de produire et d'évaluer les performances de l'Adcitmer®, un anticorps thérapeutique ciblant le CD56 bioconjugué à une molécule cytotoxique dans un modèle préclinique de CCM.

Pour ce faire, après avoir confirmé par immunohistochimie sur *tissue microarray*, l'expression fréquente du CD56 sur des tumeurs CCM issues de patients (66% de tumeurs positives), et démontré la fixation spécifique et l'internalisation du m906, un anticorps thérapeutique ciblant le CD56 par les lignées de CCM, l'Adcitmer® a été généré en couplant le m906 à une molécule cytotoxique (monomethyl auristatine E : MMAE) par le procédé de bioconjugaison innovant McSAF Inside®. L'homogénéité de l'Adcitmer® a été confirmée par chromatographie d'interaction hydrophobe. Les propriétés cytotoxiques de l'Adcitmer® médiées par l'expression du CD56 ont été démontrées *in vitro* sur des lignées de CCM. De plus, un effet *bystander* a pu être mis en évidence dans ce modèle. L'étude de l'interaction de l'Adcitmer® avec les cellules immunitaires *in vitro* a démontré que la non-reconnaissance de la partie Fc de l'anticorps par les effecteurs immuns, induite par la déglycosylation de l'anticorps, réduit la toxicité et améliore les performances de la molécule. Enfin l'Adcitmer® déglycosylé réduit significativement la croissance tumorale dans un modèle murin de CCM.

Notre étude suggère que l'Adcitmer® pourrait représenter une option thérapeutique chez les patients avec un CCM, utilisable en alternative ou en combinaison avec les inhibiteurs de point de contrôle immunitaire.

Mots clés : carcinome à cellules de Merkel, CD56, antibody-drug conjugate, immunothérapie

Abstract

Merkel cell carcinoma (MCC) is an aggressive skin cancer. While immune checkpoint inhibitors constitute a major advance in the treatment of patients with metastatic MCC, new therapeutic options are still urgently required.

CD56 is a membrane antigen expressed by MCC tumor cells and has been reported as an interesting target in solid tumors and hematological malignancies. In this context, our study objective was to evaluate the performances of Adcitmer®, a therapeutic antibody targeting CD56 conjugated to a cytotoxic molecule on a preclinical model of MCC.

To this end, after confirmation by immunohistochemistry on a tissue microarray of the frequent expression of CD56 by MCC tumors (66% of positives tumors) and demonstration of the specific fixation and internalization of m906 - a therapeutic antibody targeting CD56 by MCC cell lines -, Adcitmer® was generated by conjugating m906 to a cytotoxic molecule (monomethyl auristatin E : MMAE) through an innovative bioconjugation process, McSAF Inside®. The high homogeneity of Adcitmer® was confirmed by hydrophobic interaction chromatography. The CD56-mediated cytotoxicity of Adcitmer® was demonstrated *in vitro* in MCC cell lines. Moreover, a bystander effect was shown in this model. Investigation of Adcitmer® interaction with immune cells *in vitro* demonstrated that antibody deglycosylation, resulting in lack of Fc part recognition by immune cells, reduced the toxicity and improved performances of the molecule. Finally, deglycosylated Adcitmer® significantly reduced tumor growth in a MCC mouse model.

Our study suggests that Adcitmer® should be further assessed as a therapeutic option in patients with MCC, as an alternative therapy or combined with immune checkpoint inhibitors.

Key words : merkel cell carcinoma, CD56, antibody-drug conjugate, immunotherapy

Figures list

Figure 1. Merkel cell polyomavirus genomic organization (Becker *et al.,* 2017).___ 16
Figure 2. Hypothesis for cell of origin of MCC. _____ 21
Figure 3. Merkel cell carcinoma characteristics, clinically and histologically. _____ 23
Figure 4. Role of immunity in MCC._____ 30
Figure 5. Current treatments for advanced metastatic MCC. _____ 33
Figure 6. Schematic representation of extracellular and intracellular domains of CD56._____ 37
Figure 7. Design of an antibody-drug conjugate and recommended biological properties. _____ 48
Figure 8. Overview of ADC mode of action._____ 52
Figure 9. Main conclusions on Adcitmer® intrinsic characteristics, MCC *in vitro* and *in vivo* effects as well as interactions with immune cells. _____ 120
Figure 10. Major differences between lorvotuzumab and m906 that could explain the toxicity observed in NOD/SCID mice model. _____ 130
Figure 11. Mouse IgG receptors and expression pattern. _____ 131

Tables list

Table 1. Distinct characteristics of MCPyV-positive and MCPyV-negative MCC. __ 19
Table 2. Staging of Merkel cell carcinoma from the 8th edition of the AJCC stage from Harms *et al.* 2016. _____ 25
Table 3. List of the main tumor entities with CD56 expression._____ 41
Table 4. Clinical trials of lorvotuzumab mertansine. _____ 45
Table 5. FDA-approved ADCs. _____ 47
Table 6. Characteristics of lorvotuzumab mertansine and Adcitmer®._____ 125

Abbreviations

4EBP1 : eukaryotic translation initiation factor 4E-binding protein 1
ADC : antibody-drug conjugates
ADCC : antibody-dependent cell-mediated cytotoxicity
ADCP : antibody dependent cell-mediated phagocytosis
ADN : acide désoxyribonucléique
AJCC : american joint committee on cancer
ALTO : alternative large T antigen open reading frame protein
APC : allophycocyanin
ARF : ADP ribosylation factor
ATPase : adenosinetriphosphatase
BCL-2 : B cell lymphoma 2
BPDCN : blastic plasmacytoid dendritic cell neoplasm
C1q : complement component 1q
CAR-T : chimeric antigen receptor-T
CCL19 : chemokine (C-C motif) ligand 19
CCM : carcinome à cellules de Merkel
CD : cluster of differentiation
CDC : complement dependent cytotoxicity
CK1α : casein kinase 1 isoform alpha
CREB1 : cAMP responsive element binding protein 1
CRISPR/Cas9 : Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated protein 9
CT : computed tomography
CTLA : cytotoxic T-lymphocyte-associated
DAR : drug-to-antibody ratio
DC : dendritic cell
DLL3 : delta-like protein 3
DM1 : mertansine
DNA : deoxyribonucleic acid
EGFR : epidermal growth factor receptor
EMA : european medicines agency
EMT : epithelial-mesenchymal transition
ERK : extracellular signal-regulated kinase
Fab : fragment antigen-binding
Fc : fragment crystallizable
Fc : fragment cristallisable
FcRn : neonatal Fc receptor
FcγR : Fc gamma receptor
FDA : food and drug administration
FDG-PET : fluorodeoxyglucose positron emission tomography
FGFR : fibroblast growth factor
GPI : glycosylphosphatidylinositol
GSK3 : glycogene synthetase kinase 3
HER2 : human epidermal growth factor receptor 2
HIV : human immunodeficiency virus
HLA : human leukocyte antigen
HSPG : heparan sulfate proteoglycan
IC50 : half-maximal inhibitory concentration
ICI : immune checkpoint inhibitors
IFN : interferon
IgG : immunoglobuline G
IL : interleukine
INSM1 : insulinoma-associated 1
ITAM : immunoreceptor tyrosine-based activation motif
ITIM : immunoreceptor tyrosine-based inhibitory motif
KO : knockout
KRT : cytokeratin
LM : lorvotuzumab mertansine
LSD : LT stabilization domain
LT : large T
mAb : monoclonal antibody
MAPK : mitogen-activated protein kinases
MCC : Merkel cell carcinoma
MCPyV : Merkel cell polyomavirus
MF-TTZ-MMAE : McSAF-trastuzumab-MMAE
MFI : mean fluorescence intensity
MHC : major histocompatibility complex
MIC : MCH class 1 related protein
MMAE : monomethyl auristatin E
MMAF : monomethyl auristatin F
mRNA : messenger RNA
MSH2 : mutS homolog 2
mTOR : mammalian target of rapamycin
MUC-1 : mucin-1
MUR : Merkel unique region

NCAM : neural cell adhesion molecule
NCCN : national comprehensive cancer network
NCCR : non-coding control region
NF-κB : nuclear factor kappa light chain enhancer of activated B cells
NHS : N-hydroxysuccinimide
NK : natural killer
NOD/SCID : nonobese diabetic/severe combined immunodeficiency
NSCLC : non-small cell lung cancer
NSG : NOD/SCID gamma mice
OBD : origin-binding-domain
OMS : organisation mondiale de la santé
Ori : origin of replication
p53 : tumor protein 53
PAB : para-aminobenzylique
PBD : pyrrolobenzodiazepine
PBS : phosphate-buffered saline
PD : phamacodynamic
PD-1 : programmed cell death protein 1
PD-L1 : programmed death-ligand 1
PEG : polyethylene glycol
PET : positron emission tomography
PI3K : phosphoinositide 3-kinases
PIK3C : phosphatidylinositol 3-kinase catalytic subunit type 3
PK : pharmacokinetic
PML : progressive multifocal encephalopathy
PMS : N-methyl dibenzopyrazine methyl sulfate
PNGase : protein N-glycosidase
PNU : anthracycline
PP2A : protein phosphatase 2A
PSA : polysialic acid
RB1 : retinoblastoma protein 1
RNA : ribonucleic acid
RNAse : ribonuclease
SATB2 : special AT-rich sequence-binding protein
scFv : single-chain variable fragment
SCLC : small cell lung carcinoma
SEM : standard error of the mean
SPP : N-succinimidyl-4-(2-pyridyldithio)pentanoate
SSTR : somatostatin receptor
sT : small T
Tim-3 : T-cell immunoglobulin mucin 3
TLR : toll-like receptor
TME : tumor microenvironment
TTF1 : thyroid transcription factor 1
UV : ultraviolet
Val-Cit : valine-citrulline
VEGF : vascular endothelial growth factor
VIH : virus de l'immunodéficience humaine
VP1 : viral protein 1
WHO : world health organisation
WT : wild-type
XTT : 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
ΔLT : truncated LT

Summary

SYNTHÈSE EN FRANÇAIS 1

1. INTRODUCTION 1
2. CONTEXTE ET OBJECTIFS 3
3. RESUME DES ETUDES EXPERIMENTALES 4
   - *3.1. Génération et caractérisation de l'Adcitmer®, un anticorps thérapeutique couplé à une molécule cytotoxique pour le traitement des patients CCM* 4
   - *3.2. Évaluation de l'activité cytotoxique et de la spécificité de l'Adcitmer® in vitro* 6
   - *3.3. Évaluation de l'interaction de l'Adcitmer® avec les cellules NK* 10
   - *3.4. Efficacité in vivo de l'Adcitmer® et de l'Adcitmer® déglycosylé* 11
4. CONCLUSION GENERALE ET PERSPECTIVES 12

INTRODUCTION 13

1. MERKEL CELL CARCINOMA, A RARE AND AGGRESSIVE CUTANEOUS CANCER 13
   - *1.1. History of Merkel cell carcinoma* 13
   - *1.2. Merkel cell Polyomavirus (MCPyV)* 13
   - *1.2.1. Discovery of MCPyV* 13
   - *1.2.2. Transmission of MCPyV* 14
   - *1.2.3. Genomic organisation of MCPyV* 15
   - *1.2.4. Oncogenic property of MCPyV* 16
   - *1.3. MCPyV-negative and MCPyV-positive MCCs* 18
   - *1.4. What is the cell of origin of MCC?* 19
   - *1.5. Epidemiology of MCC* 21
   - *1.6. MCC diagnosis* 22
   - *1.7. MCC tumor staging* 24
   - *1.8. Immune response against MCC* 25
   - *1.8.1. Cancer immune responses* 25
   - *1.8.2. Impact of immunosupression in MCC developement* 26
   - *1.8.3. Humoral response against MCPyV* 26
   - *1.8.4. T-cell response* 27
   - *1.8.5. Tumor evasion mechanisms and interest for immunotherapies* 28
   - *1.9. Merkel cell carcinoma treatments* 30
   - *1.9.1. Surgery* 31
   - *1.9.2. Radiotherapy* 31
   - *1.9.3. Chemotherapies: previous standard of care* 31
   - *1.9.4. Immunotherapies* 32
   - *1.9.4.1. Avelumab* 32
   - *1.9.4.2. Pembrolizumab* 32
   - *1.9.4.3. Nivolumab* 32
   - *1.9.5. Immunotherapies limits* 33
   - *1.9.6. Other potential immunotherapies* 34
   - *1.9.7. Interest for targeted therapies* 34
2. CD56 EXPRESSION: IMPLICATIONS IN PHYSIOLOGY AND PATHOLOGY 35
   - *2.1. CD56 : structure and functions* 35
   - *2.2. CD56 expression by immune cells* 38
   - *2.3. CD56 in non cancerous diseases* 39
   - *2.4. CD56 expression in cancer* 39
   - *2.4.1. Carcinomas* 40
   - *2.4.2. Pediatric tumors* 40
   - *2.4.3. Hemopathy* 40
   - *2.5. Therapies targeting CD56* 42

3. THE ANTIBODY-DRUG CONJUGATES IN ONCOLOGY 46
3.1. Structure of ADC 47
3.2. Targets 48
3.3. Antibodies 49
3.4. Payloads 50
3.5. Linker 52
3.6. Linker-antibody conjugation 53
3.7. Strengths, weaknesses and challenges in ADC field 53

CONTEXT AND OBJECTIVES 57

EXPERIMENTALES STUDIES 59

1. ARTICLE 1: ADCITMER®, A NEW CD56-TARGETING MONOMETHYL AURISTATIN E-CONJUGATED ANTIBODY, IS A POTENTIAL THERAPEUTIC APPROACH IN MERKEL CELL CARCINOMA 59
2. ARTICLE 2: EVALUATION AND OPTIMIZATION OF ADCITMER®, A MMAE-CONJUGATED ADC TARGETING CD56-EXPRESSING CANCERS 79

GENERAL CONCLUSION AND PERSPECTIVES 119

1. RATIONAL FOR THE USE OF ADCITMER® IN MERKEL CELL CARCINOMA 119
1.1. An overview of Adcitmer® characterization 119
1.2. Rational for the use of an ADC therapy in MCC 120
1.3. Identification of CD56 as a relevant therapeutic target in MCC 123
1.4. Previous CD56 targeting ADC in MCC 124
2. CHARACTERIZATION OF ADCITMER® PROPERTIES 125
2.1. Biochemical properties 125
2.1.1. Payload 125
2.1.2. Bioconjugation 126
2.2. Interaction with immune cells 127
2.2.1. In vitro investigation 127
2.2.2. Investigations to explain NOD/SCID mice symptomatology after m906 injection 128
2.2.3. Impact of deglycosylation of Adcitmer® in tumor growth 130
2.2.4. An aglycosylated version of Adcitmer® for further study 131
3. PERSPECTIVES 132
3.1. Adcitmer®: the next steps to achieve a clinical trial 132
3.2. Adcitmer® in other CD56 expressing tumors 133
4. CONCLUSIONS 134

REFERENCES 135

APPENDIX 153

SYNTHÈSE EN FRANÇAIS

1. Introduction

Le carcinome à cellules de Merkel (CCM) est un cancer cutané neuroendocrine agressif dont la survie à 5 ans est estimée à 39% en cas de métastases ganglionnaires loco-régionales et 18% en cas de métastase à distance (Lemos et al., 2010; Paulson et al., 2013). Le CCM est un cancer induit dans 80% des cas par l'intégration du génome du polyomavirus à cellules de Merkel (MCPyV) (Feng et al., 2008) et dans 20% des cas par l'exposition aux UV (Harms et al., 2015). Bien qu'il s'agisse d'une tumeur rare, les publications récentes montrent que l'incidence de ce cancer a fortement augmenté sur les deux dernières décennies probablement du fait du vieillissement de la population et de l'amélioration des techniques diagnostiques (Schadendorf et al., 2017). Le CCM survient presque exclusivement chez les personnes âgées, avec un âge médian lors du diagnostic de 75 ans (Albores-Saavedra et al., 2010). L'immunosuppression (*i.e.* hémopathies, transplantations d'organes, et infections par le VIH) semble être associée à un risque plus élevé de CCM (Asgari et al., 2014; Cook et al., 2019).

L'organisation mondiale de la santé (OMS) définit le CCM comme un carcinome neuroendocrine primitif cutané. En effet, la différenciation neuroendocrine definit un sous-groupe de cancers, comportant des similarités phénotypiques avec les cellules du système neuroendocrine disséminé physiologique, représenté au niveau de la peau par les cellules de Merkel.

Cliniquement, le CCM se présente comme une papule ou un nodule cutané, erythémateux ou violacé, indolore et de croissance rapide. Les tumeurs sont principalement situées sur les régions photo-exposées.

Le diagnostic du CCM repose sur la biopsie cutanée et la recherche par immunohistochimie de marqueurs épithéliaux tel que la cytokératine 20 (García-Caballero et al., 2003) et neuroendocrines (*i.e.* chromagranine A, CD56 et synaptophysine) (Fernández-Figueras et al., 2007).

L'étiologie du CCM a pendant longtemps été inconnue. Dans les années 2000, une cause infectieuse a été suggérée du fait de l'augmentation de l'incidence du CCM chez les patients immunodéprimés (Shaw et al., 2006). En 2008, la mise en évidence par soustraction digitale du transcriptome de la séquence du MCPyV intégrée dans le génome des cellules tumorales de 80% des CCM constitue la première démonstration formelle du rôle oncogénique d'un polyomavirus chez l'Homme (Feng et al., 2008).

Alors que l'infection par le MCPyV est ubiquitaire (Li et al., 2013), l'intégration et les mutations du génome viral sont des événements rares et essentiels à l'oncogénèse induite par le MCPyV.

Environ 20% des cas de CCM ne sont pas liés à l'intégration virale. Plusieurs observations montrent que l'exposition aux UV est le principal facteur oncogénique des tumeurs MCPyV-négatives : 1) la charge mutationnelle élevée au sein de ces tumeurs ; 2) la signature mutationnelle avec des transitions C > T ; 3) l'association avec d'autres cancers cutanés induits par les UV notamment les carcinomes épidermoïdes.

Les deux sous-types de CCM, (MCPyV-positifs et MCPyV-négatifs), ont tous les deux un phénotype neuroendocrine car les mêmes voies de signalisation sont altérées soit par les protéines virales soit par les mutations somatiques (González-Vela et al., 2017; Starrett et al., 2017). Cependant, certaines différences cliniques sont observées entre les deux sous-types, les CCM UV-induits semblent notamment associés à un plus mauvais pronostic (Moshiri et al., 2017).

Les patients immunodéprimés ont un risque accru de développer un CCM (Clarke et al., 2015; Engels et al., 2002; Heath et al., 2008). Cette observation clinique montre un lien étroit entre le développement du CCM et le système immunitaire. Bien que l'immunité anti-cancéreuse prévient souvent la formation de tumeur, cette dernière peut échapper à la réponse immunitaire si le système immunitaire devient tolérant vis-à-vis de la tumeur (Hiam-Galvez et al., 2021). Ainsi dans le CCM, de nombreux mécanismes d'évasion immunitaire ont été mis en évidence comme la baisse d'expression membranaire du complexe majeure d'histocompatibilité, l'exclusion des effecteurs lymphocytaires ainsi que l'expression de points de contrôle immunitaire (ICI), comme par exemple PD-1 et son ligand PD-L1.

Comme pour d'autres cancers, l'utilisation des ICI a révolutionné le traitement des patients atteints de CCM au stade métastatique. Ainsi, l'avelumab, un anticorps monoclonal ciblant le point de contrôle immunitaire PD-L1, a été la première thérapeutique approuvée dans ce cancer au stade métastatique (Kaufman et al., 2016). Par la suite, le pembrolizumab, anticorps monoclonal anti-PD-1, a également été approuvé par la FDA en 2018 dans les CCM avancés (Nghiem et al., 2018). Cependant, les ICI anti-PD-1/PD-L1 ne permettent d'obtenir une réponse objective durable que chez 25% des patients soulignant la nécessité d'identifier de nouvelles stratégies thérapeutiques. L'absence d'expression des antigènes viraux à la surface

des cellules tumorales, ne permet pas le développement d'une thérapie ciblant spécifiquement les protéines du MCPyV. L'antigène membranaire CD56 a été identifié comme une cible thérapeutique fréquemment exprimée par les CCM UV- et MCPyV-induits. De manière intéressante, le CD56 est un marqueur neuroendocrine qui est exprimé par un grand nombre de tumeurs, comme le carcinome à petites cellules du poumon ou certaines hémopathies (Crossland et al., 2018; Wilson et al., 2021). Dans ce contexte, le ciblage du CD56, exprimé par les cellules tumorales du CCM apparait comme une piste thérapeutique intéressante au stade métastatique (Ollier et al., 2018).

2. Contexte et objectifs

Le CD56 est une cible membranaire exprimée par une majorité de CCM (Fernández-Figueras et al., 2007; Tetzlaff and Harms, 2020). Des *antibody-drug conjugates* (ADC) ciblant cet antigène ont déjà fait l'objet d'essais pré-cliniques (Feng et al., 2016; Yu et al., 2018a, 2018b) ou cliniques comme le lorvotuzumab mertansine (LM) qui a montré des résultats encourageants sur des patients CCM (Shah et al., 2016). Cependant, en phase I-II le LM a été testé en combinaison avec le carboplatine et l'étoposide chez des patients atteints de carcinome à petites cellules du poumon et un effet secondaire majeur (*i.e.* neutropénie sévère) est survenu (Socinski et al., 2017). Dans ce contexte, après avoir évalué le niveau d'expression du CD56 dans une cohorte de patients CCM, un immunoconjugué ciblant le CD56 couplé à la MMAE, l'Adcitmer®, a été produit et caractérisé. La cytotoxicité spécifique de l'ADC contre les cellules exprimant la cible ainsi que l'effet sur les cellules tumorales n'exprimant pas le CD56 ont été évalués *in vitro*. De plus, l'interaction de l'ADC avec les cellules immunitaires dont les cellules NK a été déterminée. Pour finir, l'ADC a été optimisé pour réduire la toxicité sur les cellules immunitaires en bloquant la reconnaissance de sa région constante par déglycosylation. Les performances de l'ADC déglycosylé ont été comparées *in vitro* et dans un modèle murin de CCM, à celles de l'ADC initialement produit.

3. Résumé des études expérimentales

3.1. Génération et caractérisation de l'Adcitmer®, un anticorps thérapeutique couplé à une molécule cytotoxique pour le traitement des patients CCM

L'expression du CD56 a été évaluée dans une cohorte multicentrique de 90 CCM et des scores immunohistochimiques d'expression du CD56 ont été établis. Soixante-six pourcents des tumeurs exprimaient le CD56 (**Figure 1A**). Le CD56 est exprimé par les 2 sous-types de CCM, MCPyV-induits et UV-induits (**Figure 1B**). Le CD56 est exprimé par 4 lignées de CCM MCPyV-positives (**Figure 1C**) sur lesquelles ont été réalisées les expériences *in vitro*.

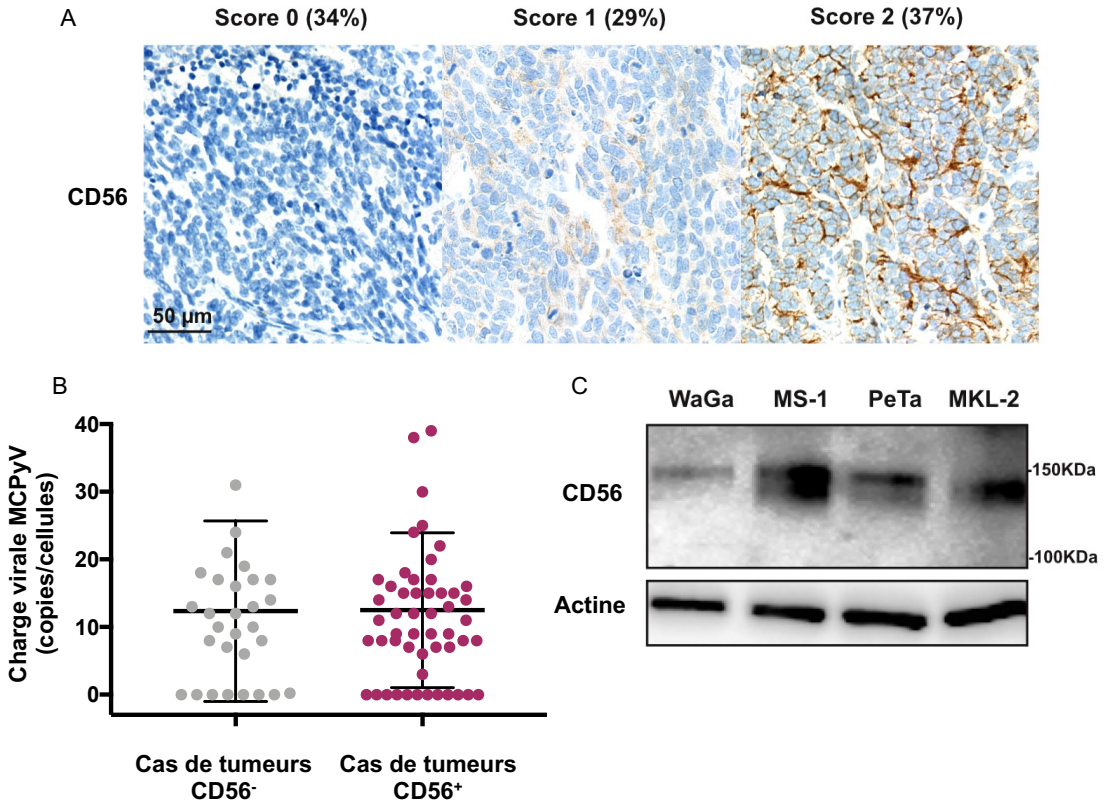

Figure 1. Expression du CD56 par les tumeurs de carcinome à cellules de Merkel (CCM) et les lignées de CCM. (A) Expression du CD56 par les tumeurs de CCM, (B) comparaison de la charge virale (MCPyV) entre les CCM $CD56^+$ et $CD56^-$ et (C) expression du CD56 par les lignées cellulaires de CCM.

Le m906 est un anticorps thérapeutique monoclonal IgG1 humain qui a été conjugué à un intercalant de l'ADN, le pyrrolobenzodiazepine (PBD) et qui a montré des résultats encourageants sur des lignées de neuroblastomes (Feng et al., 2016).

La reconnaissance spécifique du CD56 par le m906 ainsi que l'internalisation de ce dernier par les cellules tumorales de CCM, ont été démontrées par immunocytochimie (**Figure 2A**) et par cytométrie couplé à un imageur en flux (**Figure 2B**), respectivement. En conséquence, l'Adcitmer® a été généré en couplant l'anticorps m906 à la MMAE (monomethyl auristatine E : molécule inhibant la polymérisation de la tubuline). Cette bioconjugaison sur les ponts disulfures inter-chaines des anticorps a été réalisée par la technologie brevetée McSAF Inside® visant à optimiser les performances de l'ADC.

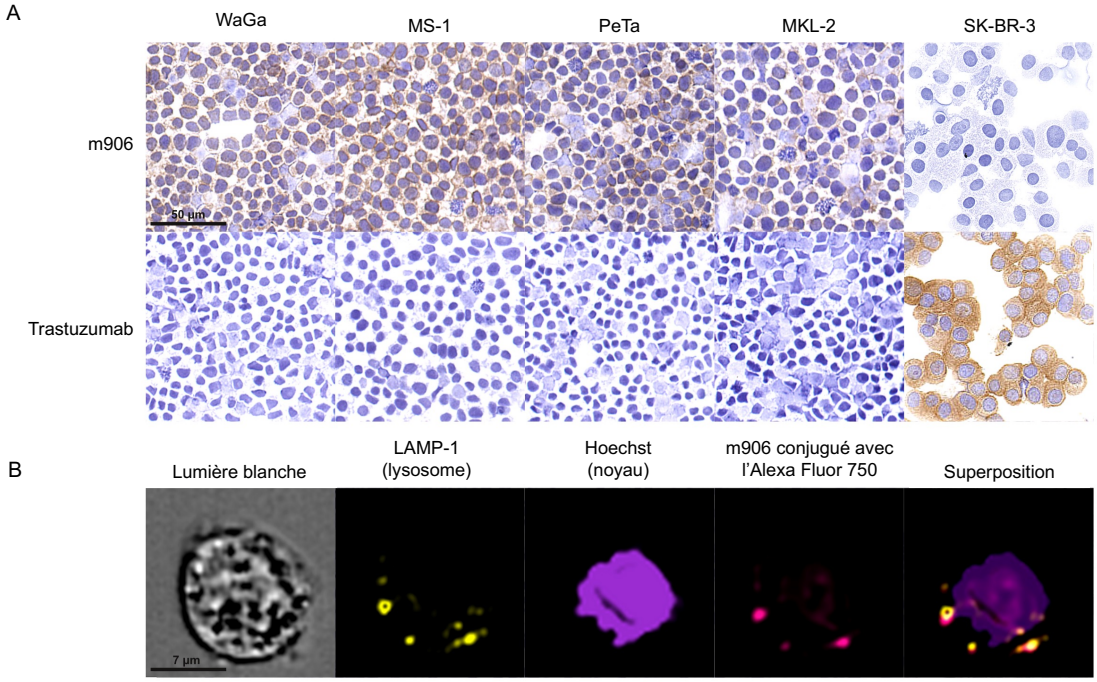

Figure 2. Fixation et internalisation du m906 par les lignées de CCM. (A) Fixation du m906 au CD56 exprimé par les lignées de CCM et (B) internalisation de l'anticorps.

L'Adcitmer® ainsi généré est un ADC vectorisant 4 molécules de MMAE (**Figure 3**). Le *linker* est conjugué aux résidus cystéines de l'anticorps et permet la reconstruction des ponts disulfures de l'anticorps. Le *linker* est composé d'une gâchette peptidique valine-citrulline (Val-Cit) clivable par la cathepsine B lysosomale et un groupement para-aminobenzylique (PAB) agissant en tant qu'espaceur auto-immolable (**Figure 3**) permettant la libération de la MMAE active après internalisation dans la cellule tumorale exprimant la cible. Ce type de *linker* permet de contrôler le nombre de molécules de MMAE par anticorps ainsi que leur position. Une analyse par

chromatographie d'interaction hydrophobe a permis d'évaluer le *drug-to-antibody ratio* (DAR) moyen de l'Adcitmer® à 3,8.

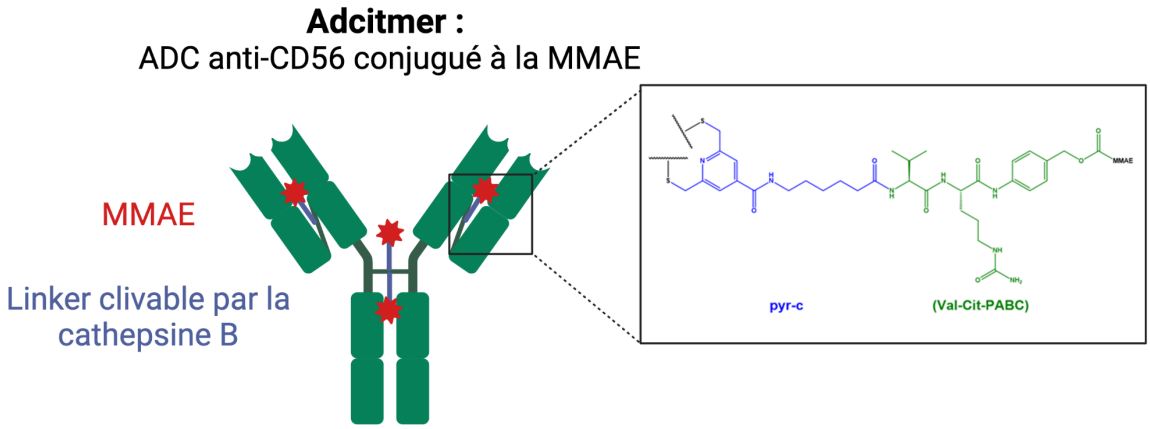

Figure 3. Structure de l'Adcitmer® et du *linker.*

3.2. Évaluation de l'activité cytotoxique et de la spécificité de l'Adcitmer® in vitro

L'évaluation *in vitro* a mis en évidence un effet cytotoxique de l'Adcitmer® sur les lignées de CCM avec des concentrations inhibitrices médiane (IC50) comprises entre 1 et 10 nM. La MMAE vectorisée présente une activité cytotoxique similaire à la MMAE seule, démontrant que la MMAE couplée a été libérée sous sa forme active. L'anticorps m906 natif n'est pas cytotoxique, démontrant l'importance de coupler l'anticorps à une molécule cytotoxique. Afin de démontrer la spécificité de l'Adcitmer®, un ADC « non relevant » a été évalué comme contrôle : un anticorps anti-HER2, le trastuzumab, couplé à la MMAE avec le même *linker* et ayant le même DAR que l'Adcitmer® (MF-TTZ-MMAE). Cet ADC n'était pas cytotoxique sur les lignées de CCM, montrant qu'en l'absence de reconnaissance de la cible tumorale et d'internalisation de l'ADC, la MMAE n'est pas libérée (**Figure 4A**).

La spécificité de l'Adcitmer® a été également évaluée par l'invalidation de l'expression du CD56 par les lignées de CCM *via* la technologie CRISPR/Cas9. Cette expérimentation a permis de confirmer que les propriétés cytotoxiques de l'Adcitmer® étaient dépendantes de l'expression de sa cible (**Figure 4B**).

Afin de caractériser cette cytotoxicité, l'effet de l'Adcitmer® sur le cycle cellulaire a été démontré. En effet, le traitement des lignées de CCM sauvages avec l'Adcitmer® et la MMAE seule induit un arrêt du cycle en G2/M puis une apoptose alors qu'aucun

effet de l'Adcitmer® n'est observé sur les lignées CD56 inactivé (*knockout* : KO) (**Figure 4C**).

Afin d'établir une preuve de concept, l'injection par voie intraveineuse de l'Adcitmer® (10 mg/kg, au jour 0, 14 et 26) a montré une réduction de la croissance tumorale dans un modèle de xénogreffe de CCM (données non présentées).

De manière intéressante, la cytotoxicité de l'Adcitmer® a été démontré *in vitro* sur des lignées cellulaires de carcinome à petites cellules du poumon et de néoplasie à cellules dendritiques plasmocytoïdes blastiques (données non présentées).

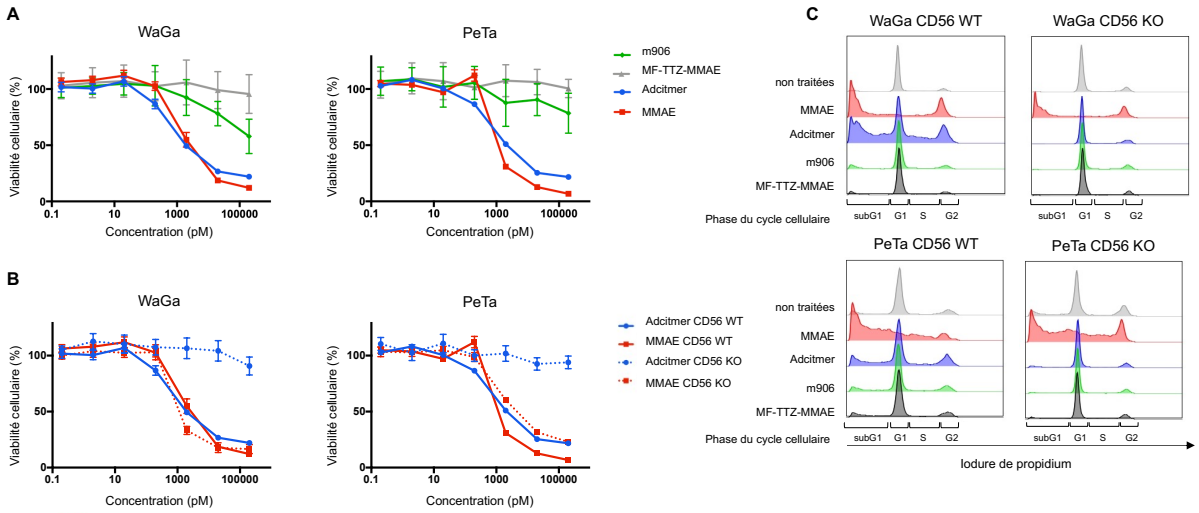

Figure 4. Évaluation de la cytotoxicité et de l'effet sur le cycle cellulaire de l'Adcitmer® sur des cellules de CCM CD56 WT ou KO (A) Cytotoxicité de l'Adcitmer® et des contrôles [m906, monomethyl auristatin E (MMAE) et MF–trastuzumab (TTZ)–MMAE sur les lignées de CCM, (B) Comparaison de la cytotoxicité de l'Adcitmer® sur les lignées de CCM CD56 sauvage (*wild-type* : WT) et CD56 inactivé (*knockout* : KO) (C) Effet sur le cycle cellulaire de l'Adcitmer® sur les lignées de CCM exprimant ou non le CD56.

La MMAE est une molécule cytotoxique qui diffuse à travers les membranes lipidiques, et ainsi peut induire un effet *bystander* (Abdollahpour-Alitappeh et al., 2019) par relargage de la molécule par les cellules ciblées (exprimant le CD56) dans leur environnement (**Figure 5A**). Afin d'évaluer si l'Adcitmer® induit un effet *bystander*, une co-culture associant des cellules de CCM WT et KO pour le CD56 a été réalisée (**Figure 5A**). En condition de co-culture, l'analyse du cycle cellulaire montrait une augmentation du nombre de cellules en phase subG1 pour les WaGa CD56 WT mais également pour les WaGa CD56 KO alors qu'en absence de co-culture ces dernières ne voyaient pas leur cycle cellulaire impacté par l'Adcitmer® (**Figure 5B**). L'Adcitmer® induit donc un effet *bystander* sur les cellules de CCM qui n'expriment pas la cible

(**Figure 5C**). L'effet *bystander* permet d'optimiser l'effet thérapeutique des ADC lors du traitement de tumeurs avec une expression hétérogène de la cible (Abdollahpour-Alitappeh et al., 2019).

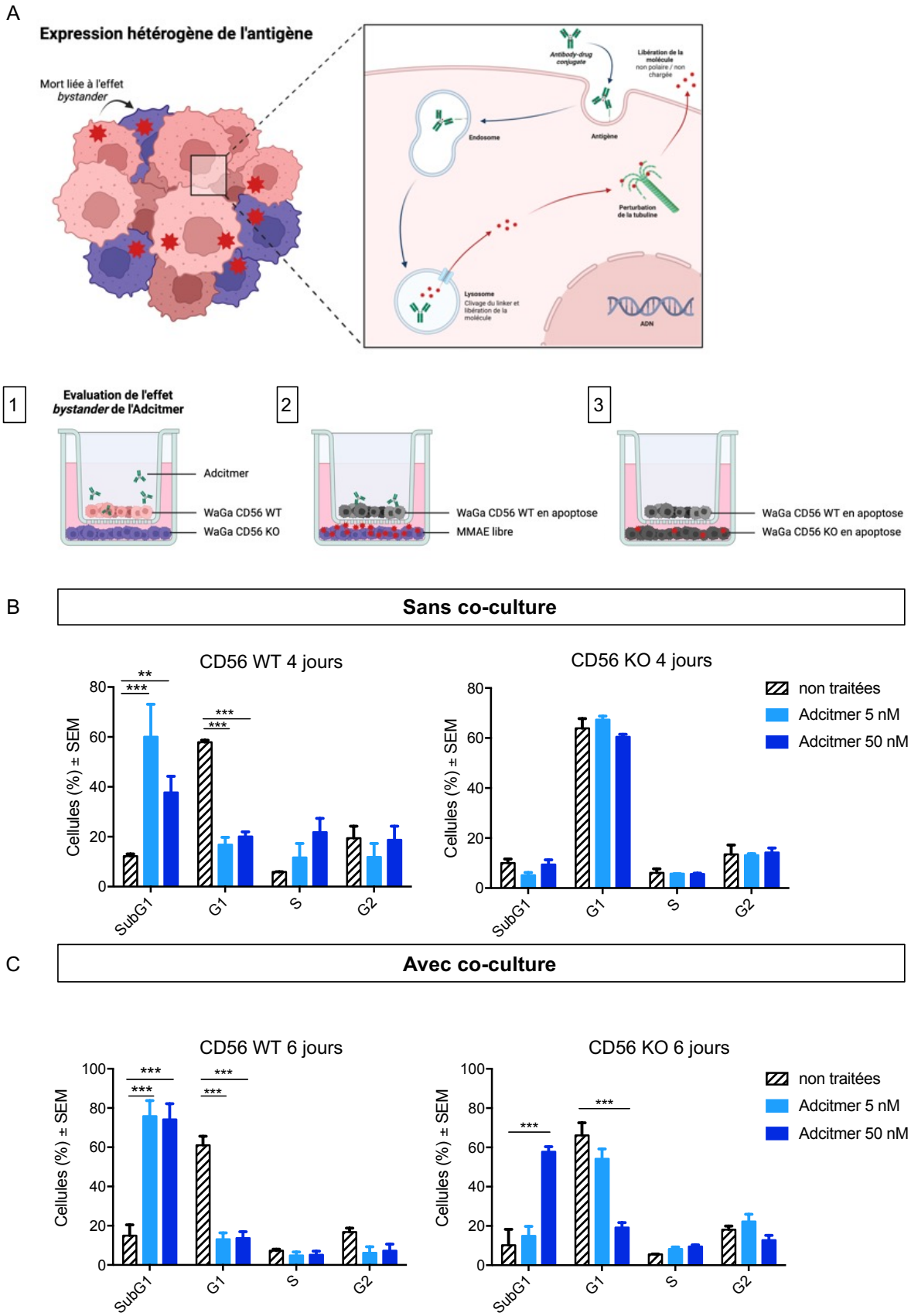

Figure 5. Évaluation de l'effet *bystander* d'Adcitmer® dans le CCM *in vitro*. (A) Représentation schématique de l'effet *bystander* dans une tumeur avec une expression

hétérogène de l'antigène ciblé. Schéma illustrant la co-culture entre une lignée de CCM, WaGa CD56 sauvage (WT) (*i.e.* cellules exprimant le CD56) et WaGa CD56 inactivé (KO) (*i.e.* cellules n'exprimant pas le CD56) pour déterminer l'effet *bystander* de l'ADC. Étape 1, traitement de l'Adcitmer® sur les cellules WaGa WT CD56 et WaGa KO CD56. Étape 2, l'Adcitmer® est internalisé dans les cellules WaGa CD56 WT, qui deviennent des cellules apoptotiques et la monomethyl auristatine E (MMAE) est libérée à l'extérieur des cellules. Étape 3, la MMAE libre traverse la membrane plasmique et devient cytotoxique pour les cellules WaGa KO CD56. (B) Effet du traitement avec l'Adcitmer® sur le cycle cellulaire des WaGa CD56 WT et CD56 KO par rapport aux cellules non traitées pendant 4 jours sans co-culture. (C) Effet du traitement avec l'Adcitmer® sur le cycle cellulaire des WaGa CD56 WT et CD56 KO par rapport aux cellules non traitées pendant 6 jours en co-culture avec des cellules WaGa CD56 WT. Le pourcentage de cellules dans chaque phase du cycle cellulaire (G1, S, G2) ou en phase subG1 est représenté.

3.3. Évaluation de l'interaction de l'Adcitmer® avec les cellules NK

Le CD56 n'est pas uniquement exprimé par les cellules tumorales mais est aussi exprimé par les cellules NK, posant la question d'un possible effet *on-target off tumor* de l'Adcitmer® sur ces cellules. Dans ce contexte, la fixation et la cytotoxicité de l'Adcitmer® sur des cellules NK ont été évaluées en utilisant une lignée de CCM comme contrôle. Le CD56 est exprimé par les cellules NK primaires. Cependant, la moyenne d'intensité de fluorescence du CD56 est plus faible sur les NK que sur les WaGa (40 000 *vs.* 170 000, respectivement p=0,029*), cette différence traduit une expression plus faible du CD56 par les NK (**Figure 6A**). Puis, la fixation de l'Adcitmer® sur des cellules NK et WaGa a été évaluée par cytométrie en flux. Pour empêcher la fixation des anticorps aux cellules immunitaires par le fragment cristallisable (Fc) de l'anticorps et ne conserver que la fixation spécifique par la cible CD56, un réactif bloquant les récepteurs Fc a été utilisé. Cette expérience a confirmé que l'anticorps nu (m906) et l'Adcitmer® se fixent sur les cellules NK *via* le CD56, avec une capacité de fixation plus faible que pour les cellules de CCM (**Figure 6B**). Par conséquent, l'Adcitmer® a été déglycosylé avec de la PNGase dans le but d'empêcher la reconnaissance du Fc par les cellules immunitaires (**Figure 6C**). La déglycosylation d'Adcitmer® n'affecte pas sa cytotoxicité sur les cellules de CCM mais permet de prévenir la toxicité sur les cellules NK suggérant un effet *off target* (**Figure 6D**). Ce résultat met en évidence que la fonctionnalité du Fc du m906 et de l'Adcitmer® est à elle seule responsable de la toxicité sur les NK.

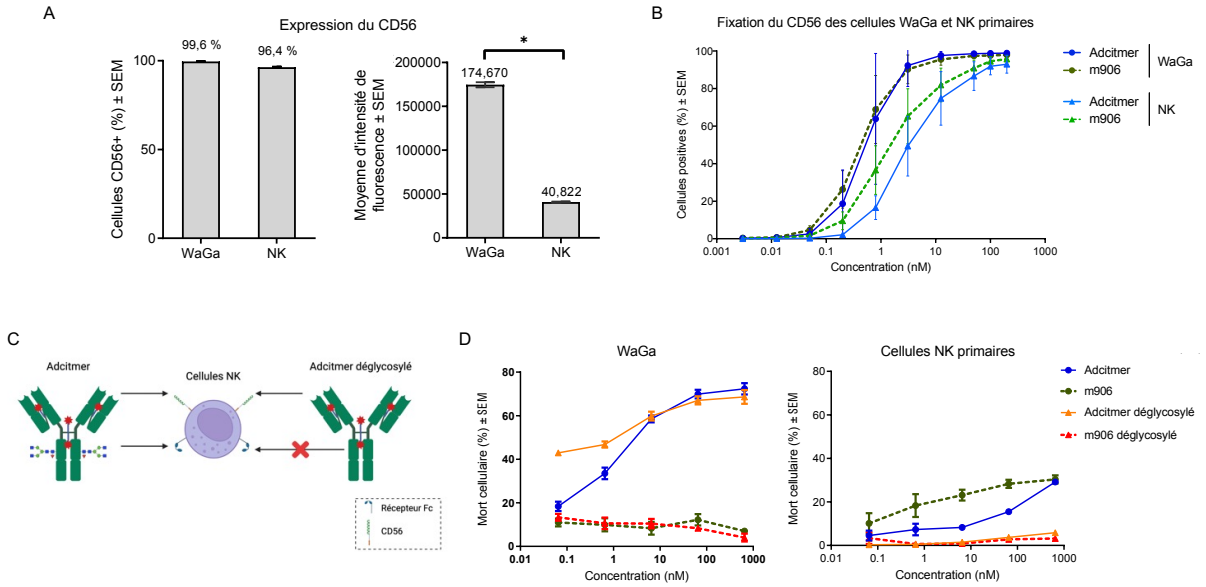

Figure 6. Interactions entre les cellules NK et l'Adcitmer® ou le m906 par rapport aux cellules de CCM, WaGa. (A) Expression du CD56 par les WaGa et les cellules NK. (B) Fixation du m906 et de l'Adcitmer® au CD56 exprimé par les cellules NK par rapport aux cellules WaGa, utilisées comme contrôle positif (cytométrie en flux). Les récepteurs Fc des cellules NK ont été bloqués par le Fc Block. (C) Représentation schématique de l'interaction de l'Adcitmer® et de l'Adcitmer® déglycosylé avec les cellules NK par le CD56 et le récepteur Fc. (D) La mortalité des cellules WaGa et NK incubées pendant 4 jours avec l'Adcitmer®, l'Adcitmer® déglycosylé, le m906 et le m906 déglycosylé a été analysée par cytométrie en flux.

3.4. Efficacité in vivo de l'Adcitmer® et de l'Adcitmer® déglycosylé

Le traitement avec l'Adcitmer® a permis de réduire la croissance tumorale dans un modèle de xénogreffe de CCM cependant une prostration des animaux a été observée rapidement après la primo-injection de l'Adcitmer® alors qu'aucun symptôme n'a été observé après l'injection de l'Adcitmer® déglycosylé ou de PBS. De plus, le traitement des souris avec l'Adcitmer® déglycosylé à 5 mg/kg 2 fois par semaine pendant 30 jours (9 injections au total) par voie intraveineuse a montré un effet anti-tumoral (**Figure 7A**) et une réduction du poids final des tumeurs (**Figure 7B**) significativement supérieurs par rapport aux souris traitées avec l'Adcitmer®. Une régression tumorale complète a été atteinte dans le groupe de souris traitées avec l'Adcitmer® déglycosylé (n= 3/7).

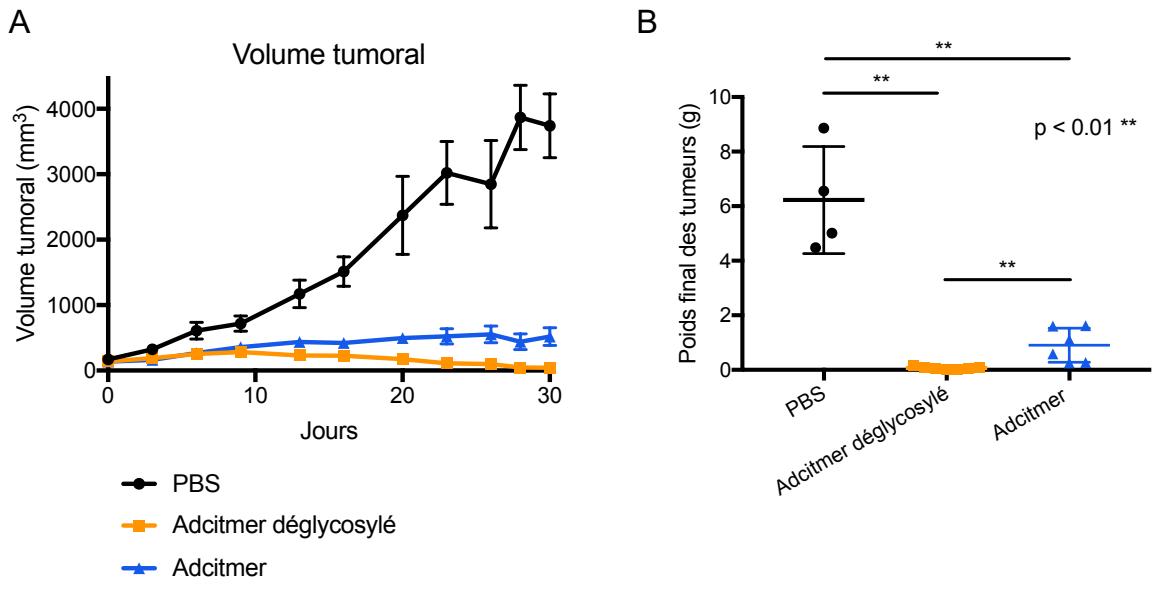

Figure 7. Traitement avec l'Adcitmer® et l'Adcitmer® déglycosylé dans un modèle murin de xénogreffe de CCM. (A) Évolution des volumes tumoraux moyens pendant 30 jours ; les injections intraveineuses ont été réalisées 2 fois par semaine à 5 mg/kg (chaque souris a reçu 9 injections au total sur 30 jours) (B) Poids des tumeurs 30 jours après le début des traitements, dans les groupes contrôle et expérimentaux.

4. Conclusion générale et perspectives

Pour conclure, l'immunoconjugué Adcitmer® est cytotoxique sur les cellules exprimant la cible CD56 et permet de réduire la croissance tumorale dans un modèle préclinique de CCM. La déglycosylation de l'Adcitmer® permet une optimisation de son efficacité et potentiellement de sa toxicité en diminuant l'effet *off-target* sur les cellules immunitaires. Cette molécule pourrait représenter une option thérapeutique prometteuse pour les patients atteints de CCM en échec de traitement ou en combinaison avec les inhibiteurs de point de contrôle immunitaire. L'Adcitmer® pourrait être également utilisé comme traitement pour d'autres cancers exprimant le CD56 comme le carcinome à petites cellules du poumon ou la néoplasie à cellules dendritiques plasmacytoïdes blastiques pour lesquels les options thérapeutiques sont actuellement limitées.

INTRODUCTION

1. Merkel cell carcinoma, a rare and aggressive cutaneous cancer

1.1. History of Merkel cell carcinoma

Merkel cell carcinoma (MCC) was first described by Toker *et al* in 1972 as the "trabecular carcinomas of the skin" (Toker, 1972). In the initial report, the authors described a tumor composed of cells nests within dermis or subcutis, without connection to the overlying epidermis. Electronic microscopy analysis further revealed the presence of neurosecretory granules in the tumor cell cytoplasm, a feature physiologicallly restricted to neuroendocrine cells. In the skin, the neuroendocrine cells *i.e.* Merkel cells, are localized in the epidermis basal layer and the hair follicles in connection to intradermal nerves. Accordingly, Merkel cells act as sensory receptors involved in touch sensitivity. Due to phenotypic similarities between such “Toker’s tumors” and physiological Merkel cells, these cancers were subsequently refered to as MCC.

In the 2000’s, epidemiological studies revealed increased MCC incidence in immunocompromised patients (Engels et al., 2002; Heath et al., 2008), raising the possibility of an infectious etiology for MCC which was finally demonstrated in 2008 (Feng et al., 2008). Indeed, in about 80% of MCC, the oncogenic driver is constituted by the genomic integration of the Merkel cell Polyomavirus (MCPyV) within host cells leading to oncoproteins expression, while 20% of MCC cases are UV-induced.

1.2. Merkel cell Polyomavirus (MCPyV)

1.2.1. Discovery of MCPyV

For a long time, the nature of the genetic alterations driving MCC oncogenesis had remained unknown (Houben et al., 2009). Indeed, activated mutations of *C-KIT* proto-oncogene was reported for other tumors, and even if a majority of MCC express KIT protein, no evidence of activated mutations has been shown (Swick et al., 2007).

Moreover, lack of MAP kinase pathway activation has been described in MCC cells (Houben et al., 2006).

Based on increased MCC incidence in immunocompromised people, an infectious etiology was suggested. However, neither human papillomaviruses nor Epstein-Barr virus were detected in MCC (Kanitakis et al., 2006; Shaw et al., 2006).

In 2008, the discovery of Merkel cell polyomavirus integrated in the genome of MCC tumor cells by Feng *et al.,* was the first identification of a polyomavirus-induced cancer in humans (Feng et al., 2008). This research group, used a new method called digital transcriptome subtraction with the aim to identify foreign non-human transcripts in MCC. Feng *et al.* identified non-human transcripts harboring high homology levels with known polyomavirus sequences by comparing MCC tumor transcripts with human sequences databases. The sequence of this new polyomavirus, therefore called MCPyV, was shown to be integrated in tumor cell genome of about 80% of MCC cases (n=10) (Feng et al., 2008).

In 1971, the two first human Polyomaviruses, BKPyV and JCPyV were identified in urine and brain of patients with kidney transplant and progressive multifocal encephalopathy (PML) respectively (Gardner et al., 1971; Padgett et al., 1971). Additional investigations revealed widespread latent infections in the healthy population by these viruses (Kardas et al., 2015) and that their reactivation under immunosuppression can result in either BK-induced nephropathy or JC related-PML. Futhermore, some recent studies suggest an oncogenic potential of BK virus in kidney-transplant patients (Müller et al., 2018; Papadimitriou et al., 2016; Sirohi et al., 2018).

1.2.2. Transmission of MCPyV

A cutaneous transmission have been suspected for MCPyV. Indeed, MCPyV DNA is frequently detected on the skin of healthy individuals but also in the environement. The transmission of MCPyV occurs probably by direct or indirect cutaneous contact although the exact mode of transmission is not yet characterized (Foulongne et al., 2010, 2011). A seroepidemiological study has shown a correlation of MCPyV infection within the brotherhoods and between the child and his mother, these results are in favor of an early intrafamilial transmission by close contacts (Martel-Jantin et al., 2013) This childhood infection followed by a latent state in a majority of healthy individuals, could be reactivated upon host immunosuppression with a potential acquisition of

pathogenic properties. MCPyV seroprevalence reaches more than 75% in healthy adults (Martel-Jantin et al., 2013; Nicol et al., 2013; Tolstov et al., 2011; Touzé et al., 2010).

1.2.3. Genomic organisation of MCPyV

The genome of MCPyV is a circular double strand DNA, of 5.4 kb including three functional regions: a non-coding control region (NCCR), an early coding region and a late coding region (**Figure 1**) (Krump and You, 2021).

1) The early region encodes genes that are expressed prior to the onset of viral DNA replication : the large T (LT) small T (sT) antigens, T for “Tumor” reffering to the oncogenic properties of these viral proteins. These proteins are commun to all the polyomaviruses and are generated by alternative splicing. These proteins are involved in viral replication, the expression of viral genes and control of the host cell cycle. The 57kT (Gjoerup and Chang, 2010; Shuda et al., 2008) and the alternate frame of the LT open reading frame antigen (ALTO) (Carter et al., 2013) proteins might be expressed in MCC although their functions are still unknown.
2) The non-coding control region contains viral origin of replication (Ori) as well as bidirectional promotors controlling gene transcription of early and late regions. The Ori contains pentanucleotidic sequences (GAGGC ou GCCTC) linking up to LT origin-binding-domain (OBD) during viral replication initiation (Gjoerup and Chang, 2010).
3) The late region encodes capsid structural proteins: the major capsid protein, VP1 and the minor capsid proteins VP2 and VP3. The 3’ region of late transcripts encode also microRNAs playing a post-transcriptional regulation role (early viral transcripts) (Bauman and Mandelboim, 2011; Lee et al., 2011).

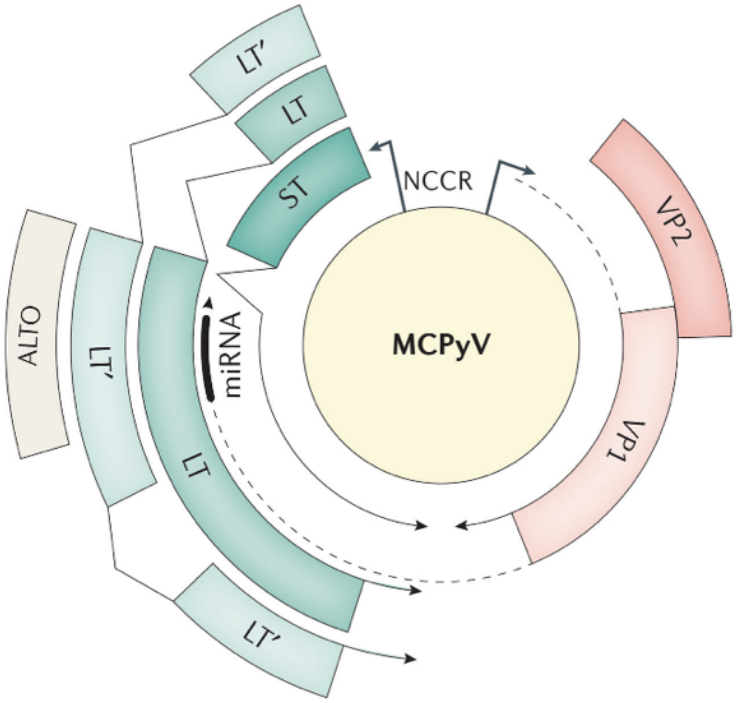

Figure 1. Merkel cell polyomavirus genomic organization (Becker *et al.,* 2017). Multiple transcripts are generated from the early region by alternative splicing and possibly alternative start sites, including large T antigen (LT), small T antigen (sT), LT' also known as 57 kT antigen (57 kT), alternative frame of the large T open reading frame (ALTO). The late region encodes two viral coat proteins, VP1 and VP2, and a microRNA targeting T antigen transcripts.

1.2.4. Oncogenic property of MCPyV

Oncogenic viruses interfere with the regulation of the host cell cycle, to promote phase S entrance and recruit cellular factors needed for viral replication and virions production (Moore and Chang, 2010). MCPyV genome integration in host cell genome is expected to be the start point of MCC oncogenesis although no integration hot spots has been described in the host cell genome (Starrett et al., 2020). Viral genome isolated from MCC tumors is characterized by a constante molecular signature (*i.e.* point mutations or deletions in the early region) leading to pre-matured non sens codons and truncated LT (ΔLT) expression while sT sequence is not affected by these alteration (Shuda et al., 2008). Both integration and mutations/deletion seem to be essential for oncogenesis. Nevertheless, these events are rare regarding MCPyV seroprevalence (Li et al., 2013). The fact that all MCPyV-positive MCC tumors harbors this molecular signature, is likely due to a selection biais. Indeed, integration of MCPyV genome with a full lenght LT could result in formation of multiple replication forks in cell

host genome and trigger DNA dammage responses and cell cycle arrest (Shuda et al., 2008) therefore resulting in cell death rather than transformation. LT truncation inhibits virus replication and induces oncogenic transformation. Moreover, truncation in the LT results in overexpression of this oncoprotein therefore promoting MCC tumor growth (Cheng et al., 2013).

The LT antigen contains the N-terminal J domain, a DNA binding stabilizer, required for viral replication. The LXCXE motif sequestrates the retinoblastoma protein (RB1) and allows the release of E2F transcription factor required for cell cycle progression. Origin binding domain (OBD), helicase domain and cell growth-inhibitory domain are lost due to the truncation (Laude et al., 2010). Merkel unique region (MUR) 1 and 2 is a specific MCPyV LT domain (Wendzicki et al., 2015).

Regarding the sT, the N-terminal J domain is dispensable for replication while protein phosphatase 2A (PP2A) targeting by sT is required to enhance viral replication (Kwun et al., 2009). Ubiquitin ligases are sequestrated by LT stabilisation domain (LSD) of sT, this interaction increase LT protein levels and induce viral replication (Kwun et al., 2013). However, the mechanism is not fully understood, since ubiquitin ligases were found to play no role in LT degradation (Dye et al., 2019). Moreover, sT activates cap-dependant translation by keeping 4EBP1 protein in an hyperphosphorylated state (Shuda et al., 2011).

Truncated LT has no C-terminal functionals domains such as OBD, helicase/ATPase leading to loss of replication ability and loss of capsid proteins expression. By contrast the binding domain to RB1 is always preserved in the truncated LT (Shuda et al., 2008), and is required for MCC cells proliferation (Houben et al., 2012).

Indeed, both T antigens (*i.e.* sT and truncated LT) are required for MCC cell lines proliferation while only sT is able to induce the cell transformation in mice fibroblasts (Shuda et al., 2011).

Additionnal mechanisms are expected to contribute to MCC tumorigenesis such as inhibition of the p53 in MCC virus-positive cell lines (Houben et al., 2013). RB1 sequestration by the truncated LT activates E2F transcription factors inducing increased ARF level, an inhibitor of the major p53-degrading E3 ligase MDM2 and trigger p53 activation (Park et al., 2019). While sT is an indirect transcriptional activator of MDM2 and also CK1α that activates MDM4. Both MDM2 and MDM4 inhibit p53

function (Park et al., 2019). The functional domains of the T antigens bind to cellular proteins to disregulate the cell cycle and inhibit apoptosis (Erovic et al., 2013).

Interestingly, UV can impact integration, viral genome mutations and are associated to an increased sT transcription (Mogha et al., 2010). Indeed, *in vitro* MCPyV early phase promotor is UV-inductible and an increased sT mRNA levels has been reported. Nevertheless, UV radiation could concomittantly activate the splicing machinery since LT mRNA level was unchanged while a commun promotor drives sT and LT transcription (Mogha et al., 2010).

1.3. MCPyV-negative and MCPyV-positive MCCs

About 20% of the MCC cases are not related to viral integration. DNA damages caused by UV exposure are regarded as the main oncogenic factor of MCPyV-negative tumors (**Table 1**), as suggested by several findings: 1) high mutation burden similar to those observed in melanoma with approximately 1,100 somatic single nucleotide variants per-exome (Carter et al., 2018; Goh et al., 2016; Starrett et al., 2017; Sunshine et al., 2018; Yarchoan et al., 2019), 2) UV-mutational signature with cytosine to thymidine transitions (Carter et al., 2018; Goh et al., 2016; Starrett et al., 2017), 3) association of MCPyV-negative MCC with other UV-induced neoplasms *i.e.* squamous cell carcinoma and precursors (Kervarrec et al., 2022; Walsh, 2001). Frequent alterations of genes involved in proliferation signaling pathways such as *PIK3C, HRAS* and *NOTCH* (Harms et al., 2015), damaging mutations of tumor suppressor *i.e. TP53* and *RB1* (Carter et al., 2013; Goh et al., 2016; Starrett et al., 2017), mutations in genes involved in chromatin modification *i.e. ASXL1*, *MLL2*, and *MLL3* and in DNA-damage repair *i.e. ATM*, *MSH2*, and *BRCA1* (Goh et al., 2016) are observed in this MCC subset.

Virus-positive and UV-induced MCC present a common neuroendocrine phenotype that could be explained by the alterations of similar oncogenic pathways by T antigens or somatic mutations, such as *RB1* and *TP53* inactivation (González-Vela et al., 2017; Starrett et al., 2017). However, variations in phenotype and behavior have been reported between both subsets. UV-induced cases seem to be associated with worst outcome (Moshiri et al., 2017). Morphologically, MCPyV-positive tumor cells typically harbor scant cytoplasm and round nuclei, with clear chromatin, whereas more irregular nuclei and more abundant clear cytoplasm are observed in MCPyV-negative cases (Iwasaki et al., 2013; Kervarrec et al., 2019; Kuwamoto et al., 2011). Futhermore,

presence of a divergent component is suggestive of MCPyV-negative tumors (Carter et al., 2018; Foschini and Eusebi, 2000; Martin et al., 2013). Ulceration, hyperkeratosis or an intraepidermal component are also associated with MCPyV-negative cases (Kervarrec et al., 2019; Nagase et al., 2019). Moreover, UV-induced cases are frequently negative for cytokeratin 20 (Miner et al., 2015). Such phenotypic variations are expected to reflect the genetic background variations between MCPyV-positive and -negative MCC cases. Harms *et al.* suggest an epidermal origin for MCC (Harms et al., 2022). Recently, this finding was further strengthened by Kervarrec *et al.* shown by whole exome sequencing many shared mutations and common copy number variation profil between the squamous cell carcinoma and virus-negative MCC components of combined tumors, further confirming a keratinocytic origin of some MCPyV-negative cases (Kervarrec et al., 2022).

Table 1. Distinct characteristics of MCPyV-positive and MCPyV-negative MCC.

Features	MCPyV-positive MCC	MCPyV-negative MCC	References
Prevalence	80%	20%	Feng *et al.,* 2008
Pronostic	Better than MCPyV-negative MCC	Unfavorable	Sihto *et al.,* 2009
Oncogenic triggers	MCPyV T antigen: sT and truncated LT	UV-induced mutations	Feng *et al.,* 2008 Harms *et al.,* 2015
Morphology Nucleus Cytoplasm	 Round Few	 Irregular/spindle More abundant	Iwasaki *et al.,* 2013
Immunohistochemical markers KRT20 KRT8 TTF1 Neurofilament	 + (dot-like pattern) + (dot-like pattern) + +	 +/- + +/- -	Kervarrec *et al.,* 2019
RB1* and *TP53	Wild-type	Inactivating mutations	Harms *et al.,* 2015
Mutation load	Low	High	Goh *et al.,* 2016

(+) frequent positivity, (-) frequent negativity, (+/-) increased or decrease expression frequency compared to MCPyV-positive MCC.

1.4. What is the cell of origin of MCC?

Although the physiological Merkel cells have been regarded for a long time as the cell of origin of MCC, whether these cells might be infected by the MCPyV and give rise to MCC is at least doubtful. In 2018, MCC international experts assumed that no

consensus has been reach for the MCC cell of origin (Harms et al., 2018). Potential candidates were epithelial progenitors, dermal mesenchymal stem cells, fibroblasts and pro/pre-B cells among others (**Figure 2**). In a transgenic mice model, conditional expression of viral antigens in transcription factor atonal homolog 1-reprogrammed epidermal cells in skin induce hair follicle-derived MCC like tumor transformation (Verhaegen et al., 2015, 2022). Our team had investigated a case of a tumor combining virus-positive MCC and trichoblastoma (*i.e.* cutaneous tumor derived from follicular germinative cells) providing evidence of an epithelial origin of MCC (Kervarrec et al., 2020a). An epithelial origin has also been suggested from investigation of tumors combining UV-induced MCC and squamous cell carcinoma (Harms et al., 2022; Kervarrec et al., 2022). Determined the cell of origin of MCC is a key question, allowing to understand tumor initiation, to develop experimental models and therefore providing new clues for diagnosis and treatments.

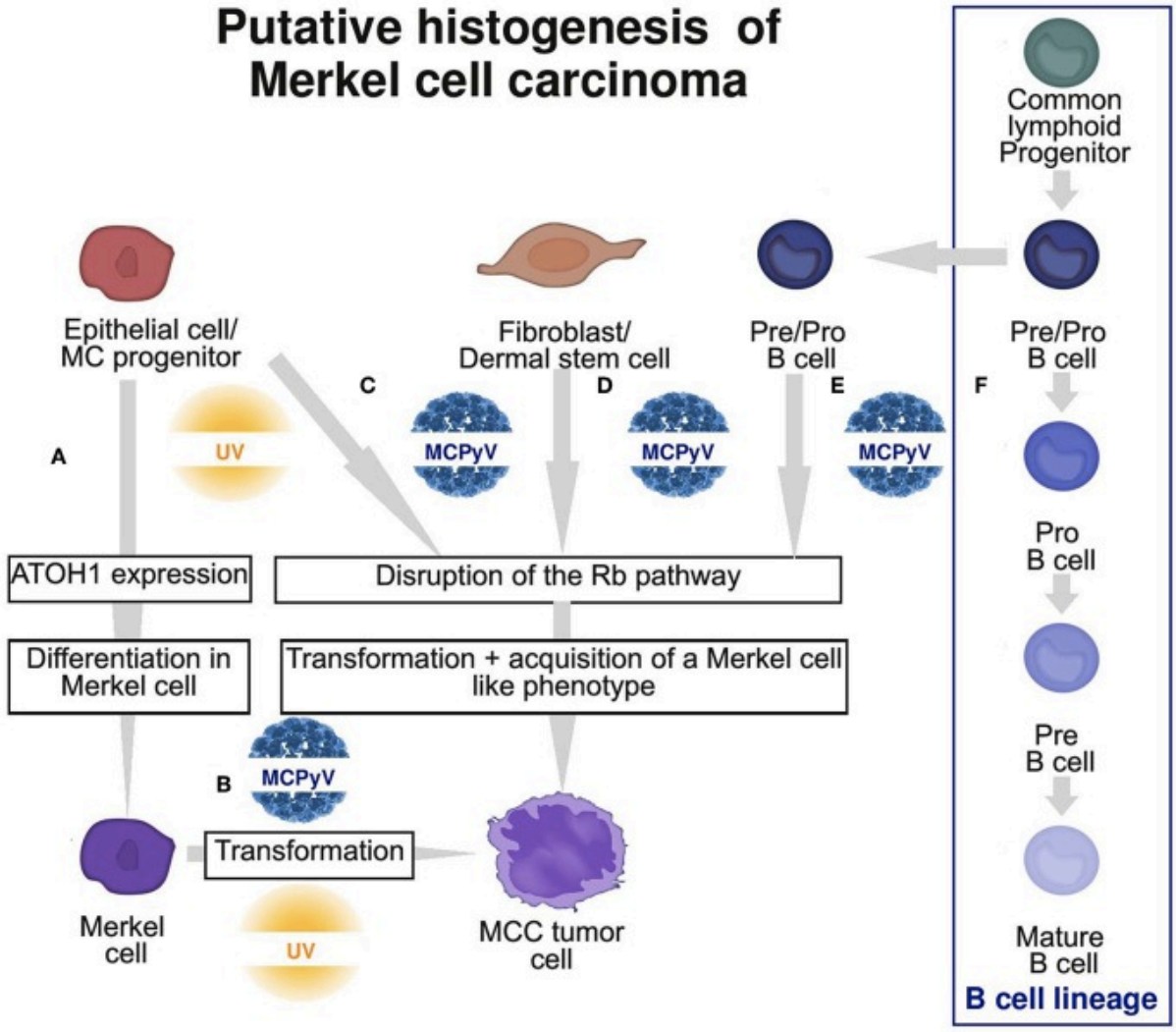

Figure 2. Hypothesis for cell of origin of MCC. (A) Physiological Merkel cell differentiation (B) First hypothesis: physiological Merkel cell as the cell of origin of MCC, suggesting that T antigens can induce transformation in this cell type. (C–E) Second hypothesis: oncogenic events occur in a non-Merkel cell and induce transformation and acquisition of a Merkel cell-like phenotype. Potential ancestries are epithelial progenitors (C), fibroblast/dermal stem cells (D) or pre/pro B cells (E) from the B cell lineage (F). MCPyV, Merkel cell polyomavirus, from Kervarrec *et al.,* 2019.

1.5. Epidemiology of MCC

Due to MCC rarity and the lack of large international registries, the exact MCC incidence is difficult to evaluate (Schadendorf et al., 2017). About 1,600 new cases are diagnosed each year in the United States (US) (Paulson et al., 2018a) and estimated incidence rates of 0.70 and 0.17-0.30 cases/100 000 persons/year have been reported in the US (Fitzgerald et al., 2015) and in Europe (Eisemann et al., 2014; Kieny et al., 2019), respectively. Most recent reports revealed an increasing MCC incidence over

the two last decades that might be explained by several factors, including population ageing and improved diagnostic tools (Schadendorf et al., 2017).

MCC arises mostly in the elderly, with a median age of 75 years at diagnosis. Less than 5% of the patients are diagnosed before 50 years (Albores-Saavedra et al., 2010). MCC predominantly occurs in white males (Schadendorf et al., 2017). Immunosuppression due to hematologic malignancies, HIV infections or immunosuppressive treatments after organ transplant is associated with an increased risk of MCC (Asgari et al., 2014; Cook et al., 2019) and an impaired outcome (Cook et al., 2019; Paulson et al., 2013). Compared to the general population, a ten-fold and a three-fold increased incidence of MCC is observed in HIV patients (Engels et al., 2002) and in patients with chronic lymphoid leukemia, respectively (Cook et al., 2019; Heath et al., 2008). As for other skin carcinomas, sun exposure constitutes a risk factor of MCC, most MCC arising in sun exposed areas (*i.e.* head and neck, lower limbs and upper extremities) (Heath et al., 2008).

1.6. MCC diagnosis

MCC appears as a rapidly growing, painless isolated nodule, frequently without alteration of skin surface (**Figure 3**). MCC characterstic key clinical features have been summarized as the “AEIOU” criteria (Heath et al., 2008): Asymptomatic, Expanding rapidly (< 3 months), Immunosuppression, Older than 50 years, UV exposed site. Even if 89% of MCC cases fulfill at least three of these criteria, MCC are frequently misdiagnosed as other skin malignancies or even benign tumors (Heath et al., 2008). Although MCC frequently arises in sun exposed area especially head and neck (Heath et al., 2008; Schadendorf et al., 2017), it can appear on other locations such as limbs, buttocks or trunk. Interestingly, a small portion of MCC might arise from mucosae (Islam et al., 2018; Lewis et al., 2010) or be diagnosed as lymph node metastasis without skin primary tumors (de Biase et al., 2012; Pan et al., 2014). At diagnosis, the median tumor size is approximately 2 cm but can vary from 0.1 to more than 7 cm (Iyer et al., 2014). Dermoscopy use can contribute to MCC diagnosis (Dalle et al., 2012), however microscopic examination of a skin biopsy is mandatory to establish the diagnosis.

Microscopically, MCC tumor cells harbor a high nucleocytoplasmic ratio, scant cytoplasm, round/ovale nucleus with frequent nuclear molding and fine, granular so called “salt and pepper” chromatin. Mitotic figures and large areas of necrosis are

frequent and reflect tumor aggressiveness. Immunohistochemical investigations confirm both epithelial and neuroendocrine differentiation of MCC tumor cells (**Figure 3**). MCC tumors express epithelial markers *i.e.* pancytokeratins (García-Caballero et al., 2003), and neuroendocrine markers *i.e.* chromogranin A, synaptophysin, CD56 (Fernández-Figueras et al., 2007) and insulinoma-associated 1 (INSM1) (Leblebici et al., 2019). Moreover, several markers are used to distinguish MCC from extracutaneous neuroendocrine carcinomas (Kervarrec et al., 2019; Thanguturi et al., 2022). Notably most MCC cases express cytokeratin 20 (KRT20) (Cheuk et al., 2001) with paranuclear dot-like pattern as well as neurofilament (Stanoszek et al., 2019) and the special AT-rich sequence-binding protein (SATB2) (Bellizzi, 2020).

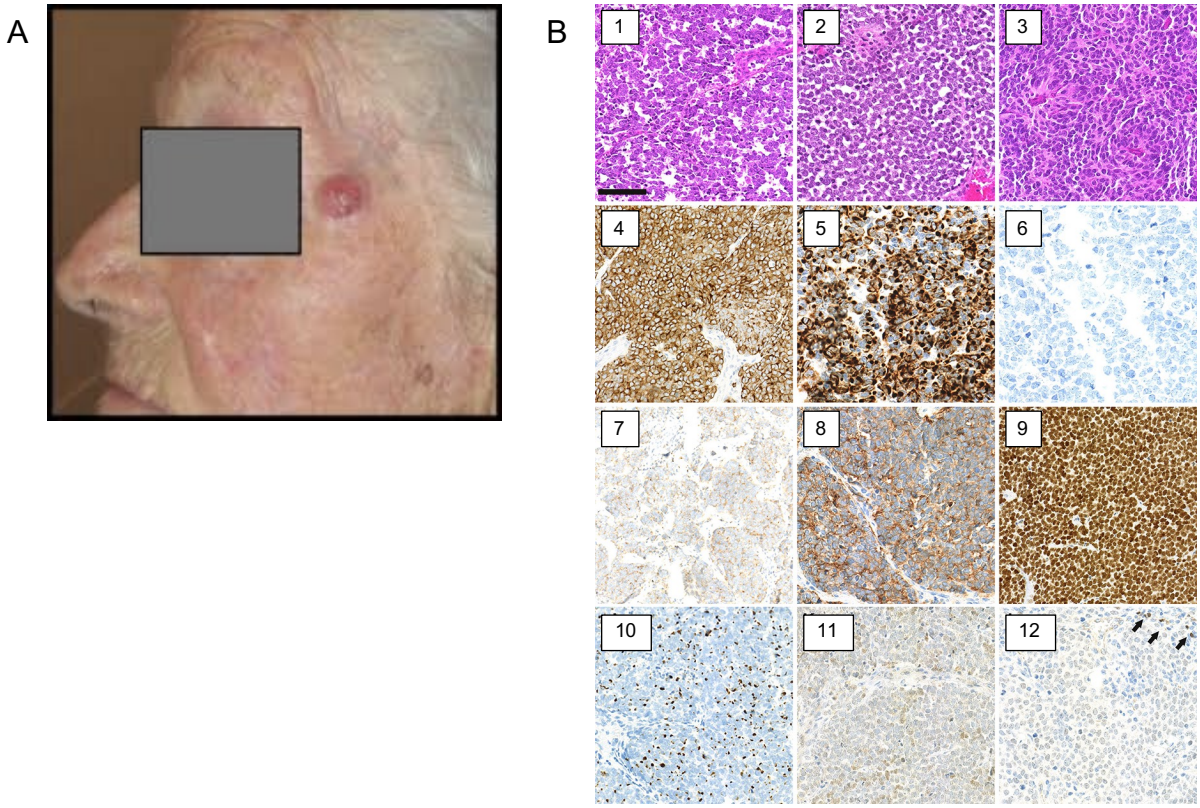

Figure 3. Merkel cell carcinoma characteristics, clinically and histologically. (A) The MCC tumor of the face of an 87-year-old woman. MCC tumors frequently develop in UV-exposed areas as a fast-growing erythematous or violaceous nodule (adapted from Samimi *et al.,* Semin. Oncol., 2014). (B) MCC morphological and histological features (adapted from Kervarrec *et al.,* Front Oncol., 2019). Panel B: (1–3) hematein-phloxin-saffron staining revealed sheet of tumor cells with high mitotic activity (scale = 100 µm). Whereas MCPyV-positive MCC (1,2) harbor scant cytoplasm, round nucleus and dusty chromatin, MCPyV-negative tumor cells have an abundant clear cytoplasm and irregular nucleus (3). (4) chromogranin A cytoplasmic positivity, (5) cytokeratin 20 expression with paranuclear dot-pattern; (6) thyroid transcription factor-1 negativity; (7) membranous synaptophysin expression; (8) membranous CD56 expression; (9) special AT-rich sequence-

binding protein 2 (SATB2) nuclear expression; (10) neurofilament expression with a dot-pattern; (11) terminal deoxynucleotidyl transferase weak/moderate expression, (12) paired box 5 weak expression in tumor cells in comparison with intratumor lymphocytes (arrows).

1.7. MCC tumor staging

At time of diagnosis, a physical examination is required to detect cutaneous metastasis, palpable regional or distant adenopathy (Gauci et al., 2022). A regional lymph node ultrasonography is recommended, and most recent data argue for systematic assessment with a whole body computed tomography (CT) scan and/or a 18-FDG-PET (Singh et al., 2021). Especially, 18-FDG-PET is more sensitive than conventional imaging technics for MCC metastasis detection (Colgan et al., 2012; George et al., 2014). If no metastasis is observed after extension work-up, resection and histological analysis of sentinel lymph node are recommended during initial surgery. Sentinel lymph node procedure allows the detection of micrometastasis in 1/3 patients (Allen et al., 2001; Fields et al., 2011; Jouary et al., 2015), this latter being associated with an impaired prognosis (Jouary et al., 2015; Lemos et al., 2010; Paulson et al., 2013; Shibayama et al., 2015).

The MCC staging according to the 8th American Joint Committee on Cancer (AJCC) classification has been established from analysis of 9,387 MCC follow-up and staging data (between 1998 to 2012) cases from the National Cancer Data Base (Harms et al., 2016). The new AJCC 8th edition MCC staging system is based on tumor size, the presence of nodal micro and macrometastasis, and distant metastases (**Table 2**).

MCC typically shows an aggressive course, with a high metastatic potential. At initial diagnosis, 65% of patients present a localized tumor, while 26% and 9% already harbor regional nodal metastasis and distant metastasis, respectively (Harms et al., 2016). MCC “with no primary” (or “occult primary”), which are diagnosed on metastasis to lymph node without any evidence of skin tumor, represent approximatly 12% of MCC cases (Chen et al., 2013; Deneve et al., 2012; Kervarrec et al., 2018a). After treatment, rates of relapse are still high, as 30 to 40% of patients present local or distant recurrence usually within the 2 years after diagnosis (Allen et al., 2005; van Veenendaal et al., 2018). The 5 years survival rates for localized, regional and distant stages are 64, 39 and 18%, respectively (Lemos et al., 2010; Paulson et al., 2013).

Table 2. Staging of Merkel cell carcinoma from the 8th edition of the AJCC stage from Harms *et al.* 2016.

Stage	Primary tumor	Regional lymph node	Distant metastasis	5-year overall survival regarding pathological stage (%)
0	Tis	N0	M0	
I	T1	N0	M0	62.8
IIA	T2-3	N0	M0	54.6
IIB	T4	N0	M0	34.8
IIIA	T1-4	N1a(sn) or N1a	M0	40.3
IIIB	T1-4	N1b-3	M0	26.8
IV	T0-4	Any N	M1	13.5

Site	Stage	Criteria
Primary tumor	T0	no evidence of primary tumor
	Tis	in situ primary tumor
	T1	maximum clinical tumor diameter ≤ 2 cm
	T2	maximum clinical tumor diameter > 2 cm but ≤ 5 cm
	T3	maximum clinical tumor diameter > 5 cm
	T4	primary tumor invades fascia, muscle, cartilage or bone
Regional lymph node	N0	no regional metastasis detected
	N1a(sn)	clinically occult nodal metastasis identified only by sentinel lymph node biopsy
	N1a	microscopic lymph node metastasis clinically occult
	N1b	microscopic confirmation of clinically detected regional lymph node metastasis
	N2	in transit metastasis without lymph node metastasis
	N3	in transit metastasis with lymph node metastases
Distant metastasis	M0	no evidence of distant metastasis by clinical or radiological examination
	M1	microscopic confirmation of metastases

1.8. Immune response against MCC

1.8.1. Cancer immune responses

Following the general concept of immunoedition in cancers, three successives stages are required before cancer establishment: i) destruction of transformed cells by the host immune system ; ii) steady state in which immune system controls the growth

of transformed cells ; iii) immune escape (Dunn et al., 2002). Both innate and adaptative immune responses are involved in tumor control. Indeed, interactions between tumor and immune cells include presentation of released tumors antigens by antigen-presenting cells, interaction and activation of T cells leading to engagement of target tumor cell by the activated effector T cell. Although anti-cancer immunity often prevents tumor growth, tumor could escape immune response through several mechanisms (Hiam-Galvez et al., 2021).

1.8.2. Impact of immunosupression in MCC developement

Immune status seems to be involved in both MCC initiation and progression. Indeed as stated above, patients with immunosuppression due to HIV infection, organ transplant or chronic lymphocytic leukemia, are at increased risk of MCC development (**Figure 4**) (Clarke et al., 2015; Engels et al., 2002; Heath et al., 2008). Moreover, immunosuppression is an independent risk factor of mortality in MCC patients (Paulson et al., 2013; Tarantola et al., 2013). Accordingly, MCC regression has been reported after withdrawal of immunosuppressive agents *i.e.* azathioprine (Muirhead and Ritchie, 2007) or ciclosporine (Friedlaender et al., 2002). Given that only 10% of MCC patients have such “obvious” immunodeficiencies, we can hypothesize that, in most patients, occurrence of the cancer is promoted rather by loss of immune surveillance with advanced age. Indeed, transcriptomic analyses of MCC tumors sampled from patients with a favorable outcome have evidenced an overexpression of T immune response-associated molecules (cytotoxic granules such as granzyme A, B, H, and K, CCL19 chemokine, lymphocyte activation gene…) (**Figure 4**) (Paulson et al., 2011). Immune response could also explain MCC spontaneous regression sometimes observed after a tumor biopsy (about 1.4% of reported cases) (Koba et al., 2012). An enhanced post-biopsy intratumoral lymphocytic infiltrate was hypothesized to explain this phenomenon, but was not confirmed by immunohistochemical studies (Koba et al., 2012).

1.8.3. Humoral response against MCPyV

Humoral immune response against MCPyV proteins (T-antigens) is detected specifically in approximately 50% of MCC patients and track disease burden. Indeed, the T-antigens are expressed in the nucleus of tumoral cells and are detected by the immune system when tumor cells are in apoptosis (Nakamura et al., 2010).

Interestingly, in some MCPyV-positive MCC patients, anti-T antigens circulating antibodies are highly detected at diagnosis while the antibody titers drop rapidly after successful treatment of MCC tumors (**Figure 4**), such finding arguing for their use for monitoring patients during follow-up (Paulson et al., 2010; Samimi et al., 2016). The viral capsid proteins, which constitute the structural proteins of MCPyV viral particules, also induce an host immune response which occurs during childhood, when primo-infection by the ubiquitary MCPyV occurs. As such, antibodies targeting capsid proteins are detected in the general population as well as MCC patients, but at higher titers in the latter. In addition, high level of anti-capsid proteins have been associated with progression-free survival in MCC patients (**Figure 4**) (Samimi et al., 2016; Touzé et al., 2011). One hypothesis is that MCC patients are exposed to a greater virus burden at replicative state preceeding MCC development.

1.8.4. T-cell response

A cellular immune response against MCPyV antigens is also present in MCC patients. In a pilot study, circulating CD4+ and CD8+ T cells recognizing the MCPyV proteins epitopes have been identified in the blood of 14/27 MCC patients and of 5/13 healthy donors (**Figure 4**). These lymphocytes were specific of the viral capsid epitopes (2 epitopes) or MCPyV T antigens (24 epitopes) (Iyer et al., 2011). From blood of 38 MCC patients and 30 healthy donors, 53 CD8+ T cell responses have been identified against 35 differents MCPyV peptides. Both circulating and intratumoral T-cells directed against T antigens were detected in MCC patients (Lyngaa et al., 2014) and were associated with high tumor burden (Afanasiev et al., 2013a). Although, UV-induced tumors have a high mutational burden, which is associated with increased probability of presenting neoantigens derived from mutated peptides that can be targeted by the immune system (Goh et al., 2016).

Intratumor infiltrate is associated to MCC natural history (**Figure 4**). A brisk CD8+ cells intratumor infiltrate was confirmed to be a good prognostic factor in independants large MCC series (**Figure 4**) (Paulson et al., 2011, 2014a). Sihto *et al.* characterized intratumor immune infiltrate in 116 MCC and demonstrated that dense intratumor CD3+, CD8+, or FoxP3+ T cells infiltrates and high CD8+/CD4+ ratio was associated with a favorable prognosis regardless the MCC MCPyV status (Sihto et al., 2012). However, programmed death-ligand 1 (PD-L1) and PD-L2 ligands are expressed by MCC tumor cells and/or by tumor microenvironement cells (*e.g.* dendritic cells and

macrophages) (Afanasiev et al., 2013a; Dowlatshahi et al., 2013; Lipson et al., 2013). *Ex vivo,* MCC tumor infiltrating CD8+ T cells showed an exhausted phenotype notably through the expression of programmed cell death protein 1 (PD-1) and Tim-3 (**Figure 4**) and the reduced production of IFNγ in response to LT specific peptides. Nevertheless their cytotoxic properties were restored after incubation with antibodies targeting PD-1 and Tim-3 constituting a rationnal for the use of immunotherapy in MCC.

1.8.5. Tumor evasion mechanisms and interest for immunotherapies

As for many other tumors, MCC is able to escape from the host immune response by several strategies.

First, the tumor can hide itself from the immune system by downregulating the cell surface expression of major histocompatibility complex class I, which is necessary for tumor antigen presentation to the adaptive immune system. The suppression of all class I human leukocyte antigen (HLA) genes must be distinguished from differential suppression of a single HLA allele targeted by specific CD8 T-cells. Both mechanisms have been demonstrated in MCC (Paulson et al., 2014b, 2018b).

For the rare patients with brisk intratumor CD8+ cells, infiltrate is restricted to the junction between tumor and stroma in most of the cases (Wheat et al., 2014). This phenomenon of lymphocytes exclusion was described as an impaired T cell migration due to the downregulation of intratumoral vascular E-selectin, an adhesion molecule that elicits the migration of cutaneous T cells, and may represent a tumor escape mechanism (Afanasiev et al., 2013b; Paulson et al., 2011; Wheat et al., 2014).

Another immune evasion mechanism has been identified by *ex vivo* characterization of MCC patients T cells infiltrate. These lymphocytes expressed inhibition markers (PD-1, Tim-3 and CTLA-4), consistent with a lymphocyte phenotype exhaustion (**Figure 4**) (Afanasiev et al., 2013a). The PD-1 ligands, PD-L1 and PD-L2, are found in both MCC tumor cells and the microenvironment (macrophages and dendritic cells) (Afanasiev et al., 2013a; Dowlatshahi et al., 2013; Lipson et al., 2013). As expected, these T cells did not induce cytotoxic responses against autologous tumor cells *ex vivo*, despite *in vitro* treatment with immunostimulating cytokines *i.e.* IL-2 and IL-15, or with anti-PD-1 and Tim-3 antibodies enabling activation, proliferation and recovery of lymphocytes cytotoxic functions (Dowlatshahi et al., 2013). These

results support the use of immune checkpoint inhibitors (ICI) to restore T-cell responses.

Importantly, MCC tumors are not only infiltrated by T-cells but also by macrophages (CD68+ and CD163+), evidenced as the main source of PD-L1 expression within the microenvironement (Kervarrec et al., 2018b; Lipson et al., 2013; Sihto et al., 2012; Wheat et al., 2014). Tumor-associated macrophages usually belong to the M2 tolerogenic subset exert immunosuppressive effect by modulating immune response. Moreover, these tumor-associated macrophages enhance vascular density by promoting vascular endothelial growth factor (VEGF) production (Elliott et al., 2017). By contrast to T-cell infiltrates, CD68+ or CD163+ myeloid infiltrates have not been associated to MCC prognosis (Lipson et al., 2013; Sihto et al., 2012), whereas intratumoral infiltration by cells expressing the early myeloid differentiation marker CD33+ was associated with improved outcome (Kervarrec et al., 2018b). An improved outcome was also observed in MCC cases infiltrated by natural killer (NK) cells (Laniosz et al., 2017). Although most MCC tumors evade NK-cell mediated lysis by downregulating the MHC class I chain-related protein (MIC) A and B (Ritter et al., 2016).

Altogether, the immune microenvironment plays a crucial role in controlling MCC development, paving the way for several therapies aiming to restore and/or activate such immune responses.

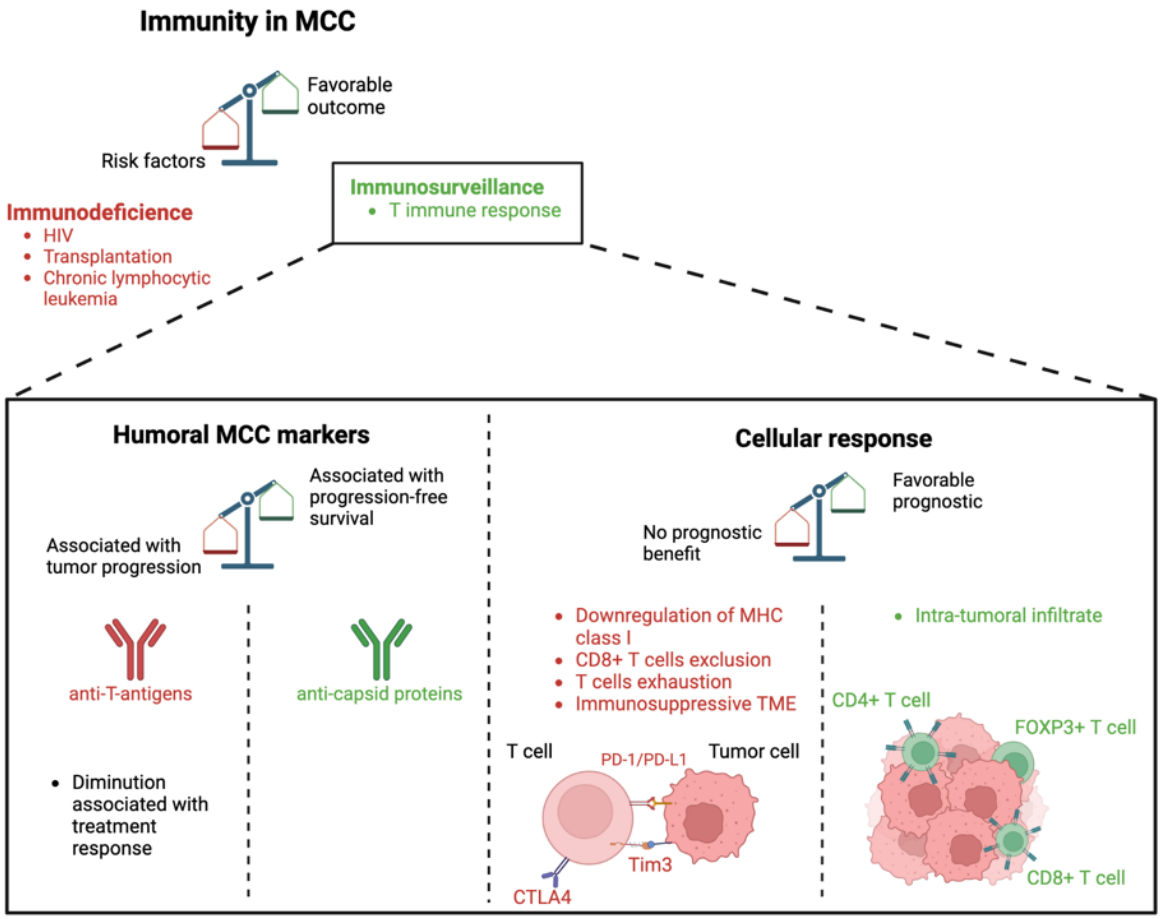

Figure 4. Role of immunity in MCC. MCC risk factors (*i.e.* human immunodeficiency virus (HIV), organ transplantation, chronic lymphocytic leukemia) and immunosurveillance associated with favorable outcome for patients with MCC. Both humoral and cellular responses could be correlated with favorable (in green, antibodies targeting capsid proteins / intratumoral infiltrate) or poor prognostic (in red, antibodies targeting T-antigens / downregulation of MHC class I, T cells exclusion, T cells exhaustion or immunosuppressive tumor microenvironment (TME)).

1.9. Merkel cell carcinoma treatments

The following sections are written in line with the last european recommendations for MCC management (Gauci et al., 2022) and the National Comprehensive Cancer Network guidelines, although evidence-based data remains sparse because of the rarity of MCC. Treatment depends on initial staging of MCC, follow-up can be based on clinical examinations and nodal ultrasound every 4 months during the first 3 years then every 6 months for up to 5 years (Lebbe et al., 2015). CT or PET CT might be proposed every year for 5 years. Advanced MCC are manage following decision of dermatologist, surgeon and radiotherapist (Gauci et al., 2022).

1.9.1. Surgery

At the localized stage, treatment consists in a complete excision surgery with histologically clear margins combined with sentinel lymph node procedure (Becker et al., 2017). The complete excision with clinical safety margins of 1 cm are followed by adjuvant radiotherapy. Interestingly, an alternative surgery, which consists in the progressive removal and examination of the cancer tissue until only cancer-free tissue remains, called Mohs micrographic surgery, has been compared to the classical local excision and similar overall survival and cancer-specific survival rates was reported (Shaikh et al., 2018; Singh et al., 2018; Su et al., 2018).

Sentinel lymph node biopsy is recommend for the staging procedure of patients with stage I-II MCC in order to identify lymph node metastasis (Gauci et al., 2022). If a macroscopic nodal metastasis is detected the recommendation is to proceed to a complete lymph node dissection (Gauci et al., 2022).

1.9.2. Radiotherapy

MCC is sensitive to radiation (Leonard et al., 1995), indeed, radiotherapy is an interesting therapeutic at various MCC stage. After surgical excision, adjuvant radiotherapy improved overall survival in stage I-II MCC compared to surgery alone without adjuvant radiotherapy (Bhatia et al., 2016). Adjuvant radiotherapy of primary tumor and lymph node area should be considered on an individual basis (Becker et al., 2017; Tseng et al., 2017).

1.9.3. Chemotherapies: previous standard of care

Historically, advanced metastatic MCC *i.e.* stage III or stage IV, have been treated with platinum-based drugs, etoposide, taxanes and anthracyclins (such as cyclophosphamide, vincristine, doxorubicin, and topotecan), either alone or in various combinations in first line setting, for which a median response rates of 57% were observed (Nghiem et al., 2017). Conventional chemotherapies were associated with poor outcomes. Moreover, responders patients present a short term recurrence (*i.e.* median response duration: 3 months) and important adverse effects were also reported such as myelosuppression, sepsis, fatigue, alopecia, nausea/vomiting and renal injury (Nghiem et al., 2017; Samimi et al., 2019). Therefore, chemotherapy should be consider for patients with immunotherapy contraindications or as a palliative strategy (Gauci et al., 2022).

1.9.4. Immunotherapies

The low efficiency of these therapies in advanced stages led to several single-arm trials investigating ICI, either blocking PD-1 (pembrolizumab and nivolumab) or PD-L1 (avelumab) (**Figure 5A**).

1.9.4.1. Avelumab

Since 2017, avelumab is approved in US and Europe for the treatment of advanced MCC patients. Avelumab is a fully human IgG1 monoclonal antibody that binds PD-L1 and therefore inhibits its interaction with PD-1. Avelumab has also been shown to contributes to disease control through antibody-dependent cell-mediated cytotoxicity (ADCC) on PD-L1+ tumor cells *in vitro* (Boyerinas et al., 2015). In first line, avelumab allow responses in 50% of metastatic stages (D'Angelo et al., 2018). In second line, 30% of MCC patients responded to avelumab (**Figure 5B**) (D'Angelo et al., 2020; Kaufman et al., 2018). After avelumab approval, a phase IV study including 494 patients with metastatic MCC treated with avelumab aimed to confirm previous clinical observations in a larger cohort. The objective response rate was of 46.7% in 240 evaluable patients (Walker et al., 2020).

1.9.4.2. Pembrolizumab

In addition to avelumab, the first approved drug in this settings, other ICI have shown promising results. In 2018, pembrolizumab was FDA-approved in the same setting. Pembrolizumab is an IgG4 humanized monoclonal antibody targeting PD-1. In 2018, the US NCCN and in 2019 S2K German guidelines recommended ICI as first line therapies for all patients with advanced MCC (Becker et al., 2019; Bichakjian et al., 2018). Pembrolizumab was evaluated in patients with untreated advanced MCC and the objective response rate was 56% with 24% of complete response and 32% of partial response (Nghiem et al., 2019).

1.9.4.3. Nivolumab

Nivolumab is a fully human monoclonal IgG4 antibody that also targets PD-1. Nivolumab is approved by the FDA as monotherapy for treating advanced melanoma, lung cancer, Hodgkin's lymphoma, colorectal cancer, renal cell carcinoma, urothelial carcinoma, head and neck cancer and hepatocellular carcinoma. A clinical study that

evaluate nivolumab for untreated advanced MCC patients or MCC patients which received 1 or 2 previous systemic therapies is ongoing (NCT02488759).

To note, avelumab, pembrolizumab and nivolumab have shown comparable response rate, approximately 50 to 60% in a first line setting.

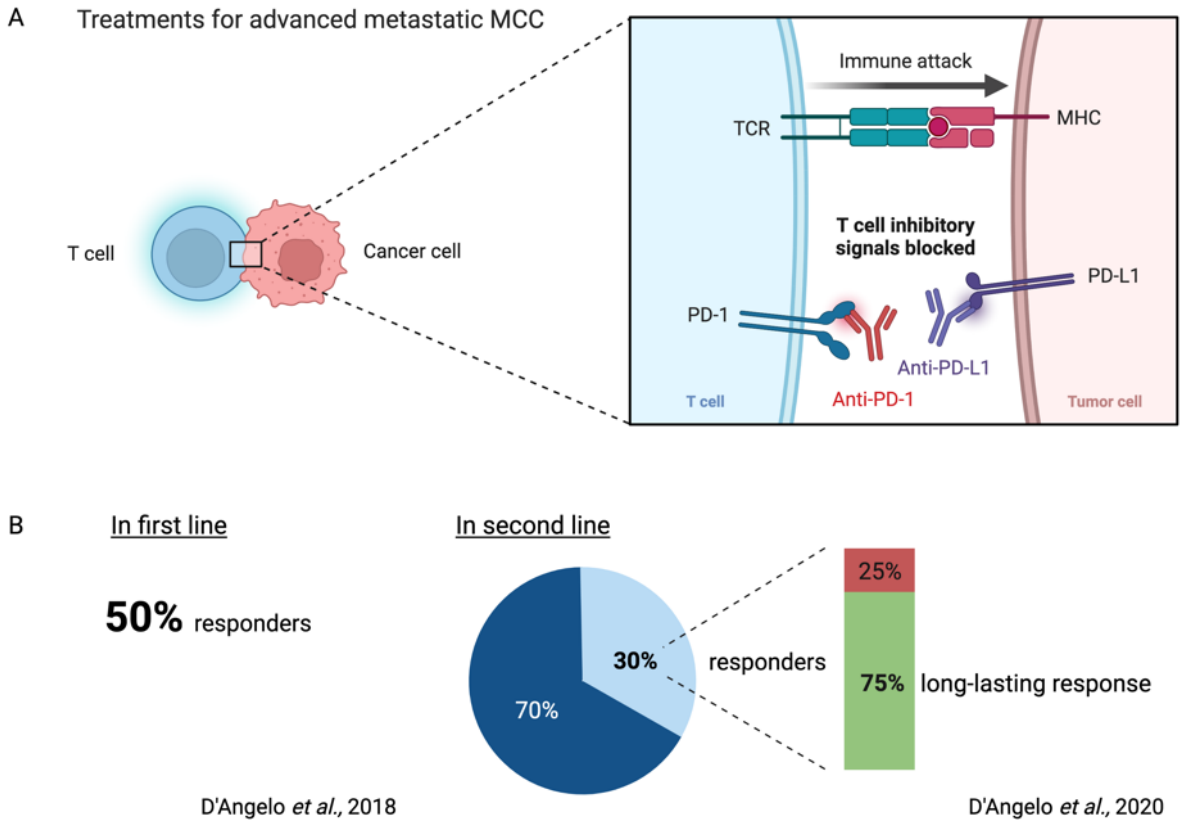

Figure 5. Current treatments for advanced metastatic MCC. (A) Mode of action of antibody targeting PD-1 and PD-L1 express by T cells and tumor cells respectively. (B) Percentage of response of patients with metastatic MCC treated with immune checkpoint inhibitors in first and second line adapted from D'Angelo *et al.,* 2018 and 2020.

1.9.5. Immunotherapies limits

In MCC, PD-1/PD-L1 inhibitors were most of the time well-tolerated, 11.4 to 28% of patients had grade 3-4 adverse events and treatment-related adverse events leading to treatment discontinuation occur in 9.1 to 15.4% of patients (D'Angelo et al., 2018, 2020; Nghiem et al., 2019). No biomarker (*i.e.* PD-L1 expression, tumor mutational burden or MCPyV status) has been associated to ICI clinical response (D'Angelo et al., 2020). Consequently, avelumab treatment is not restricted by any prior assessment. Approximately 50% of patients do not respond to anti-PD-1/PD-L1

antibodies in a first-line setting, underlying the needs of alternative options for patients who do not respond to ICI.

1.9.6. Other potential immunotherapies

For advanced MCC, several other immune-based strategies are being investigated, some examples are explain below. Intratumoral immune infiltration, immune recognition and activation are regulated by pro- and anti-inflammatory molecules within the tumor-immune microenvironment. Therefore, intratumoral electroporation of DNA encoding the potent pro-inflammatory cytokine IL-12 has demonstrated that local expression of IL-12 induces inflammation and led to systemic immune responses and clinically meaningful benefit in some patients (Bhatia et al., 2015, 2020). Importantly, this study has shown that *in vivo* electroporation was safe, indeed, systemic administration of IL-12 leads to severe adverse events, including death.

Recently, to increase the activity of anti-tumoral immune response, intratumoral administration of a toll-like receptor 4 (TLR4) agonist on superficial MCC has shown partial responses and one complete response (Bhatia et al., 2019).

Type I interferons (IFNs) are immunomodulatory molecules able to reinduce MHC class I expression or activate immune competent cells. Therefore, treatment of MCC with type I IFNs appears as a promising therapeutic option for patients with MCC. In adddition, IFNs were directly cytotoxic on MCC cell lines *in vitro* and *in vivo* (Willmes et al., 2012).

A majority of MCC (84% n=114) evades immune detection through downregulation of HLA class I molecules required for antigen presentation (Paulson et al., 2014b). NK cells typically target cells that downregulate HLA class I expression. Interestingly, NK-cell therapy is currently under investigation (NCT02465957), moreover, adoptive T-cell therapy with MCPyV T antigen-specific polyclonal autologous CD8-positive T cells is assessed in combination with avelumab, localized radiation and recombinant IFN-β (NCT02584829).

1.9.7. Interest for targeted therapies

In ICI-refractory MCC, the identification of non-immune targets which target tumor is of interest because it could prevent from immune related adverse events. Indeed, targeted therapies are an emerging approach to treat MCC alone or in combination

with ICI. The major aim of targeted therapy is to prevent patients from adverse events due to toxicity of drugs on non-tumor cells and to polarize drugs effects on tumor cells. Somatostatin receptors are expressed by MCC (Gardair et al., 2015), activation of somatostatin receptors lead to an inhibition of cell proliferation, therefore somatostatin analogues have been suggested in MCC treatment (Akaike et al., 2021). Protein kinase inhibitors of PI3K/AKT/mTOR pathway (Fang et al., 2020; Wu et al., 2021), glycogen synthase kinase 3 (GSK3) inhibitor (Houben et al., 2022) and vatalanib, a VEGF receptor, platelet-derived growth factor receptor, and c-Kit inhibitor (NCT00655655) were also proposed as therapeutic approaches. MCC express delta-like ligand 3 (DLL3), this protein was used as target for antibody-drug conjugate (ADC), rovalpituzumab tesirine, an antibody targeting DLL3 conjugated to a cross-linking DNA strands drug (Xie et al., 2020). Interestingly, CD56 was also used as another tumor target to develop another ADC conjugated to microtubule inhibitor, lorvotuzumab mertansine (LM). CD56 is expressed by a majority of MCCs on tumor cell surface (85% (n=169) and 88% (n=34) of positive cases (Mendoza et al., 2020; Tetzlaff and Harms, 2020)). Indeed, CD56 was reported as the neuroendocrine marker with the highest expression in MCC (Fernández-Figueras et al., 2007; Kurokawa et al., 2003). Nevertheless, no correlation between CD56 expression and survival was reported (Fernández-Figueras et al., 2007). Moreover, *in vitro,* a screening of 332 cell surface antigens on a pool of the five MCC cell lines CD56 was found to be an homogenously and highly expressed antigen (Daily et al., 2015; Ollier et al., 2018). In this regard, CD56 has been suggested as a relevant therapeutic target in MCC (Ollier et al., 2018).

2. CD56 expression: implications in physiology and pathology

2.1. CD56 : structure and functions

CD56 or neural cell adhesion molecule (NCAM) was the first cell adhesion molecule identified in the nervous system. CD56 plays a key role in neurogenesis for neurite outgrowth and synaptic plasticity (Rønn et al., 2000). Under physiological conditions, CD56 is expressed by neurons and neuroendocrine cells among others (Lanier et al., 1989).

CD56 is a glycoprotein belonging to the immunoglobulin superfamily including cadherins, selectins, and integrins. CD56 extracellular domain is composed by five immunoglobulin (Ig) domains and two fibronectin type III domains. The Ig domains are involved in homophilic interactions (*i.e.* dimerization) with other CD56 molecules on

the same cell (*i.e cis*-interactions) or another cell (*i.e trans*-interactions) (**Figure 6A**). The fibronectin domains of CD56 allow interaction with other cell membrane receptors (Bencharit et al., 2007). CD56 is encoded by a unique gene (*NCAM1*) containing 25 exons on chromosome 11. CD56 is physiologically involved in cell adhesion, migration, communication, differentiation and survival (Rønn et al., 2000) through intercellular homophilic interactions. Moreover, CD56 functions are also mediated by CD56 heterophilic binding to different membranes, cytoplasmic proteins (*i.e.* fibroblast growth factor receptor, non-receptor kinases Fyn, mitogen-activated protein kinase pathway) or extracellular matrix proteins (*i.e.* collagen) (Ditlevsen et al., 2008).

CD56 can be post-translationally modified by Ser/Thr phosphorylation, palmitoylation, sulfation and *N*- or *O*-linked glycosylation. Indeed, the CD56 extracellular domain can be modified by glycosylation, 6 sites can carry *N*-glycosylation in the immunglobulin domains 1 glycosylation site in the Ig 3 domain, 2 glycosylation sites in the Ig 4 domain and 3 glycosylation sites in the Ig 5 domain. The addition of α2,8-linked N-acetylneuraminic acids called polysialic acid (PSA) occurs at 2 of 3 glycosylation sites in the Ig 5 domain (Sato and Kitajima, 2021) (**Figure 6B**).

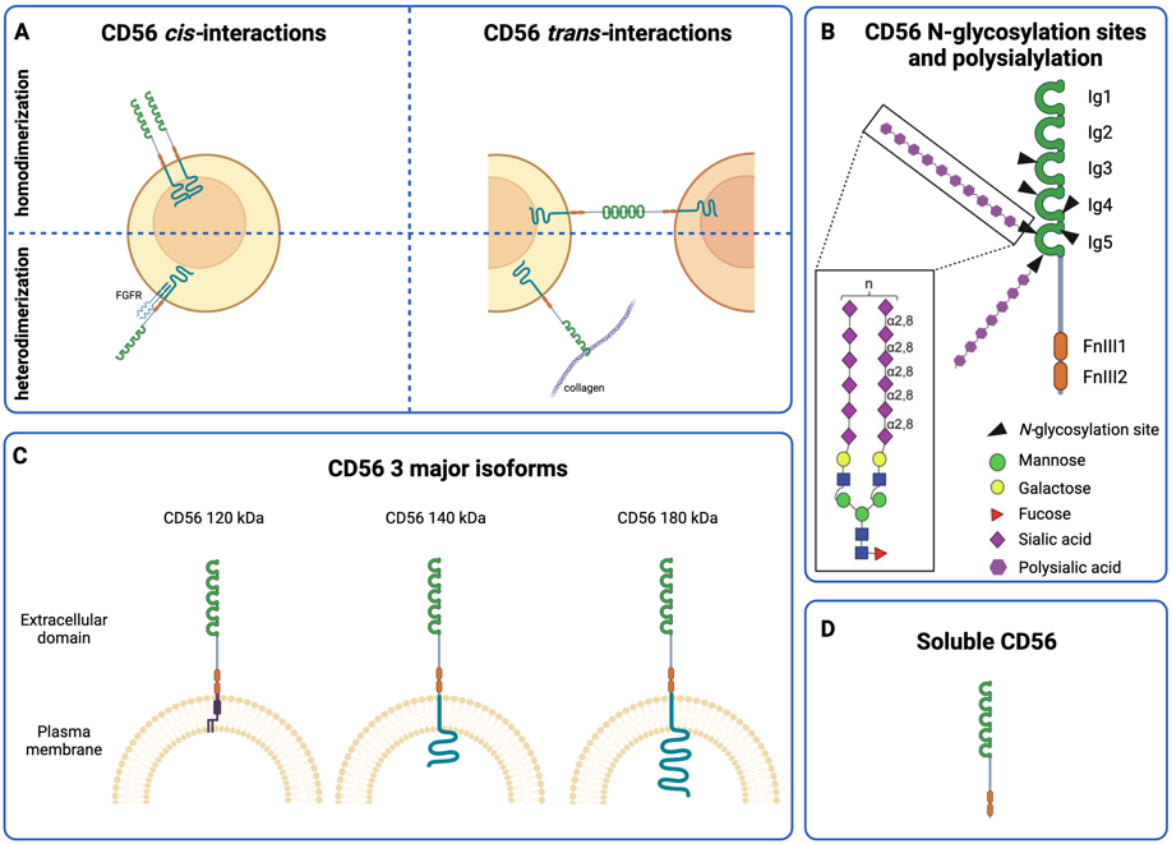

Figure 6. Schematic representation of extracellular and intracellular domains of CD56. (A) CD56 homophilic and heterophilic *cis*- *trans*-interactions. (B) CD56 *N*-glycosylation sites and polysialylation. (C) Structure of CD56 major isoforms (120, 140 and 180 kDa). (D) Soluble CD56. Representation of five immunoglobulin domains (green) and glycosylation sites (black triangles), two fibronectin domains (orange), transmembrane domain (purple) and intracellular domain (blue) adapted from Gattenlöhner *et al.,* 2009.

Polysialylation is characterized by the addition of long linear polymers of sialic acid (over 50 sialic acid residues) to a protein (Seifert et al., 2012). Polysialylation is catalyzed by polysialyltransferases *i.e.* ST8Sia II and ST8Sia IV, and occurs in the Golgi apparatus (Sato and Kitajima, 2021). Polysialylation physiological functions have been demonstrated in neural cell migration, myelination and synapse formation (Bonfanti, 2006). Sialic acids are negatively charged and inhibit cell-cell interactions through an anti-adhesive effect, promoting metastasis of cancer cells (Yang et al., 1994). CD56 homophilic binding properties are modulated by the differential expression of polysialic acid (Rutishauser et al., 1988). CD56 polysialylated form has been associated with tumor progression, for example, in neuroblastoma, polysialic acid expression by tumor cells *in vitro* interferes with adhesion and promotes invasion and

metastasis (Scheer et al., 2020). Polysialic acid and CD56 expression are prognostic markers for neuroblastoma (Korja et al., 2009). Moreover, in glioblastoma, an increased expression of sialyltransferase has been shown to increase invasiveness (Baeza-Kallee et al., 2019).

CD56 extracellular domain is mainly conserved for the three major isoforms, *i.e.* CD56 180 kDa, CD56 140 kDa, CD56 120 kDa. To note, few differences (*i.e.* mutations and deletions) are observed on the fibronectin domain between CD56 120 and 140 kDa. Isoforms harbour distinct intracellular domains (**Figure 6C**) (Gattenlöhner et al., 2009). Indeed, CD56 180 kDa and 140 kDa are both transmembrane isoforms. CD56 120 kDa is a glycosylphosphatidylinositol (GPI)-anchored isoform. To note, CD56 140 kDa isoform has been described to be more often express by tumor cells (Van Acker et al., 2017).

Interestingly, there is a soluble form of CD56 (**Figure 6D**) originating from enzymatic processing, *i.e.* matrix metalloproteases membrane-proximal cleavage or phosphatidylinositol-specific phospholipase C cleavage of CD56 120 kDa (He et al., 1987). Alternative splicing could result in a secreted isoform (with only extracellular domain CD56 115 kDa) due to the insertion of a stop codon (Gower et al., 1988). Soluble CD56 can be polysialylated similarly to the membrane CD56 (Secher, 2010). Its expression has been reported in cerebrospinal fluid, serum and amniotic fluid and has been shown to modulate neurite outgrowth (Ibsen et al., 1983; Secher, 2010). This soluble protein has also been suggested to interfere with homophilic interactions between different cells expressing CD56 (Olsen et al., 1993).

2.2. CD56 expression by immune cells

CD56 expression indicates a transition of immature NK cells into mature NK cells (Abel et al., 2018). Six distinct developmental stages have been described for NK cells. CD56 is expressed at stage 4a, 4b, 5 and 6 of NK maturation (Abel et al., 2018). Mature NK cells are divided into 2 subsets based on surface density of CD56 antigen: $CD56^{bright}$ cells, are predominantly observed in secondary lymphoid tissues, and $CD56^{dim}$ cells, are mainly found in peripheral blood (Moretta, 2010). Importantly, the majority of peripheral blood NK cells are $CD56^{dim}$ (Farag and Caligiuri, 2006). Their functions depend on their maturation stage, $CD56^{dim}CD16^{+}$ NK (*i.e.* stage 5 and 6) are cytotoxic cells but produce low level of immunoregulatory cytokines, contrarily,

$CD56^{bright}CD16^{-}$ NK (*i.e.* stage 4) are immunoregulatory NK cells that produce high level of immunoregulatory cytokines, have low cytotoxicity and low ADCC ability (Farag and Caligiuri, 2006). Nevertheless, CD56 is not only a phenotypic marker of NK cells. Indeed, activated immune cells exhibiting cytotoxic properties express CD56 referred as CD3+ CD56+ cells, NK-like T cells and cytokine-induced killer cells (Schmidt-Wolf et al., 1993). Immune cells, such as, αβ T cells, γδ T cells, dendritic cells (DCs), monocytes, and possibly even more cells of the immune system can upregulate or neo-express CD56 when activated (Van Acker et al., 2017). Moreover, Ziegler *et al.* elucidate CD56 role on NK cells during innate immune response and has demonstrated in the context of *Aspergillus fumigatus* infection that CD56 acts as a pathogen recognition receptor on human NK cells (Ziegler et al., 2017).

2.3. CD56 in non cancerous diseases

CD56 is involved in neurodegenerative disorders such as Alzheimer's disease. Indeed, higher soluble CD56 concentration has been detected in serum from Alzheimer's patients compared to patients without neurodegenerative disorders (Todaro et al., 2004). Indeed, CD56 cleavage resulting in soluble CD56, avoid axons growth and synaptogenesis. CD56 has also a role in normal cognitive functions and is dysregulated in neuropsychiatric and neurodegenerative diseases such as Alzheimer's disease, schizophrenia and bipolarity (Gilabert-Juan et al., 2012; Hane et al., 2012; Lyons et al., 1988; Murray et al., 2016).

Furthermore, CD56 has been identified as rabies virus receptor; its membrane expression has been reported on suceptible cell lines and a decreased CD56 expression has been observed on rabies virus resistant cells (Thoulouze et al., 1998). In 2020, CD56 has been identified as a receptor of zika virus (Srivastava et al., 2020). The authors showed that treatment of zika sensitive cells with an anti-CD56 antibody before the addition of zika virus reduced the binding and entry of the virus into the cells.

2.4. CD56 expression in cancer

Recurrent CD56 expression is observed and might affect tumor behavior differently in several neoplasia such as in carcinomas (notably small cell lung carcinoma (SCLC), ovarian high-grade serous adenocarcinoma) (Whiteman et al., 2014a; Zecchini et al., 2011), pediatric tumors (*i.e.* neuroblastoma, nephroblastoma) (Heinly and Grant, 2022; Mengelbier et al., 2014; Yap et al., 2017) (**Table 3**) and haematological neoplasm

(acute myeloid leukemia, acute lymphoblastic leukemia and multiple myeloma) (Cottini et al., 2022; Fischer et al., 2009; Gattenlöhner et al., 2009; Sasca et al., 2019) (**Table 3**).

2.4.1. Carcinomas

CD56 expression is observed in 93% of SCLC (n=117), and is used as a diagnostic marker of this entity (Kontogianni et al., 2005; Whiteman et al., 2014a). Interestingly, CD56 positive circulating tumor cells have been detected in SCLC patients and might be used for patient monitoring (Messaritakis et al., 2017).

CD56 positivity in epithelial ovarian carcinoma correlate with tumor grade (n=458) (Zecchini et al., 2011). CD56 interacts and activates fibroblast growth factor receptor (FGFR) and is shown to promote migration and invasion (Zecchini et al., 2011).
Moreover, CD56 is also expressed by 88% (n=34) of MCC (Tetzlaff and Harms, 2020).

2.4.2. Pediatric tumors

Polysialilated CD56 is express in 85% of neuroblastoma (n=20) (Heinly and Grant, 2022). In neuroblastoma, drug resistant tumor cells acquire increased invasive capacity compared to sensitive tumor cells due to a decreased CD56 expression (Blaheta et al., 2006). High CD56 level is detected in serum of patients with metastasis neuroblastoma (Heinly and Grant, 2022).

2.4.3. Hemopathy

In haematological malignancies, CD56 is expressed in 70% of multiple myeloma and Cottini *et al.* have shown that CD56 promote multiple myeloma cell growth, survival, and adhesion to stromal cells (Cottini et al., 2022) through activation of CREB1 anti-apoptotic target genes *BCL2* and *MCL1*. CD56 expression was associated with a poor prognosis in multiple myeloma patients and has been suggest as a potential therapeutic target in this setting (Cottini et al., 2022).

Interestingly, 24% of acute myeloid leukemia (n=152) are positive for CD56, specifically CD56 140 kDa isoform, that is associated with disease progression and drug resistance through NF-κB/BCL2 pathway (Gattenlöhner et al., 2009; Sasca et al., 2019).

For acute lymphoblastic leukemia, 14% of tumors are CD56-positive (n=452) and this expression is associated lower rate of complete remission and higher rate of

resistant disease (Fischer et al., 2009). Moreover, 100% (n=16) of blastic plasmacytoid dendritic cell neoplasm (BPDCN) are CD56-positive but its biological role is unkwown (Garnache-Ottou et al., 2009).

Table 3. List of the main tumor entities with CD56 expression. The percentage of CD56 expression, its association with tumor aggressivity, activated pathways and expressed isoforms are depicted.

Cancer classification	Cancer type	CD56 expression level (%) (number of evaluated cases)	CD56 association with tumor aggressivity	Activated pathway	Expressed isoforms	Publications
Hematologic malignancies	Multiple myeloma	70% (N/A)	- CD56 expression associated with poor prognosis	- overexpression resulted in activation of RSK2 and transcription factor, CREB1. CREB1 binds to DNA consensus CRE elements, and promoted transcription of the anti-apoptotic genes BCL2 and MCL1	N/A	Cottini *et al.,* Mol Cancer Res, 2022.
	Acute myeloid leukemia	24% (n= 152)	- confers drug resistance - associated with disease progression	- increased proliferation and decreased apoptosis *via* the NF-κB/BCL2 pathway	140 kDa	Sasca et al., Blood, 2019 and Gattenloehner et al.,Blood, 2007.
	Acute lymphoblastic leukemia	14% (n=452)	- lower rate of complete remission - higher rate of resistant disease	N/A	N/A	Fischer et al., Haematologica, 2009.
	Malignant NK/T-cell lymphoma	76% (n=118)	- not reported to be significant upon prognosis	N/A	N/A	Li *et al.,* Leuk Lymphoma, 2010.
	Blastic plasmacytoid dendritic cell neoplasm	100% (n=16)	N/A	N/A	N/A	Garnache-Ottou *et al.,* Br J Haematol, 2009.

Cancer classification	Cancer type	CD56 expression level (%) (number of evaluated cases)	CD56 association with tumor aggressivity	Activated pathway	Expressed isoforms	Publications
Blastema tumors	Neuroblastoma	- 85% express PSA-CD56 (n=20) - high serum level in metastatic cases	- low CD56 expression is associated with high metastatic potential - PSA-CD56 associated with an increased migration and invasion - PSA-CD56 marker of disease progression during treatment	N/A	180 kDa associated with cell motility	Heinly *et al.,* Front Oncol, 2022.
	Nephroblastoma	74% (n=30)	- proliferation - survival - tumorigenesis - blastemal marker	- MAPK, ERK1/2 and PI3K/Akt are activated - CD56 interaction with HSPG promote metastasis though vascular endothelium binding - PSA on CD56 140 kDa impair cell-to-cell adhesion and increased EMT and metastasis	140 kDa	Yap *et al.,* Fetal Pediatr Pathol, 2017 and Sehic *et al.,* Am J Clin Pathol, 2014.
Carcinomas	Small cell lung carcinoma	93% (n=117)	N/A	N/A	N/A	Whiteman *et al.,* Mabs, 2014.
	Merkel cell carcinoma	88% (n=34)	N/A	N/A	N/A	Tetzlaff *et al.,* Mod Pathol, 2020.
	Ovarian carcinoma	- CD56 level increased at invasive state, 35% vs. 24% at primary state - correlate with tumor grade (n=458)	- promote progression	- interaction of CD56 with FGFR	N/A	Zecchini *et al.,* EMBO Mol Med., 2011.

RSK2: ribosomal protein S6 kinase A3, CREB1: cAMP responsive element binding protein 1, HSPG: heparan sulfate proteoglycans, EMT: Epithelial-Mesenchymal Transition, N/A: undetermined

2.5. Therapies targeting CD56

In oncology, CD56 was used as a target in case of adoptive immune effector cell therapy. Particularly, chimeric antigen receptor-T (CAR-T) cell therapy was efficient in rhabdomyosarcoma, neuroblastoma and SCLC (Crossland et al., 2018; Jiang et al.,

2019). Nevertheless, CAR-T cell therapy is really expensive due to their manufacturing and autoimmunity remain CAR-T cells challenges nowadays (Patel et al., 2021).

Three other CD56-targeting ADCs *i.e.* m906 conjugated to pyrrolobenzodiazepine dimer, promiximab-duocarmycin and promiximab-monomethyl auristatin E (MMAE) (Feng et al., 2016; Yu et al., 2018a, 2018b) have been developed and investigated *in vitro* or in preclinical neuroblastoma and SCLC models, respectively. Nevertheless, these three ADCs have not been assessed in clinical trial.

In preclinical neuroblastoma model, m906 was identified and binds to N-terminal IgG-like domains of CD56 and trigger CD56 downregulation (Feng et al., 2016). m906 was conjugated to a highly potent drug that induce DNA alkylation, named pyrrolobenzodiazepine (PBD) dimer. The half-maximal inhibitory concentration (IC50) in neuroblastoma cell lines was comprised between 0.05 and 1.7 pM (IMR-05, NGP, SK-N-FI and SK-N-AS cell lines) (Feng et al., 2016).

The average drug-to-antibody ratio (DAR) of promiximab-MMAE was 3.13 MMAE per antibody. The IC50 was evaluated on SCLC cell lines H524, H526 and H69 and was of 19, 5 and 0.3 nM, respectively. In H69 and H526 xenograft models, a total of three injections every three days of promiximab-MMAE at 10 mg/kg lead to tumor regression and at 5 mg/kg tumor growth was strongly inhibited (Yu et al., 2018a).

Morevover, the same antibody, promiximab was conjugated with another drug with a different mechanism of action, a DNA alkylating agent *via* reduced interchain disulfides to produced promiximab-duocarmycin and an average DAR 2 has been obtained. The IC50 on SCLC cell lines H526, H524 and H69 was of 0.07, 0.2 and 0.3 nM, respectively. In H526 xenograft model, a total of three injections every three days of promiximab-duocarmycin at 5 and 10 mg/kg lead to sustained regression. Interestingly, in the other xenograft model, with H69 tumors in the same therapeutic settings, a significant inhibition of tumor growth was shown, which corroborate the IC50 result.

Lorvotuzumab mertansine (LM) also known as IMGN901 or BB-10901, is an anti-CD56 DM1-conjugated ADC in which the drug is linked to lysins of humanized IgG1 antibody (huN901) using a disulfide linker, the N-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP). This first generation bioconjugation technology involved NHS-ester bioconjugation heads binding to lysins residues. These bioconjugation head led to the generation of heterogeneous ADCs, with a high DAR

variability ranging between 0 to 8, and multiple different linker positions (Leung et al., 2020). Moreover, no precise chemical characterization in term of homogeneity has been provided for LM, but it has been suggested that the DAR was around 3.3 and 3.6 (Whiteman et al., 2014b).

Until now, seven clinical trials have been conducted with LM (**Table 4**). Several phases I-II clinical trials in $CD56^+$ cancers demonstrated favorable toxicity profiles of LM as monotherapy with low incidence of grade 3/4 adverse events (Ailawadhi et al., 2019; Geller et al., 2020; Shah et al., 2016). Moreover, signs of clinical activity were especially detected in the four included MCC patients with two objective responses (Shah et al., 2016). A subsequent phase I-II clinical trial investigated the combination with carboplatine/etoposide combined with LM versus carboplatine/etoposide alone in patients with small-cell lung cancer (Socinski et al., 2017). Treatment related adverse events appeared when LM was tested in combination with etoposide/carboplatine in SCLC patients. The toxicity issues were peripheral neuropathy (29% of the patients), grade 3 (74%) and grade 4 (48%), such as pneumonia and sepsis, leading to death (Socinski et al., 2017). This trial was first modified by reduction of the dose of carboplatine/etoposide (Socinski et al., 2017) (NCT01237678). Overall, there was no improved efficacy of etoposide/carboplatine and LM compared to etoposide/carboplatine alone for SCLC patients, which is perhaps the reason why the ADC development was stopped by the company Immunogen. Such increased haematological toxicity could be rather related to an off-target effect of LM, as only NK cells expressed CD56 in blood cells. Interestingly, safety concerns of LM could be due to unspecific cleavage of SPP linker and drug release into blood circulation as observed with trastuzumab (Lewis Phillips et al., 2008). Nowadays, ADC knowledges allows optimizations of ADC components that could lead to a reduction of toxicity.

Table 4. Clinical trials of lorvotuzumab mertansine.

Indication	Trial phase	Conclusion	Year end of the clinical trial	Trial identifier
Neuroblastoma; Peripheral Nerve Sheath Tumor (Neurofibrosarcoma); Peripheral Nervous System (PNS) Tumor; Rhabdomyosarcoma; Synovial Sarcoma; Wilms' Tumor (Nephroblastoma)	Phase II	The trial was completed. Based on the results reported, LM is tolerated in children at the adult recommended phase II dose.	2022	NCT02452554
Acute Lymphocytic Leukemia (ALL, Acute Lymphoblastic Leukemia); Acute Myelocytic Leukemia (AML, Acute Myeloblastic Leukemia); Blood Cancer; Chronic Myelocytic Leukemia (CML, Chronic Myeloid Leukemia); Leukemia; Myelodysplastic Syndrome; Myelofibrosis; Natural Killer Cell Lymphomas; Post-Essential Thrombocythemia Myelofibrosis (Post-ET MF); Post-Polycythemia Vera Myelofibrosis (PPV-MF)	Phase II	The trial was completed. Based on the study results published, due to insufficient data, it could not draw any conclusion.	2018	NCT02420873
Small-Cell Lung Cancer; Solid Tumor	Phase I/II	The trial was terminated due to lack of efficacy signal and safety concerns. Based on the results reported, LM with carboplatin and etoposide is not safe in subjects with solid tumors and extensive stage small cell lung cancer.	2018	NCT01237678
Neuroendocrine Tumors; Ovarian Cancer; Small-Cell Lung Cancer; Solid Tumor	Phase I	The trial was completed. Based on results reported, the treatment with LM is found to be effective with acceptable safety and tolerability in subjects with Merkel cell carcinoma (MCC).	2015	NCT00346385
Multiple Myeloma (Kahler Disease); Refractory Multiple Myeloma; Relapsed Multiple Myeloma	Phase I	The trial was completed. Based on the results reported, LM is well tolerated, safe, effective and had acceptable pharmacokinetic profile in the treatment of subjects with multiple melanoma.	2015	NCT00346385
Multiple Myeloma (Kahler Disease); Refractory Multiple Myeloma; Relapsed Multiple Myeloma	Phase I	The trial was completed. Based on the results reported, LM in combination with lenalidomide and dexamethasone was found to be effective and safe in patients with relapsed or relapsed/refractory multiple myeloma.	2014	NCT00991562
Small-Cell Lung Cancer; Solid Tumor	Phase I/II	The trial was completed. Based on the results reported, weekly dosing of LM was found to be safe and well tolerable in subjects with relapsed small cell lung cancer.	2012	NCT00065429

3. The antibody-drug conjugates in oncology

ADC caracteristics as well as ADC indication in oncodermatology has been decribed in a review paper. This review has been published in the international peer-review journal Cancers:
Esnault, C., Schrama, D., Houben, R., Guyétant, S., Desgranges, A., Martin, C., Berthon, P., Viaud-Massuard, M.-C., Touzé, A., Kervarrec, T., Samimi., M. (2022). Antibody-Drug Conjugates as an Emerging Therapy in Oncodermatology. Cancers (Basel) 14, 778. https://doi.org/10.3390/cancers14030778.
This review is presented in appendix.

This review shed light on the interest of ADC in oncology, in era of targeted therapies. Then, the caracteristics and indications of approved ADC in oncology were reported. This review provides the detailled of ADC structure and major results obtained preclinically and throughout clinical evaluations in oncodermatology, specifically for cutaneous T-cell lymphoma, melanoma and skin carcinoma *i.e.* squamous cell carcinoma and MCC. An overview of ADC structure, design and development was presented. The importance of target choice, its specific antibody, the linker and payload options as well as the importance of linker-antibody conjugation part were highlighted in this study. The ADC improvement opportunities were also detailled.

Among targeted therapies, ADCs combine a monoclonal antibody (mAb) with a highly cytotoxic molecule allowing its specific delivery to tumor cells (Chau et al., 2019). Over the last past years, optimization of ADC technologies in order to increase their therapeutic performances and overcome their limitations, as well as evaluation of their effects in combination with currently approved drugs, has significantly expanded their use in oncology (Joubert et al., 2020; Tong et al., 2021). Indeed, twelve ADCs have been approved by the FDA for treatment of hematologic malignancies (**Table 5A**) and solid tumors (**Table 5B**). Moreover, the number of ADCs in development is steadily increasing with 195 ongoing clinical trials (https://www.beacon-intelligence.com/). Overall, ADC are currently being applied in all oncology areas including skin cancers.

Table 5. FDA-approved ADCs.

A. Hematological malignancies

Commercial name	International nonproprietary names (INN)	Target	Antibody isotype	Bioconjugaison head (antibody amino acid)	Linker	Drug (therapeutic class)	Indication
Adcetris®	brentuximab vedotin	CD30	Chimeric IgG1	Maleimidocaproyl (Cysteine)	Cleavable / proteolytic (cathepsin B)	auristatin	anaplastic large cell lymphoma + Hodgkin's lymphoma
Polivy®	polatuzumab vedotin-piiq	CD79b	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable / proteolytic (cathepsin B)	auristatin	relapsed or refractory diffuse large B-cell lymphoma
Mylotarg®	gemtuzumab ozogamicin	CD33	Humanized IgG4	Acetyl butyrate (Lysine)	Cleavable / hydrazone	calicheamicin	CD33-positive acute myeloid leukemia
Beponsa®	inotuzumab ozogamicin	CD22	Humanized IgG4	Acetyl butyrate (Lysine)	Cleavable / hydrazone	calicheamicin	lymphoblastic leukemia
Lumoxiti®	moxetumomab pasudotox	CD22	N/A	N/A	Cleavable / proteolytic (furin)	*Pseudomonas* endotoxin A	hairy cell leukemia
Zynlonta®	loncastuximab tesirine-lpyl	CD19	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable / proteolytic (cathepsin B)	pyrrolobenzodiazepine dimer (PBD)	relapsed/refractory diffuse large B-cell lymphoma
Blenrep®	belantamab mafodotin	CD38	Humanized IgG1k	Maleimidocaproyl (Cysteine)	Uncleavable	auristatin	relapsed/refractory multiple myeloma

B. Solid tumors

Commercial name	International nonproprietary names (INN)	Target	Antibody isotype	Bioconjugaison head (antibody amino acid)	Linker	Drug	Indication
Kadcyla®	ado-trastuzumab emtansine	HER2	Humanized IgG1	Maleimidocaproyl (Lysine)	Uncleavable	maytansine	HER2/neu positive breast cancer
Padcev®	enfortumab vedotin	Nectin-4	Human IgG1	Maleimidocaproyl (Cysteine)	Cleavable / proteolytic (cathepsin B)	auristatin	locally advanced or metastatic urothelial cancer
Enhertu®	trastuzumab deruxtecan	HER2	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable / proteolytic (cathepsin B)	deruxtecan (topoisomerase inhibitor)	breast cancer HER2 positive after two or more lines of anti-HER2 therapy
Trodelvy®	sacituzumab govitecan	TROP-2	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable / hydrazone	topoisomerase inhibitor	metastatic triple-negative breast cancer
Tivdak®	tisotumab vedotin	Tissue factor	Human IgG1	Maleimidocaproyl (Cysteine)	Cleavable / proteolytic (cathepsin B)	auristatin	cervical cancer

Unprovided data: N/A

3.1. Structure of ADC

ADC consist in a cytotoxic drug *i.e.* a payload bioconjugated through a linker to mAb targeting tumor cell antigens (Beck et al., 2017) (**Figure 7**). The specific combination of these components determines both therapeutic performances and safety of the ADC (Chau et al., 2019). The following part aims to provide a brief overview on the features of the different ADC components and how these can impact efficacy and safety.

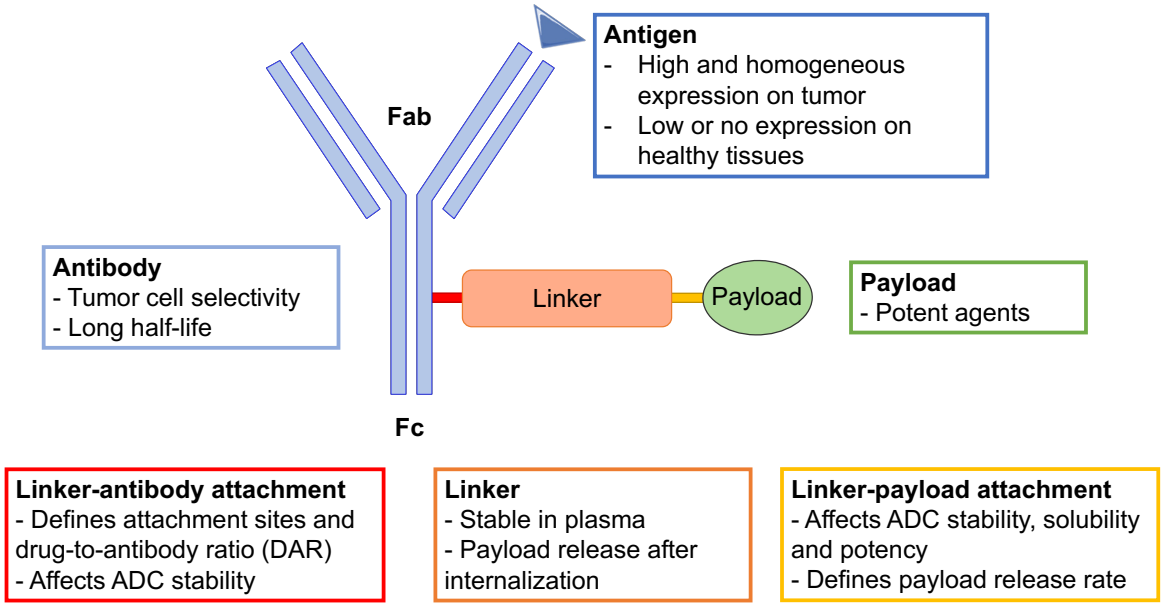

Figure 7. Design of an antibody-drug conjugate and recommended biological properties. Characteristic of antigen, antibody, linker-antibody attachment, linker, linker-payload attachment and payload are detailed. The antibody Fc part is implied in half-life and immunogenicity. The Fab part controls affinity and avidity to the targeted antigen.

3.2. Targets

Target identification is a crucial point in ADCs development (Amani et al., 2020; Boni et al., 2020). Tumor specific biomarkers previously used for diagnosis, as well as proteins overexpressed due to gene amplification or proteins involved in tumor aggressiveness (Teicher, 2009), might be considered as potential ADC targets. Most ADCs bind to proteins expressed on the cell surface while targeting of intracellular tumor-associated proteins can also be achieved by using T-cell-receptor-like antibodies recognizing peptides in the context of presentation by MHC-I complexes (Lai et al., 2018). In both cases, *i.e.*, extra- and intracellular proteins, the specificity of the tumor antigen, antigen expression levels and antigen/ADC internalization (Staudacher and Brown, 2017) determine the performances of an ADC target (Amani et al., 2020).

To provide optimal payload delivery, high and homogenous expression of targeted antigens on tumor cell surface is required (Damelin et al., 2015). By contrast, absent or low expression in healthy tissues (Hoffmann et al., 2018) is expected in order to limit toxicity on physiologic cells (*i.e.* in order to avoid on-target off-tumor cytotoxicity). Nevertheless, several clinically approved ADCs such as brentuximab vedotin,

targeting CD30 (Vaklavas and Forero-Torres, 2012), polatuzumab vedotin, targeting CD79 (Sehn et al., 2020), or inotuzumab ozogamicin, targeting CD22 (Fayad et al., 2013), actually engage with ligands expressed by immune cells but still harbor therapeutic efficiency and acceptable tolerance. Moreover, most ADCs require internalization of the targeted surface antigen through receptor-mediated endocytosis and lysosomal degradation of the linker in order to deliver the payload (Sievers and Senter, 2013). One evasion mechanism that limits ADC performances is the downregulation of the targeted antigen expression, which obviously affects ADC binding to the target cells (Amani et al., 2020).

While antigens present on cell surface are the most frequent candidates for ADCs targets (Thomas et al., 2016), secreted proteins/soluble antigens might additionally offer new opportunities by being more accessible. As a consequence, linker and drug have to be designed to be active in this context (Hoffmann et al., 2018). Some ADCs targeting non-internalizing soluble antigens - such as Tenascin-C splice variants or fibronectin - with maytansinoid and auristatins as payloads have already demonstrated anti-tumor activity (Gébleux et al., 2017; Perrino et al., 2014). Importantly, targeting soluble antigens in the tumor microenvironment (TME) might impact not only tumor cells but also tumor stroma, extracellular matrix and blood vessels. Moreover, an association between bevacizumab, an anti-VEGF monoclonal antibody, and an ADC has provided improved outcomes compared to a combination of bevacizumab with standard chemotherapy, in a phase Ib clinical study including patients with platinum-resistant ovarian cancer (O'Malley et al., 2020).

3.3. Antibodies

mAbs used in the ADC structure are mostly of IgG1 or IgG4 subtypes, and are chimeric or humanized in order to decrease their immunogenicity (Chau et al., 2019). Both parts of the mAbs *i.e.* fragment antigen-binding (Fab) and fragment crystallizable region (Fc region) contribute to the performances of an ADC. While affinity of a mAb for its epitope is determined by the Fab portion (Teicher, 2009), the Fc part is determinant for mAb stability and interactions with immune cells such as antibody dependent cell-mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) and antibody dependent cell-mediated phagocytosis (ADCP). To this end, the Fc part interacts with neonatal Fc receptor (FcRn) (Castaneda et al.), a recycling

receptor determining both IgG half-life and biodistribution (Chen et al., 2014), or complement (C1q) implied in CDC or Fc receptors on immune effector cells (FcγR), a family of receptors which upon Fc binding may trigger an immune response (Nimmerjahn and Ravetch, 2008). The ADC interactions with immune cells might either constitute an opportunity to induce anti-tumor response, or represent a potential side effect resulting in toxicity on immune cells (Hoffmann et al., 2018). Accordingly, to improve or avoid immune functions (ADCC, CDC, ADCP), genetic engineering introducing mutations in the Fc domain of ADCs is used (Aguiar et al., 2018; Hoffmann et al., 2018). The Fc mutations aim to promote or impair interaction between ADCs and immune cells, Fc receptors or complement (C1q) in order to prevent or improve their activation, such as increased ADCC with ADC glyco-engineering (*i.e.* afucosylation) (Carter, 2006; Gauzy-Lazo et al., 2020). On the other hand, other Fc modifications have been proposed to increase ADC half-life *via* FcRn-mediated ADC recycling (Amani et al., 2020).

Additional factors related to the payload and linker affect ADC pharmacokinetic (PK) and pharmacodynamic (PD) (Lucas et al., 2019). Notably, ADC hydrophobicity is associated with shorter half-life in serum (Hamblett et al., 2004) and because most of the payloads are hydrophobic, attachment to a hydrophilic antibody remains a challenge (Buecheler et al., 2020). To overcome hydrophobicity and ADC aggregation, the most common ADCs modification consists in glycosylation, a naturally occurring post-translational modification influencing solubility, antigenicity and stability of proteins (Buettner et al., 2018). PEGylation, addition of polyethylene glycol (PEG) to the linker in order to mask payloads hydrophobicity and to improve PK of ADCs (*i.e.* reduction of aggregation, improvement of solubility and half-life (Buecheler et al., 2020)) has also been proposed (Buettner et al., 2018).

3.4. Payloads

While radionucleotides, toxins (Schrama et al., 2006) and cytokines (King et al., 2004) have been suggested as ADC payloads (Teicher and Chari, 2011; Wu and Senter, 2005; Milenic et al., 2004), most preclinical and FDA/EMA-approved ADCs are bioconjugated with small cytotoxic molecules (Leung et al., 2020) with a broad range of structures and mechanisms of action (Chau et al., 2019). Among these, duocarmycins and pyrrolobenzodiazepines (PBD) dimers induce DNA alkylation by

linking alkyl residues to AT-rich regions or guanine leading to cell apoptosis (Leung et al., 2020). Cell death can also be induced by calicheamicins inducing DNA double-strand breaks (Leung et al., 2020). Maytansine derivatives (including DM1 and DM4) (Yaghoubi et al., 2020) and auristatins (MMAE), monomethyl auristatin F (MMAF) (Beck et al., 2017) are poisons which inhibit microtubule polymerization by targeting tubulins resulting in G2/M arrest and apoptosis. A third family of cytotoxic agent inhibit the topoisomerase enzymes, which control DNA structure changes. Topoisomerase inhibitors block the cell cycle ligation step, which generates DNA single- and double-strand breaks, leading to apoptotic cell death (Thomas and Pommier, 2019). Trastuzumab deruxtecan and sacituzumab govitecan are both approved topoisomerase inhibitors ADCs (Thomas and Pommier, 2019). Currently, auristatins, maytansine derivatives and PBD / PNU (anthracyclines family) represent 29%, 25% and 2% of the global active ADCs respectively (https://www.beacon-intelligence.com/). Overall, MMAE remains the most frequently payload used for ADCs development, involved in 19% of the active ADCs for which the payload is disclosed (https://www.beacon-intelligence.com/).

Beyond their direct cytotoxic tumor-targeted activity, highly membrane-permeable payloads, such as MMAE or calicheamicin, are able to induce a bystander effect (Abdollahpour-Alitappeh et al., 2019) due to drug release from the primary target cells into the TME. The bystander effect enhances ADC efficacy by targeting not only stromal cells, which do not express the targeted antigen, but also antigen-negative tumor cells in cases of intratumoral heterogeneity (Staudacher and Brown, 2017) (**Figure 8**). By contrast, MMAF, another auristatin derivate, has low cell permeability due to its negative charge. While such properties limit the off-target toxicity of MMAF ADCs, their bystander activity remains low (Staudacher and Brown, 2017). Regarding this limit, MMAF ADCs require high tumor expression of target antigen (Smith et al., 2006). Nevertheless, a MMAF-conjugated ADC, belantamab mafodotin, was approved in 2020 by FDA and EMA for relapsed or refractory multiple myeloma treatment (Lonial et al., 2021; Tai et al., 2014).

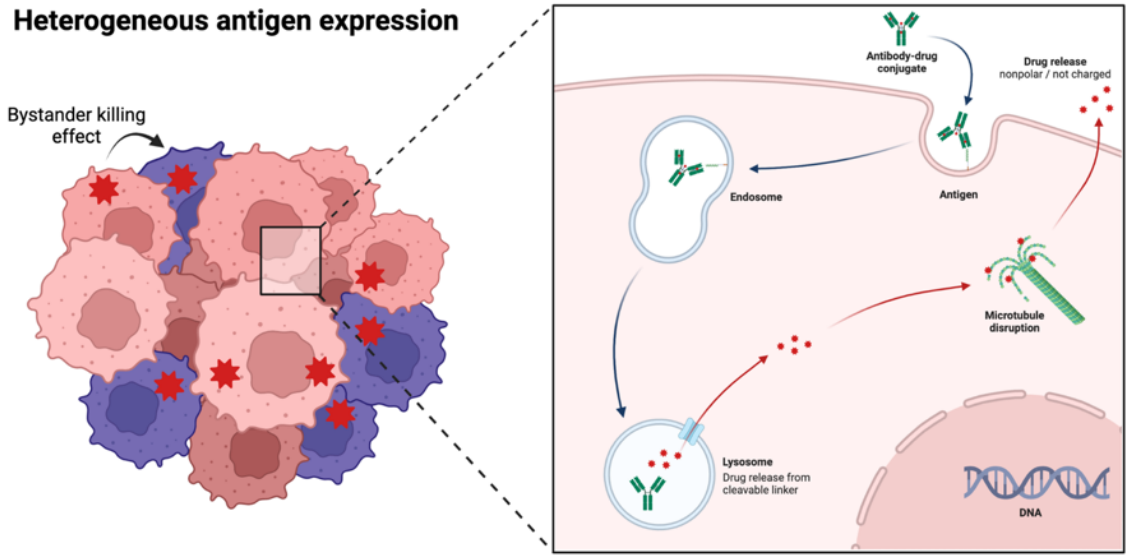

Figure 8. Overview of ADC mode of action. Binding of ADC to antigen expressed by cancer cell. Internalization of ADC. Degradation of the linker or antibody inside the lysosome induces the release of an active form of the payload. The payload exerts a cellular toxicity depending on its mode of action. A bystander effect can occur.

3.5. Linker

The linker is the ADC portion that connects antibody and drug. Depending on the payload and antibody properties, either cleavable or non-cleavable linkers are used. Cleavable linkers are used in combination with payloads being only fully active after lysosomal degradation (*e.g.* MMAE or calicheamicin) (Leung et al., 2020). To prevent the cleavage of the linker in blood circulation resulting in unspecific cytotoxicity, the ADC linker has to be stable until the ADC reaches its target cell. To this end, cleavable linkers are usually sensitive to conditions specifically found in lysosomes (*e.g.* acidic condition or lysosomic proteases (cathepsin B, glycosidase, phosphatase)) or high intracellular glutathione concentrations (Leung et al., 2020) and thereby release the payload only after endocytosis is achieved, leading to death of the target cell (Leung et al., 2020) (**Figure 8**).

Regarding non-cleavable linkers, the drug is active after internalization and enzymatic digestion of the antibody into the lysosome, allowing the release of an active metabolite, amino acid-linker-drug complex (*e.g.* trastuzumab emtansine) (Beck et al., 2017). Non-cleavable linkers allow increased ADC stability leading to reduced toxicity in non-target tissues (Beck et al., 2017). Payloads such as MMAF or DM1 are classically used in this setting.

3.6. Linker-antibody conjugation

The drug to antibody ratio (DAR), defined as the number of drug molecules attached to one mAb, is another important determinant for ADC performance (Leung et al., 2020). The optimal DAR is depending to the payload nature (J. Walsh et al., 2021). The paradigm that a DAR value of 4 is optimal for pharmacokinetics has been challenged by the recent approval of DAR 8 antibodies, in particular in light of improved hydrophobicity-masking technologies (Viricel et al., 2019). The technology connecting the mAb to the linker is crucial to obtain a homogeneous and controlled DAR (*i.e.* position of drugs and loading) (Junutula et al., 2008). Chemical linkers involving functional groups (*i.e.* bioconjugation head) are used to bind to mAb amino acids. First generation bioconjugation technologies involved either N-hydroxysuccinimide (NHS) ester bioconjugation heads binding to lysine residues, or hydrazones, maleimides and thioethers binding to cysteines (Bargh et al., 2019). However those bioconjugation heads led to the generation of heterogeneous ADCs, presenting a high variability of the DAR in the range of 0 to 8, and to multiple different linker positions (Leung et al., 2020).

More recently, site-specific conjugation, *i.e.* engineered cysteine residues, unnatural amino acids, or enzymatic conjugation through glycosyltransferases, has been applied to obtain more homogeneous ADCs (Beck et al., 2017). Indeed, site-specific conjugation of antibodies has been shown to improve the ADC therapeutic index because it improve pharmacokinetic by reducing hydrophobicity of the linker-payload as well as prevent a release of payload in blood (Junutula et al., 2008). Moreover, site-specific conjugation with rebridging of antibody disulfide bonds results in stable and homogeneous ADCs with a control number and position of payload, and higher stability characteristics (Juen et al., 2021).

Interestingly, Van Geel *et al.* has shown that it was possible to link a payload to the native N-glycan residing at asparagine 297 and demonstrated *in vitro* and *in vivo* efficacy (van Geel et al., 2015).

3.7. Strengths, weaknesses and challenges in ADC field

When compared to other cancer therapies, notably conventional cytotoxic treatment, the use of ADCs is a vectorized therapy with low off-target toxicity. Nevertheless, ADCs could also have some drawbacks, due to recognition of the target

on non-tumor cells leading to on-target off-tumor cytotoxicity limiting the therapeutic window in which ADC can be applied (Hoffmann et al., 2018). Moreover, unspecific linker cleavage can cause drug delivery without antibody recognition resulting in systemic toxicity, which represents another important limitation of ADCs (Szijj et al., 2018). Improvements in the production process as well as modifications in the ADC structure may counteract these constraints (Hoffmann et al., 2018). In order to obtain stable ADCs, engineering of the bioconjugation head might contribute to linker stability improvement (Amani et al., 2020). As an example, maleimide residue either includes in the linker or the bioconjugation head might react with free thiol of plasma, *e.g.* albumin resulting in the so called retro-Michael reaction. Hence, this retro-Michael induces unspecific release of the linker and drug in the plasma which not only impacts the efficacy of the ADCs but also leads to off-target toxicity (Szijj et al., 2018).

Several resistance mechanisms to ADCs have been described such as target antigen downregulation or mutations, impairment of lysosomal degradation pathways. Moreover ADC payloads can be rejected of tumor cells through multidrug resistance transporter efflux (Parslow et al., 2016). For instance, loss of CD30 expression can be observed in cutaneous CD30-positive lymphoid neoplasm treated with brentuximab vedotin (Chen et al., 2015; Goyal et al., 2019). Trogocytosis, *i.e.* the extraction of the antibody-epitope complexes by monocytes may induce resistance to the ADC in CD30-positive lymphoma (Goyal et al., 2019).

A wide number of ADC optimizations have been developed to improve ADC performances. A new class of therapy aiming to improve tumor-specificity of mAbs, named probodies, consist in the optimization of mAb used for ADC technology (Desnoyers et al., 2013). These antibodies are modified in order to mask the Fab paratopes, thereby limiting their activity in healthy tissue. By contrast, in the TME, high protease activity removes the masking peptide, allowing paratope-epitope recognition. Enhancement of the therapeutic index by such probody-based therapy was demonstrated in an epidermal growth factor receptor (EGFR)-overexpressing mouse model (Desnoyers et al., 2013). The use of probodies in ADCs to enhance tumor cell-specific delivery of drugs has recently been tested (Autio et al., 2020). In this respect, an anti-CD166 probody-drug conjugate has shown on-target on-tumor specificity in a preclinical study on lung cancer (Chomet et al., 2020).

ADC antitumoral effect could be optimized by the use of innovative payload or new bioconjugation strategy. As an example, trastuzumab emtansine is indicated as second line treatment for HER2-positive metastatic breast cancer. Interestingly, a second anti-HER2 ADC, trastuzumab deruxtecan (Modi et al., 2020), with eight topoisomerase inhibitors conjugated to trastuzumab through a cleavable linker was recently approved by the FDA and EMA - this ADC has shown higher efficacy than trastuzumab emtansine demonstrating the involvement of payload and bioconjugation strategy in ADC performances (Modi et al., 2020).

Among the challenges ADCs toxicity (*i.e.* thrombocytopenia, neutropenia or peripheral neuropathy) should be known and control, for example by dose modulation (Donaghy, 2016). Moreover, unexpected combinatorial toxicities of bleomycin and brentuximab vedotin observed for Hodgkin's lymphoma should be consider for future combination (Donaghy, 2016).

To conclude, ongoing optimization of ADC technology as well as promising combination with already existing drugs, are likely to expand their application in oncology in the upcoming years, as illustrated below with ADCs currently approved or investigated in the field of oncodermatology.

CONTEXT AND OBJECTIVES

Since 2008, the main research focus of the team “biology of polyomavirus infections” is the MCC, with the aim to identify diagnostic and prognostic markers as well as to develop new therapeutic option for MCC patients. In this context, a collaboration was established between this research team and the dermatology and pathology departments of Tours hospital. Due to the MCC rarity, a multicentric network has been established allowing to the generation of a large MCC cohort from 9 french hospitals (Tours, Orléans, Besançon, Angers, Le Mans, Poitiers, Rennes, Ambroise Paré and Strasbourg) (RCB 2009-A01056-51). For each patient, the MCC histological diagnosis was validated and then clinical and following data were collected and updated frequently. In one hand, patient’s serum and tumor samples are collected and included in the cohort. Moreover, *in vitro* and *in vivo* preclinical MCC models were established to assessed anti-tumoral effect of drugs.

The starting point of the present project came from the observation of frequent CD56 expression by a majority of MCC, suggesting that this membrane antigen could be used as a target to develop a vectorized therapy. A collaboration with Dr. Feng, NIH, allowed to obtain an antibody targeting CD56 that was previously evaluated in neuroblastoma. Various anti-CD56 ADCs have been developed in other indications including one ADC, LM evaluated in clinical trials with severe haematological toxicity. Therefore, in collaboration with the company McSAF, a French company with expertise in the development of innovative ADCs linker, we aimed to develop a new generation CD56-targeting ADC with improved safety and enhanced therapeutic performances. To this purpose, Adcitmer® was generated using a McSAF Inside® patented linker and performances of this ADC were evaluated *in vitro* and *in vivo* in a MCC preclinical models. The results of these experiments led to a patent registration (WO2021170961) and a publication of an original article in the *British Journal of Dermatology* in 2021.

To provide the first proof of concept of this project, funding was obtained from the French Society of Dermatology in 2018, and from the “*Cancéropôle Grand-Ouest*” in 2019. Additional funding from the “*region Centre*” were then obtained to further characterized the product (“*ARD CVL Biomédicaments*” 2020-2022).

I started to work on this project during the year of my master thesis during which the first proof of concept was done. Then, I started as a PhD student in the same team and went to Dr. Roland Houben and Dr. David Schrama's research team in Würzburg, Germany for 3 months, to learn experimental procedures such as CRISPR/Cas9 gene knockout (KO) and to perform additional characterization of the ADC. The initial description of Adcitmer® properties was published in the *British Journal of Dermatology* in August 2021. In parallel, I wrote a general review regarding the interest of ADCs in oncodermatology. During the last year of my PhD, with the support of several research teams (Dr. Gouilleux-Gruart, FRAME, Pr. Garnache-Ottou UMR 1098 RIGHT, Dr. Aubrey, UMR ISP1282 BIOMAP), we conducted further experiments to characterize Adcitmer® including *in vivo* comparison of the first and optimized version of Adcitmer® as explained in the article 2.

This project led to the publication of a patent, an original article, and a review, a second original article (Article 2) being in preparation. Moreover, I presented our results through oral communications and posters (*Labex MabImprove 2020, 2021 and 2022*, *AIS 2022, ESDR 2021*, *Journées biomédicales Tours-Angers 2021*, *Biotechnocentre 2021*, *CARD 2020*, *Cancéropôle Grand-Ouest 2020*, *Journée immunité et cancer 2019*).

The major objectives of this work are:

1) first, to demonstrate therapeutic performances of Adcitmer® in MCC preclinical models (Article 1).
2) to bring extensive characterization and optimization of the product with the goal to finally test Adcitmer® in clinical trial (Article 2).

EXPERIMENTALES STUDIES

1. Article 1: Adcitmer®, a new CD56-targeting monomethyl auristatin E-conjugated antibody, is a potential therapeutic approach in Merkel cell carcinoma

This study was carried out between 2018 and 2021 and has been supported by the French Society of Dermatology and the "*Cancéropôle Grand-Ouest*".

The goal was to make a proof of concept of a new CD56-targeting ADC in MCC. The major objectives of this study were:

1) to assess CD56 expression in MCC tumors.
2) to generate Adcitmer® through the bioconjugation of m906 with MMAE using McSAF Inside® specific bioconjugation head.
3) to provide chemical characterization of Adcitmer®.
4) to evaluate therapeutic performance of Adcitmer® in MCC *in vitro*.
5) to determine the potential effect of Adcitmer® treatment on tumor growth in a xenograft model of MCC.

The major results of article 1 are summarized as follows:

1) CD56 was expressed by 66% of MCC tumors in our cohort, in line with previous reports, confirming its relevance as a therapeutic target. The CD56 expression was unchanged between both MCPyV-positive and -negative subgroups of MCC.
2) the high homogeneity of the newly generated Adcitmer® with an average DAR 4 was confirmed by hydrophobic interaction chromatography.
3) specific binding and internalization of the m906 antibody was validated in MCC cell lines. The CD56-mediated cytotoxicity of Adcitmer® was demonstrated *in vitro* in MCC cell lines.
4) finally, Adcitmer® significantly reduced tumor growth in a MCC xenograft model.

This work has been published in the international peer-review journal *British Journal of Dermatology*:

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This article is presented from page 61 to page 78. To note, in the following section, “Figure X” refers to article’s figures.

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Adcitmer®, a new CD56-targeting monomethyl auristatin E-conjugated antibody, is a potential therapeutic approach in Merkel cell carcinoma

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Summary

Background Merkel cell carcinoma (MCC) is an aggressive skin cancer, whose tumour cells often express CD56. While immune checkpoint inhibitors constitute a major advance for treating patients with MCC with advanced disease, new therapeutic options are still urgently required.

Objectives To produce and evaluate the therapeutic performance of a new antibody–drug conjugate (Adcitmer®) targeting CD56 in preclinical models of MCC.

Methods CD56 expression was evaluated in a MCC cohort (immunohistochemistry on a tissue microarray of 90 tumour samples) and MCC cell lines. Interaction of an unconjugated CD56-targeting antibody with CD56$^+$ MCC cell lines was investigated by immunohistochemistry and imaging flow cytometry. Adcitmer® product was generated by the bioconjugation of CD56-targeting antibody to a cytotoxic drug (monomethyl auristatin E) using the McSAF Inside® bioconjugation process. The chemical properties and homogeneity of Adcitmer® were characterized by hydrophobic interaction chromatography. Adcitmer® cytotoxicity was evaluated in vitro and in an MCC xenograft mice model.

Results Similar to previous reports, CD56 was expressed by 66% of MCC tumours in our cohort, confirming its relevance as a therapeutic target. Specific binding and internalization of the unconjugated CD56-targeting antibody was validated in MCC cell lines. The high homogeneity of the newly generated Adcitmer® was confirmed by hydrophobic interaction chromatography. The CD56-mediated cytotoxicity of Adcitmer® was demonstrated in vitro in MCC cell lines. Moreover, Adcitmer® significantly reduced tumour growth in a MCC mouse model.

Conclusions Our study suggests that Adcitmer® should be further assessed as a therapeutic option in patients with MCC, as an alternative therapy or combined with immune checkpoint inhibitors.

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Conflicts of interest
C.M., A.D., C.B.B., M.C.V.-M. are employees of McSAF, which is the owner of the patent. V.L., M.S., A.T. and T.K. filed a patent on Adcitmer® in collaboration with McSAF. T.K. is A.D.'s husband. The other authors have no conflicts of interest to declare.

Data availability
The data supporting this study are available in the Supporting Information.

M.S., A.T. and T.K. contributed equally.

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What is already known about this topic?

- Merkel cell carcinoma (MCC) is an aggressive skin cancer.
- While immune checkpoint inhibitors constitute a major advance in treating patients with MCC with advanced disease, new therapeutic options are still urgently required.
- CD56, which is expressed by most MCC tumours, might constitute a therapeutic target for the development of antibody–drug conjugate therapies.

What does this study add?

- We generated a CD56-targeting monomethyl auristatin E (MMAE) bioconjugate antibody (Adcitmer®) and demonstrated its optimal biochemical properties, specific binding and cytotoxicity *in vitro* in MCC cell lines, and its capacity to reduce tumour growth *in vivo* in a MCC mouse model.

What is the translational message?

- Adcitmer®, a new CD56-targeting MMAE bioconjugate antibody, may represent a potential therapeutic option to be investigated as an alternative approach or in combination with immune checkpoint inhibitors in patients with inoperable MCC.

Merkel cell carcinoma (MCC) is a rare and aggressive cancer of the skin with a 5-year overall survival rate of 40%.[1] In 2008, Feng *et al.* identified the integration of a previously unknown virus – the Merkel cell polyomavirus (MCPyV) – in the genome of MCC tumour cells.[2] Subsequent studies established that about 80% of cases of MCC are associated with MCPyV, while the remaining virus-negative cases of MCC harbour a prominent ultraviolet (UV)-signature.[1,3] In the past, patients with an unresectable metastatic tumour received platinum-based chemotherapy without any benefit to survival.[4,5] Then came immune checkpoint inhibitors, which revolutionized MCC therapy. In this regard, the programmed cell death-ligand 1 inhibitor avelumab has been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency for the treatment of metastatic MCC in 2017, followed by approval of pembrolizumab – an antibody targeting programmed cell death 1 (PD-1) – by the FDA in 2018.[6] When used in a first-line setting, immune checkpoint inhibitors provide objective response rates in 50% of patients with MCC and, more importantly, sustained responses are achieved in 50% of responders.[5,7,8] Nevertheless, approximately half of patients do not respond to immune checkpoint inhibitors,[7,8] underlining the need for new therapeutic options.

Despite the identification of several potential molecular targets (such as prosurvival Bcl-2 family members, receptor tyrosine kinases or somatostatin receptors), the targeted therapy approach has, until now, been disappointing in MCC,[9] with only a small proportion of patients demonstrating clinical responses to various targeted therapies in clinical trials.[10–14] Antibody–drug conjugates (ADCs) represent one targeted therapy approach that allows for specific delivery of a drug to cancer cells.[15–19] Importantly, recent technical progress in the production of ADCs allow the achievement of optimal levels of cytotoxic drug conjugation [referred to as the drug-to-antibody ratio (DAR)], low level of unconjugated antibody (DAR homogeneity) and stable link between drug and antibody have been identified as three crucial determinants for ADC performances and reduced toxicity.[20–24] CD56, also called neural cell adhesion molecule (NCAM), has been suggested as a relevant therapeutic target in MCC.[25,26] A CD56-targeting ADC, lorvotuzumab mertansine (IMGN901), has been developed by covalently coupling the cytotoxic drug – a maytansinoid – to the humanized anti-CD56 monoclonal antibody huN901 (lorvotuzumab). This drug has demonstrated safety and acceptable tolerability as a monotherapy in different CD56-expressing tumours in phases I/II clinical trials,[27–30] including objective responses in two of four patients with MCC.[27] However, toxicity rates of lorvotuzumab mertansine combined with conventional chemotherapy in a phase II trial involving patients with small cell lung cancer led to the discontinuation of the drug development.[31] Moreover, three other CD56-targeting ADCs (i.e. m906 conjugated to pyrrolobenzodiazepine dimer, promiximab–duocarmycin and promiximab–monomethyl auristatin E [MMAE])[32–34] have been developed and investigated *in vitro* or in preclinical small cell lung cancer and neuroblastoma models.

In this study, taking advantage of the recent technical progress in improving the performance of ADCs, we generated a CD56-targeting ADC called Adcitmer® (PCT/FR2021/050332), comprising the previously described m906 antibody and an antimitotic drug, MMAE.[32] Subsequently, the performances of Adcitmer® were evaluated *in vitro* and in a xenograft mouse model of MCC.

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Materials and methods

Patients and samples

Tumour samples were selected from a historical/prospective cohort of patients with MCC from 10 French hospital centres; tumour tissues were collected between 1998 and 2017 [local ethics committee (Tours, France) approval no. RCB2009-A01056-51]. The inclusion criteria have been described previously.[35] Among the cohort, only cases with available formalin-fixed paraffin-embedded tumours and interpretable CD56 immunohistochemical staining of tissue microarrays (TMAs) were included.

Clinical, follow-up data and Merkel cell polyomavirus status determination

Age, sex, American Joint Committee on Cancer (AJCC) stage at the time of surgery and the location of the primary tumour (primary tumour vs. metastasis) were collected from the patient files. MCPyV status was determined using real-time polymerase chain reaction (PCR) as previously described.[36]

Cell lines

The following MCC cell lines were used: WaGa (RRID: CVCL_E998), MS-1 (RRID: CVCL_E995), PeTa (RRID: CVCL_LC73) and MKL-2 (RRID: CVCL_D027). In addition, SK-BR-3 (RRID: CVCL_0033), a human epidermal growth factor receptor 2 $(HER2)^{+}$ $CD56^{-}$ breast cancer cell line, was included as the negative control. MCC cell lines were grown in Roswell Park Memorial Institute medium containing 10% fetal calf serum (FCS), 1% penicillin, streptomycin and amphotericin B. SK-BR-3 were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FCS and 1% penicillin, streptomycin and amphotericin B.

Plasmids and lentiviral transduction

Plasmid encoding the CD56-targeting m906 antibody was kindly provided by Dr Feng.[32] Lentiviral plasmids encoding a clustered regularly interspaced short palindromic repeats (CRISPR) guide targeting three different CD56 exons and Cas 9 were used. Guides were designed using the CRISPOR website (Appendix S1; see Supporting Information). A CD56 expression construct was generated by cloning the human CD56 sequence in a pCDH backbone (System Biosciences, Palo Alto, CA, USA) containing puromycin resistance.

Evaluation of CD56 expression by reverse transcription polymerase chain reaction and immunoblot

For reverse transcription PCR (RT-PCR), total cellular RNA was isolated using the peqGOLD total RNA kit (Peqlab, Erlangen, Germany) with a subsequent DNaseI digestion step according to the manufacturer's instructions, followed by cDNA synthesis with the Superscript II RT First Strand Kit (Invitrogen, Karlsruhe, Germany). *CD56* and *RPLP0* transcripts were amplified using the previously described primers,[37,38] and PCR products were separated on agarose gel. CD56 protein expression was detected by immunoblot using an anti-CD56 antibody (sc-7326, 1/200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Appendix S1).

Immunohistochemistry

Immunohistochemical staining for CD56 (123C3, prediluted; Ventana, Oro Valley, AZ, USA) on TMA was performed using a BenchMark XT Platform according to the manufacturer's instructions. Immunocytostainings were performed using m906 (650 ng mL^{-1}) or trastuzumab [TTZ; 40 ng mL–1 (Roche, Basel, Switzerland)]. CD56 was evaluated by one pathologist (T.K.) who was blinded to the clinical data using the following semi-quantitative score: 0, lack of expression; 1, weak and/or heterogenous positivity; 2, intense and diffuse positivity.

Production of antibody–drug conjugates

Antibody disulfide bridges, m906 or TTZ were reduced and allowed to react with bis(bromomethyl)pyridine-caproic-valine-citrulline-*p*-aminobenzylcarbonate-monomethyl auristatin E, following the McSAF Inside® patented protocol. ADCs were purified by size-exclusion chromatography [PD-10 desalting column (GE Healthcare, Chicago, IL, USA) and PBS pH 7·4 (Gibco, Waltham, MA, USA)] to remove residual small molecules. Protein concentration was determined by UV spectroscopy [280 nm; NanodropOne® (Thermo Fisher Scientific, Waltham, MA, USA)]. Hydrophobic interaction chromatography (HIC), size exclusion chromatography and mass spectrometry are described in Appendix S1.

Cytotoxicity assay

To evaluate cell viability and cellular metabolic activity, 2,3-Bis(2-methyoxy-4-nitro-5-sulfophenyl) (XTT) assays were performed according to standard protocols. Cells were plated in six replicates in a 96-well plate with 50 000 cells per well. Adcitmer®, m906 and MF–TTZ–MMAE were added in incremental concentrations, considering that each antibody bears four MMAE molecules. Untreated cells were used as viability reference. In addition, cells treated with Triton X100 1% were included to confirm that cell death could be detected. After 4 days, 25 µL XTT reagent at 1 mg mL^{-1} (Alfa Aesar; Thermo Fisher Scientific) with N-methyl dibenzopyrazine methyl sulfate activator (25 µmol L^{-1}) was added per well and the absorbance was read at 450 nm after 4 h of incubation. Absorbance at 620 nm was used as a reference. Three independent experiments were performed. Half-maximal inhibitory concentration (IC_{50}) was determined with GraphPad Prism (GraphPad, La Jolla, CA, USA).

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Flow cytometry and cell cycle analysis

For CD56 staining, after washing cells with bovine serum albumin (BSA) 0·1% (Sigma-Aldrich, St Louis, MO, USA) in PBS, cells were incubated with CD56 Alexa Fluor 647 antibody (NCAM 123C3 Alexa Fluor 647; Santa Cruz Biotechnology) for 20 min on ice. After two washes with PBS 0·1% BSA, cells were analysed by flow cytometry. For DNA staining, cells were fixed using 90% ice-cold ethanol for at least 1 h, followed by treatment with a propidium iodide (PI) solution (PBS supplemented with 1% FCS, 0·1 mg mL^{-1} PI and 0·1 mg mL^{-1} RNAse A) at 37°C for 1 h. The cell cycle was then analysed by flow cytometry (Cytoflex; Beckman Coulter, Brea, CA, USA). All details regarding imaging flow cytometry analysis (combination of fluorescence microscopy and flow cytometry analysis) are provided in Appendix S1.

Mice model

Eighteen 7-week-old female nonobese diabetic/severe combined immunodeficiency mice (Janvier Labs, Le Genest-Saint-Isle, France) were maintained under aseptic conditions. All animal procedures were approved by the local ethics committee (Apafis#10076-2017053015488124v4). The WaGa CD56-expressing MCC cell line was used for tumour induction.[39] Isoflurane-anaesthetized mice received one subcutaneous injection of 10^7 cells associated with 10% Matrigel on the right flank. The general state of the animals was monitored every 2 days during the entire procedure. Tumour volume was measured with a caliper as $\pi/6 \times$ width $\times$ length $\times$ height. When the tumour volume reached 50 mm^3, mice were included in the study and randomly assigned to the different groups.

After inclusion, animals (six per group) received intravenous (IV) injections of ADC (either Adcitmer® or MF-TTZ–MMAE 10 mg kg^{-1}) or a volume-equivalent injection of PBS. As no dose regimens are available for such therapy, we planned for additional injections in each group whenever the mean tumour volume of the experimental group had doubled in size. During the experiment the mice received a total of three injections (on days 0, 14 and 26). Mice were sacrificed 30 days after inclusion or when they reached a predefined endpoint (tumour ulceration, weight loss > 20% or prostration). Tumours, lungs and liver were removed for macroscopic examination. After dissection, tumour weight and volume were assessed. In order to detect metastatic spread and the potential toxicity of Adcitmer®, whole tumours and organs (lung and liver) were formalin-fixed and paraffin embedded for microscopic evaluation of architecture changes, inflammatory infiltrate, necrosis, vascular changes and fibrosis. In addition, livers were analysed for signs of steatosis.

Statistical analysis

Continuous data are described as mean (range). Categorical data are described as number (percentage) of interpretable cases. Associations were assessed by the two-tailed Fisher exact test for categorical data, and Mann–Whitney or Kruskal–Wallis tests for continuous data. Statistical analysis was conducted using XLStat (Addinsoft, Paris, France). $P < 0{\cdot}05$ was considered to be statistically significant.

Results

The majority of Merkel cell carcinoma cases express CD56

Previous studies have demonstrated CD56 expression in MCC tumours.[26,40] To confirm these findings and to detect any potential association with patient characteristics, we analysed a set of 90 MCC tumours [Figure 1a (Table S1; see Supporting Information)]. Overall, 66% of samples expressed CD56 ($n = 59$), with strong expression (defined as a score of 2) detected in 37% of analysed cases ($n = 33$). Age, sex, AJCC staging and prognosis did not differ between CD56 high and low expressing cases. Importantly, CD56 was expressed in both MCPyV-positive and MCPyV-negative tumours (cutoff 1·2 copies per cell) suggesting that a CD56-targeting therapeutic approach might be suitable for both subsets (Figure 1b).

Accordingly, CD56 expression was confirmed in four MCPyV-positive MCC cell lines by PCR, immunoblot and flow cytometry [Figure 1 c–e (Figure S1; see Supporting Information)], demonstrating those as appropriate *in vitro* models with which to analyse CD56-based drug conjugates. Owing to the lack of a validated MCPyV-negative MCC cell line,[41] such cell lines were not included in the experiments.

m906 interacts with Merkel cell carcinoma cells

As the expression of surface markers can be used to deliver cytotoxic drugs to cancer cells by ADC,[24] the interaction of a noncytotoxic, unconjugated CD56-targeting antibody (m906)[32] with MCC cell lines was established as a first validation step. Binding of m906 and TTZ, a HER2-targeting antibody used as a nonrelevant control, was evaluated on the four CD56-expressing MCC cell lines and one CD56-negative, HER2-overexpressing SK-BR-3 breast cancer cell line (Figure 2).

Immunohistochemistry demonstrated that m906 bound to all MCC cell lines and not to SK-BR-3, while the opposite was seen with TTZ, confirming the specificity of antibody/cell binding (Figure 2a). Rapid internalization of m906 and its co-localization with lysosomes, a crucial step in ADC drug release, was evidenced by imaging flow cytometry in MCC cell lines [Figure 2b; Figure S2 and Table S2 (see Supporting Information)].

McSAF Inside® bioconjugation process allows the production of a highly homogenous antibody–drug conjugate

Next, m906 and TTZ were site-specifically conjugated with a potent antimitotic, MMAE (Figure 3a), using McSAF Inside®

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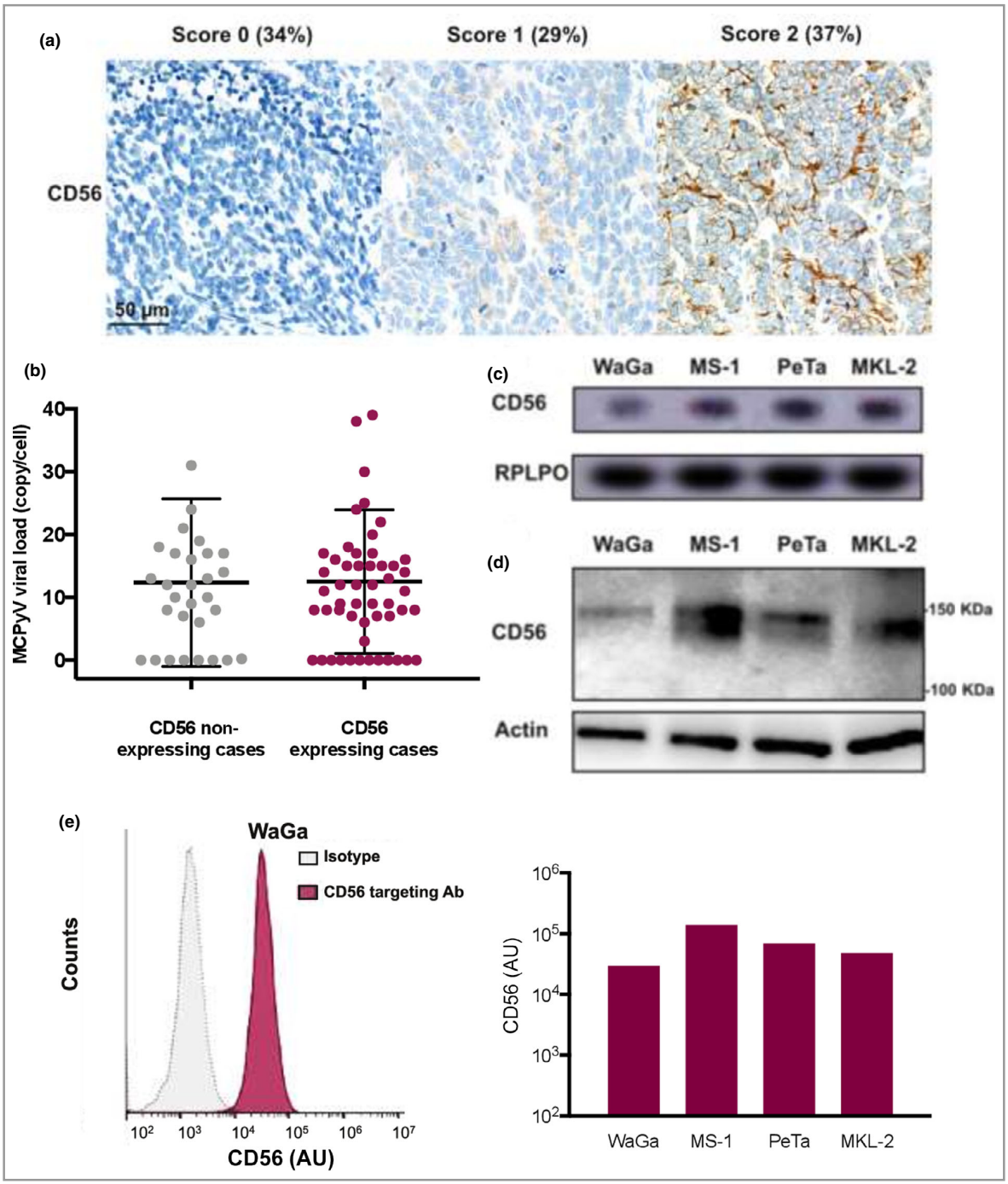

Figure 1 Expression of CD56 in Merkel cell carcinoma (MCC) tumours and cells lines. (a) Representative microscopic view of CD56 immunohistochemical detection in MCC tumour samples (scale bar = 50 µm). Representative MCC cases with none (score 0), low (score 1) and high (score 2) expression are depicted. Percentages of cases with the respective CD56 expression levels are given in brackets. (b) Merkel cell polyomavirus (MCPyV) viral load determined by real-time polymerase chain reaction (PCR) according to CD56 expression (results are expressed in MCPyV copies per cell). Horizontal line is the median, box edges are the interquartile range and whiskers are ranges. (c–e) Confirmation of CD56 expression in four MCPyV-positive MCC cell lines (WaGa, MS-1, PeTa and MKL-2) assessed by (c) reverse transcription PCR, (d) immunoblot (predominant band size 140 kDa isoform) and (e) flow cytometry. Ab, antibody.

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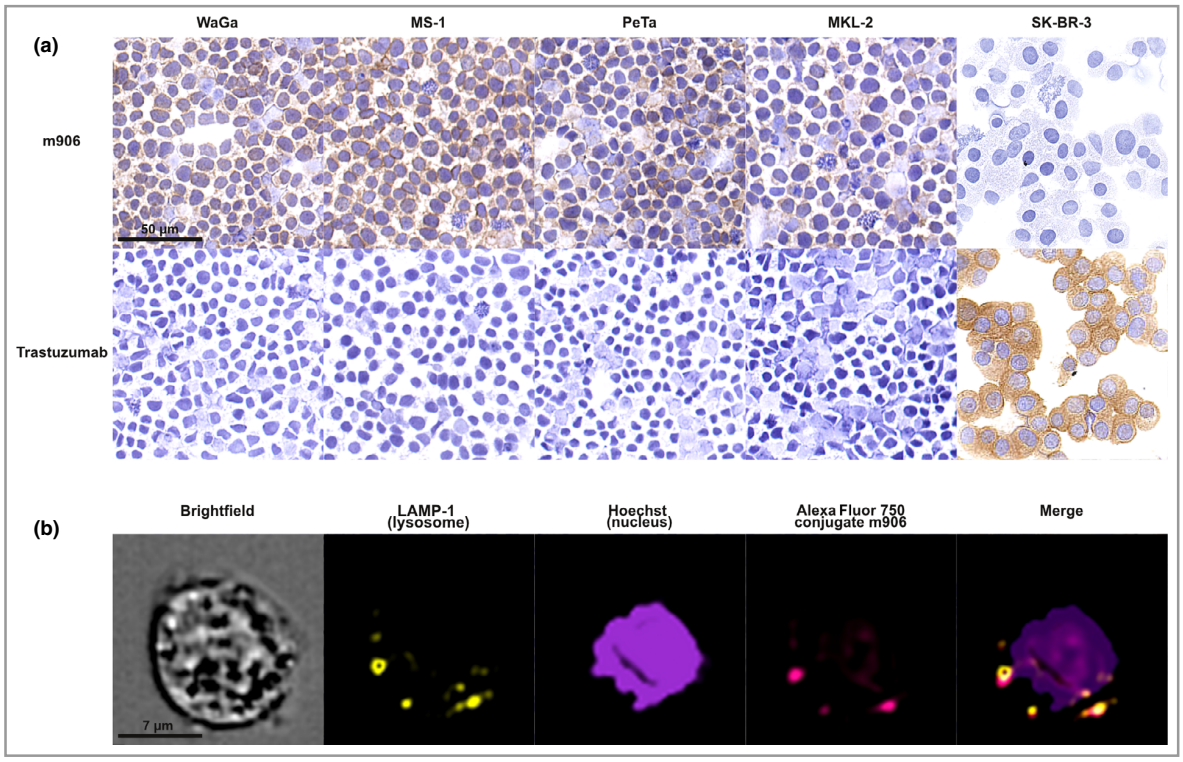

Figure 2 Binding and internalization of m906 by Merkel cell carcinoma (MCC) cell lines. (a) Binding assessment of m906 and trastuzumab [human epidermal growth factor receptor 2 (HER2)-targeting antibody] to MCC cell lines and SK-BR-3 ($CD56^{-}$ $HER2^{+}$ breast cancer cell line used as a control) evaluated by immunocytochemistry (scale bar = 50 µm). (b) Representative example of co-localization of Alexa Fluor-conjugated m906 with the lysosome marker lysosomal-associated membrane protein 1 (LAMP-1) in WaGa tumour cells (after 30 min incubation). Quantification of this analysis is provided in Table S2 (see Supporting Information).

technology (Figure 3b).[42] Levels of cytotoxic drug conjugation, as well as DAR homogeneity – two biochemical determinants of ADC performances[20–23] – were evaluated for both products (Adcitmer® and MF-TTZ–MMAE) and compared to a representative first-generation ADC (Figure 3c). Adcetris is an FDA-approved CD30-targeting MMAE-conjugated ADC; the DAR ranges from 0 to 8. The Adcetris linker is comparable to Adcitmer®'s linker (i.e. valine-citrulline-*p*-aminobenzyloxycarbonyl–MMAE), while the bioconjugation head differs in the two products. Optimal performances are seen with homogenous ADCs with four MMAE molecules per antibody.[21] While hydrophobic interaction chromatography revealed a mean DAR that was suboptimal for first-generation Adcetris (mean DAR of 3·4 and only 35% of species with a DAR of 4), the newly generated Adcitmer® and MF-TTZ–MMAE reached a mean DAR of 3·9 and 3·8, with 60% and 64% of species having a DAR of 4, respectively [Figure 3d; Figure S3 (see Supporting Information)]. Moreover, using size-exclusion chromatography analysis, Adcitmer® and MF-TTZ–MMAE were almost exclusively constituted of monomers (99% and 98% for m906- and TTZ-MMAE, respectively) (Figure 3e), confirming the absence of aggregation. These findings indicate that the chemical properties of Adcitmer® overcame those of commercially available ADC and therefore might result in stronger therapeutic performances and reduced toxicities.

Adcitmer® exerts CD56-specific cytotoxicity towards Merkel carcinoma cells *in vitro*

To investigate whether Adcitmer® affects the viability of MCC cell lines, dose–response curves were determined by assessing viability after 4 days of drug exposure (Figure 4). Adcitmer® was found to be as cytotoxic as unbound MMAE on MCC cell lines (IC_{50} 2.5–30.7 nmol L^{-1}). Cellular viability was not affected by control treatments, with either unconjugated m906 or MF-TTZ–MMAE, suggesting that unconjugated CD56-specific antibody lacks intrinsic cytotoxicity but is capable of delivering linked MMAE to MCC cells.

To confirm that the cytotoxicity of Adcitmer® was dependent on CD56 expression, CD56 knockout (KO) was obtained in two MCC cell lines (WaGa and PeTa) by CRISPR/Cas9 technology (Figures S4 and S5; see Supporting Information). Accordingly, no cytotoxicity was induced by Adcitmer® in CD56 KO cell lines, while ectopic re-expression of CD56 restored the therapeutic effect of Adcitmer® (Figure 5). Indeed, cell cycle analysis after treatment with MMAE alone or Adcitmer® (Figure 5a) revealed a statistically significant

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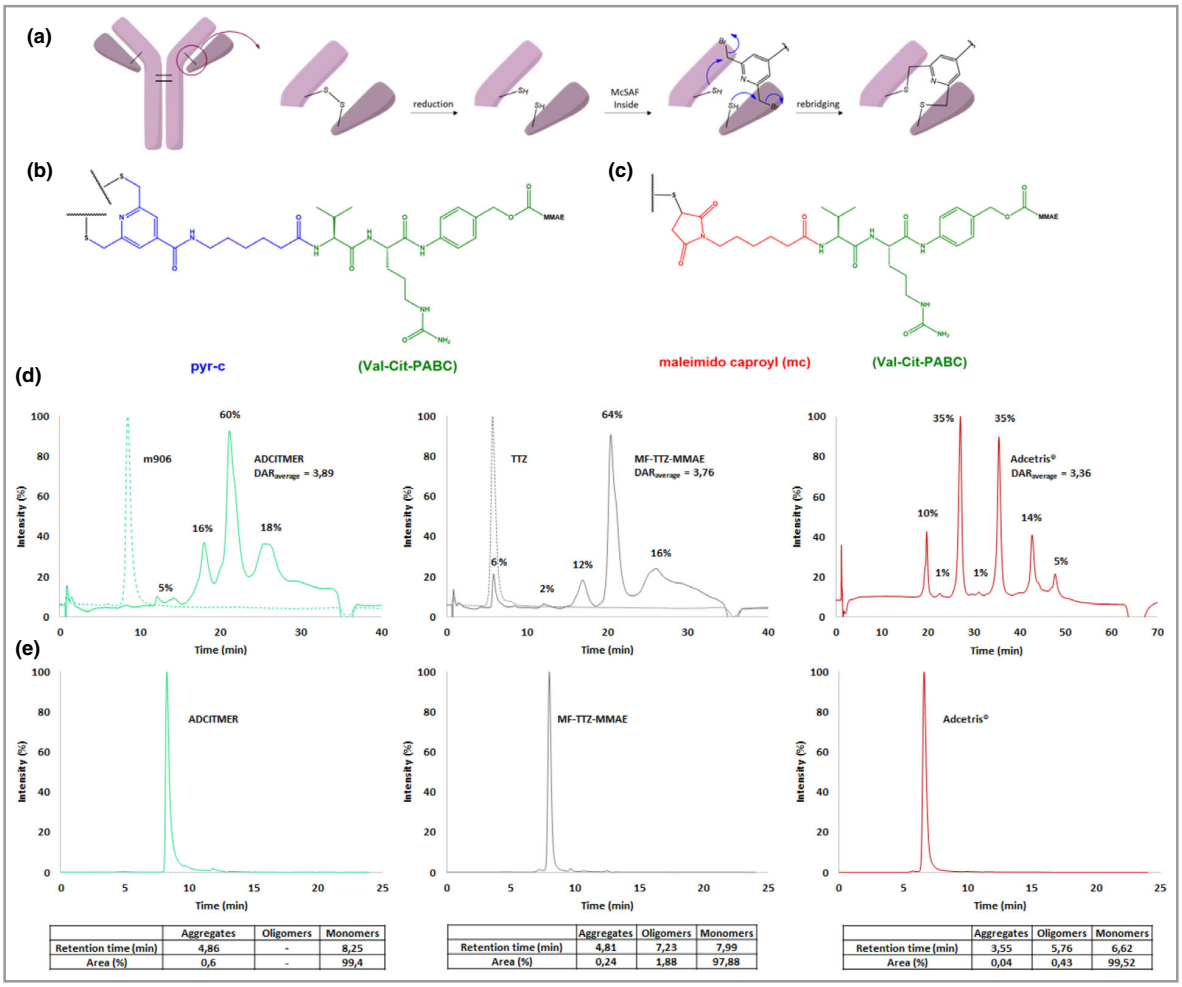

	Aggregates	Oligomers	Monomers
Retention time (min)	4,86	-	8,25
Area (%)	0,6	-	99,4

	Aggregates	Oligomers	Monomers
Retention time (min)	4,81	7,23	7,99
Area (%)	0,24	1,88	97,88

	Aggregates	Oligomers	Monomers
Retention time (min)	3,55	5,76	6,62
Area (%)	0,04	0,43	99,52

Figure 3 Chemical characterization of Adcitmer® and controls. (a) Representation of the rebridging mechanism using a McSAF Inside® bioconjugation head on an IgG1 antibody. (b, c) Chemical structure of the bioconjugation linkers valine (Val)–citrulline (Cit)–human anti-PfCSP recombinant antibody (PABC)–monomethyl auristatin E (MMAE) containing (b) a bifunctional pyridino-caproic head used on m906 and trastuzumab and (c) a maleimido-caproic head used in Adcetris®. (d) Hydrophobic interaction chromatography. The dashed and solid lines correspond to antibody alone and the antibody–drug conjugate (ADC), respectively. (e) Size-exclusion chromatography analysis of Adcitmer®, MF–trastuzumab (TTZ)–MMAE and Adcetris®, an FDA-approved, CD30-targeting, MMAE-conjugated antibody used as representative of MMAE delivering a first-generation ADC. DAR, drug-to-antibody ratio.

increased frequency of cells in G2/M phase and subG1 population compared with untreated cells ($P < 0{\cdot}05$) reflecting cell cycle arrest and cell death, respectively. In contrast, unconjugated m906 did not impair MCC cell viability in this setting. Furthermore, in line with our cytotoxic data (Figure 5b, c), the impact of Adcitmer® was drastically reduced in CD56 KO cells, confirming that CD56 expression is essential for Adcitmer®-mediated cytotoxicity.

Adcitmer® reduces tumour growth in a Merkel cell carcinoma xenograft mouse model

To evaluate the *in vivo* performance of Adcitmer®, an MCC xenograft was performed. Subsequently, animals were treated with IV injections (10 mg kg^{-1}) of Adcitmer®, while mice in the control groups received either MF-TTZ–MMAE (10 mg kg^{-1}) or PBS. All animals received a total of three injections (days 0, 14 and 26). In the PBS and MF-TTZ–MMAE groups (Figure 6a), similar tumour growth was seen [mean growth curve slope = 105% of initial tumour volume per day (range 35–166%) and 108% per day (range 33–318%), respectively], whereas in the experimental group, tumour volume increase was significantly delayed (mean growth curve slope = 18% per day (range 1–53%); Kruskal Wallis test $P = 0{\cdot}01$). Accordingly, final median tumour weight was statistically significantly reduced in the Adcitmer® group compared with the control mice [mean weight 0·7 g (range 0·4–1·3) vs. 2·03 g (range 1·3–3·7) and 1·78 g (range

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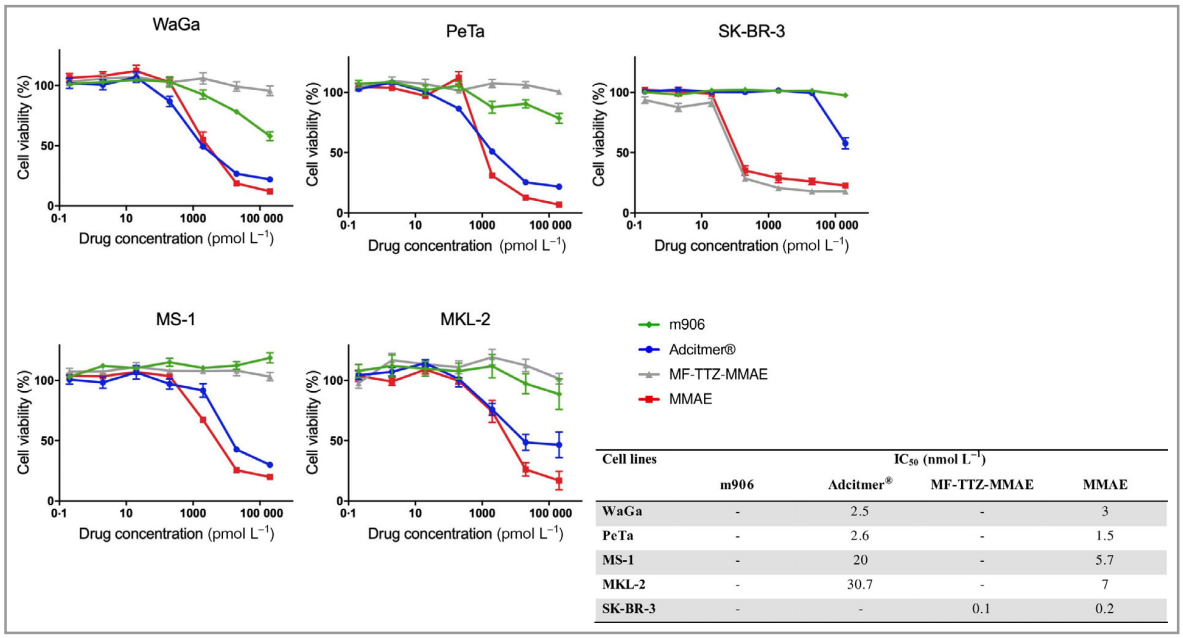

Cell lines	IC_{50} (nmol L^{-1})			
	m906	Adcitmer®	MF-TTZ-MMAE	MMAE
WaGa	-	2.5	-	3
PeTa	-	2.6	-	1.5
MS-1	-	20	-	5.7
MKL-2	-	30.7	-	7
SK-BR-3	-	-	0.1	0.2

Figure 4 Evaluation of Adcitmer® cytotoxicity and controls [m906, monomethyl auristatin E (MMAE) and MF–trastuzumab (TTZ)–MMAE] in Merkel cell carcinoma and SK-BR-3 cell lines *in vitro*. After 4 days of exposure to drugs, viability of WaGa, PeTa, MS-1, MKL-2 and SK-BR-3 cell lines was assessed by an 2,3-Bis(2-methyoxy-4-nitro-5-sulfophenyl) (XTT) assay. Untreated cells were used as a reference. Experiments included six replicates and were performed three times independently. Data are expressed as mean ± SEM of percentages. The half-maximal inhibitory concentration (IC_{50}) value was determined for each molecule and cell line. (–), IC_{50} was not reached with the evaluated doses.

1·0–2·7); Kruskal–Wallis test $P = 0·02$] (Figure 6b, c). After the mice were euthanased, distant metastases were not observed in the experimental or control groups. Importantly, although the study was primarily designed to determine efficacy, no animal demonstrated significant weight loss or reached the predefined endpoint within 30 days. Accordingly, no obvious toxicity of Adcitmer® was seen at necropsy.

Discussion

In this study, we preclinically evaluated Adcitmer®, a new CD56-targeting MMAE conjugated antibody, as a possible therapeutic approach for MCC. Adcitmer® was able to target $CD56^+$ MCC cells specifically and subsequently induce cell death *in vitro*, and to control tumour growth in an MCC xenograft mice model.

CD56 is a membranous adhesion molecule that is physiologically involved in cell adhesion, migration, communication, differentiation and survival.[43] Under physiological conditions, CD56 is mostly expressed by neuroendocrine cells, neurons and natural killer (NK) cells.[44–46] In tumours, CD56 positivity is regarded as a diagnostic marker in several haematological malignancies, some paediatric tumours and neuroendocrine neoplasms, including MCC.[47–49] In the present study, we confirmed CD56 positivity in 66% of cases of MCC, which is comparable to results reported in previous studies.[26,40] Such frequent expression, together with its membranous location, suggest CD56 as a relevant target for the development of an ADC to target MCC. Accordingly, we demonstrated the efficiency of Adcitmer® *in vitro* and in a MCC mice model. Further investigations, including pharmacodynamic and cytotoxic evaluations, are obviously required; however, such an approach is already a potential option for patients with metastatic MCC. Given its cytotoxic activity in MCC preclinical models, Adcitmer® could be assessed as an alternative option or in association with immunotherapy. Interestingly, such a combination of ADC and an immune checkpoint inhibitor has already been investigated in other cancers such as $CD30^+$ lymphomas.[50] Indeed, cytotoxic molecules, either conventional or ADCs, have been shown to improve immune antitumour response via the release of tumour antigens and stimulation of both innate and adaptive immunity,[51,52] and therefore might exert synergistic effects with immune checkpoint inhibitors.

Of note, three CD56-targeting ADCs have been developed and investigated *in vitro* or in preclinical small cell lung cancer and neuroblastoma models, but these have not been developed further.[32–34] Lorvotuzumab mertansine has already been assessed in CD56-expressing tumours, including small cell lung cancer, ovarian carcinoma, neuroblastoma, myeloma and MCC.[27,28,31] In this setting, several phase I and II clinical trials have demonstrated acceptable tolerance and revealed clinical activity, especially in patients with MCC. Importantly, while CD56-targeting strategies might result in a reduction of CD56-expressing NK cells,[29] it should be noted that no clinically

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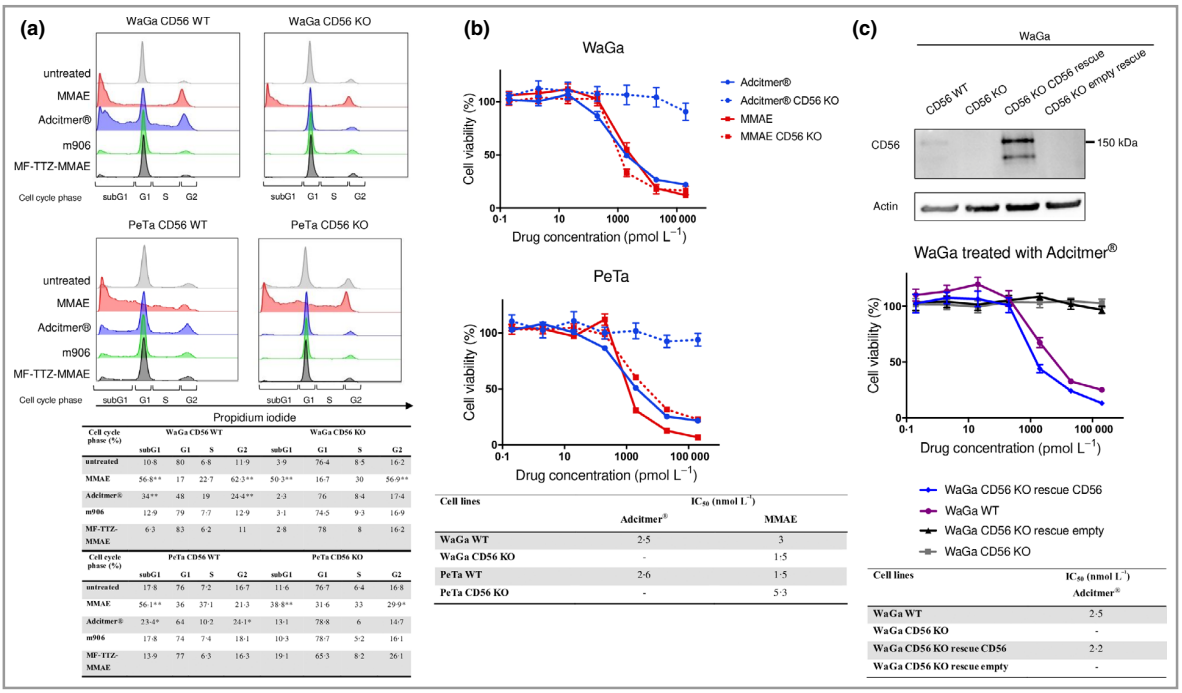

Cell cycle phase (%)	WaGa CD56 WT				WaGa CD56 KO			
	subG1	G1	S	G2	subG1	G1	S	G2
untreated	10·8	80	6·8	11·9	3·9	76·4	8·5	16·2
MMAE	56·8**	17	22·7	62·3**	50·3**	16·7	30	56·9**
Adcitmer®	34**	48	19	24·4**	2·3	76	8·4	17·4
m906	12·9	79	7·7	12·9	3·1	74·5	9·3	16·9
MF-TTZ-MMAE	6·3	83	6·2	11	2·8	78	8	16·2

Cell cycle phase (%)	PeTa CD56 WT				PeTa CD56 KO			
	subG1	G1	S	G2	subG1	G1	S	G2
untreated	17·8	76	7·2	16·7	11·6	76·7	6·4	16·8
MMAE	56·1**	36	37·1	21·3	38·8**	31·6	33	29·9*
Adcitmer®	23·4*	64	10·2	24·1*	13·1	78·8	6	14·7
m906	17·8	74	7·4	18·1	10·3	78·7	5·2	16·1
MF-TTZ-MMAE	13·9	77	6·3	16·3	19·1	65·3	8·2	26·1

Cell lines	IC_{50} (nmol L^{-1})	
	Adcitmer®	MMAE
WaGa WT	2·5	3
WaGa CD56 KO	-	1·5
PeTa WT	2·6	1·5
PeTa CD56 KO	-	5·3

Cell lines	IC_{50} (nmol L^{-1}) Adcitmer®
WaGa WT	2·5
WaGa CD56 KO	-
WaGa CD56 KO rescue CD56	2·2
WaGa CD56 KO rescue empty	-

Figure 5 Adcitmer® exerts activity only when MCC cell lines express CD56. (a) Cell cycle analysis of Adcitmer® and control treatments on WaGa and PeTa CD56 wild type (WT) or knockout (KO). (b) WaGa and PeTa CD56 WT or KO were exposed to Adcitmer® or monomethyl auristatin E (MMAE) alone to evaluate the cytotoxic effect. The dose–response curves for CD56 KO cells are depicted as dashed lines. (c) Evaluation of CD56 expression of WaGa CD56 WT, KO, ectopic re-expression of CD56 (CD56 rescue) and empty rescue. Adcitmer® cytotoxicity was evaluated in WaGa CD56 WT, KO, CD56 rescue and empty rescue. Dose–response curves for CD56 KO or rescue empty cells are depicted as grey and black lines, respectively. The half-maximal inhibitory concentration (IC_{50}) value was determined for each molecule and cell line. (–), IC_{50} was not reached with the evaluated doses. TTZ, trastuzumab. (*P < 0·05, **P < 0·005).

relevant side-effects related to NK depletion were seen with CD56-targeting therapies.[27,28,31] Furthermore, mature NK cells are mostly non- or slowly dividing cells,[53] and as MMAE only affects cells in division, Adcitmer® may have only limited toxicity to NKs.[34] Accordingly, several clinically approved ADCs such as brentuximab vedotin (targeting CD30),[54] polatuzumab vedotin (targeting CD79)[55] or inotuzumab ozogamicin (targeting CD22)[56] actually engage with ligands expressed by immune cells but still harbour therapeutic efficiency and acceptable tolerance.

By contrast, most common ADC toxicities are related to unspecific cytotoxic drug release. In a phase II clinical trial evaluating a CD56-targeting ADC, lorvotuzumab mertansine in combination with cisplatin and etoposide in small cell lung cancer,[31] increased mortality due to severe neutropenia was observed. As CD56 is absent on neutrophils, such a side-effect is probably due to the cumulative toxicity of chemotherapy and ADC, or might be related to an unspecific drug release, which is dependent on the bioconjugation process. The generation of ADC results from the bioconjugation of a monoclonal antibody with a highly cytotoxic molecule using a spacer arm (i.e. linker), according to various grafting technologies. First-generation bioconjugation, consisting of the engraftment of cytotoxic molecules either on cysteine (after a reduction in disulfide bonds) or on lysine, led to heterogeneous ADCs with variation in the number and location of engrafted molecules.[57] In contrast, optimal performances are seen with a homogenous ADC with four MMAE molecules per antibody.[21] In addition, first-generation bioconjugation frequently involved the use of first-generation maleimide, which induced linker instability and potential unspecific release of MMAE in the blood.[20,21] In the present study, the bioconjugation technology was devoid of a first-generation maleimide motif, leading to the production of a homogeneous ADC with an optimal average DAR close to 4, which might result in stronger therapeutic activity and reduced toxicities. Moreover, ADC produced with our bioconjugation technology recently demonstrated enhanced stability under thermal stress conditions or in the presence of human serum albumin, when compared to first-generation ADCs.[42] Thus, the use of site-specific bioconjugation technology for the generation of Adcitmer® is likely to improve the toxicity rates of Adcitmer® over lorvotuzumab mertansine. Further toxicity studies, such as the determination of the maximum tolerated dose and optimization of the therapeutic scheme, have to be conducted during further development.

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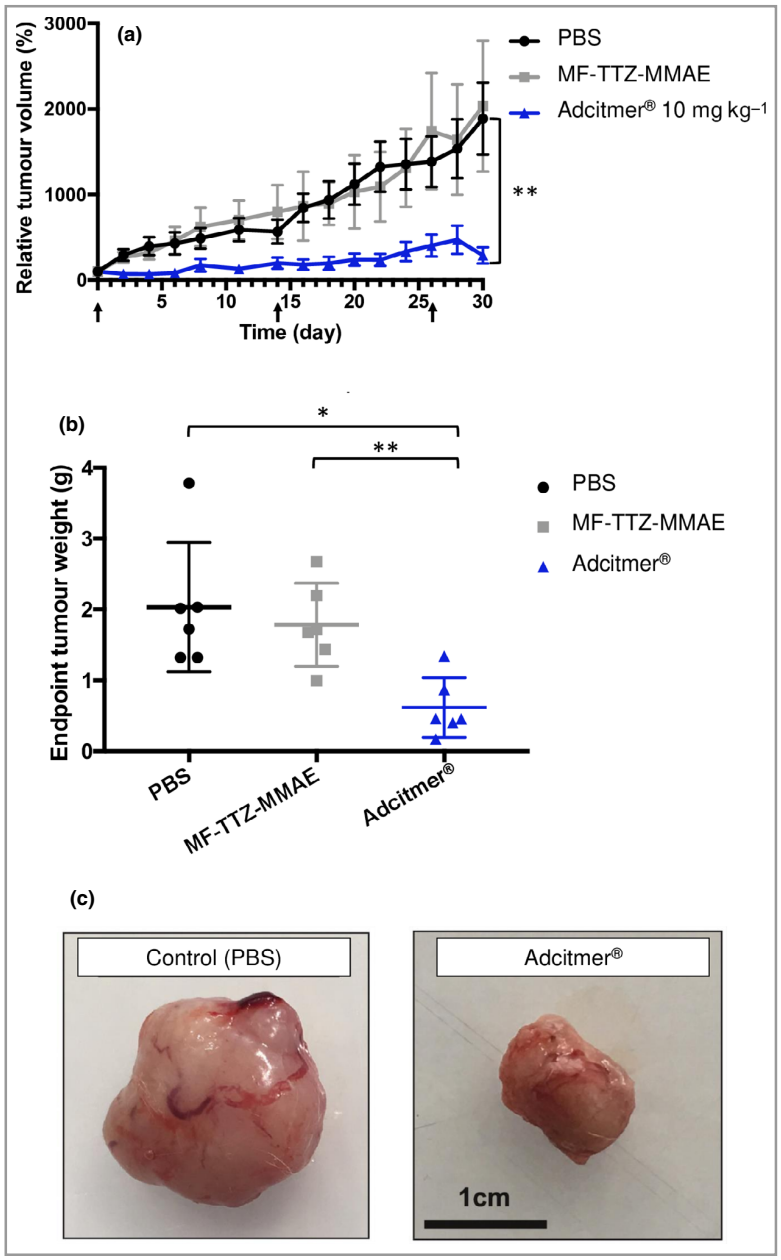

Figure 6 Adcitmer® significantly impairs tumour growth in a Merkel cell carcinoma xenograft. (a) Mean relative tumour volume curves ± SEM during the experiment. Relative tumour volumes (RTVs) were calculated using start point tumour volume as the reference (volume day 0 = 100%). Each point represents mean RTVs in Adcitmer®, MF–trastuzumab (TTZ)–monomethyl auristatin E (MMAE) and phosphate-buffered saline (PBS)-treated groups. Each injection time point is indicated by a black arrow (days 0, 14 and 26). (b) Endpoint tumour weights in the experimental and control groups (*$P < 0{\cdot}05$, **$P < 0{\cdot}005$). Horizontal lines are mean ± SEM. (c) Representative macroscopic views of tumours in the Adcitmer®- and PBS-treated groups (tumours representative of the median group volume are depicted).

To conclude, CD56-targeting MMAE-conjugated Adcitmer® appears to be a promising therapeutic option in preclinical models of MCC. Such treatment might be investigated as an alternative option or in combination with immunotherapy in patients with advanced MCC.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1 CRISPR guides targeting CD56.
Table S1 Clinical and virological characteristics of the patient cohort according to CD56 expression.
Table S2 Internalization of m906 and lysosomal localization scores.
Figure S1 Expression of CD56 in primary fibroblasts compared to PeTa MCC cell line, and evaluation of Adcitmer® cytotoxicity and controls.
Figure S2 Binding of Alexa Fluor 750-conjugated m906 to WaGa CD56 wild type (WT) and WaGa CD56 knockout (KO) by flow cytometry, and internalization of Alexa Fluor 750-conjugated m906 to WaGa CD65 KO by imaging flow cytometry.
Figure S3 Mass spectrometry characterization of Adcitmer® and control (raw data and deconvoluted spectra).
Figure S4 CD56 expression of WaGa and PeTa wild type (WT) and knockout using three different CRISPR guides.
Figure S5 Evaluation of CD56 expression of WaGa CD56 wild type (WT), knockout (KO), ectopic re-expression of CD56 (CD56 rescue) and empty rescue by immunoblot and quantification using ImageJ, and evaluation of CD56 expression of WaGa and PeTa WT and KO using three different CRISPR guides and quantification using ImageJ.

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Appendix S1. CRISPR guides targeting CD56.

CD56 Guides	Target	Sequence
A	Exon 2	GGATATTGTTCCCAGCCAGG
B	Exon 3	AAACCAGCAGCGGATCTCAG
C	Exon 7	AAAGGTGGATAAGAACGACG

Supplementary Table 1. Clinical and virological characteristics of the patient cohort according to CD56 expression.

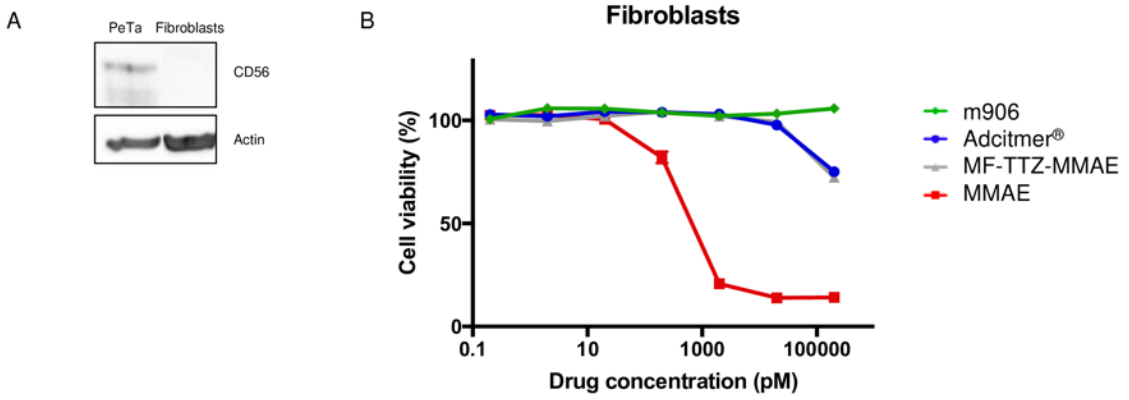
CD56 expression	Score 0 (n=31)	Score 1 (n=26)	Score 2 (n=33)
Age, years			
mean, ranges	75 (50-95)	78 (57-91)	71 (50-96)
Unavailable data	4	4	7
Sex			
F/M	20/11	14/11	18/13
Unavailable data	0	1	2
AJCC Stage, n (%)			
I	8 (33%)	5 (28%)	7 (32%)
II	7 (29%)	10 (55%)	4 (18%)
III	9 (38%)	3 (17%)	10 (45%)
IV	0	0	1 (5%)
Unavailable data	7	8	11
Tumor site, n (%)			
Primary	14 (67%)	14 (78%)	14 (48%)
Metastasis	7 (33%)	4 (22%)	15 (52%)
Unavailable data	10	8	4
MCPyV status, n, (%)			
Negative	8 (26%)	3 (12%)	9 (28%)
Positive	23 (74%)	22 (88%)	23 (72%)
Unavailable data	0	1	1

Supplementary Table 2. Internalization of m906 and lysosomal localization scores.

Incubation period (minutes)	Focused cells count	Surface fluorescence of m906 (MFI*[1])	Intracellular fluorescence of m906 (MFI*[1])	Internalization score of m906	BDS*[2] score (LAMP1/m906)
5	8617	930	50046	7.08	1.4
30	5223	580	50464	7.96	1.4

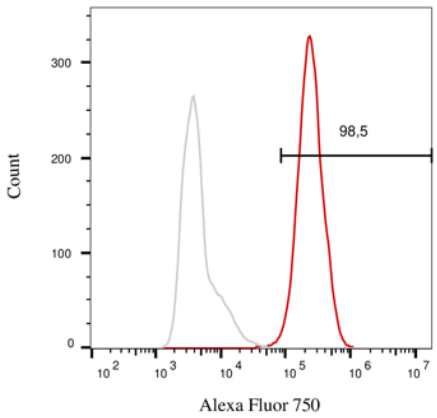
*[1] Mean Fluorescence Intensity

*[2] Bright Detail Similarity



Supplementary Figure 1. (A) Expression of CD56 in primary fibroblasts compared to PeTa MCC cell line. **(B)** Evaluation of Adcitmer® cytotoxicity and controls (m906, MMAE and MF-TTZ-MMAE) in primary fibroblasts. After four days of exposure to drugs, viability of primary fibroblasts was assessed by XTT assay. Untreated cells were used as reference. Experiments included 6 replicates and were performed three times independently. Data are expressed as mean (+/- SEM) of percentages.

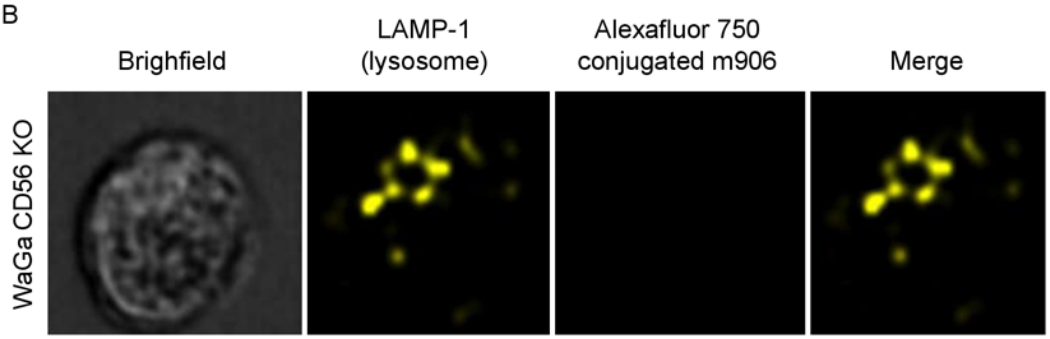
A



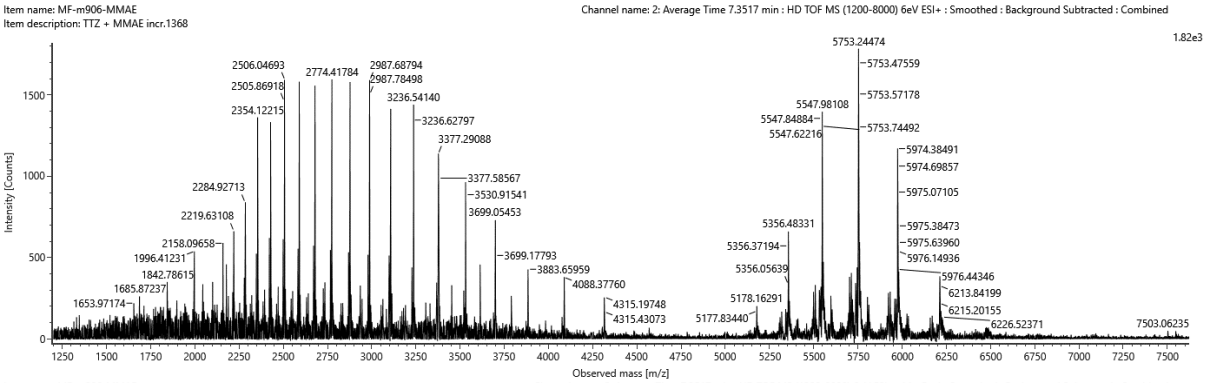
WaGa CD56 KO
WaGa CD56 WT

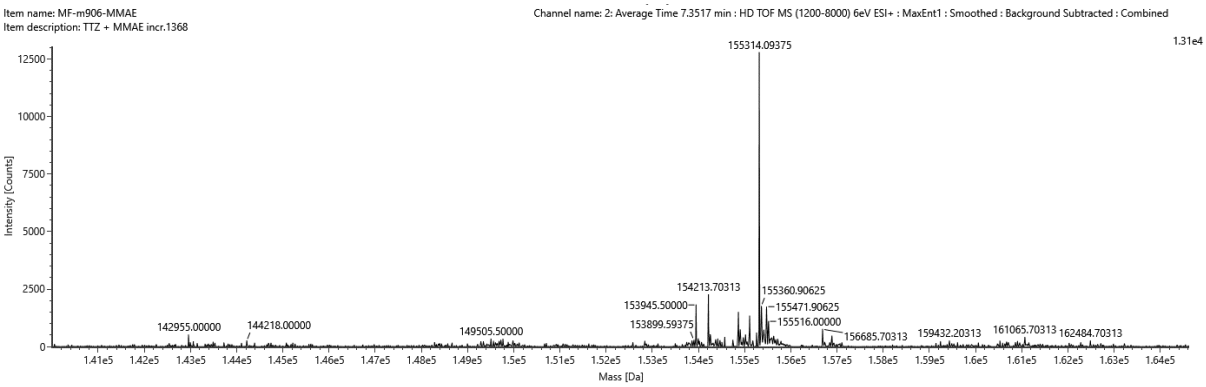
Cells	antibody anti-CD56 (MFI)
WaGa CD56 WT	236361
WaGa CD56 KO	3937

B

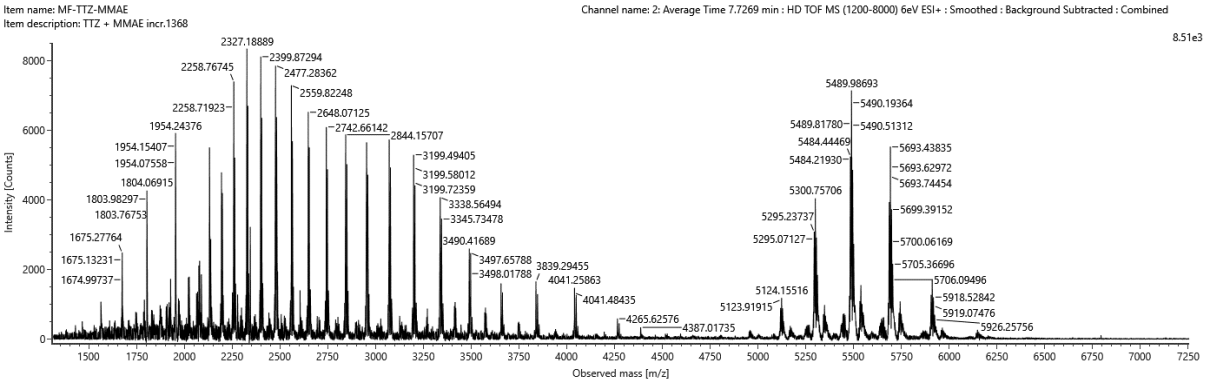


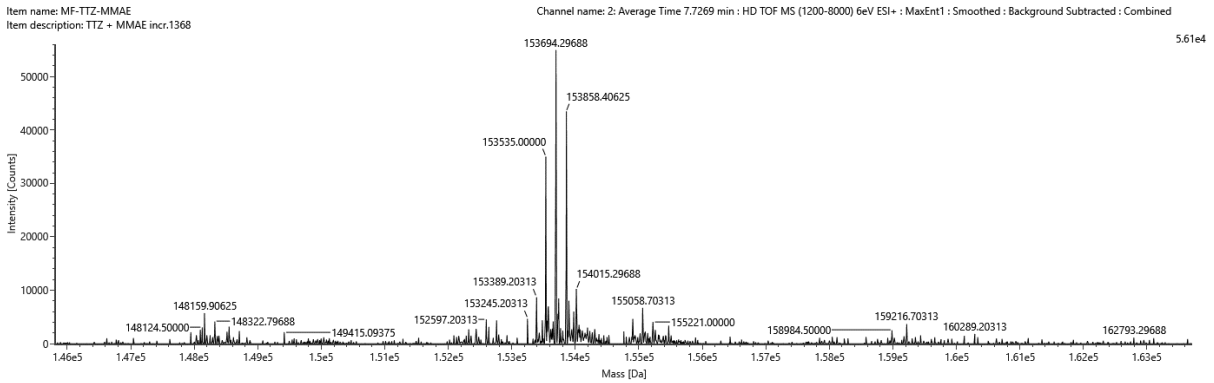
Supplementary Figure 2. Binding of Alexa Fluor 750-conjugated m906 to WaGa CD56 WT and WaGa CD56 KO by flow cytometry **(A)** and internalization of Alexa Fluor 750-conjugated m906 to WaGa CD56 KO by imaging flow cytometry **(B)**.





Adcitmer® (MF-m906-MMAE)	DAR 0	DAR 1	DAR 2	DAR 3	DAR 4	DAR 5
MW (Da)[1]	n.o.[2]	n.o.[2]	152572	153945	155315	156686
Area (%)	0	0	0.7	17.0	74.5	7.7
Mean DAR	**3.89**					

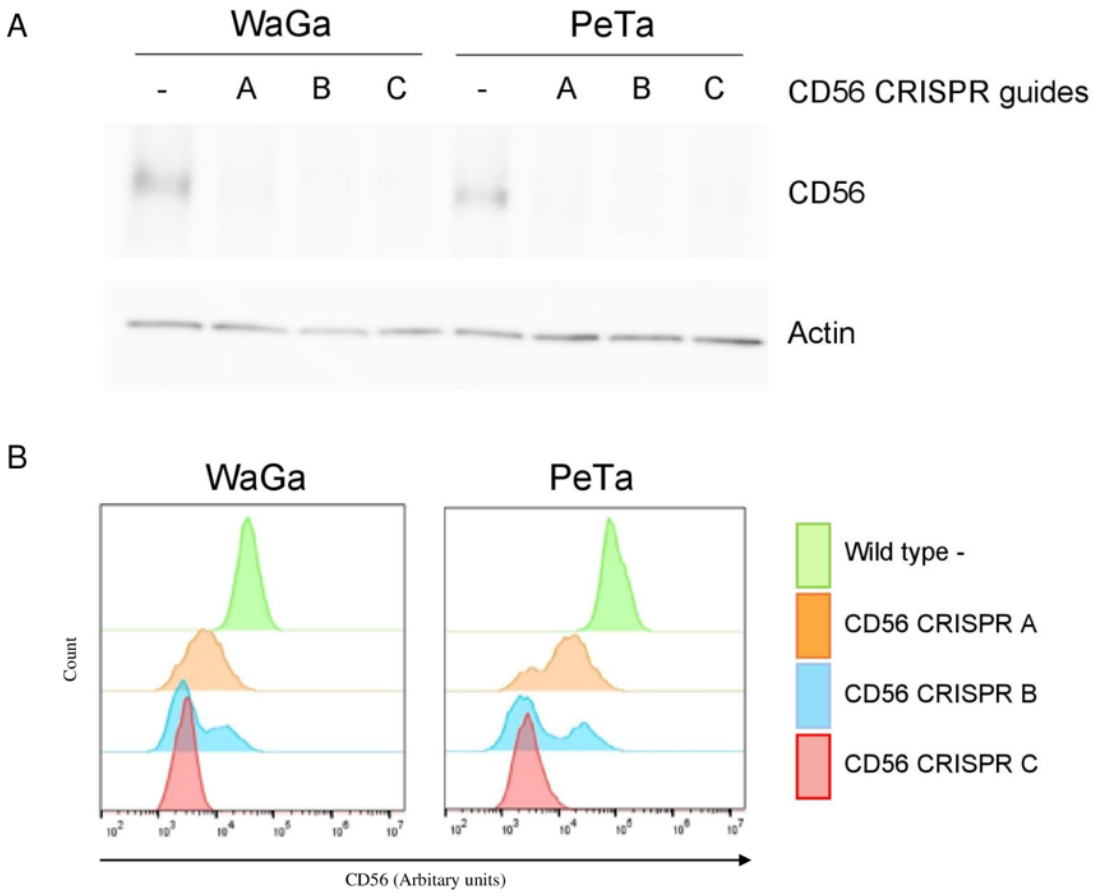




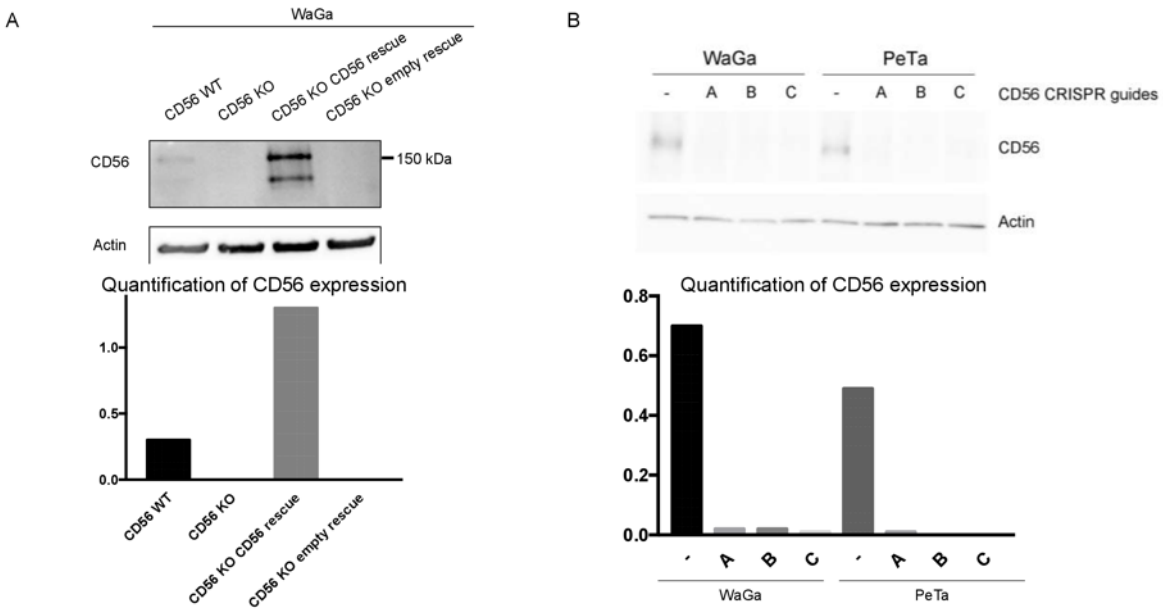
MF-TTZ-MMAE	DAR 0	DAR 1	DAR 2	DAR 3	DAR 4	DAR 5
MW (Da)[1]	148032	n.o.[2]	n.o.[2]	152165	153535	154903
Area (%)	2.7	0	0	4.8	79.6	12.9
Mean DAR	**3.97**					

Supplementary Figure 3. Mass spectrometry characterization of Adcitmer® and control (raw data and deconvoluted spectra).

[1] Area of the first intense glycosylation was used to calculate area percentage.[2] Not observed



Supplementary Figure 4. CD56 expression of WaGa and PeTa wild type and knock-out using 3 different CRISPR guides. **(A)** Immunoblot **(B)** Flow cytometry.



Supplementary Figure 5. (A) Evaluation of CD56 expression of WaGa CD56 WT, KO, ectopic re-expression of CD56 (CD56 rescue) and empty rescue by immunoblot and quantification using ImageJ. **(B)** Evaluation of CD56 expression of WaGa and PeTa wild type and knock-out using 3 different CRISPR guides and quantification using ImageJ.

2. Article 2: evaluation and optimization of Adcitmer®, a MMAE-conjugated ADC targeting CD56-expressing cancers

This study was carried out since 2021 and has been supported by the "*ARD CVL Biomédicaments*".

The goal was to optimally characterize Adcitmer® in MCC and in other CD56-positive neoplasms. The major objectives of this study were:

1) to assess Adcitmer® cytotoxicity *in vitro* on CD56-positive tumors such as SCLC, BPDCN and neuroblastoma.
2) to determine Adcitmer® effect on tumoral phenotype (*i.e.* expression of targeted antigen and bystander potential).
3) to evaluate Adcitmer® interactions with immune cells.
4) to improve Adcitmer® structure considering the previous results.
5) to compare the anti-tumoral effect of Adcitmer® and improved Adcitmer® version in a preclinical MCC model.

The major results of article 2 are summarized as follows:

1) first, Adcitmer® cytotoxicity has been confirmed in SCLC and BPDCN cell lines *in vitro* (with IC50 6.8 and 27 nM, respectively).
2) Adcitmer® induces a CD56 downregulation, which nevertheless, might be couterbalanced by a bystander effect.
3) Adcitmer® interaction with immune cells (both CD56-positive or -negative cells) are related to its interactions with Fc receptors.
4) deglycosylation of Adcitmer® prevents its interaction with immune cells.
5) finally, deglycosylated Adcitmer® significantly reduces tumor growth compared to Adcitmer® in a MCC xenograft model at 10 mg/kg twice a week for 30 days.

Article under preparation:

Evaluation and optimization of Adcitmer®, a MMAE-conjugated ADC targeting CD56-expressing cancers. Clara Esnault[1], Aurélie Drouin[1], Morgane Fouché[2], Shawk Karim[2], Laurine Durand[1], Christine B. Baltus[3], Pauline Gaboriaud[1], Patricia Berthon[1], Valérie Leblond[1], Christine Dhommée[2], Maxime Fredon[3], Camille Martin[4], Fanny Boursin[5], Nicolas Aubrey[5], Roland Houben[6], David Schrama[6], Serge Guyétant[1,7], Francine Garnache-Ottou[3], Audrey Desgranges[4], Marie Claude Viaud-Massuard[4,8], Valérie Gouilleux-Gruart[2], Mahtab Samimi[1,9*], Antoine Touzé[1*], Thibault Kervarrec[1,7*]

This article is presented from page 81 to page 115. To note, in the following section, “Figure X” refers to article’s figures.

Evaluation and optimization of Adcitmer®, a MMAE-conjugated ADC targeting CD56-expressing cancers

Clara Esnault[1], Aurélie Drouin[1], Morgane Fouché[2], Shawk Karim[2], Laurine Durand[1], Christine B. Baltus[3], Pauline Gaboriaud[1], Patricia Berthon[1], Valérie Leblond[1], Christine Dhommée[2], Maxime Fredon[3], Camille Martin[4], Fanny Boursin[5], Nicolas Aubrey[5], Roland Houben[6], David Schrama[6], Serge Guyétant[1,7], Francine Garnache-Ottou[3], Audrey Desgranges[4], Marie Claude Viaud-Massuard[4,8], Valérie Gouilleux-Gruart[2,9], Mahtab Samimi[1,10*], Antoine Touzé[1*], Thibault Kervarrec[1,7*]

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Abstract

Background. NCAM, *i.e.* CD56 is a membrane antigen identified as a potential therapeutic target in several solid tumors and hematological malignancies. Recently we generated an antibody-drug conjugate targeting CD56, called Adcitmer® to treat Merkel cell carcinoma (MCC), a rare and aggressive skin cancer and demonstrated its antitumor properties in MCC preclinical models.

Objectives. In this context, this study aimed to bring further characterization of Adcitmer®. First, Adcitmer® cytotoxicity was assessed *in vitro* in non-MCC CD56-positive cell lines. Then, interactions between Adcitmer® and tumor cells and immune cells were characterized *in vitro*.

Methods. To evaluate the effect of Adcitmer® on other CD56 expressing cancers, cytotoxic assays were conducted on a small cell lung carcinoma (SCLC) cell line - H69, on a blastic plasmacytoid dendritic cell neoplasm (BPDCN) cell line - GEN2.2, and on a neuroblastoma cell line - SK-N-FI. To further characterize how Adcitmer® impacts tumor cell biology, regulation of its target *i.e.* CD56 and induction of a bystander effect upon treatment were evaluated by combining flow cytometry, immunohistochemistry and cytotoxic assays. Interaction with immune cells (*i.e.* binding to CD56 or FcγR and induction of apoptosis) was characterized with the native Adcitmer® as well as a deglycosylated form of Adcitmer®, in order to avoid the interaction between its Fc part and immune cells. Therapeutic performances of Adcitmer® and its deglycosylated form were assessed in an MCC xenograft mice model.

Results. Adcitmer® exerted a cytotoxic effect *in vitro* in SCLC and BPDCN cell lines (IC50 H69: 6.8 nM, GEN2.2: 27 nM). Upon Adcitmer® treatment, CD56 was transitory dowregulated and a bystander effect was highlighted on MCC tumor cells *in vitro*. Most interactions of Adcitmer® with immune cells were related to Fc recognition. Accordingly, Adcitmer® deglycosylation reduced its toxicity on immune cells and improved its therapeutic performances in a MCC mouse model.

Conclusions. Our study suggests that the deglycosylated form of Adcitmer® is a candidate drug for further evaluation as a therapeutic option in patients with advanced MCC or other CD56-positive tumors.

Key words: merkel cell carcinoma, CD56, antibody-drug conjugate, deglycosylation

Introduction

Antibody-drug conjugates (ADCs), combining a monoclonal antibody to a cytotoxic agent, represent an emerging class of therapeutics with fourteen FDA-approved molecules in oncology for both heamatological and solid tumors (Fu et al., 2022). ADC performances are related on one hand to the antibodies properties such as affinity and internalization abilities (Feng et al., 2016; Khongorzul et al., 2020; Rudnick et al., 2011) and on the other hand to the nature of the cytotoxic drug and bioconjugation technology (Esnault et al., 2022; Juen et al., 2021; Martin et al., 2018). Notably, new generation bioconjugation technologies enhance homogeneity and stability of the linker-payload, leading to an optimized therapeutic index and also prevent payload release in blood (Juen et al., 2021; Junutula et al., 2008). An established mechanism of resistance of tumor cells to ADCs treatment is the downregulation of the targeted antigen (Chen et al., 2015). Interestingly, the bystander effect *i.e.* drug release from the primary targeted cells to neighboring tumor cells and microenvironment, could overcome the antigen downregulation and is another determinant of ADC performances (Abdollahpour-Alitappeh et al., 2019).

Beyond their direct cytotoxic activity, ADCs also affect tumor biology by impacting immune responses (Drago et al., 2021). First, captation of the ADC by the immune system (through the recognition of the Fc region or expression of the ADC target by immune cells) might affect immune effectors in the tumor microenvironement. Second, ADC-induced tumor cell lysis might boost tumor immunogenicity, as previously demonstrated by the synergistic association of cytotoxic ADC and immune checkpoint inhibitors in CD30-expressing lymphoma (Zinzani et al., 2019).

Besides the antigens targeted by the FDA-approved ADC, *i.e.* HER2, CD30, CD79 and CD22, several other membrane antigens have been identified as relevant candidates for ADC-based strategy. Among these, CD56, also known as neural cell adhesion molecule (NCAM), is expressed in various neoplasms, *e.g.* small cell lung cancer (SCLC), blastic plasmacytoid dendritic cell neoplasm (BPDCN) and neuroblastoma (Crossland et al., 2018; Heinly and Grant, 2022; Wilson et al., 2021). Accordingly, a CD56-targeting ADC, lorvotuzumab mertansine (LM) was previously generated to treat CD56 expressing tumors. This ADC demonstrated acceptable tolerability profile and promising efficacy in a phase I trial (Ailawadhi et al., 2019; Shah et al., 2016). However, the cytotoxicity observed when the ADC was combined to

chemotherapy led to the discontinuation of the product after a phase II trial (Socinski et al., 2017).

Recently, using an optimized bioconjugation technology, our group generated a new CD56-targeting monomethyl auristatin E (MMAE)-conjugated ADC (PCT/FR2021/ 050332) called Adcitmer® for treatment of Merkel cell carcinoma (MCC), a rare and aggressive CD56-positive skin cancer. Adcitmer® is an homogeneous ADC with an optimal average drug-to-antibody ratio close to 4, which might result in stronger therapeutic activity and reduced toxicity. After bringing confirmation of specific recognition of MCC tumor cells by Adcitmer®, we demonstrated its cytotoxic effect *in vitro* and in a MCC xenograft mice model (Esnault et al., 2021).

In this context, the goal of the present study was to bring further characterization of Adcitmer®. First, Adcitmer® cytotoxicity was assessed *in vitro* in other CD56-positive cell lines. Then, interactions between Adcitmer® and tumor cells and immune cells were characterized *in vitro*.

Materials and Methods

Tumor cell lines

MCC cell lines WaGa (RRID: CVCL_E998) and PeTa (RRID: CVCL_LC73) were grown in RPMI 1640 containing 10% fetal calf serum (FCS), 1% penicillin, streptomycin and amphotericin B. WaGa CD56 knockout (KO) has been obtained as previously described (Esnault et al., 2021).

The human SCLC cell line, H69 (RRID: CVCL_1579) was kindly provided by Dr. Iochmann and Dr. Reverdiau, from the *centre d'étude des pathologies respiratoires* (CEPR), UMR 1100 in the University of Tours. H69 were grown in RPMI 1640 containing 10% FCS, 30 mg/L sodium bicarbonate, 70 mg/L glucose, 0.1 mM HEPES, 10 µM sodium pyruvate, 1% penicillin, streptomycin and amphotericin B.

The GEN2.2 cell line (RRIC: CVCL_5G44) (patent #0215927, EFS, France) (Chaperot et al., 2004) was used as a representative human BPDCN cell line. The GEN2.2 cell line was cultured with MS5 feeder cells in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 µM non-essential amino acid, 1 mM sodium pyruvate, 10 mM HEPES, 1% penicillin and streptomycin.

The SK-N-FI cell line (RRID: CVCL_1702) was used as a representative neuroblastoma cell line, cultured in DMEM supplemented with 10% FCS, 100 µM non-essential amino acid and 1% penicillin and streptomycin.

Primary NK cells and monocytes

Primary natural killer cells (NK) and monocytes were obtained from blood of healthy adult volunteers at the *Etablissement Français du Sang*, according to institutional research protection guidelines agreement N° CA-REC-2019-188, Centre Val de Loire, France. Ficoll density centrifugation step (Eurobio) was performed to isolate peripheral blood mononuclear cells (PBMC).

NK cells were isolated from PBMC by negative selection (NK cell isolation kit human, Miltenyi Biotec) with a purity higher than 95%. NK cells were cultured for up to 36 h in RPMI 1640 supplemented with 10% FCS, 1% penicillin and streptomycin,1% L-glutamine and 100 UI/mL IL-2 at 37°C, 5% CO_2 at 1.10^6 cells/mL.

Monocytes were isolated from PBMC by positive selection using anti-CD14 MicroBeads (Miltenyi Biotec) according to manufacturer recommendations. Monocytes were cultured in serum-free X-VIVO-15 medium (Lonza) at 1.10^6 cells/mL.

Antibodies and ADCs production

m906 is a human IgG1 specific for CD56 immunoglobulin part and m906 binds murine CD56 (Feng et al., 2016). This monoclonal antibody was produced in ExpiCHO-S cell line (ThermoFisher). The cells were transfected under manufacturer guidelines. Briefly, on the day prior to transfection (Day -1), ExpiCHO-S cells were split to a final density of 4.10^6 viable cells/mL and incubated overnight at 37°C and 8% CO_2 under shaking. On the next day (Day 0), cell culture was diluted to 6.10^6 cells/mL and transfected by 0.8 µg/mL of plasmid encoding CD56 targeting m906 antibody, previously mixed to ExpiFectamine CHO reagent. On the day after transfection, max titer protocol was performed. ExpiFectamine CHO Enhancer and ExpiCHO Feed were added, and cells were incubated at 32°C with 5% CO_2 under shaking. On day 5 post-transfection, a second volume of ExpiCHO Feed was added to the flask. After 12 days post-transfection, supernatant containing m906 antibody was harvested and purified with protein A affinity chromatography (Akta, Cytivia). The antibody was eluted by a linear pH gradient in 0.1 M citrate buffer running from pH 6 to pH 2 and buffer was removed by desalting column. Antibody concentration was determined with a UV detector at 280 nm.

Then, m906 disulfide bridges were reduced and allowed to react with bis(bromomethyl)pyridine-caproic-valine-citrulline-p-aminobenzylcarbonatemonomethyl auristatin E, following the McSAF Inside® patented protocol to obtain Adcitmer®.

Lorvotuzumab (Evitria, Suisse) disulfide bridges were reduced and allowed to react with bis(bromomethyl)pyridine-caproic-valine-citrulline-p-aminobenzylcarbonatemonomethyl auristatin E, following the McSAF Inside® patented protocol to obtain lorvotuzumab-MMAE.

Both ADCs were purified by size-exclusion chromatography [PD-10 desalting column (GE Healthcare, Chicago, IL, USA) and PBS pH 7.4 (Gibco, Waltham, MA, USA)] to remove residual small molecules. Protein concentration was determined by UV spectroscopy [280 nm; NanodropOne® (Thermo Fisher Scientific, Waltham, MA, USA)].

Antibodies and ADCs mass spectrometry and size exclusion chromatography analysis are reported in supplementary materials and methods.

m906 and Adcitmer® binding to CD56-positive cells

The m906 and Adcitmer® were labelled with Alexa Fluor 488 (Alexa Fluor 488 Protein Labeling Kit, Invitrogen) to allow their detection by flow cytometry. The m906 and Adcitmer® Alexa Fluor 488 antibodies were added to 5.10^4 NK cells at different concentrations ranging from 0.003 to 200 nM in the presence of Fc blocking reagent (Fc Blocking Reagent human,

Miltenyi Biotec). As control, WaGa cells were incubated at the same density. The mean fluorescence intensity (MFI) of Alexa Fluor 488 was measured by Cytoflex (Beckman Coulter).

Lorvotuzumab and m906 binding to MCC cells

WaGa and PeTa cells were washed with PBS 1% FCS, lorvotuzumab or m906 were incubated at 5 µg/mL for 1 hour on ice. After two washes with PBS 1% FCS, cells were stained for 30 minutes on ice with an anti-human antibody conjugated with APC (clone IS11-12E4.23.20 Miltenyi Biotec). Finally, cells were analyzed by Cytoflex (Beckman Coulter).

Assessment of CD56 expression on tumoral and immune cells by flow cytometry

CD56 expression was assessed on WaGa and PeTa cells upon m906 or lovotuzumab treatment. Cells were seeded in a 6-well plate at a density of 1.10^6 cells/well and incubated with m906 or lorvotuzumab at 5 nM. Untreated cells were used as a control of basal CD56 expression. After 1, 2, and 3 incubation days, 1.10^5 cells were stained with an Alexa Fluor 647 mouse anti-human CD56 antibody (clone B159, BD Biosciences). To prevent steric hindrance with m906, this antibody recognizes the CD56 fibronectin domains since m906 binds a CD56 immunoglobulin domain. Indeed, Feng *et al.* have shown that two antibodies specific for immunoglobulin and fibronectin domain respectively, can bind CD56 without competition (Feng et al., 2016). The percentage of Alexa Fluor 647 positive cells was measured by Cytoflex (Beckman Coulter) for each condition. Immunohistochemistry CD56 staining details are presented in supplementary materials and methods.

CD56 antigen was detected on primary NK cells using an anti-CD56 mouse monoclonal antibody (clone 123C3, Santa-Cruz Biotechnology). The percentage and intensity of CD56-positive cells was measured by Cytoflex (Beckman Coulter).

Co-culture between WaGa CD56-positive and -negative cells: assay for ADC bystander effects

A co-culture of WaGa CD56-positive, *i.e.* CD56 wild-type (WT) cells, and WaGa CD56-negative *i.e.* WaGa CD56 knockout (KO) cells, separated by an insert with 0.4 µm pore-size (Corning). In 6-well cell culture plates, 1.10^6 CD56-negative cells per well were seed in the bottom chamber and the same number of CD56-positive cells were seed in the upper chamber. Cells were incubated with Adcitmer® or deglycosylated Adcitmer® (described in supplementary materials and methods) at 5 or 50 nM for 6 days, and untreated cells were used as control. Cell cycle analysis was performed on cells in the upper and in the bottom inserts separately.

Cell cycle analysis

Cells were fixed using ice-cold ethanol (90%) for at least 1 h followed by treatment with a propidium iodide solution (PBS supplemented with 1% FCS, 0.1 mg/mL propidium iodide, and 0.1 mg/mL RNAse A) at 37°C for 1 h. Cell cycle was then analyzed by flow cytometry on Cytoflex.

Metabolic cell activity

To evaluate cell viability and cellular metabolic activity, XTT assays were performed according to standard protocols. WaGa, H69, GEN2.2 and SK-N-FI were plated in 3 replicates in a 96-well plate with 5.10^4 cells/well. Lorvotuzumab-MMAE, Adcitmer® and MMAE were added in incremental concentrations, considering that each antibody bears 4 MMAE molecules. Untreated cells were used as viability reference. Cells treated with 1%Triton X100 were used as cell death positive control. After 4 days, 25 µL XTT reagent at 1 mg/mL (Alfa Aesar, Thermo Fisher) with N-methyl dibenzopyrazine methyl sulfate (PMS) activator (25 µM) was added per well and absorbance was measured at 450 nm after 4h incubation. Absorbance at 620 nm was used as a reference. Three independent experiments have been performed.

Detection of apoptotic cells

NK cells and monocytes were incubated in the presence of Adcitmer®, deglycosylated Adcitmer®, m906 and deglycosylated m906 at 37°C 5% of CO_2. Cell mortality induced by the different treatments was revealed by APC-Annexin V and 7-AAD staining (APC Annexin V Apoptosis Detection Kit with 7-AAD, Biolegend) and was measured by flow cytometry. m906, deglycosylated m906, Adcitmer® and deglycosylated Adcitmer® were tested after 4 days treatment on NK cells and 4h on monocytes.

m906 and Adcitmer® interaction with FcγR as well as NK cells functional responses are available in supplementary materials and methods.

Xenograft mice model

Eighteen 7-week-old females NOD/SCID (Janvier Labs) mice were maintained under aseptic conditions. All animal procedures were approved by local ethics committee (Apafis#26772-2020072715262737 v2). WaGa cell line was used for tumor induction, as previously described (Esnault et al., 2021). Isofluran-anaesthetized mice received one subcutaneous injection of 10^7 cells with 10% matrigel on the back. General state and weight were monitored twice a week

during the procedure. Tumor volume was measured with a caliper and tumor volume was calculated according to π/6 x width x length x height. When tumor volume reached 150 mm^3, mice were randomly assigned to the different groups. After inclusion, animals (n=6/group) received intravenous injections into the tail vein of ADC at 5 mg/kg (either Adcitmer® or deglycosylated Adcitmer®) or volume-equivalent injection of PBS twice a week. Mice were sacrificed 30 days after inclusion or when reaching one predefined endpoint (tumor ulceration, weight loss >20%, or prostration). Tumors, heart, lungs, spleen, and liver were removed for macroscopic examination. After dissection, tumor weight and volume were assessed. To detect metastatic spread and potential toxicity of Adcitmer® and deglycosylated Adcitmer®, whole tumors and organs (heart, lungs, spleen, and liver), were formalin-fixed and paraffin embedded for microscopic evaluation to evaluate architecture changes, inflammatory infiltrate, necrosis, vascular changes, fibrosis and liver steatosis.

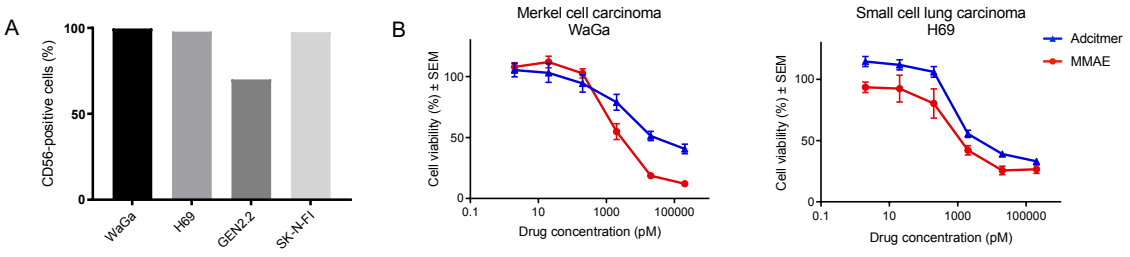
Statistical analysis.

Continuous data are described as mean (range). Categorical data are described as number (percentage) of interpretable cases. Two-way Anova was used to analyze the difference between the means of more than two groups with two independent variables. Comparisons were assessed by the Mann-Whitney test for independent samples and Wilcoxon test for dependent samples. Statistical analysis was conducted using GraphPad Prism (GraphPad, La Jolla, CA, USA). *$p< 0.05$, **$p<0.01$,***$p<0.001$ were statistically significant.

Results

Adcitmer® as a potential therapeutic option in CD56-positive tumors

Cytotoxic properties of Adcitmer® have previously only been demonstrated in MCC (Esnault et al., 2021). Therefore, we aimed to validate its performances in other CD56-expressing cancers, for which therapeutic options are limited until now (Wilson et al., 2021).



IC50 (nM)	WaGa	H69	GEN2.2	SK-N-FI
Adcitmer	31	6.8	27	-
MMAE	3	1.5	3.8	1

Figure 1. CD56 expression and cytotoxicity of Adcitmer® in vitro in MCC, SCLC, BPDCN and neuroblastoma cell lines. (A) CD56 expression in MCC, SCLC, BPDCN and neuroblastoma cell lines. (B) In vitro evaluation of cytotoxicity of Adcitmer® (blue) and free monomethyl auristatin E (MMAE) (red) cytotoxicity in a MCC cell line, WaGa, in a SCLC cell line, H69, in BPDCN cell line GEN2.2 and in neuroblastoma cell line, SK-N-FI in vitro. After 4 days of exposure to drugs, viability of WaGa, H69, GEN2.2 and SK-N-FI cell lines was assessed by XTT assay. Untreated cells were used as a reference. Experiments included six replicates and were performed three times independently. Data are expressed as mean ± SEM of percentages. The half-maximal inhibitory concentration (IC50) value was determined for Adcitmer® and MMAE. (-): incalculable.

To this aim, Adcitmer® cytotoxicity was assessed *in vitro* on four CD56-positive cell lines (**Figure 1A**), a SCLC cell line, H69, a BPDCN cell line, GEN2.2 and a neuroblastoma cell line, SK-N-FI after 4 incubation days (**Figure 1B**). Adcitmer® was found to be efficiently cytotoxic on these cell lines, and IC50 were comparable between H69, GEN2.2 and the MCC WaGa cell line (6.8, 27, 31 nM, respectively). By contrast, while high cytotoxicity (IC50 = 1 nM) was

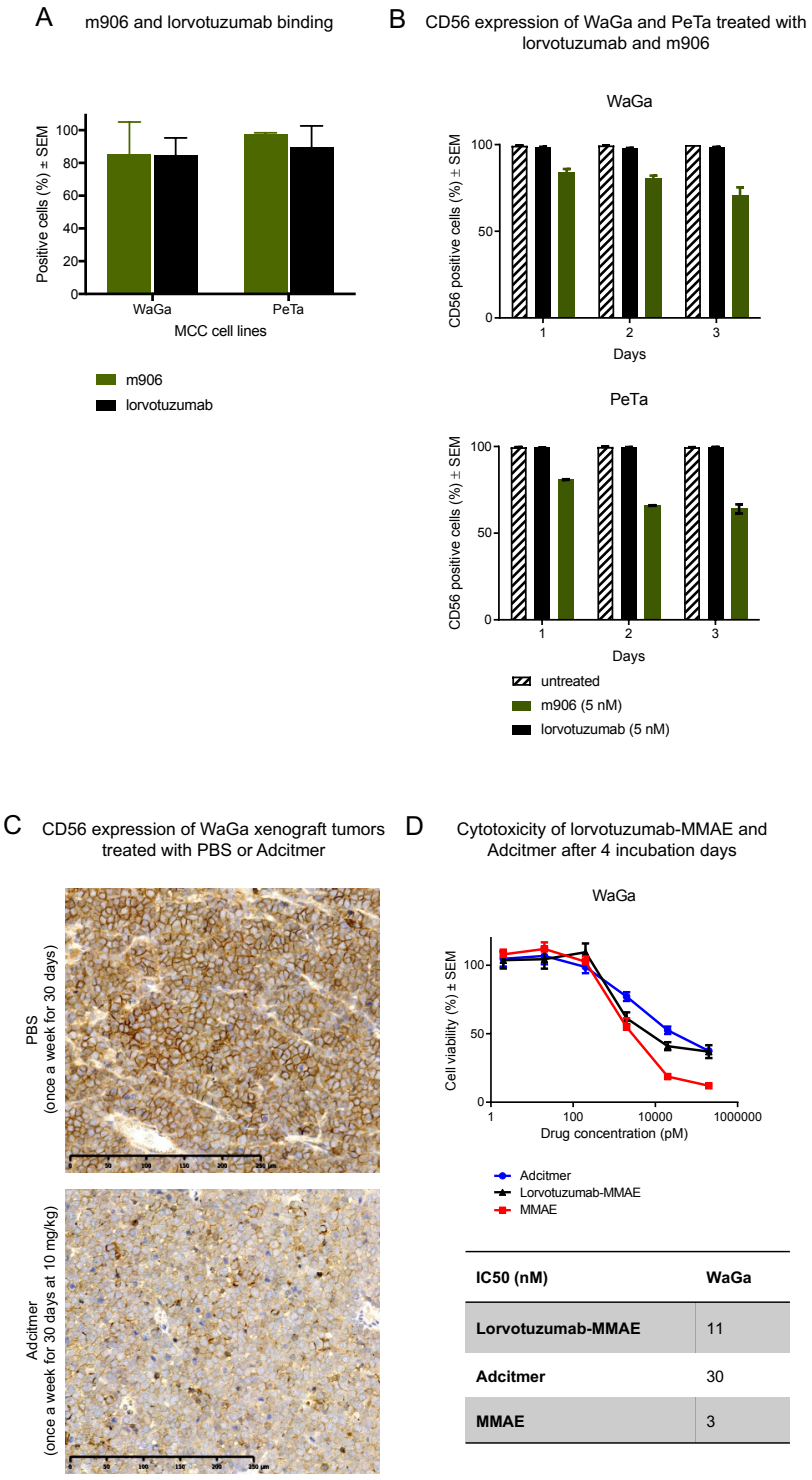
observed on the neuroblastoma cell line with free MMAE, Adcitmer® IC50 could not be determined since more than 50% of cells were still alive at the highest dose, 2,000 nM. To note CD56 expression was still confirmed in this model, while a reduced binding of both m906 and Adcitmer® were observed (**Figure S1**) probably excluding Adcitmer® as a therapeutic option in this setting.

These results indicate that in addition to MCC, Adcitmer® might constitute a potential therapeutic option in some other CD56-expressing neoplasms including SCLC and BPDCN.

Adcitmer® and m906 treatment reduce CD56 expression *in vitro* and *in vivo*

The affinity between the antibody and its target is expected to be a determinant factor of ADC performances (Esnault et al., 2021; Guillemard et al., 2005). Therefore, we aimed to compare the affinity properties of m906 and lorvotuzumab, the two antibodies involved in Adcitmer® and LM constructs respectively.

First, high levels of binding capacities of both lorvotuzumab and m906 antibodies were shown on CD56 expressed by MCC cells lines (**Figure 2A**). To note, Adcitmer® and unconjugated m906 harbored a similar binding ability suggesting that bioconjugation does not affect CD56 recognition (data not shown).



IC50 (nM)	WaGa
Lorvotuzumab-MMAE	11
Adcitmer	30
MMAE	3

Figure 2. CD56 expression upon m906, lorvotuzumab treatments *in vitro*, Adcitmer® treatment *in vivo* and comparison of cytotoxicity of Adcitmer® and lorvotuzumab mertansine treatment in MCC cell line. (A) Evaluation of m906 (green) and lorvotuzumab (black) binding to the MCC cell lines WaGa and PeTa (B) CD56 expression of WaGa and PeTa cells at 1, 2 or 3 days upon treatment with lorvotuzumab (black) or m906 (green) at 5 nM

compared to untreated cells (striped black and white). CD56 positive cells were determined by flow cytometry and were expressed as mean ± SEM of percentages. (C) Representative immunohistochemical view of CD56 in MCC tumor samples of NOD/SCID mice treated once a week with PBS or Adcitmer® at 10 mg/kg (Esnault et al., 2021). (D) Evaluation of Adcitmer® (blue) and lorvotuzumab-MMAE (black) and MMAE (red) cytotoxicity in the MCC cell line WaGa after 4 incubation days. Untreated cells were used as a reference. Experiments included six replicates and were performed three times independently. Data are expressed as mean ± SEM of percentages. The half-maximal inhibitory concentration (IC50) value was determined for Adcitmer® and lorvotuzumab-MMAE and MMAE.

A transitory downregulation of the ADC target is expected to reflect internalization of the ADC (Feng et al., 2016; de Goeij et al., 2014), therefore we then aimed to determine how CD56-targeting antibodies would affect CD56 expression. Thus, CD56 expression was determined on WaGa and PeTa cells, by flow cytometry, at different time-points upon lorvotuzumab or m906 treatment and compared to untreated cells. m906 induced a transitory decrease of CD56 expression on WaGa and PeTa cells from day 1 to day 3 incubation days (**Figure 2B**) as previously described (Feng et al., 2016), however these differences were not significative. By contrast no CD56 downregulation was observed upon lorvotuzumab treatment on MCC cell lines *in vitro* (**Figure 2B**). In line with the transitory decrease of CD56 expression observed upon m906 treatment *in vitro*, the immunohistochemical investigation of CD56 expression in xenograft tumor tissues of mice treated with Adcitmer® (previously described in (Esnault et al., 2021)) demonstrated a reduction in CD56 expression when compared to tumors from untreated mice (**Figure 2C**).

These results might suggest that m906 and lorvotuzumab harbor the same binding ability to their target CD56, while m906 seems to induce higher CD56 downregulation than lorvotuzumab. However, the evaluation of Adcitmer® and lorvotuzumab-MMAE cytotoxic properties on the WaGa cell line *in vitro* revealed similar cytotoxic effects after 4 incubation days (30 and 11 nM, respectively) (**Figure 2D**) suggesting that at least in our model, CD56 downregulation did not significantly impair ADC performances.

Adcitmer® exerts a bystander effect

MMAE is a highly membrane-permeable payload that can trigger bystander effect, due to drug release from the primary targeted cells to close tumor cells and microenvironment (TME) (Abdollahpour-Alitappeh et al., 2019) (**Figure 3A**).

To evaluate whether Adcitmer® might exert a bystander effect in MCC, the effect of Adcitmer® was evaluated on wild-type (WT) (*i.e.* CD56-positive) and CD56 CRISPR knockout (KO) (*i.e.* CD56-negative) MCC tumor cells (WaGa) alone or in combination (co-culture assay) (**Figure 3B**). This *in vitro* model potentially allows the diffusion of free MMAE released by CD56-positive cells after Adcitmer® internalization, through insert permeable membrane to the CD56-negative cells (**Figure 3B**).

Under these conditions, cell cycle analysis revealed an increased number of cells in subG1 phase in WaGa WT in comparison to untreated cells after 4 days of Adcitmer® incubation with 5 and 50 nM (2-way Anova, $p<0.001$*** and $p<0.01$** respectively), (**Figure 3C**). By contrast, no changes were observed on WaGa CD56 KO at the same time point as previously described (Esnault et al., 2021) (**Figure 3C**). Importantly, in the co-culture experiments, an increased number of cells in subG1 phase was observed in both WaGa CD56 WT and KO cells. Furthermore, an increased mortality in CD56 KO cells was detected after 6 days at the highest dose of ADC (2-way Anova, $p<0.001$***) (**Figure 3D**). These results demonstrate that Adcitmer® exerts a bystander effect in a dose dependent manner.

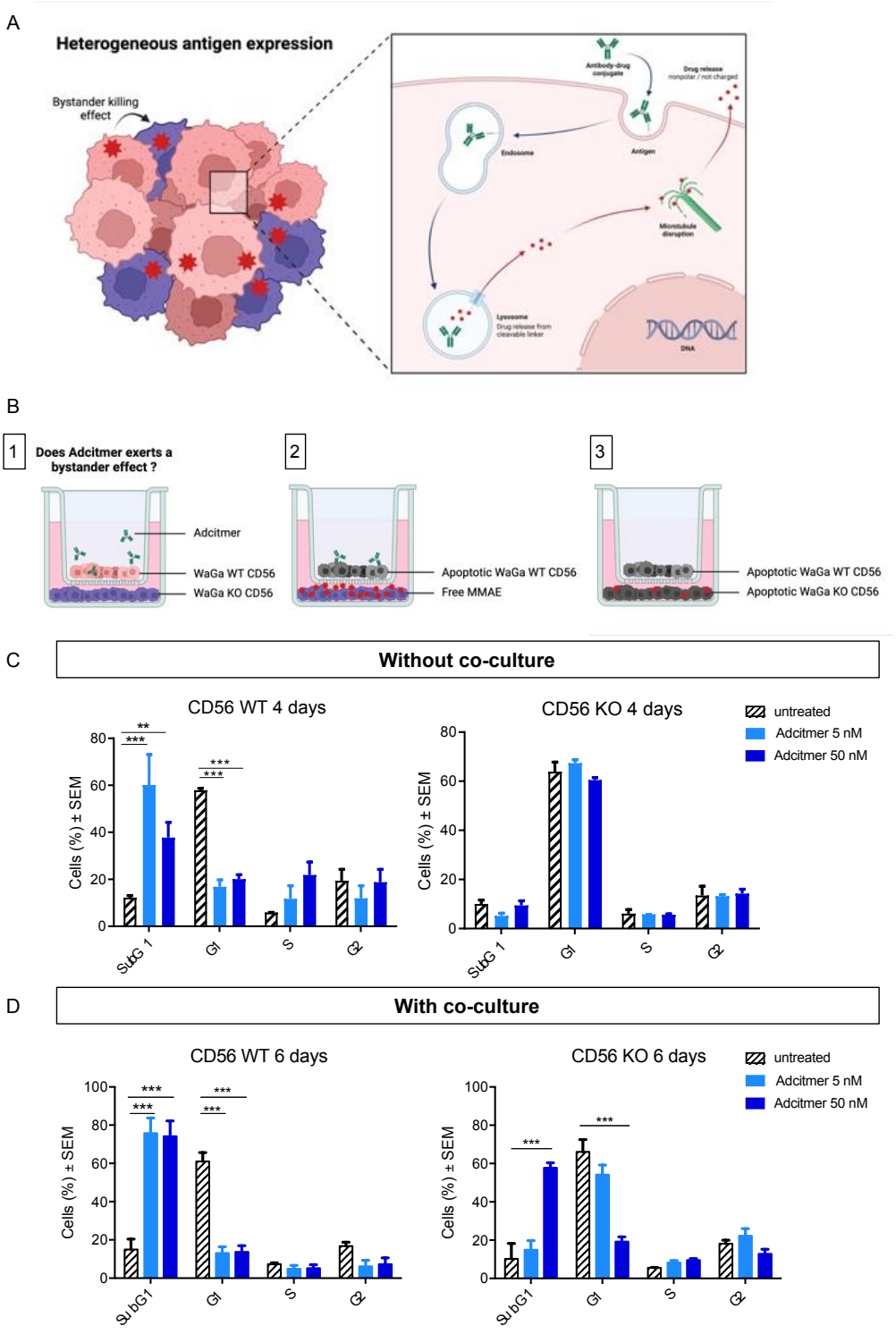


Figure 3. Adcitmer® bystander effect in MCC *in vitro*. (A) Schematic representation of bystander effect into a tumor with heterogeneous antigen expression. (B) Illustration of the co-culture between the MCC cell line, WaGa CD56 wild-type (WT) (*i.e.* CD56-positive cells) and WaGa CD56 knock-out (KO) (*i.e.* CD56-negative cells) to determine ADCs bystander effect. Step 1, Adcitmer® treatment on WaGa CD56 WT and WaGa CD56 KO. Step 2, Adcitmer® is internalized by WaGa CD56 WT cells and induce apoptosis, leading to the release of

monomethyl auristatin E (MMAE) outside the cells. Step 3, free MMAE crosses plasma membrane and induces cytotoxicity on WaGa CD56 KO (C) Cell cycle analysis of WaGa CD56 WT and KO untreated cells compared to cells treated with two Adcitmer® doses (5 nM, light blue and 50 nM, dark blue) for 4 days without co-culture. (D) Cell cycle analysis of WaGa CD56 KO untreated cells compared to cells treated with two Adcitmer® doses (5 nM, light blue and 50 nM, dark blue) for 6 days in co-culture with WaGa CD56 WT cells. The percentages of cells in each cell cycle phase (G1, S, G2) or in subG1 phase are depicted. Experiments were performed three times independently. Results are expressed as mean ± SEM of percentages of cells in each cell cycle phase. Statistical significance between untreated cells and cells treated with Adcitmer® are represented with **p<0.01 and ***p<0.001.

Interactions between immune cells and Adcitmer® and generation of deglycosylated Adcitmer®

ADCs might also affect tumor biology by impacting immune response (Drago et al., 2021) through specific or unspecific targeting of immune cells. Importantly, CD56 expression is not restricted to tumor cells but also reported in NK cells (Robertson and Ritz, 1990). Accordingly, flow cytometry analysis revealed a CD56 expression in primary human NK cells but with a lower mean fluorescence intensity (MFI) than CD56 expression on WaGa MCC cells (40,000 and 170,000 respectively p= 0.029*) (**Figure 4A**).

In this context, we aimed to evaluate binding and cytotoxicity of Adcitmer® on primary human NK cells using MCC tumor cells as control. To evaluate Adcitmer® binding through CD56 recognition only and to avoid unspecific binding of the antibodies to immune cells through their Fc region, an Fc receptor blocking reagent was used (Andersen et al., 2016). Such experiment confirmed that both m906 and Adcitmer® bind to NK cells, however with a lower binding ability than for the MCC cell line WaGa (Wilcoxon test, Adcitmer® binding on WaGa vs NK p=0.002** and m906 binding on WaGa vs NK p=0.002**) (**Figure 4B**).

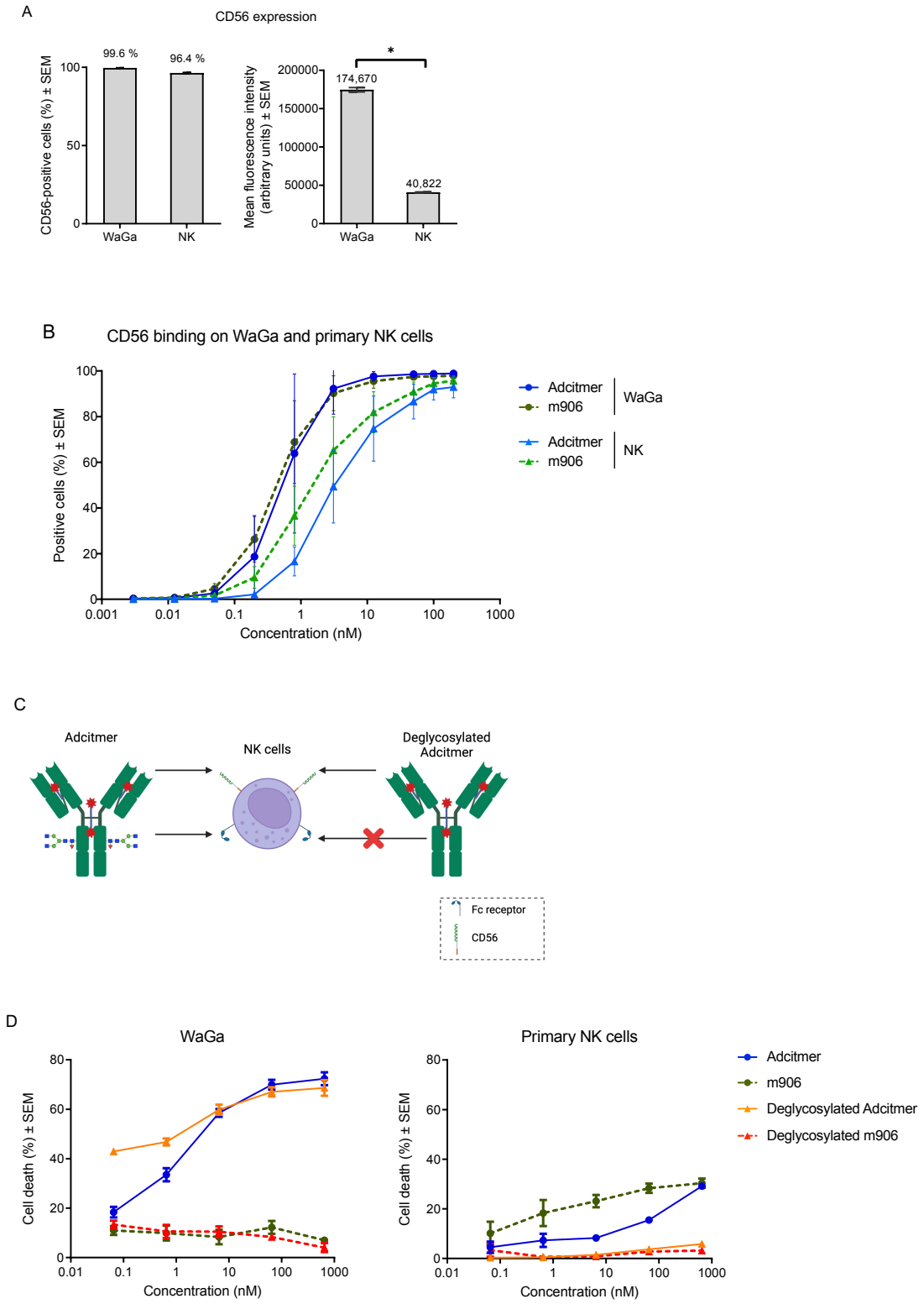


Figure 4. Interactions of Adcitmer® and m906 with NK cells compared to the MCC cell line, WaGa. (A) CD56 expression on WaGa and NK cells expressed with percentage of CD56-positive cells and mean fluorescence intensity. (B) m906 and Adcitmer® binding to CD56 on primary NK cells compared to the MCC cell line, WaGa, used as positive control, detected by

flow cytometry. m906 and Adcitmer® were labelled by Alexa Fluor 488. m906 and Adcitmer® were used at different concentrations on WaGa cells or on NK cells, in presence of Fc blocking reagent. Adcitmer® and m906 on WaGa cells are represented with dark blue circle curve and dark green circle dashed curve, respectively. Adcitmer® and m906 on NK cells are represented with light blue triangle curve and light green triangle dashed curve respectively. Results are expressed as mean ± SEM of percentages of CD56 positive cells. Experiments were performed four times independently. (C) Schematic representation of Adcitmer® and deglycosylated Adcitmer® interactions with NK cells through CD56 and Fc receptor. (D) Cell death of WaGa and NK cells with different concentrations of Adcitmer® (dark blue circle curve), deglycosylated Adcitmer® (orange triangle), m906 (dark green circle dashed curve) and deglycosylated m906 (red triangle). Cell death was analyzed after 4 days of incubation by flow cytometry. Experiments were performed three times independently. Results are expressed as mean ± SEM of percentages of late apoptotic cells.

We then evaluated whether Adcitmer® might impair NK cell viability. To determine if the potential cytotoxic effect is related to CD56 recognition or to the Fc binding to the NK cells, we generated a deglycosylated Adcitmer® (**Figure 4C**) therefore avoiding its Fc recognition by immune cells (Radaev and Sun, 2001). Chemical characterization of the deglycosylated Adcitmer® is available in **Figure S2**. WaGa and primary human NK cells were then exposed to increasing concentrations of Adcitmer®, deglycosylated Adcitmer®, as well as m906 or deglycosylated m906 used as controls, and cell survival was analyzed after 4 days.
Such analysis demonstrated that Adcitmer® and deglycosylated Adcitmer® exerted similar cytotoxic effects on WaGa cells (IC50 31 and 56 nM, respectively) whereas m906 and deglycosylated m906 did not induce cell death, as expected (**Figure 4D**). By contrast, a cytotoxic effect of Adcitmer® was observed on NK cells although to a lesser degree than on the MCC cell line (29% and 72% of cell death at 650 nM respectively, Mann-Whitney, p=0.01*). Importantly, similar cytotoxicity was observed on NK cells upon treatment with Adcitmer® and m906 (Mann-Whitney, p=0.15 n.s.) while no cytotoxic effect was observed with the deglycosylated molecules strongly suggesting that cytotoxicity of these antibodies on NK is related to antibody-dependent cell-mediated cytotoxicity (ADCC) through Fc recognition and not to CD56 specific binding. Accordingly, interactions between m906 and Adcitmer® and several Fc receptors (*i.e.* CD16, CD32, CD64), were confirmed by FcγR binding inhibition assay (**Figure S3**) and an enhanced expression of activation markers in NK cells after m906 or Adctimer® exposure was observed (**Figure S4**).

To finally evaluate whether Adcitmer® recognition through its Fc region might affect other myeloid population, a similar experiment was conducted on human monocytes. Incubation of

monocytes with the highest dose (2.6 µM) of Adcitmer® induced approximately 10% of cell death whereas no cell death was observed with m906, deglycosylated Adcitmer® or deglycosylated m906 (**Figure S5**).

Therefore, our results strongly suggest that deglycosylation of Adcitmer® did not impair its cytotoxic effect on MCC tumor cells but avoided off-target effect on NK cells and monocytes by preventing Fc region recognition by immune cells. We therefore investigated further the performances of this deglycosylated Adcitmer®.

Deglycosylation of Adcitmer® enhances antitumor growth effect and reduces toxicity in a MCC xenograft mice model

Finally, we aimed to compare the performances of Adcitmer® and its deglycosylated form *in vivo* in a previously described MCC tumor xenograft mice model (Esnault et al., 2021). To this aim, MCC cell line WaGa was subcutaneously injected to NOD/SCID mice. Mice received intravenous injections of Adcitmer®, deglycosylated Adcitmer® (5 mg/kg) or PBS twice a week for 30 days (*i.e.* mice received a total of 9 injections).

By contrast to our initial experiment (Esnault et al., 2021) during which no toxicity was detected, prostration was observed for all animals briefly after Adcitmer® primo-injection (n=7) while no symptoms were observed after injection of deglycosylated Adcitmer® or PBS. The acute reaction upon injection with Adcitmer® was characterized by orbital tightening, piloerection, decreased activity, led to the death of one mouse injected with Adcitmer® and did not recur after the following injections. A similar symptomatology was detected after injection of m906 in an independent cohort of mice (n=7) (data not shown) suggesting that this reaction was due to an antibody property and not due to linker-payload activity. Interestingly, no symptoms occurred after injection of deglycosylated Adcitmer® suggesting that glycosylation and even Fc recognition might be involved in mice symptomatology.

Importantly, although a reduced tumor growth was observed upon Adcitmer® treatment as previously described, the anti-tumor effect was significantly higher in deglycosylated Adcitmer® treated group when compared to Adcitmer® treated mice (5 mg/kg) (Mann-Whitney, $p<0.01$**) (**Figure 5A**). A complete regression of the tumor was even achieved in the group of mice treated with the deglycosylated Adcitmer® (n= 3/7) (**Figure 5B**).

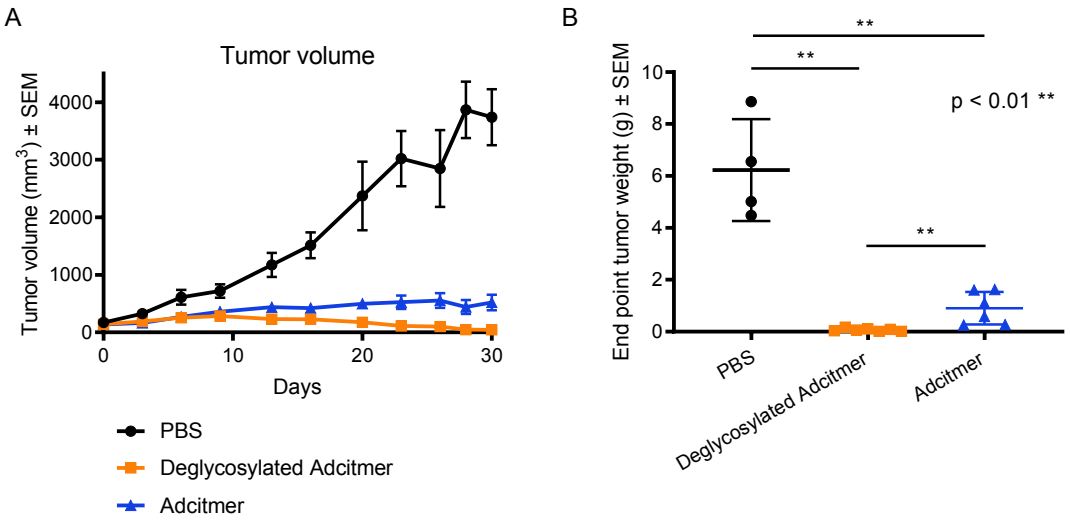


Figure 5. Adcitmer® and deglycosylated Adcitmer® anti-tumoral evaluation in a MCC xenograft mice model. (A) Mean tumor volume curves ± SEM during the experiment. Each point represents mean tumor volume in PBS, Adcitmer®, deglycosylated Adcitmer®-treated groups (black, blue and orange, respectively). Intravenous injections were done twice a week at 5 mg/kg (each mouse received 9 injections for 30 days). (B) Endpoint tumor weights in the experimental and control groups (**p<0.01). Horizontal lines are mean ± SEM.

No weight loss was observed in the three treatment groups (**Figure S6**). After the mice were euthanized, distant metastases were not observed. Accordingly, no obvious toxicity of Adcitmer® and deglycosylated Adcitmer® was evidenced at necropsy.

Although unexpected toxicities related to the m906 were detected during the present study, our results demonstrate that the deglycosylated Adcitmer® was well tolerated and exerted performant antitumor activity *in vivo* with complete tumor regression in nearly half of the cases.

Discussion

In the present study, we further characterized Adcitmer®, a recently generated CD56-targeting MMAE-conjugated ADC, designed to treat metastatic MCC. As a preliminary step, we confirmed the cytotoxic effect of Adcitmer® on CD56 expressing tumor cell lines other than MCC, such as SCLC and BPDCN, suggesting that Adcitmer® also constitutes a therapeutic option in the broad spectrum of CD56-positive tumors including hematologic malignancies for which therapeutic options are frequently limited (Wilson et al., 2021). Further *in vitro* characterization indicates that m906 triggers a CD56 downregulation while lorvotuzumab did not. Nevertheless, these differences do not seem to substantially impact ADC cytotoxicity *in vitro*. Interestingly, Adcitmer® can trigger a bystander effect. Therefore, although CD56 downregulation may constitute a resistance mechanism to Adcitmer®, the bystander effect may counterbalance this target downregulation. Subsequently, we confirmed that Adcitmer® interacts with immune cells and impairs NK cell viability, such effect being related to the Fc binding on NK cells and not to CD56 targeting. Then, to avoid Fc recognition by immune cells we generated a deglycosylated Adcitmer®. Deglycosylation of Adcitmer® did not impair cytotoxicity on MCC tumor cells *in vitro* and interestingly prevented off target effect on immune cells. Finally, the evaluation of the deglycosylated Adcitmer® in a MCC xenograft mice model confirmed that deglycosylated Adcitmer® reduced tumor growth with higher performances than with the glycosylated form of Adcitmer®.

Since CD56 is expressed by several aggressive neoplasms, other ADC targeting CD56 have previously been generated including one molecule *i.e.* LM evaluated in human (Feng et al., 2016; Yu et al., 2018a, 2018b). Indeed, LM demonstrated acceptable tolerability profile and signal efficacy in two phase I clinical trials (Ailawadhi et al., 2019; Shah et al., 2016) while toxicities notably severe neutropenia were observed when combined with etoposide/cisplatin for treatment of SCLC patients (Socinski et al., 2017). Accordingly, used as a monotherapy, LM was well tolerated in a recent phase II trial including CD56-positive pediatric tumors with a partial and a delayed complete response observed in one patient respectively (Geller et al., 2020). To note, LM also demonstrated signs of efficacy in refractory multiple myeloma patients with stable disease or responses detected in 42.9% of patients (Ailawadhi et al., 2019; Berdeja, 2014). In line with these results, our data suggest that Adcitmer® could be an interesting therapeutic option to evaluate, not only in MCC patients but also in patients with other CD56

expressing tumors, notably SCLC and BPDCN, a rare but aggressive tumor, with limited therapeutic options until now (Wilson et al., 2021).

ADCs therapeutic performances are related to antibody's affinity and internalization abilities (Feng et al., 2016; Khongorzul et al., 2020; Rudnick et al., 2011). Indeed, an high level of ADCs internalization is associated with enhanced tumor penetration, this characteristic combined to a potent payload being required for optimal ADCs antitumor activities (Beck et al., 2017). Notably, Feng *et al.* suggested that downregulation of CD56 upon m906 exposure as also observed for Adcitmer® may indicate a high level of internalization of the drug (Feng et al., 2016). However, formal demonstration of the internalization levels in this setting would require assessment with imaging flow cytometry or confocal microscopy.

Another determinant of ADC efficiency is the bystander effect, which might enhance the ADC effect in tumors with heterogeneous expression of the targeted antigen (Kovtun et al., 2006). In our previous study, among CD56-expressing tumors, low or heterogeneous expression of CD56 was observed in 44% cases underlying the interest of a bystander effect in this settings (Esnault et al., 2021). Notably, such properties have already been documented in some of the FDA approved ADC. In HER2-positive breast cancer model, the trastuzumab deruxtecan which was found to be effencient in patients with metastatic breast cancer with low HER2 expression (Modi et al., 2020) induces bystander killing effect while the first generation of HER2 targetting ADC *i.e.* trastuzumab emtansine does not. Similarly, brentuximab vedotin, a CD30-targeting MMAE-conjugated ADC approved for Hodgkin lymphoma or anaplastic large-cell lymphoma, was found to induce a bystander effect that is beneficial in tumors with heterogeneous CD30 expression (Masuda et al., 2015).

Although ADC therapy aims to a specific delivery of the drug to the tumor cells, ADCs are also likely to affect other cell populations since 1) expression of the ADC target is almost never completely restricted to tumor cells, 2) ADC interact with immune cell through their Fc portion (Drago et al., 2021). Indeed, the antibody Fc part interacts with Fc receptors on immune effector cells which may trigger an immune response (Nimmerjahn and Ravetch, 2008). Moreover, Fc region interacts with neonatal Fc receptor (FcRn), a recycling receptor determining both IgG half-life and biodistribution (Chen et al., 2014; Roopenian and Akilesh, 2007). The ADC interactions with immune cells might either constitute an opportunity to induce an antitumor response, or represent a potential side effect, resulting in toxicity on immune cells (Hoffmann et al., 2018). Accordingly, genetic engineering of the ADCs Fc was

developed to promote or impair interactions between ADCs and immune cells, such as increased or decreased ADCC by glycosylation engineering (*i.e.* afucosylation and aglycosylation, respectively) (Carter, 2006; Gauzy-Lazo et al., 2020). Accordingly, we demonstrated that Adcitmer® interacts with immune cells mostly through its recognition by the Fc receptors expressed by NK cells and monocytes. Even if CD56 is also expressed by NK cells, the toxicity observed on immune cells was shown to be related to Fc receptor binding since toxicity was also detected on monocytes without CD56 expression and disappears after Adcitmer® deglycosylation. Interestingly, although in our initial publication (Esnault et al., 2021), no toxicity was detected after intravenous injection of Adcitmer® to NOD/SCID immunodeficient mice, in the present study, we observed upon the same experimental conditions an acute reaction after Adcitmer® or m906 injection. Such effect may be related to an off-target effect (Mahalingaiah et al., 2019) of the m906, notably the glycosylated form might be competent to bind FcγR-positive cells and induce a cross-linking between effector cells (FcγR-positive) and murine circulating CD56-positive cells. Accordingly, the improved anti-tumor effect of deglycosylated Adcitmer® might be explained by the Fc deglycosylation that prevent the interaction of Adcitmer® with Fc receptor-positive cells in mice, therefore increasing its biodisponibility and enhancing anti-tumor effect. To note, while deglycosylation is not appropriate for industrial production (Zheng et al., 2011), aglycosylated antibodies generated by Fc portion mutagenesis might be suitable for this purpose. Indeed although no aglycosylated ADC has been approved until now, six therapeutic aglycosylated antibodies are currently approved by the FDA (Kang and Seong, 2020).

To conclude, the deglycosylated Adcitmer® appears as a promising therapeutic option in preclinical models of MCC, with limited toxicity and confirmed antitumor activities. Such treatment might be used as a relevant alternative or in combination in advanced MCC patients and in other CD56-positive malignancies.

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Supplementary materials and methods

Deglycosylation of m906 and Adcitmer®

1 mL at 1 mg/mL of Adcitmer® in PBS 1X pH 7.4 was incubated with 15 µL of PNGase F of *Flavobacterium meningosepticum*, recombinant from *E. coli* (Sigma) at 37°C for 16 h. PNGase F was then removed by centrifugal concentration on Vivaspin® PES 50 kDa (Sartorius) at 4°C and 5 000 g with buffer replacement with DPBS 1X pH 7.4 (4 cycles of 5 min). The protein concentration was determined by UV spectroscopy at 280 nm with NanoDrop™ One/OneC (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to the desired concentration.

Mass spectrometry and size exclusion chromatography

Denaturing mass spectrometry (MS) analysis of Adcitmer® and deglycosylated Adcitmer® were performed on a Bruker maXis mass spectrometer coupled to a Dionex Ultimate 3000 RSLC system. Prior to MS analysis, samples (*ca.* 5 µg) were desalted on a MassPREP desalting cartridge (2.1 × 10 mm, Waters) heated at 80°C using 0.1% formic acid as solvent A and 0.1% formic acid in MeCN as solvent B at 500 µL/min. After 1 min, a linear gradient from 5 to 90% B in 1.5 min was applied; the first 1.5 min were diverted to waste. HRAM data were acquired in positive mode with ESI source over the m/z range from 900 up to 5000 at 1 Hz and processed using DataAnalysis 4.4 software (Bruker) and MaxEnt algorithm for spectral deconvolution.

For size exclusion chromatography, both Adcitmer® and deglycosylated Adcitmer® were filtered on a 0.22 µm membrane (PVDF). A sample of 50 µg was injected on an AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm from Agilent Technologies, connected to a Thermo Scientific Vanquish Flex apparatus equipped with a photodiode array detector (Thermo Scientific) set for detection at 280 nm. Samples were run with an isocratic gradient (1 mM potassium phosphate monobasic, 155 mM sodium chloride, 3 mM sodium phosphate dibasic, 3 mM sodium azide, pH 7.0) over 20 min at a flow rate of 1 mL/min. The column oven temperature was maintained at 25 °C.

Immunohistochemistry

CD56 expression was analyzed *in vivo* on WaGa xenograft. Mouse tumor sections were deparaffinizated and rehydrated, then, they were submitted to an antigen retrieval step, in 10 mM citrate buffer, pH 6.1, for 20 min at 121°C. Endogenous peroxidase was inhibited by a 1/100 dilution of 30% hydrogen peroxide (Sigma) in methyl alcohol for 30 min at room temperature. Non-specific binding sites were blocked by a 45 min incubation with 20% normal

goat serum diluted in phosphate buffered saline (PBS). CD56 was immunolabeled using an anti-CD56 mouse monoclonal antibody (clone 123C3, Santa-Cruz Biotechnology), 1/50 diluted and incubated overnight at 4°C, followed by a 30 min incubation at room temperature with the universal immunoperoxidase polymer anti-mouse N-Histofine (Nichirei Biosciences, MMFrance). Enzymatic activity was revealed by using diaminobenzidine and sections were counterstained with a Harris hematoxylin solution.

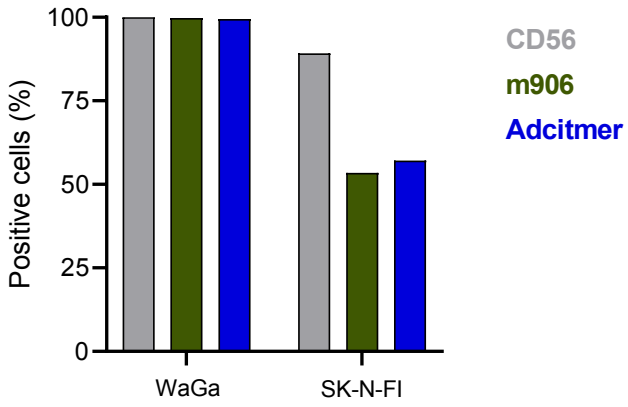
m906 and Adcitmer® interaction with FcγR

m906 and Adcitmer® interactions with FcgR (*i.e.* CD16, CD32, CD64) was evaluated using Lumit™ FcγR Binding Immunoassay (Promega). A human IgG1 labeled with LgBiT (Tracer-LgBiT) is used as the tracer. A C-terminal biotinylated human FcγR bound to Streptavidin-SmBiT (FcγR-Biotin-SA-SmBiT) is used as the target. In the absence of antibody, Tracer-LgBiT binds to the FcγR-SmBiT target, resulting in maximum luminescence signal. In samples, unlabeled IgG will compete with Tracer-LgBiT for binding to the FcγR target, resulting in a concentration-dependent decrease in luminescent signal. Substrate detection was performed by luminometer (GloMax® System).

NK cells functional responses

Adcitmer® and m906 were tested at different concentrations for 4h and functional NK responses, indicated by CD16 engagement, were evaluated by flow cytometry. The degranulation marker, CD107a (PE-Cy5 mouse anti-human CD107a, BD Biosciences), IFN-γ production (IFN-γ PE, Beckman Coulter) and CD16 shedding (CD16 3G8 APC-Alexa Fluor 750, Beckman Coulter) was assessed. The fluorescence was detected by flow cytometry (Cytoflex).

Supplemental figures



Supplemental Figure 1. CD56 expression and m906 and Adcitmer® binding on SK-N-FI, a neuroblastoma cell line and the MCC, WaGa cell line.

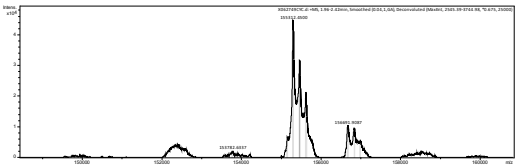
Size-exclusion chromatography analysis

ADC monomer:

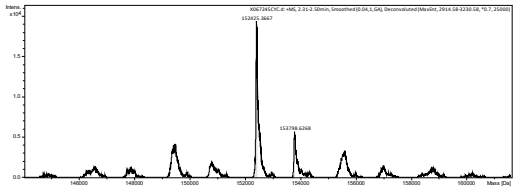
	Monomer (%)
Adcitmer	92.81
Deglycosylated Adcitmer	90.57

Mass spectrometry analysis

Adcitmer: zoom 150 000-160 000 m/z



Deglycosylated Adcitmer: zoom 146 000-160 000 m/z



Drug-to antibody ratio:

	LHHL DAR4 (%)
Adcitmer	81.2
Deglycosylated Adcitmer	77.3

Glycoform analysis:

	LHHL DAR4 species molecular weight (Da)
Adcitmer	155,313
Deglycosylated Adcitmer	152,425
Difference of molecular weight (Adcitmer - deglycosylated Adcitmer)	2,888

Adcitmer : 2 x 1,445 Da = 2,888 Da **G0F major Adcitmer glycoform**

G0F = 4 x GlcNAc + 3 x Mannose + 1 Fucose

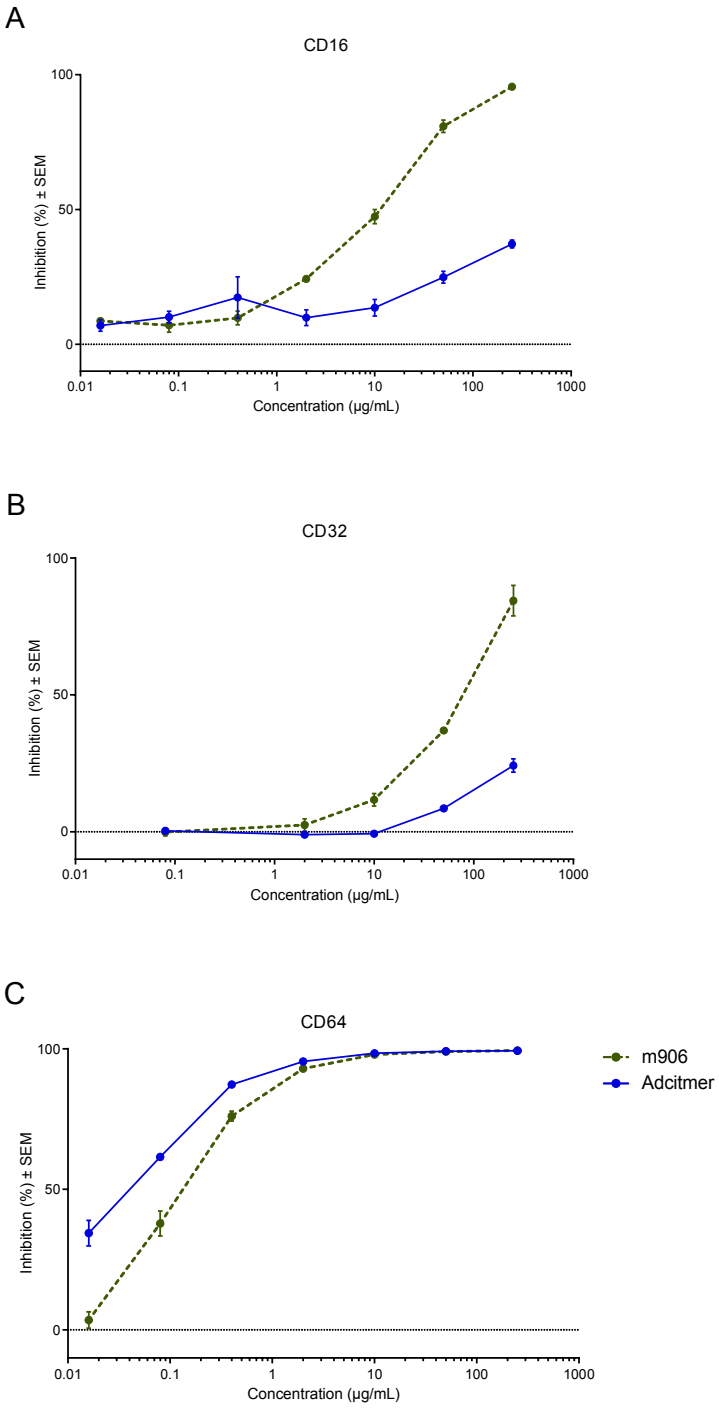
GlcNAc = 203 Da
Fucose = 146 Da
Mannose = 162 Da

Glycoform G0F structure

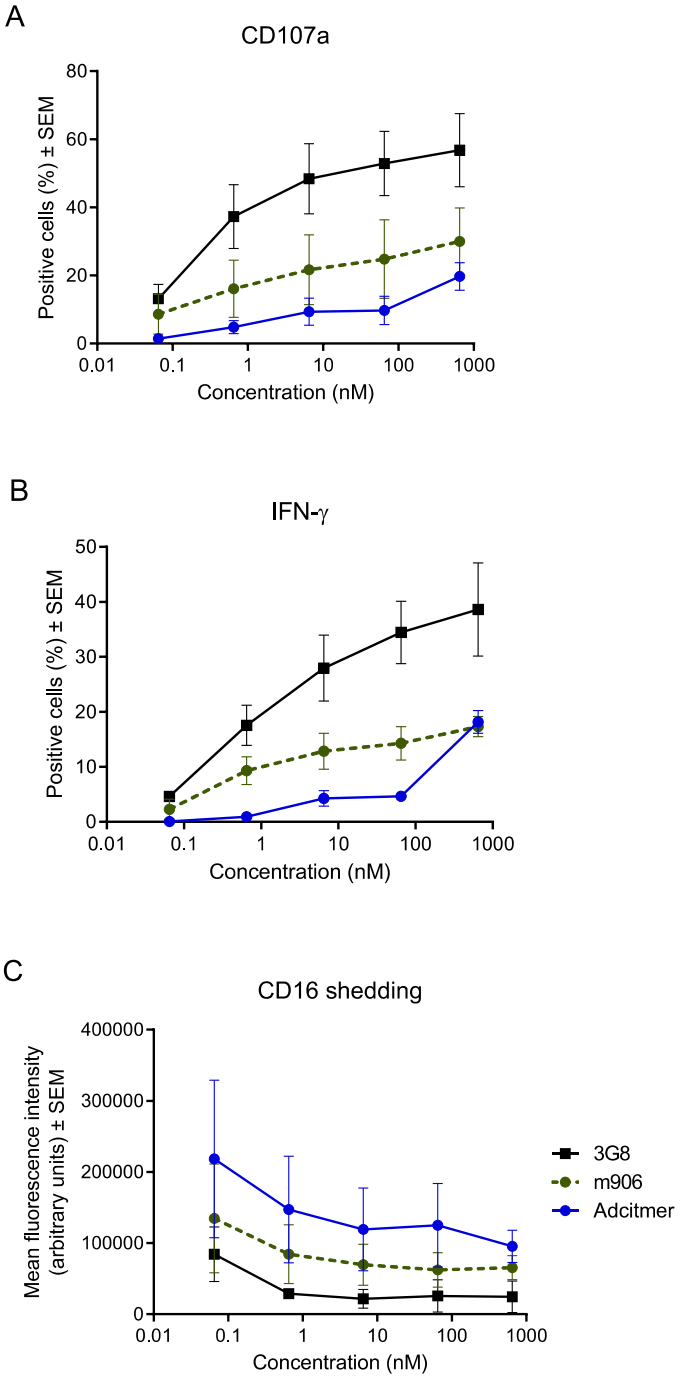


Adapted from Sha *et al.*, 2016

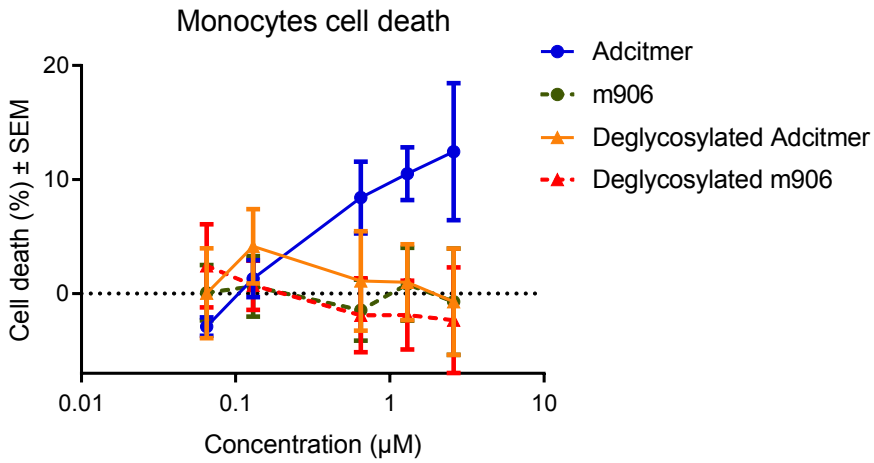
Supplemental Figure 2. Biochemical characterization of Adcitmer® and its deglycosylation form.



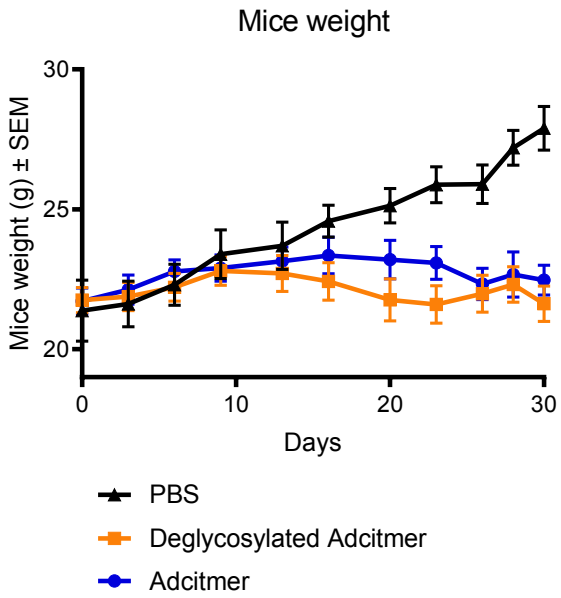
Supplemental Figure 3. m906 and Adcitmer® binding to FcγR (*i.e.* CD16, CD32, CD64). Results are presented as percentages of FcγR binding inhibition with Adcitmer® (blue curve) or m906 (green dashed curve) concentration range at different substract incubation times: (A) CD16 binding (2 min), (B) CD32 binding (10 min), (C) CD64 binding (15 min). Experiments were performed two times independently. Data are expressed as mean ± SEM of percentages.



Supplemental Figure 4. Functional responses of NK cells exposed 4h with different concentrations of m906 and Adcitmer®. NK cell functional responses were assessed by (A) the degranulation marker, CD107a, (B) INF-γ production (C) CD16 shedding indicated CD16 engagement, were measured by flow cytometry. NK cells were incubated with 3G8 antibody as NK activation control (black curve), with m906 (green dashed) or with Adcitmer® (blue). Experiments were performed three times independently. Data are expressed as mean ± SEM of percentages or fluorescence intensity.



Supplemental Figure 5. Monocytes cell death was assessed by flow cytometry after 4h at 37°C, with different concentrations of m906 (dashed green), Adcitmer® (blue), deglycosylated m906 (dashed red), and deglycosylated Adcitmer® (orange). Experiments were performed three times independently. Data are expressed as mean ± SEM of percentages.



Supplemental Figure 6. Evolution of mice body weights for 30 days between the beginning of the treatment and experiment endpoint. Mice treated with PBS are represented in black, deglycosylated Adcitmer® is depicted in orange, blue curve is the mice treated with Adcitmer®. Mice were injected twice a week at 5 mg/kg by intravenous route. Data are expressed as mean ± SEM.

Published articles :

Esnault, C., Leblond, V., Martin, C., Desgranges, A., Baltus, C.B., Aubrey, N., Lakhrif, Z., Lajoie, L., Lantier, L., Clémenceau, B., et al. (2021). Adcitmer®, a new CD56-targeting monomethyl auristatin E-conjugated antibody, is a potential therapeutic approach in Merkel cell carcinoma. Br J Dermatol https://doi.org/10.1111/bjd.20770.

Esnault, C., Schrama, D., Houben, R., Guyétant, S., Desgranges, A., Martin, C., Berthon, P., Viaud-Massuard, M.-C., Touzé, A., Kervarrec, T., et al. (2022). Antibody-Drug Conjugates as an Emerging Therapy in Oncodermatology. Cancers (Basel) 14, 778. https://doi.org/10.3390/cancers14030778.

Article in preparation :

Evaluation and optimization of Adcitmer®, a MMAE-conjugated ADC targeting CD56-expressing cancers. **Clara Esnault**, Aurélie Drouin, Laurine Durand, Pauline Gaboriaud, Valérie Leblond, Patricia Berthon, Shawk Karim, Morgane Fouché, Christine Dhommée, Christine B. Baltus, Camille Martin, Fanny Boursin, Nicolas Aubrey, Roland Houben, David Schrama, Serge Guyétant, Audrey Desgranges, Marie Claude Viaud-Massuard, Valérie Gouilleux-Gruart, Mahtab Samimi*, Antoine Touzé*, Thibault Kervarrec*

GENERAL CONCLUSION AND PERSPECTIVES

1. Rational for the use of Adcitmer® in Merkel cell carcinoma

1.1. An overview of Adcitmer® characterization

In this work, we preclinically evaluated Adcitmer®, a new CD56-targeting MMAE conjugated antibody, as a possible therapeutic approach for metastatic MCC (**Figure 9**). CD56 is a diagnostic marker for MCC and has also been proposed as a potential therapeutic target (Fernández-Figueras et al., 2007; Ollier et al., 2018; Tetzlaff and Harms, 2020). In our study, we confirmed CD56 expression in 66% of MCC cases (n=90) in both MCPyV-positive and -negative tumors. Since technical progresses in bioconjugation are likely to improve the performances of ADCs (Junutula et al., 2008), Adcitmer® was generated using an innovative McSAF Inside® bioconjugation technology that allows to control number and position of the MMAE molecules (**Figure 9**). Adcitmer® was able to target and internalize specifically in MCC cells expressing CD56 and induce cell cycle arrest and cell death *in vitro* (**Figure 9**). Interestingly, *in vitro*, Adcitmer® can trigger a bystander effect (*i.e.* drug release from the primary targeted cells to neighboring tumor cells) in MCC CD56-positive cells and subsequently induce cell death of tumor cells that does not express CD56 (**Figure 9**). This bystander effect is likely to increase the ADC efficiency in case of tumors with heterogeneous expression of the target (Kovtun et al., 2006). Since in our previous study, among CD56-expressing tumors, low or heterogeneous expression of CD56 was observed in 44% the cases (Esnault et al., 2021), Adcitmer® could be of interest for MCC patients. Although Adcitmer® treatment controls tumor growth in an MCC xenograft mice model, it exerts toxicity *in vitro* on immune cells expressing CD56 and/or Fc receptors (*i.e.* NK cells and monocytes) (**Figure 9**). This observation led us to generate a deglycosylated form of Adcitmer® which prevented fixation and toxicity of Adcitmer® on immune cells *in vitro*. Moreover, the deglycosylated form of Adcitmer® led to a higher anti-tumor effect compared to the same therapeutic scheme with Adcitmer® in a MCC mice model (complete tumor reduction was observed in nearly half of the cases treated with deglycosylated Adcitmer®) (**Figure 9**).

To conclude, deglycosylated Adcitmer® appears as a promising therapeutic option to treat MCC tumors with limited toxicity against immune cells. Such treatment might be used as a relevant alternative or in combination with immunotherapy in advanced MCC patients and in other CD56-positive malignancies.

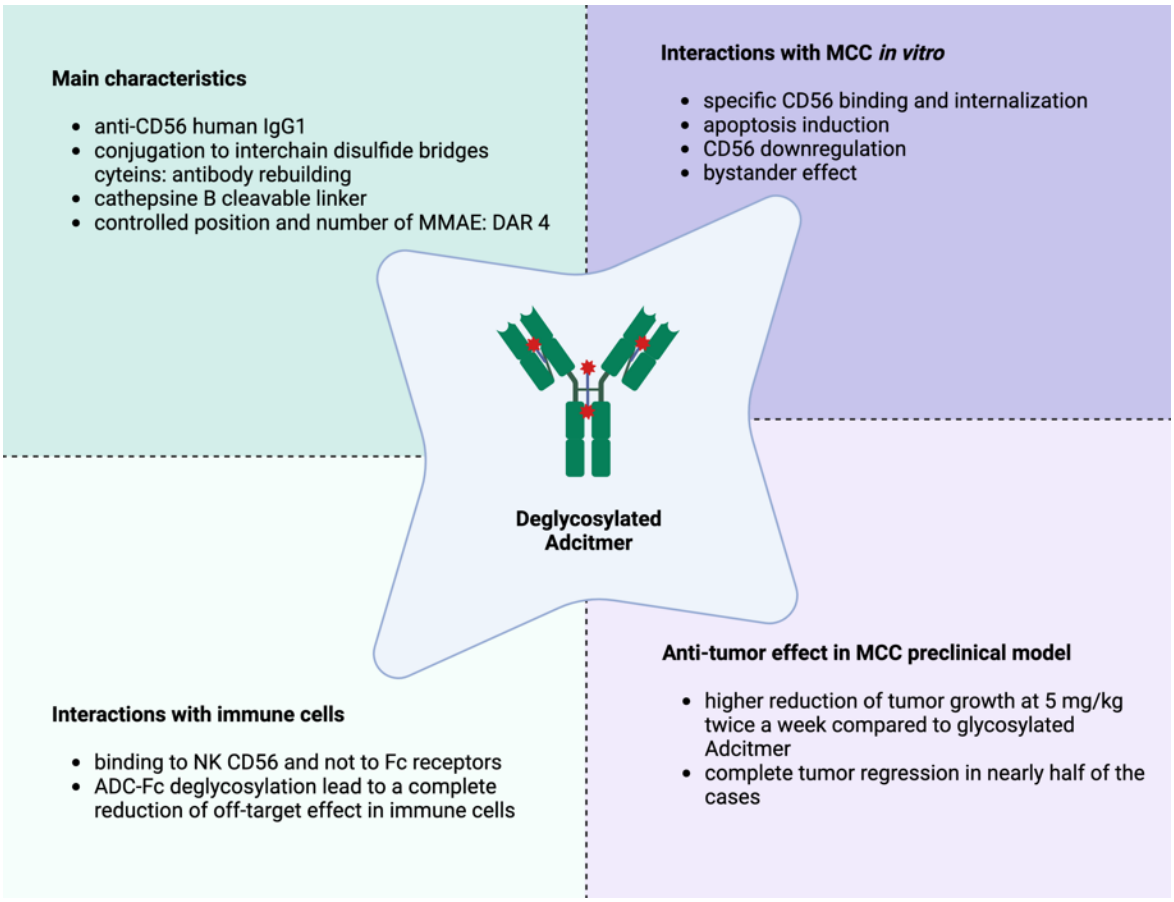


Figure 9. Main conclusions on Adcitmer® intrinsic characteristics, MCC *in vitro* and *in vivo* effects as well as interactions with immune cells.

1.2. Rational for the use of an ADC therapy in MCC

Previously, the only available therapeutic option for metastatic MCC patients was conventional chemotherapy *i.e.* platinum-based drugs, etoposide, taxanes and anthracyclins, without proven benefit on survival (Nghiem et al., 2017). Indeed, responders patients presented short term recurrences and important adverse effects (Nghiem et al., 2017; Samimi et al., 2019).

Among the arsenal of treatment currently available in oncology, targeted therapies represent a promising option allowing to induce specific cytotoxic effects with a reduced toxicity on non-tumor cells. Among this therapeutic class, small molecules allow a specific blockage of intracellular signaling pathways, while monoclonal antibodies are used for the targeting of cell surface membrane antigens or components of the microenvironment (Lee et al., 2018). However, several studies using wide

sequencing failed to identify a drug targetable signaling pathway in MCC (Carroll et al., 2017; Starrett et al., 2020), ruling out the potential use of small molecules. Accordingly use of small inhibitor molecules in monotherapy, such as a multiple-tyrosine kinase inhibitor of oncogene c-KIT inhibitor (Samlowski et al., 2010), vascular endothelial growth factor receptor (VEGF-R) inhibitor (Rabinowits et al., 2018) or bcl-2 antisense agent (Shah et al., 2009), have shown only limited clinical benefit in MCC patients.

Moreover no drug directly targeting the viral proteins is currently available, although a recent preclinical study demonstrated that treatment of MCC cell lines with a glycogen synthase kinase 3 (GSK3) inhibitor led to a repression of viral oncogenes transcription and cell growth inhibition (Houben et al., 2022).

The targeting of somatostatin receptors (SSTRs) has also been proposed. Activation of SSTRs frequently results in inhibition of cell proliferation and therefore somatostatin analogues have been used in cancer treatment or diagnostic (Florio, 2008). Indeed, MCC tumors may express SSTR type 2 (Buder et al., 2014; Gardair et al., 2015), and responses following treatment with lanreotide, a somatostatin analogue, have been observed (Fakiha et al., 2010). However, preliminary results of phase II study for advanced MCC treated with lanreotide reported that only 20% of patients had a clinical benefit for more than 6 months (NCT02351128) (Akaike et al., 2021).

Immune checkpoints inhibitors revolutionized MCC therapy. In this regard, the programmed cell death-ligand 1 inhibitor avelumab has been approved in 2017, followed by approval of pembrolizumab. When used in a first-line setting, ICI provide objective response rates in 50% of patients with MCC. Nevertheless, approximately half of patients do not respond to ICI, underlining the need for new therapeutic options.

Regarding the potential use of other antibody-based strategies for treatment of MCC patients, it should be noted that MCC tumor cells were demonstrated to be resistant to the antibody-dependent cellular cytotoxicity (ADCC) ruling out the use of an ADCC based antibody in this context. Therefore, ADC allowing to vectorize and trigger directly tumor cell toxicity appears as relevant therapeutic option in this context and might be used as an alternative, but more important in combination with ICI, in MCC metastatic patients.

Indeed, ICI and cytotoxic agents have shown a synergic effect, for example in triple-negative breast cancer and in non-small cell lung cancer (NSCLC). In triple-negative breast cancer, treatment with doxorubicin and cisplatin induces an upregulation of immune-related genes and triggers inflammation in the tumor microenvironment resulting in an increased response to the anti-PD-1 antibody (Voorwerk et al., 2019). In metastatic NSCLC patients resistant to platinum-based chemotherapy, a longer overall survival and higher response was observed with a combination of anti-PD-1 and platinum-based chemotherapy than with ICI monotherapy (Zhang et al., 2020). To improve the response rates, the combination of domatinostat, a histone deacetylase inhibitor, and avelumab is currently investigated in MCC resistant to ICI therapy (NCT04393753). Indeed, domatinostat increased the expression of MHC class I and II molecules and cytotoxic T lymphocytes infiltration in tumors. In addition, enhanced antitumor effects were observed with the combination of domatinostat and anti-PD-L1 antibody when compared to the ICI treatment alone (Bretz et al., 2019). Moreover, combination of first-line radiotherapy and avelumab led to complete clinical regression of MCC with minimal adverse effects in a patient (Principe et al., 2019). The synergistic effect of radiation therapy and ICI is currently under investigation in clinical trials in advanced MCC (NCT03304639). A phase II study has evaluated the combination of nivolumab and ipilimumab with or without radiation therapy as a first-line therapy or following previous treatments with anti-PD-1 and PD-L1 monotherapy in advanced MCC. However this study failed to demonstrate the benefit of the adjunction of radiation therapy in this setting (Kim et al., 2022).

Interestingly, some ADCs payloads are able to induce an immunogenic cell death and to trigger dendritic cell activation and maturation (D'Amico et al., 2019; Galluzzi et al., 2017; Rios-Doria et al., 2017) and therefore might contribute to overcome ICI resistance (Gerber et al., 2016). In CD30-expressing lymphoma, a synergistic therapeutic effect has been evidenced with the association of brentuximab vedotin and nivolumab (Zinzani et al., 2019).

Therefore, in the present study we aimed to develop an ADC based therapy for the treatment of metastatic MCC patients.

1.3. Identification of CD56 as a relevant therapeutic target in MCC

Development of an ADC based therapy for MCC patients first required the identification of a membranous target. As such, mucin-1 (MUC-1) has been suggested as a relevant antibody-targetable cellular protein with frequent expression observed in both virus-positive and -negative MCC (76% positive cases) (Kervarrec et al., 2019; Morimoto et al., 2022). In our hands, a first exploratory MUC-1 staining in MCC virus positive cell lines (*i.e.* WaGa, PeTa and MKL-1) confirmed MUC-1 expression in MCC tumor cells (data not shown), however MUC-1 expression by epithelial non-tumor cells of the lung, kidney, stomach, female reproductive tract, and pancreas (Gendler and Spicer, 1995) constitutes an obvious limitation for an ADC based therapy.

Similarly, epithelial cell adhesion molecule (EpCAM) is frequently expressed by MCC (65% of MCC cases n=103) (Kervarrec et al., 2020b) tumors but also by most physiologic epithelial cells, and therefore does not represent an interesting therapeutic target (Ollier et al., 2018; Schmelzer and Reid, 2008).

Delta-like protein 3 (DLL3), has been proposed as a relevant target for ADC therapy in SCLC (Owen et al., 2019) and a recent study demonstrated its expression in 91% of MCC patients notably in virus-positive cases (Xie et al., 2020). However, evaluation of DLL3 expression in MCC cell lines (*i.e.* WaGa, PeTa and MKL-1) in our lab did not reveal expression of this target (data not shown).

In order to identify ADCC-target antigens, our group previously conducted - in collaboration with Dr. H. Vié team, UMR 1232 - a large antigens screening of five MCC cell lines by flow cytometry (*i.e.* MKL-1, MKL-2, MS-1, PeTa, and WaGa) (Ollier et al., 2018). This ADCC-screening model is based on the detection of cytotoxicity triggered by murine monoclonal antibodies between human effector cells (human NK-92 cell line armed with the murine Fc receptor) and target cells (tumor cells) (Clémenceau et al., 2013). Among forty-one antigens, five were identified as potential ADCC targets (*i.e.* CD9, CD24, CD47, CD326 and SSEA-5), whereas no ADCC was observed in this model, despite high level of target expression (Ollier et al., 2018). These results suggest that CD56 could not be a target for naked monoclonal antibodies but nevertheless, CD56 remains an interesting therapeutic target for ADCs.

CD56 has been reported to be associated with tumor progression in multiple myeloma, acute myeloid leukemia, acute lymphoblastic leukemia, neuroblastoma, nephroblastoma and ovarian carcinoma (Cottini et al., 2022; Fischer et al., 2009; Heinly and Grant, 2022; Sasca et al., 2019; Yap et al., 2017; Zecchini et al., 2011). However, in our MCC tumors cohort (n=90) CD56 expression was not associated with higher rates of tumor recurrence or death. Moreover, no association has been observed between CD56 expression and MCPyV status. In addition, in exploratory experiments *in vitro*, CD56 knockdown with either RNA interference or CRISPR-Cas9 did not significantly impaired MCC tumor cell proliferation *in vitro* (data not shown). Therefore, although the contribution of CD56 expression to MCC tumor cell biology remains to determine, this molecule appears as a relevant therapeutic target for the development of an ADC-based strategy.

1.4. Previous CD56 targeting ADC in MCC

A CD56-targeting antibody, LM, has been assessed in seven clinical trials including multiple CD56-positive tumors such as small cell lung cancer, multiple myeloma, ovarian cancer, neuroendocrine tumors, MCC, neuroblastoma, nephroblastoma, acute T cell lymphoblastic leukemia, acute myeloid leukemia and malignant NK/T-cell lymphoma (Ailawadhi et al., 2019; Shah et al., 2016). Several phases I-II clinical trials demonstrated acceptable tolerability profiles of LM when used in monotherapy (Ailawadhi et al., 2019; Geller et al., 2020; Shah et al., 2016). Moreover, objective responses were detected in three metastatic MCC patients including two complete responses (n=23) (Shah et al., 2016). Nevertheless, in SCLC patients, LM was tested in combination with etoposide/carboplatine and induced severe toxicities (Socinski et al., 2017) which led the company Immunogen to stop this molecule development.

LM is a humanized IgG1 monoclonal antibody targeting CD56, conjugated to the mertansine (DM1), a cytotoxic drug which inhibits tubulin polymerization (Whiteman et al., 2014a). DM1 is attached to the antibody's lysins using a technology that does not allow the control of the sites of fixation and the number of attached DM1 molecules on the antibody (Juen et al., 2022). By contrast, Adcitmer® is composed of a human IgG1 monoclonal antibody conjugated to the MMAE, a cytotoxic drug which also impairs tubulin polymerization. MMAE is conjugated to the antibody interchain cysteins, allowing a controlled position and number of MMAE on the antibody (**Table 6**).

Table 6. Characteristics of lorvotuzumab mertansine and Adcitmer®.

Characteristics	Lorvotuzumab mertansine	Adcitmer®
Monoclonal antibody Isotype	Lorvotuzumab Humanized IgG1	m906 Human IgG1
Monoclonal antibody amino acid used for bioconjugation	Lysines	Cysteins disulfide bridges
Chemical linker	Cleavable linker N-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP)	Cleavable linker Dipeptides valine-citrulline (Val-Cit-PABC)
Payload	DM1	MMAE
Position and number of drugs	Heterogeneous DAR ≤ 4	Homogeneous DAR = 4
Fc CH2 with glycosylation site Asparagine 297	Yes	Yes

2. Characterization of Adcitmer® properties

2.1. Biochemical properties

2.1.1. Payload

Among the 13 FDA-approved ADCs, five ADCs are conjugated to MMAE including the most recent one (brentuximab vedotin, polatuzumab vedotin, tisotuzumab vedotin, enfortuzumab vedotin and disitamab vedotin). The first approved-ADC conjugated to MMAE was brentuximab vedotin in 2011. Building on this decade of clinical experience, MMAE-bioconjugated ADCs have demonstrated their therapeutic performances in solid and haematological malignancies with acceptable toxicities, mostly neutropenia and peripherical neuropathy (Donaghy, 2016). Moreover, chemical and pharmacologic properties of this therapeutic class have been extensively characterized with notably a DAR 4 required for optimal therapeutic effect (Bryant et al., 2015). For all these reasons, MMAE payload has been chosen to develop Adcitmer®.

2.1.2. Bioconjugation

The drug to antibody ratio (DAR), defined as the number of drug molecules attached to a monoclonal antibody, is an important determinant for ADC performance (Leung et al., 2020). The technology connecting the monoclonal antibody to the linker is crucial to obtain a homogeneous and controlled DAR (*i.e.* position of drugs and loading) (Junutula et al., 2008). Chemical linkers are used to bind a cytotoxic payload to amino acids of the monoclonal antibody. First generation bioconjugation technologies involved either N-hydroxysuccinimide (NHS) ester bioconjugation heads binding to lysine residues, or hydrazones, maleimides and thioethers binding to cysteines (Bargh et al., 2019). However, those bioconjugation heads led to the generation of heterogeneous ADCs, presenting a high variability of the DAR in the range of 0 to 8, and to multiple different linker positions (Leung et al., 2020).

More recently, site-specific conjugation, involving engineered cysteine residues, unnatural amino acids, or enzymatic conjugation through glycosyltransferases, have been applied to obtain more homogeneous ADCs (Beck et al., 2017). Indeed, site-specific conjugation of antibodies improve the ADC therapeutic index by reducing hydrophobicity of the linker-payload as well as preventing a release of payload into the blood (Junutula et al., 2008). Indeed, maleimide residue, either included in the linker or the bioconjugation head might react with free thiol of plasma resulting in the so called retro-Michael reaction. Hence, this retro-Michael reaction induces unspecific release of the linker and drug which not only impacts the efficacy of the ADCs but also leads to off-target toxicity (Szijj et al., 2018).

To overcome stability and heterogeneity issues of ADCs incorporating a maleimide motif, we used a new technology based on a trifunctionalized di(bromomethyl)pyridine scaffold (developed by McSAF Inside®) to develop Adcitmer®. This type of conjugation leads to highly stable and homogeneous ADC with a control linker-payload number and position (DAR 4). Interestingly, a higher anti-tumor activity of these ADCs was demonstrated in comparison with the approved-ADCs in preclinical models (Juen et al., 2021, 2022). Indeed, in a HER2-positive breast cancer xenograft model, this linker associated to the trastuzumab leads to a significantly higher reduction of tumor growth compared to the clinically approved ADC, trastuzumab emtansine.

2.2. Interaction with immune cells

ADC clinical off-target effect has been widely described. Dose-limiting toxicities of ADCs are frequently unrelated to the targeted antigen (de Goeij and Lambert, 2016). ADCs could have a target-independent toxicity (off-target off-tumor) due to mAb Fc region that interacts with effector immune cells (Donaghy, 2016). This interaction could result in adverse events due to toxicities on non-tumor cells through ADCC, complement-dependent cytotoxicity, antibody-dependent cellular phagocytosis (Donaghy, 2016). ADC interaction with Fc receptor was hypothesized as another mechanism of endocytosis that could explain ADCs off-target haematological toxicity (Uppal et al., 2015). This off-target off-tumor toxicity has been illustrated by trastuzumab emtansine Fc recognition by immune cells that induces thrombocytopenia (Hoffmann et al., 2018). Accordingly, to avoid interaction with the immune effectors (*i.e.* ADCC, complement-dependent cytotoxicity, antibody-dependent cellular phagocytosis), genetic engineering introducing mutations in the Fc domain of ADCs has been proposed (Aguiar et al., 2018; Hoffmann et al., 2018).

Interestingly, several currently FDA approved ADCs are targeting ligands expressed by immune cells but still harbor therapeutic efficiency and acceptable tolerance such as brentuximab vedotin, targeting CD30 (Vaklavas and Forero-Torres, 2012), polatuzumab vedotin, targeting CD79 (Sehn et al., 2020), or inotuzumab ozogamicin, targeting CD22 (Fayad et al., 2013).

2.2.1. *In vitro* investigation

In our study, we confirmed interaction of Adcitmer® with immune cells through both CD56 and Fc recognition by Fc receptors. Moreover, we demonstrated that most of the Adcitmer® toxicity on NK cells was related to the Fc binding and not to CD56 targeting. Nevertheless, Adcitmer® toxicity could be related to both CD56 and Fc binding on the NK cells: to assess this hypothesis *in vitro* a glycosylated IgG1 that did not target CD56 could be a relevant model. Importantly, the antibody glycosylation is required for Fc binding on immune cells (Cymer et al., 2018). Then, to avoid Fc recognition by immune cells we generated a deglycosylated Adcitmer®. Interestingly, deglycosylation of Adcitmer® prevented off target effect on immune cells.

Fc recognition by immune cells has also been described for others ADCs, notably for trastuzumab emtansine induced thrombocytopenia (Uppal et al., 2015). In this setting *in vitro* experiments have shown a trastuzumab emtansine cytotoxicity on

mature platelets independent from HER2 recognition but mediated by FcγRIIa internalization through trastuzumab Fc binding. Accordingly, ADC internalization was significantly inhibited by using FcγRII blocking antibodies or by mutations of trastuzumab Fc and led to decreased FcγR binding.

2.2.2. Investigations to explain NOD/SCID mice symptomatology after m906 injection

In our initial experiments (Esnault et al., 2021), no toxicity was detected with intravenous injection of Adcitmer® in MCC xenograft mice model. By contrast an unexpected prostration was observed for all animals briefly after Adcitmer® primo injection in latter *in vivo* experiments. The acute reaction upon injection with Adcitmer® was characterized by orbital tightening, piloerection and decreased activity. This reaction led to the death of one mouse injected with Adcitmer® (n=7) and did not recur after the following injections (n=6). A similar symptomatology was detected after injection of m906, suggesting that this reaction was due to an antibody property and not due to linker-payload activity. To explain this adverse effect, we first considered the hypothesis of an infectious contamination of the monoclonal antibody batch. However, several independent m906 batches were generated and similar symptoms appeared with all. Moreover, this symptomatology was not related to mice strain, since it was also observed in Bab/c and NOD scid gamma (NSG) mice. The dose modulation did not solve the problem neither.

Interestingly, the symptoms occurred only after the first injection or when the time frame between injections was longer than two weeks. The same symptomatology was also observed following intraperitoneal injection of the drug. Then, we wondered whether the symptoms might be dependent on CD56 epitope recognition, since m906 cross-reacts with murine CD56 (Feng et al., 2016), however anti-CD56 single-chain variable fragment (scFv) based on m906 structure was injected and no symptoms were observed. We then hypothesized that recognition of the Fc part by murine immune cells might contribute to this process and therefore generated a deglycosylated Adcitmer® avoiding Fc recognition. The symptoms that occurred after injection of Adcitmer® or m906 were not observed with deglycosylated Adcitmer® strongly suggesting that Fc recognition might be involved in mice symptomatology. To note, comparison of the glycosylation profiles by mass spectrometry of the initial batch of

ADC used without toxicity with other batches did not demonstrate significant variations. To test whether toxicities observed with the glycosylated form of Adcitmer® is only related to the Fc portion recognition or might require both CD56 and Fc parts targeting, the generation of an antibody construct based on m906 backbone but lacking the CD56 targeting part is ongoing.

Indeed, our main hypothesis is that glycosylated m906 could trigger ADCC through a cross-linking between effector cells (FcγR-positive) and murine CD56-positive cells in circulation of mice. In line with this view, a clinical trial conducted in 2006 and evaluating an anti-CD28 antibody, TGN1412, has shown a rapid T cells activation, severe cytokine release and T cells depletion after injection of this antibody. This adverse effect was related to a binding of the antibody to both FcγR-positive myeloid cells and CD28-positive T cells and is currently known as the TeGenero incident. Importantly, these observations were also reported in mice, rats and monkeys (Horvath and Milton, 2009). Therefore, a potential dual interaction of m906 with immune cells through both Fc and CD56 targeting region may explain the off-target effect observed in mice. This double interaction might trigger cytokine release by effector cells activation and consequently induce an off-target toxicity in mice (**Figure 10**) (Gorovits and Krinos-Fiorotti, 2013). Importantly, no symptomatology was observed after lorvotuzumab injection in mice, bearing in mind that lorvotuzumab does not bind murine CD56.

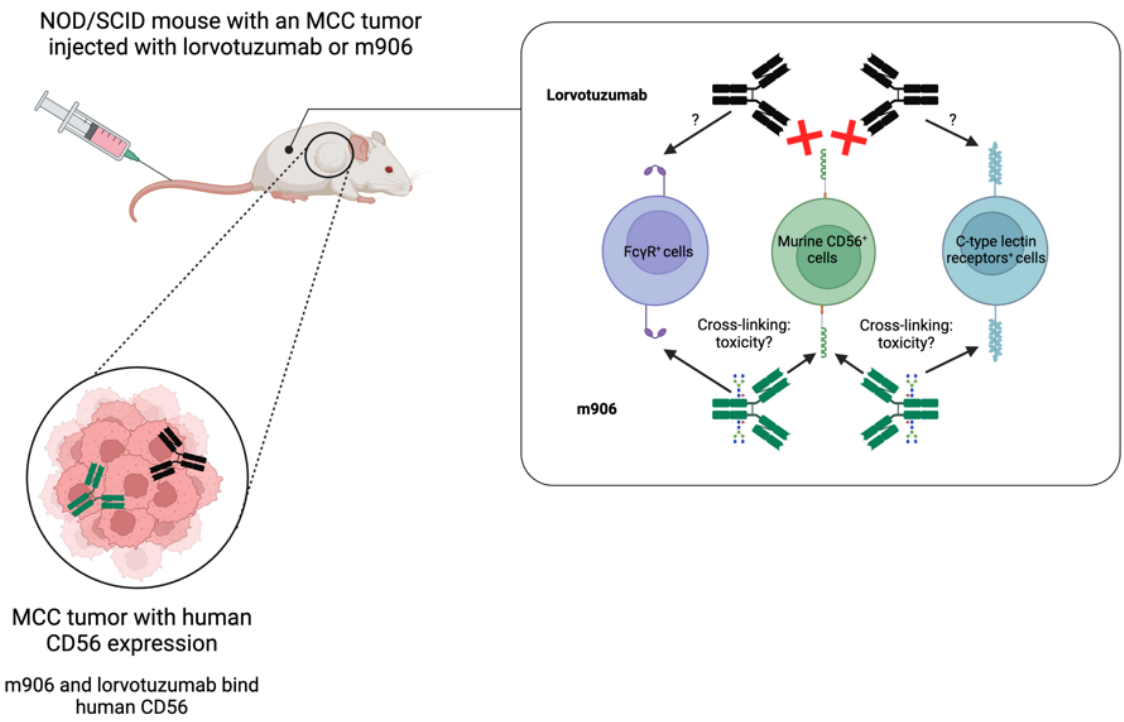
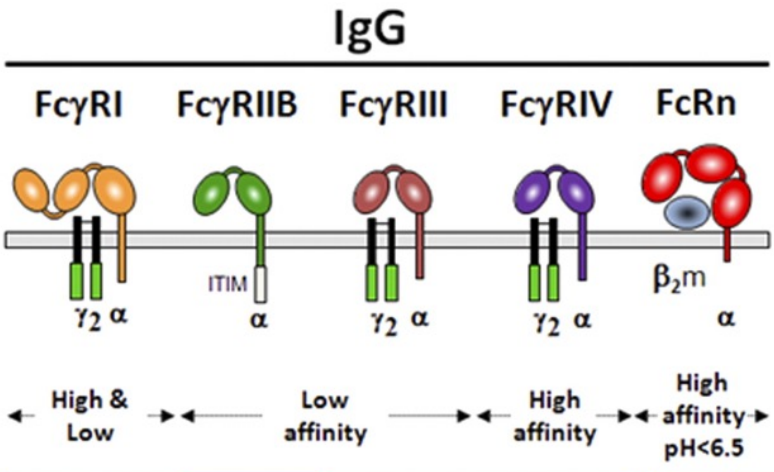


Figure 10. Major differences between lorvotuzumab and m906 that could explain the toxicity observed in NOD/SCID mice model. Both m906 and lorvotuzumab bind human CD56 expressed by tumor cells. Lorvotuzumab does not bind murine CD56 while m906 does. Glycosylated m906 could interact with cells that express FcγR or C-type lectin receptors and cross-link CD56-positive cells to FcγR or C-type lectin receptors-positive cells that could possibly induce a toxicity in mice. Glycosylation of lorvotuzumab was not described but nevertheless, a glycosylation site is present in the lorvotuzumab CH2 domain (IMGT data base). Since lorvotuzumab is produced in CHO cells (IMGT data base), we can suppose that lorvotuzumab present mammalian glycosylation that could potentially interact with FcγR or C-type lectin receptors-positive cells.

2.2.3. Impact of deglycosylation of Adcitmer® in tumor growth

The anti-tumor effect was significantly higher in mice treated with deglycosylated Adcitmer® than in the Adcitmer® treated group. A complete regression of the tumor was even achieved in the group of mice treated with the deglycosylated Adcitmer® (n= 3/7). As Adcitmer® is a human IgG1 that could interact with mice Fc receptors (Bruhns, 2012) (**Figure 11**), deglycosylation of Adcitmer® is likely to avoid interaction with Fc receptor-positive cells and could increase biodisponibility, resulting in higher anti-tumoral effect.



Function	Activation	Inhibition	Activation	Activation	Recycling Transport Ag uptake
IgG subclass binding	- **2a** 2b (3)	1 2a 2b -	1 2a 2b -	- **2a** **2b** -	**1** **2a** **2b** 3

	FcγRI	FcγRIIB	FcγRIII	FcγRIV	FcRn
B cells	–	+	–	–	+†
T cells	–	–	–	–	–
NK cells	–	–	+	–	–
Monocytes/macrophages	–	+	+	+	+
Neutrophils	–	+	+	+	+
DCs	+*	+	+	–	+
Basophils	–	+	+	–	–
Mast cells	–	+	+	–	?
Eosinophils	–	+	+	–	–
Endothelium	–	–	–	–	+
Intestinal epithelium	–	–	–	–	+

Figure 11. Mouse IgG receptors and expression pattern. Schematic representation of mouse IgG receptors at the cell membrane (gray bar) and their association or not to the FcγR-chain dimer (black). Green boxes represent ITAMs and the white box, the ITIM. Binding of a mouse IgG subclass is indicated in bold (high affinity), plain (low affinity), or between parentheses (very low affinity). - indicates no binding. The table represents expression pattern of mouse IgG receptors. * Monocyte-derived DCs, but not conventional DCs. † On splenic B cells only. Adapted from Bruhns *et al.,* Blood, 2012.

2.2.4. An aglycosylated version of Adcitmer® for further study

Although our experiments suggest efficacy and safety of the deglycosylated Adcitmer®, this form is not suitable for industrial development (Zheng et al., 2011), as deglycosylation adds a step in the bioproduction of an ADC. Interestingly, aglycosylated therapeutic antibodies are comparable to deglycosylated antibodies (Cymer et al., 2018). Therefore, further developments require mutagenesis of the

m906 sequence to produce an aglycosylated Adcitmer® (with a change from an asparagine to an alanine N297A) (Lo et al., 2017).

Although no aglycosylated ADC has been approved until now, aglycosylated therapeutic antibodies have already been approved in clinics (Hale et al., 2010). Moreover, aglycosylated antibodies assessed in preclinical and clinical studies display similar pharmacokinetics to glycosylated IgG (Jung et al., 2011). Aglycosylated antibodies have several advantages, including 1) reduction of adverse clinical reactions due to cytokine release through binding of antibody to complement or Fc receptors (Hale et al., 2010), 2) control of glycan isoforms (Jung et al., 2011). Therefore, use of an aglycosylated form of Adcitmer® appears as a promising option in this setting.

3. Perspectives

3.1. Adcitmer®: the next steps to achieve a clinical trial

Next steps of Adcitmer® characterization will require additional preclinical studies, including maximal effective dose determination and therapeutic scheme adjustment. Moreover, the drug should be successfully produced in large amount, following appropriate manufacturing practices for human use (Matsuda and Mendelsohn, 2021). The determination of the maximal tolerated dose in mice and rats is necessary to determine the therapeutic index of Adcitmer®, that corresponds to the ratio between lethal dose for 50% of individuals by the minimum effective dose for 50% of individual. Therapeutic index is a dose range in which a drug is effective without unacceptable adverse events. Noteworthy, due to the specific delivery of the drug, ADCs have a larger therapeutic index than conventional chemotherapy (Panowski et al., 2014). Moreover, a pharmacokinetic (PK) evaluation based on preclinical studies will also be conducted to determine Adcitmer® half-life and linker stability. The relationship between Adcitmer® concentration in blood and its clinical effects *in vivo*, will be establish with a pharmacodynamic model and is necessary to transpose it to human heathy volunteers - these data being required for human clinical trial design.

3.2. Adcitmer® in other CD56 expressing tumors

Importantly, CD56 expression is not restricted to MCC tumor cells but is also observed in a large range of other neoplasms. Indeed, neuroendocrine neoplasms (*i.e.* SCLC), neuroblastoma and nephroblastoma as well as a number of haematological malignancies, such as acute myeloid leukemia, T cell acute lymphoblastic leukemia and multiple myeloma express CD56. In the present study, Adcitmer® was cytotoxic *in vitro* against SCLC and BPDCN cell lines and therefore could be a potential therapeutic option for these CD56-positive neoplasms.

Although such *in vitro* investigations might contribute to determine the design of a clinical trial, notably Adcitmer® indications, previous clinical experience obtained with LM might also help in this setting. For example, LM was well tolerated in a recent phase II trial including CD56-positive pediatric tumors (neuroblastoma and nephroblastoma among others) with a partial and a delayed complete response observed in one patient respectively (Geller et al., 2020). By contrast, the association between LM and etoposide and cisplatin in small cell lung patients has led to severe toxicities. Although Adcitmer® was optimized to minimize haematological toxicities, these observations should probably lead to avoid association of Adcitmer® with conventional chemotherapy.

LM also demonstrated a signal of efficacy in refractory multiple myeloma patients, with disease stabilization or responses detected in 42.9% of patients (Ailawadhi et al., 2019; Berdeja, 2014). In line with these results, an anti-CD56 ADC could be an interesting option for haematological malignancies (Fu et al., 2022).

BPDCN is a rare but aggressive haematological malignancy, with a 10-14 months median survival (Guolo et al., 2020). Clinical presentation of BPDCN includes involvement of skin, bone marrow, lymph nodes, spleen, liver, and peripheral blood. BPDCN is characterized by the co-expression of CD123, CD4, and CD56, this latter marker being expressed in all BPDCN cases (n=16) (Garnache-Ottou et al., 2009). First-line treatment with methotrexate and asparaginase chemotherapies followed by allogeneic stem cell transplantation achieved long-term remission, although approximately 30% of patients relapsed (Garnache-Ottou et al., 2019). In this context, an ADC targeting CD123, previously developed for the treatment of acute myeloid leukemia, is currently assessed in BPDCN patients (NCT03386513) suggesting a potential interest of ADC therapeutics in this disease. In this context, a collaboration

with Pr. Garnache-Ottou's team (Besançon hospital, France UMR 1098 *Interactions Hôte-Greffon-Tumeur & Ingénierie cellulaire et Génique* (RIGHT)) expert in BPDCN, has been initiated to investigate the drug *in vitro*. Moreover *in vivo* evaluation of deglycosylated Adcitmer® in mice model of BPDCN will be conducted next year. Thus, Adcitmer® will be assessed in preclinical studies beyond MCC.

4. Conclusions

In this work we developed Adcitmer®, a new CD56-targeting MMAE conjugated antibody. Adcitmer® was able to target specifically CD56 expressing MCC cells and subsequently to induce cell death *in vitro.* Interestingly, we also demonstrated that Adcitmer® could induce a bystander effect on CD56-non expressing MCC cells. As Adcitmer® exerts toxicity *in vitro* on immune cells through its Fc portion recognition, we generated a deglycosylated form of Adcitmer® preventing fixation and toxicities of the ADC on immune cells *in vitro*. Moreover, the deglycosylated form of Adcitmer® led to a higher anti-tumor effect in a MCC mice model, in comparison with the native molecule.

To conclude, deglycosylated Adcitmer® appears as a promising therapeutic option to treat CD56 expressing cancers. Such treatment might be used as a relevant alternative or in combination with immunotherapy in advanced MCC patients but also to treat other CD56-expressing tumors.

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APPENDIX

cancers

MDPI

Review

Antibody–Drug Conjugates as an Emerging Therapy in Oncodermatology

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Simple Summary: Currently, the therapeutic arsenal to fight cancers is extensive. Among these, antibody–drug conjugates (ADCs) consist in an antibody linked to a cytotoxic agent, allowing a specific delivery to tumor cells. ADCs are an emerging class of therapeutics, with twelve FDA- and EMA-approved drugs for hematological and solid cancers. In recent years, tremendous progress has been observed in therapeutic approaches for advanced skin cancer patients. ADCs appear as an emerging therapeutic option in oncodermatology. After providing an overview of ADC design and development, the goal of this article is to review the potential ADC indications in the field of oncodermatology.

Abstract: Antibody–drug conjugates (ADCs) are an emerging class of therapeutics, with twelve FDA- and EMA-approved drugs for hematological and solid cancers. Such drugs consist in a monoclonal antibody linked to a cytotoxic agent, allowing a specific cytotoxicity to tumor cells. In recent years, tremendous progress has been observed in therapeutic approaches for advanced skin cancer patients. In this regard, targeted therapies (e.g., kinase inhibitors) or immune checkpoint-blocking antibodies outperformed conventional chemotherapy, with proven benefit to survival. Nevertheless, primary and acquired resistances as well as adverse events remain limitations of these therapies. Therefore, ADCs appear as an emerging therapeutic option in oncodermatology. After providing an overview of ADC design and development, the goal of this article is to review the potential ADC indications in the field of oncodermatology.

Keywords: antibody–drug conjugates; oncodermatology; melanoma; skin squamous cell carcinoma; cutaneous T-cell lymphoma and Merkel cell carcinoma

1. Introduction

In 1909, the German chemist Paul Ehrlich [1] provided the first description of "targeted therapy". He proposed the so called "magic bullet" concept, allowing the delivery of a therapeutic molecule to a specific target without affecting healthy tissues [2]. Currently, targeted therapies are used in daily oncology practice including oncodermatology. As such,

tyrosine kinase inhibitors (TKIs) targeting BRAF and MEK proteins block the constitutive activation of the MAPK pathway in patients with BRAF-mutated melanoma [3], therefore reducing maintenance, development or dissemination of the cancer. Similarly, sonic hedgehog pathway inhibitors (sonidegib and vismodegib) act by inhibiting the smoothened protein involved in hedgehog signal transduction which plays a crucial role in basal cell carcinoma (BCC) development.

Among targeted therapies, antibody–drug conjugates (ADCs) combine a monoclonal antibody (mAb) with a highly cytotoxic molecule, allowing its specific delivery to tumor cells [4]. In recent years, optimization of ADC technologies in order to increase their therapeutic performances and overcome their limitations as well as evaluation of their effects in combination with currently approved drugs have significantly expanded their use in oncology [5,6]. Indeed, twelve ADCs have been approved by the FDA for treatment of hematologic malignancies ((A) in Table 1) and solid tumors ((B) in Table 1). Moreover, the number of ADCs in development is steadily increasing, with 195 ongoing clinical trials (https://www.beacon-intelligence.com/, accessed on 8 December 2021). Overall, ADCs are currently being applied in all oncology areas including skin cancers.

Table 1. FDA-approved ADCs. A. Hematological malignancies. B. Solid tumors.

Hematological Malignancies							
Commercial Name	**International Non-Proprietary Names (INN)**	**Target**	**Antibody Isotype**	**Bioconjugation Head (Antibody Amino Acid)**	**Linker**	**Drug (Therapeutic Class)**	**Indication**
Adcetris®	brentuximab vedotin	CD30	Chimeric IgG1	Maleimidocaproyl (Cysteine)	Cleavable/proteolytic (cathepsin B)	auristatin	anaplastic large cell lymphoma + Hodgkin's lymphoma
Polivy®	polatuzumab vedotin-piiq	CD79b	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable/proteolytic (cathepsin B)	auristatin	relapsed or refractory diffuse large B-cell lymphoma
Mylotarg®	gemtuzumab ozogamicin	CD33	Humanized IgG4	Acetyl butyrate (Lysine)	Cleavable/hydrazone	calicheamicin	CD33-positive acute myeloid leukemia
Beponsa®	inotuzumab ozogamicin	CD22	Humanized IgG4	Acetyl butyrate (Lysine)	Cleavable/hydrazone	calicheamicin	lymphoblastic leukemia
Lumoxiti®	moxetumomab pasudotox	CD22	N/A	N/A	Cleavable/proteolytic (furin)	*Pseudomonas* endotoxin A	hairy cell leukemia
Zynlonta®	loncastuximab tesirine-lpyl	CD19	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable/proteolytic (cathepsin B)	pyrrolobenzodiazepine dimer (PBD)	relapsed/refractory diffuse large B-cell lymphoma
Blenrep®	belantamab mafodotin	CD38	Humanized IgG1k	Maleimidocaproyl (Cysteine)	Uncleavable	auristatin	relapsed/refractory multiple myeloma
Solid Tumors							
Commercial Name	**International Non-Proprietary Names (INN)**	**Target**	**Antibody Isotype**	**Bioconjugation Head (Antibody Amino Acid)**	**Linker**	**Drug**	**Indication**
Kadcyla®	ado-trastuzumab emtansine	HER2	Humanized IgG1	Maleimidocaproyl (Lysine)	Uncleavable	maytansine	HER2/neu positive breast cancer
Padcev®	enfortumab vedotin	Nectin-4	Human IgG1	Maleimidocaproyl (Cysteine)	Cleavable/proteolytic (cathepsin B)	auristatin	locally advanced or metastatic urothelial cancer
Enhertu®	trastuzumab deruxtecan	HER2	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable/proteolytic (cathepsin B)	deruxtecan (topoisomerase inhibitor)	breast cancer HER2 positive after two or more lines of anti-HER2 therapy
Trodelvy®	sacituzumab govitecan	TROP-2	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable/hydrazone	topoisomerase inhibitor	metastatic triple-negative breast cancer
Tivdak®	tisotumab vedotin	Tissue factor	Human IgG1	Maleimidocaproyl (Cysteine)	Cleavable/proteolytic (cathepsin B)	auristatin	cervical cancer

Unprovided data: N/A.

The aim of the present review is to highlight current developments of ADCs in onco-dermatology and identify potential opportunities in this field.

2. ADCs in Oncology

2.1. Structure of ADCs

ADCs consist in a cytotoxic drug, i.e., a payload bioconjugated through a linker to mAb-targeting tumor cell antigens [7] (Figures 1 and 2). The specific combination of these components determines both the therapeutic performance and safety of the ADC [4]. The following parts aim to provide a brief overview of the features of the different ADC components and how these can impact efficacy and safety.

2.2. Targets

Target identification is a crucial point in ADC development [8,9]. Tumor-specific biomarkers previously used for diagnosis, as well as proteins overexpressed due to gene amplification or proteins involved in tumor aggressiveness [10] might be considered as potential ADC targets. Most ADCs bind to proteins expressed on the cell surface while targeting of intracellular tumor-associated proteins can also be achieved by using T-cell-receptor-like antibodies recognizing peptides in the context of presentation by MHC-I complexes [11]. In both cases, i.e., extra- and intracellular proteins, the specificity of the tumor antigen, antigen expression levels and antigen/ADC internalization [12] determine the performance of an ADC target [9].

To provide optimal payload delivery, high and homogenous expression of targeted antigens on the tumor cell surface is required [13]. By contrast, absent or low expression in healthy tissues [14] is expected in order to limit toxicities on physiologic cells (i.e., in order to avoid on-target off-tumor cytotoxicity). Nevertheless, several clinically approved ADCs such as brentuximab vedotin, targeting CD30 [15], polatuzumab vedotin, targeting CD79 [16], and inotuzumab ozogamicin, targeting CD22 [17], actually engage with ligands expressed by immune cells but still harbor therapeutic efficiency and acceptable tolerance. Moreover, most ADCs require internalization of the targeted surface antigen through receptor-mediated endocytosis and lysosomal degradation of the linker in order to deliver the payload [18]. One evasion mechanism that limits ADC performance is the downregulation of the targeted antigen expression, which obviously affects ADC binding to the target cells [9].

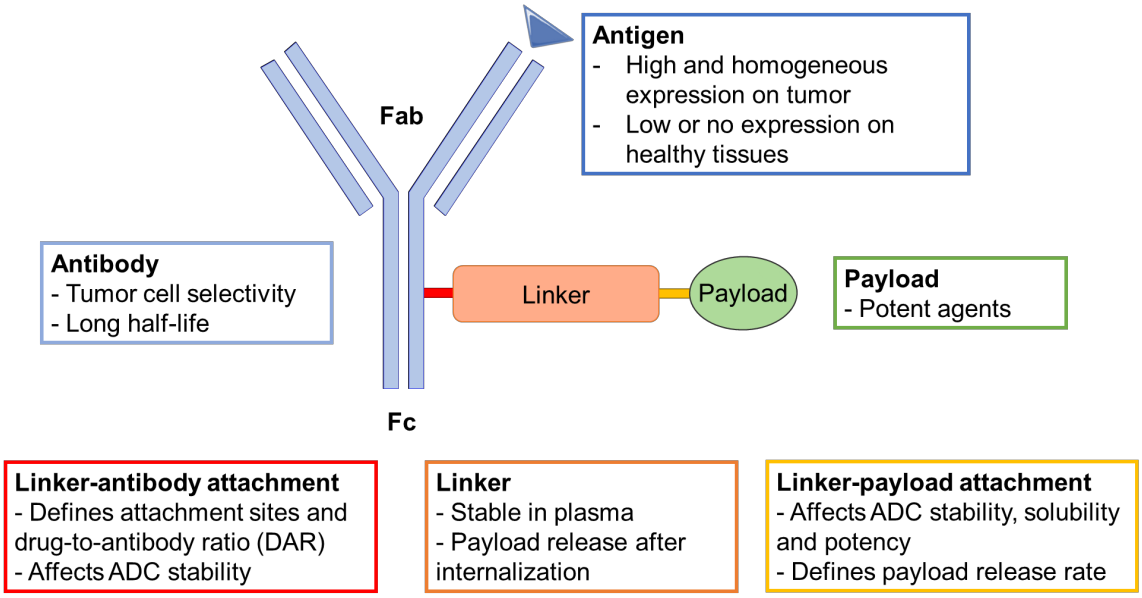

Figure 1. Design of an antibody–drug conjugate and recommended biological properties. Characteristic of antigen, antibody, linker–antibody attachment, linker, linker–payload attachment and payload are detailed. The antibody Fc part is implied in half-life and immunogenicity. The Fab part controls affinity and avidity to the targeted antigen.

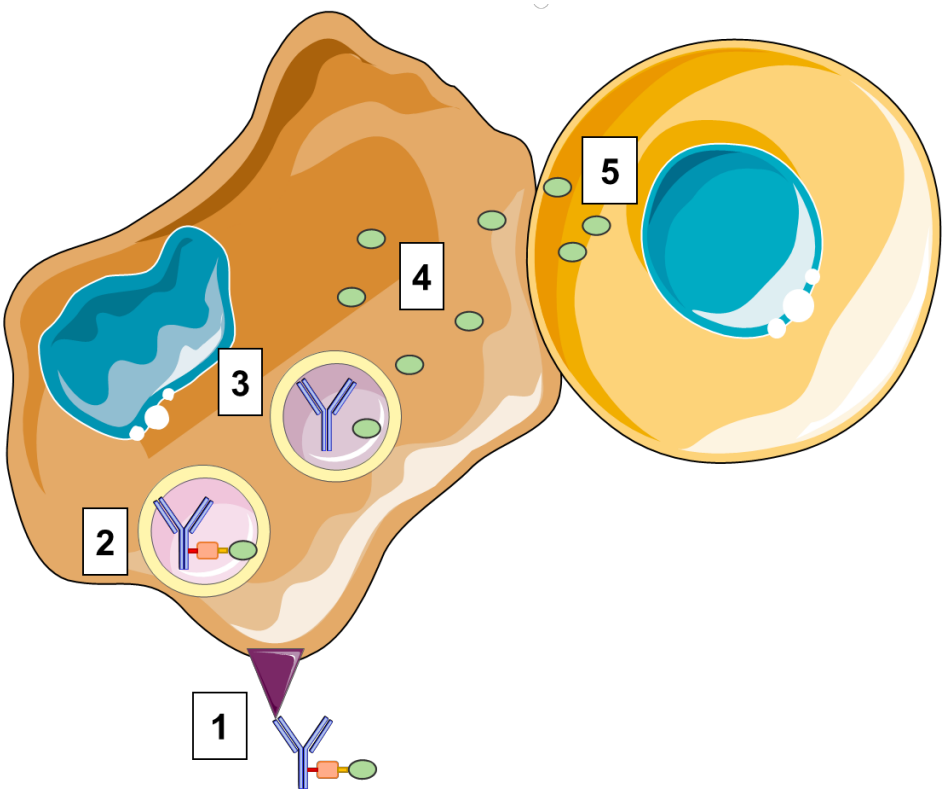

Figure 2. Overview of ADC mode of action. 1. Binding of the ADC to the antigen expressed by the cancer cell. 2. Internalization of the ADC. 3. Degradation of the linker or antibody inside the lysosome induces the release of an active form of the payload. 4. The payload exerts cellular toxicity depending on its mode of action. 5. A bystander effect can occur.

While antigens present on the cell surface are the most frequent candidates for ADC targets [19], secreted proteins/soluble antigens might additionally offer new opportunities by being more accessible. As a consequence, linker and drug have to be designed to be active in this context [14]. Some ADCs targeting non-internalizing soluble antigens, such as Tenascin-C splice variants or fibronectin, with maytansinoid and auristatins as payloads, have already demonstrated antitumor activity [20,21]. Importantly, targeting soluble antigens in the tumor microenvironment (TME) might impact not only tumor cells but also tumor stroma, extracellular matrix and blood vessels. Moreover, an association between bevacizumab, an antivascular endothelial growth factor (VEGF) monoclonal antibody, and an ADC has provided improved outcomes compared to a combination of bevacizumab with standard chemotherapy, in a phase Ib clinical study including patients with platinum-resistant ovarian cancer [22].

2.3. Antibodies

mAbs used in ADC structures are mostly of the IgG1 or IgG4 subtypes, and are chimeric or humanized in order to decrease their immunogenicity [4]. Both parts of the mAbs, i.e., the fragment antigen-binding (Fab) and fragment crystallizable regions (Fc region), contribute to the performance of an ADC. While affinity of a mAb for its epitope is determined by the Fab portion [10], the Fc part is determinant for mAb stability and interactions with immune cells such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated phagocytosis (ADCP). To this end, the Fc part interacts with neonatal Fc receptor (FcRn) [23], a recycling receptor determining both IgG half-life and biodistribution [24], or complement (C1q) implied in CDC or Fc receptors on immune effector cells (FcγR), a family of receptors

which may trigger an immune response upon Fc binding [25]. The ADC interactions with immune cells might either constitute an opportunity to induce an antitumor response, or represent a potential side effect, resulting in toxicity on immune cells [14]. Accordingly, to improve or avoid immune functions (ADCC, CDC, and ADCP), genetic engineering introducing mutations in the Fc domain of ADCs is used [14,26]. The Fc mutations aim to promote or impair interactions between ADCs and immune cells, Fc receptors or complement (C1q) in order to prevent or improve their activation, such as increased ADCC with ADC glyco-engineering (i.e., afucosylation) [27,28]. On the other hand, other Fc modifications have been proposed to increase ADC half-life via FcRn-mediated ADC recycling [9].

Additional factors related to the payload and linker affect ADC pharmacokinetics (PK) and pharmacodynamics (PD) [29]. Notably, ADC hydrophobicity is associated with shorter half-life in serum [30] and because most of the payloads are hydrophobic, attachment to a hydrophilic antibody remains a challenge [31]. To overcome hydrophobicity and ADC aggregation, the most common ADC modification consists in glycosylation, a naturally occurring post-translational modification influencing the solubility, antigenicity and stability of proteins [32]. PEGylation, the addition of polyethylene glycol (PEG) to the linker in order to mask payloads hydrophobicity and to improve the PK of ADCs (i.e., reduction in aggregation, improvement in solubility and half-life [31]), has also been proposed [32].

2.4. Payloads

While radionucleotides, toxins [33] and cytokines [34] have been suggested as ADC payloads [35–37], most preclinical and FDA/EMA-approved ADCs are bioconjugated with small cytotoxic molecules [38] with a broad range of structures and mechanisms of action [4]. Among these, duocarmycins and pyrrolobenzodiazepines (PBD) dimers induce DNA alkylation by linking alkyl residues to AT-rich regions or guanine, leading to cell apoptosis [38]. Cell death can also be induced by calicheamicins inducing DNA double-strand breaks [38]. Maytansine derivatives (including DM1 and DM4) [39] and auristatins (monomethyl auristatin E (MMAE), monomethyl auristatin F (MMAF)) [7] are poisons which inhibit microtubule polymerization by targeting tubulins, resulting in G2/M arrest and apoptosis. A third family of cytotoxic agents inhibit the topoisomerase enzymes, which control DNA structure changes. Topoisomerase inhibitors block the cell cycle ligation step, which generates DNA single- and double-strand breaks, leading to apoptotic cell death [40]. Trastuzumab deruxtecan and sacituzumab govitecan are both approved topoisomerase inhibitor ADCs [40]. Currently, auristatins, maytansine derivatives and PBD/PNU (anthracyclines family) represent 29%, 25% and 2% of the global active ADCs, respectively (https://www.beacon-intelligence.com/, accessed on 8 December 2021). Overall, MMAE remains the most frequent payload used for ADC development, involved in 19% of active ADCs for which the payload is disclosed (https://www.beacon-intelligence.com/, accessed on 8 December 2021).

Beyond their direct cytotoxic tumor-targeted activity, highly membrane-permeable payloads, such as MMAE or calicheamicin, are able to induce a "bystander effect" [41] due to drug release from the primary target cells into the TME. The bystander effect enhances ADC efficacy by targeting not only stromal cells, which do not express the targeted antigen, but also antigen-negative tumor cells in cases of intratumoral heterogeneity [12] (Figure 2). By contrast, MMAF, another auristatin derivate, has low cell permeability due to its negative charge. While such properties limit the off-target toxicity of MMAF ADCs, their bystander activity remains low [12]. Regarding this limit, MMAF ADCs require high tumor expression of target antigen [42]. Nevertheless, a MMAF-conjugated ADC, belantamab mafodotin, was approved in 2020 by the FDA and the EMA for relapsed or refractory multiple myeloma treatment [43,44].

2.5. Linker

The linker is the ADC portion that connects antibody and drug. Depending on the payload and antibody properties, either cleavable or non-cleavable linkers are used. Cleavable

linkers are used in combination with payloads being only fully active after lysosomal degradation (e.g., MMAE or calicheamicin) [38]. To prevent the cleavage of the linker in blood circulation resulting in unspecific cytotoxicity, the ADC linker has to be stable until the ADC reaches its target cell. To this end, cleavable linkers are usually sensitive to conditions specifically found in lysosomes (e.g., acidic condition or lysosomic proteases (cathepsin B, glycosidase, phosphatase)) or high intracellular glutathione concentrations [38] and thereby release the payload only after endocytosis is achieved, leading to death of the target cell [38] (Figure 2).

Regarding non-cleavable linkers, the drug is active after internalization and enzymatic digestion of the antibody into the lysosome, allowing the release of an active metabolite, amino acid–linker–drug complex (e.g., trastuzumab emtansine) [7]. Non-cleavable linkers allow increased ADC stability, leading to reduced toxicity in non-target tissues [7]. Payloads such as MMAF or DM1 are classically used in this setting.

2.6. Linker–Antibody Conjugation

The drug to antibody ratio (DAR), defined as the number of drug molecules attached to one mAb, is another important determinant for ADC performance [38]. The optimal DAR depends on the nature of the payload [45]. The paradigm that a DAR value of 4 is optimal for pharmacokinetics has been challenged by the recent approval of DAR 8 antibodies, in particular in light of improved hydrophobicity-masking technologies [46,47]. The technology connecting the mAb to the linker is crucial to obtain a homogeneous and controlled DAR (i.e., position of drugs and loading) [48]. Chemical linkers involving functional groups (i.e., bioconjugation head) are used to bind to mAb amino acids. First-generation bioconjugation technologies involved either N-hydroxysuccinimide (NHS) ester bioconjugation heads binding to lysine residues, or hydrazones, maleimides and thioethers binding to cysteines [49]. However, those bioconjugation heads led to the generation of heterogeneous ADCs, presenting a high variability of the DAR in the range of 0–8, and to multiple different linker positions [38].

More recently, site-specific conjugation, i.e., engineered cysteine residues, unnatural amino acids, or enzymatic conjugation through glycosyltransferases have been applied to obtain more homogeneous ADCs [7]. Indeed, site-specific conjugation of antibodies has been shown to improve the therapeutic index because it improves ADC pharmacokinetics by reducing the hydrophobicity of the linker–payload as well as prevent the release of payload in blood [48]. Moreover, site-specific conjugation with rebridging of antibody disulfide bonds results in stable and homogeneous ADCs with a controlled number and position of payload, and higher stability characteristics [50].

Interestingly, Van Geel et al. has shown that it is possible to link a payload to the native N-glycan residing at asparagine 297 and demonstrated in vitro and in vivo efficacy [51].

To conclude, ongoing optimization of ADC technology is likely to expand their application in oncology in the upcoming years, as illustrated below with ADCs currently approved or investigated in the field of oncodermatology.

3. ADCs in Cutaneous T-Cell Lymphoma

Cutaneous T-cell lymphoma (CTCL) was the first skin cancer for which a therapeutic ADC was approved. CTCLs are a group of extra-nodal non-Hodgkin's lymphomas with primary cutaneous infiltration of malignant monoclonal T lymphocytes. Mycosis fungoides (MF) is the most common form of CTCLs, characterized by slow progression from patches to infiltrating plaques and eventually to cutaneous and extracutaneous tumors. Among CTCLs, Sézary syndrome (SS) is characterized by erythroderma, lymphadenopathy and systemic dissemination of malignant Sézary T cells [52]. Although rare, SS displays an aggressive course, with a median survival of 1 to 5 years [53]. In the case of advanced MF and SS, the only potentially curative treatment is allogenic hematopoietic stem cell transplantation [54]. CD30, a proliferation-promoting member of the TNF receptor family [54], is expressed in 12 to 23% of SS and MF cases [55]. Since CD30 expression is

restricted in healthy human tissues to activated T, B and NK cells, it was chosen as a potential ADC target for treating advanced CTCLs [10] **(**Figure 3A). Accordingly, brentuximab vedotin (adcetris®), consisting of an anti-CD30 MMAE-conjugated ADC, with a cathepsin B cleavable linker, was developed (Table 2). Clinical benefit of brentuximab vedotin was demonstrated in CTCLs (stage IV SS and MF) with clinical objective response rates (ORR) of 70% [56,57]. Moreover, brentuximab vedotin achieved more durable responses (56% ORR lasting 4 months) than conventional treatments with methotrexate or bexarotene (12% ORR lasting 4 months) (Table 3). Consequently, brentuximab vedotin was approved by the FDA and the EMA in 2017 to treat CD30+ CTCLs after at least one line of systemic therapy.

However, resistance to MMAE was observed upon brentuximab vedotin treatment [58]. To overcome this resistance mechanism, a DM1-conjugated anti-CD30 ADC is currently in development. The anti-CD30 antibody is conjugated to DM1, a tubulin inhibitor, through antibody lysines by NHS ester non-cleavable linker (Table 2). This ADC has shown similar antitumoral effects to brentuximab vedotin without acquisition of payload resistance mechanism; moreover, the payload-bioconjugation modifications may have safety advantages comparing to brentuximab vedotin, without retro-Michael instability of maleimide. This ADC is currently evaluated in an ongoing clinical trial of patients with recurrent or refractory CD30+ hematological malignancies (NCT03894150).

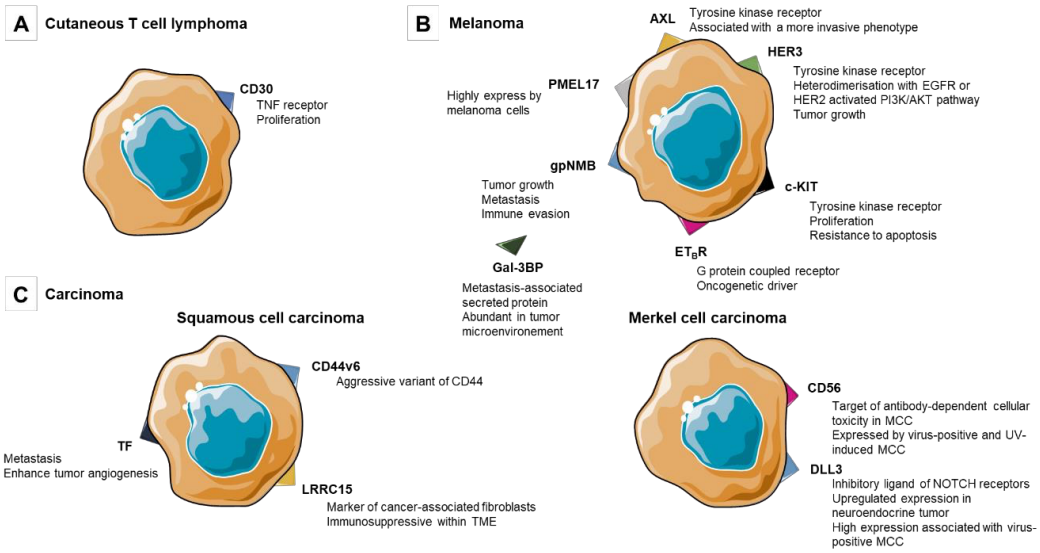

Figure 3. ADC targets in oncodermatologic indications and implication of antigen expression in cancer aggressiveness. (**A**) Cutaneous T-cell lymphoma. (**B**) Melanoma. (**C**) Carcinoma: squamous cell carcinoma and Merkel cell carcinoma.

Table 2. Preclinical and clinical trials of ADCs in cutaneous lymphoma.

Commercial Name	Target	Percentage of Positivity	Expression in Healthy Tissues	Antibody	Antibody Isotype	Bioconjugation Head (Antibody Amino Acid)	Linker	Drug	Phase
Brentuximab vedotin	CD30	75%	Activated T, B and NK cells	Brentuximab	Chimeric IgG1	Maleimidocaproyl (Cysteine)	Cleavable/ proteolytic (cathepsin B)	MMAE	Approved
F0002ADC	CD30	75%	Activated T, B and NK cells	Brentuximab	Chimeric IgG1	Ester NHS (Lysine)	Uncleavable	DM1	Phase I
N/A	Inducible co-stimulator	MF: 61% (n = 23), SS: 88% (n = 17)	Lymph node, kidney, liver	Mogamulizumab	Murine monoclonal antibody	N/A	N/A	MMAE	Preclinical
N/A	Cell surface heat shock protein 70	N/A	N/A	239-87	N/A	N/A	N/A	MMAE	Preclinical

Unprovided data: N/A.

Table 3. Results of brentuximab vedotin clinical trials in cutaneous lymphoma.

Study	Phase	Patient Number	Response Rate	Survival
Duvic et al.	II	48 (28 with MF, 9 with LyP, 2 with pcALCL)	ORR 73% ORR 54% in MF group ORR 100% in other subgroups CR 2/28, PR 13/28 in MF subgroup	PFS 1.1 years (95% CI 0.9−1.4)
Kim et al.	II	32 (MF and SS)	ORR in 21/30 (70%, 90% CI 53−83) CR in 1/30 PR in 20/30SD in 4/30	Median PFS not reached at 12 months Median EFS > 6 months 61% event free at 6 months 28% event free at 12 months
Prince et al.	III Comparison of BV with physician's choice of either bexarotene or methotrexate	123 (97 with MF, 31 with pcALCL) with 64 in BV group, 64 in PC group	ORR 56.3% (BV group) versus 12.5% (PC group), with $p < 0.0001$ ORR 67% in BV group CR 16% ORR 20% in PC group CR 2%	Median PFS 16.7 months (BV group) versus 3.5 months (PC group), with $p < 0.0001$

Objective response rate: ORR; mycosis fungoides: MF; Sézary syndrome: SS; complete response: CR; partial response: PR; lymphomatoid papulosis: LyP; primary cutaneous anaplastic large cell lymphoma: pcALCL; progression-free survival: PFS; brentuximab vedotin: BV; physician's choice: PC; event-free survival: EVS.

Another approach includes the combination of brentuximab vedotin with other therapeutics in order to improve efficacy and/or circumvent resistance. For instance, combination with the PD-1 blocking antibody nivolumab is currently being investigated in two phase I/II trials [59] (NCT02581631 and NCT01703949). Similarly, a phase I study is currently investigating the association between brentuximab vedotin and romidepsin, a histone deacetylase inhibitor which is FDA approved as a monotherapy for CTCL patients (NCT02616965).

Since patients treated with brentuximab vedotin do not achieve improvements in long-term outcomes, new CTCL targets were identified and new ADCs were developed. Inducible Co-Stimulator (ICOS) is a T-cell costimulatory receptor involved in the development of CTCLs. High ICOS expression in CTCLs suggested it as a suitable target to develop an anti-ICOS MMAE-conjugated ADC [60]. Interestingly, a comparison between brentuximab vedotin and an anti-ICOS MMAE-conjugated ADC in a preclinical model of a CTCL xenograft showed a longer overall survival of mice [60]. Recently, cell surface proteome analysis on CTCL cell lines identified cell surface heat shock protein 70 (scHSP70) as highly expressed compared with normal T cells [61]. Then, an anti-scHSP70 MMAE-conjugated ADC was developed, and in vitro comparison with brentuximab vedotin showed similar activity against MS and SS cell lines [61]. However, under hypoxic conditions, such as a tumor microenvironment, scHSP70 expression was higher and cells were obviously more sensitive to the ADC—this result suggests an advantage over brentuximab vedotin [61].

4. ADCs in Melanoma

While BRAF/MEK inhibitors or immune checkpoint inhibitors (ICI) have revolutionized the treatment of patients with metastatic melanoma, approximately 50% of patients still experience fatal outcomes. Although 70% of patients with metastatic melanoma respond upon treatment with a combination of BRAF and MEK inhibitors [62], acquired resistance is frequent, especially in those with high tumoral burden at baseline. Acquired resistance to MAPK pathway inhibition is frequently associated with reactivation of the MAPK pathway due to secondary genetic (NRAS mutation, BRAF amplification, and MEK mutations) or epigenetic changes (Akt amplification, loss of PTEN, amplification of HGF, RTK (receptor tyrosine kinase), PDGFRβ, and IGF 1R) [3]. Metastatic melanoma patients can be treated with ICI such as the anti-PD1 antibodies pembrolizumab or nivolumab as monotherapies [63] or nivolumab combined with ipilimumab, an antibody targeting CTLA-4 on T cells [63]. Overall, primary resistance occurs in 40 to 65% of metastatic melanoma cases either treated

with targeted therapies, i.e., BRAF/MEK inhibitors or ICI [64], highlighting the need for new therapeutic options [65]. In this context, several ADC strategies targeting tumor cell membrane proteins, mostly tyrosine kinase receptors, or soluble proteins, have been developed in recent years (Table 4). ADCs developed against melanoma are presented in three parts depending on their target types (Figure 3B).

Table 4. Preclinical and clinical trials of ADCs in melanoma.

Commercial Name	Target	Percentage of Positivity	Expression in Healthy Tissues	Antibody	Antibody Isotype	Bioconjugation Head (Antibody Amino Acid)	Linker	Drug	Phase
Glembatumumab vedotin	gpNMB	86% (n = 21)	Skin, bones	Glembatumumab	Human IgG2	Maleimidocaproyl (Cysteine)	Cleavable/ proteolytic	MMAE	Phase I/II
N/A	PMEL17	64% (n = 58)	Melanocytes	17A9	Mouse N/A	N/A	Cleavable/ proteolytic	MMAE	Preclinical
EV20/MMAF ADC	HER3	65% (n = 130)	Liver, pancreas, epithelial cells	EV20	Humanized IgG1	Maleimidocaproyl (Cysteine)	Uncleavable	MMAF	Preclinical
DEDN6526A	ET_BR	Majority of tumors (% N/A)	Liver, cortex, medulla	MEDN6000A	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable/ proteolytic	MMAE	Phase I
LOP628	c-KIT	66% to 88%	Skin epithelial cells, breast, neurons	LMJ	Humanized IgG1	Ester NHS (Lysine)	Uncleavable	DM1	Phase I (stopped)
Enapotamab vedotin	AXL	N/A	Muscle, testis	Enapotamab	Human IgG1	Maleimidocaproyl (Cysteine)	Cleavable/ proteolytic	MMAE	Phase I/II

Unprovided data: N/A. gpNMB: glycoprotein-NMB; PMEL17: premelanosome protein 17; HER3: human epidermal growth factor receptor 3; ET_BR: endothelin B receptor; c-KIT: tyrosine-protein kinase; AXL: Anexelekto.

4.1. Membrane Protein as Targets

Glycoprotein-NMB (gpNMB) is a transmembrane glycoprotein involved in tumor growth, tumor invasion and metastasis. Moreover, gpNMB exerts a direct inhibitory effect on activated T cells, impairing antitumor immunity and allowing immune evasion [66]. Accordingly, high gpNMB tumor expression levels are associated with poor clinical outcome [66]. In melanoma, 87% of tumors demonstrate membrane expression of gpNMB [67,68]. By contrast, in normal cells, gpNMB is restricted to intracellular compartments, therefore suggesting gpNMB as a promising ADC target in melanoma [66]. Accordingly, glembatumumab vedotin, a gpNMB-targeted MMAE-conjugated ADC [69], resulted in a 39% ORR in a phase I/II study conducted among patients with advanced melanoma refractory to ICI and BRAF/MEK inhibition, with acceptable toxicity (NCT00412828) [70]. In line with the observation that gpNMB expression is induced by the inhibition of MAPK pathway [71], combination of glembatumumab vedotin with MAPK pathway inhibitors demonstrated a synergistic therapeutic effect in a melanoma mouse model [71].

High and homogeneous expression levels of HER3, a tyrosine kinase receptor, have been observed in 65% of cutaneous melanomas [72]. HER3 is a member of the HER/EGFR family, and heterodimerization of HER3 with other HER family members (e.g., EGFR or HER2) leads to the activation of the PI3K/AKT pathway [73]. Accordingly, HER3 expression promotes tumor growth, and its overexpression was associated with impaired survival in melanoma [8,73]. Moreover, BRAF/MEK inhibitors induce FOXD3 expression, a transcription factor triggering HER3 expression. Hence, upregulation of HER3 by FOXD3 can promote adaptive resistance to BRAF inhibitors [74]. As a consequence, co-targeting HER3 and BRAF/MEK appears as an interesting option to overcome therapeutic resistances in BRAF-mutated melanoma [74]. However, unconjugated anti-HER3 antibodies have only shown limited antitumor activity justifying the use of anti-HER3 ADCs [75]. Indeed, an anti-HER3 MMAF-conjugate exhibited complete and durable antitumor responses in xenograft mice models [76]. Another anti-HER3 topoisomerase I inhibitor-conjugated ADC [77] is evaluated as monotherapy in phase I/II clinical trials for melanoma (NCT02980341).

Finally, Chen and collaborators identified the melanosomal protein, PMEL17, as a highly expressed marker restricted to melanoma cells. Accordingly, they generated an anti-PMEL17 MMAE-conjugated ADC which was cytotoxic in vitro and in vivo in a preclinical model [78].

4.2. Tyrosine Kinase Receptor

Endothelin B receptor (ET_BR) is a member of the G protein-coupled receptor superfamily mediating tissue differentiation, growth, and repair, through the MAPK pathway [79]. ET_BR has been reported to be overexpressed in metastatic melanoma compared to normal melanocytes [80]. ET_BR signaling has been implicated in malignant transformation of melanocytes, suggesting it as an oncogenic driver for melanoma development [81]. Importantly, ET_BR blockade results in inhibition of melanoma growth in vitro and in vivo [81], rendering ET_BR as a possible target for an ADC [82]. Accordingly, an anti-ET_BR MMAE-conjugated ADC is currently evaluated in a phase I clinical trial in patients with metastatic or unresectable melanoma (n = 53) (NCT01522664) [83]. Upon treatment, 32% of patients had stable disease beyond 6 months, irrespective of BRAF status [83]. Interestingly, in a preclinical model, combination of a single dose of ADC and daily intake of MAPK pathway inhibitors increased expression of ET_BR and enhanced the therapeutic response [84].

Anexelekto (AXL) is a tyrosine kinase receptor frequently expressed in melanoma [85]. Its tumoral expression has been associated with a more invasive phenotype [85]. Antitumor activity of an anti-AXL MMAE-conjugated ADC was observed in a preclinical model, and again combination with BRAF/MEK inhibitors improved antitumoral cytotoxicity [86]. Importantly, a BRAF/MEK inhibitor-resistant but high-AXL-expressing tumor sub-population was killed by the anti-AXL ADC, while BRAF/MEK inhibition was specifically cytotoxic for the low-AXL-expressing tumor cells [86]. Following this promising preclinical study, a phase I/II clinical trial is ongoing to evaluate this ADC (called enapotamab vedotin) in solid tumors including melanoma (NCT02988817). Moreover, AXL could also be an interesting target for squamous cell carcinoma [87].

c-KIT is a tyrosine kinase receptor engaged by a ligand called stem cell factor. c-KIT is involved in regulation of apoptosis, differentiation and proliferation [88]. In cancer, c-KIT mutation or gene amplification results in uncontrolled proliferation and resistance to apoptosis [88]. High c-KIT expression is observed in 64 to 88% of melanomas [88]. So far, c-KIT-targeted tyrosine kinase inhibitors only showed limited clinical benefit in *Kit*-mutated metastatic melanoma, due to the emergence of secondary mutations [89]. Preclinical studies revealed a potent antitumor activity of an anti-c-KIT DM1-conjugated ADC on several c-KIT-positive solid cancers including melanoma and leukemia [90]. Nevertheless, in a phase I clinical trial (NCT02221505), acute hypersensitivity reactions triggered by degranulation of c-KIT-expressing mast cells led to the termination of the trial [90].

4.3. Soluble Target for ADCs

Non-internalizing ADCs are also investigated for melanoma treatment using an ADC targeting the galectin-3-binding protein (Gal-3BP), a metastasis-associated secreted protein [91] which is abundant in the TME [92]. Following treatment with anti-Gal-3BP ADC, complete tumor regression was achieved in a xenograft mouse melanoma model [91].

5. ADCs in Skin Carcinomas

ADC strategies have also been developed for treating skin carcinomas which are prone to display an aggressive course, mostly squamous cell carcinoma (SCC) and Merkel cell carcinoma (MCC).

5.1. Squamous Cell Carcinoma

SCC is a common skin cancer frequently induced by cumulative exposure to ultraviolet radiation [93]. SCC prognosis is mostly favorable after complete tumor resection while poor outcome is observed at the metastatic stage, with no demonstrated benefit of conventional chemotherapies or EGFR inhibitors on patient survival [93]. Recently, one PD-1 inhibitor, cemiplimab, was shown to provide a 47% ORR in patients with advanced or metastatic cutaneous SCCs, with durable responses in 61% of them [94,95]. PD-1 inhibitors are currently recommended as first-line treatment of advanced SCCs which are not candidates for surgery or radiation therapy [96].

Several targets have been identified as candidates for ADC therapy in lung or cervical SCCs [97] (Figure 3C). Most of the studies evaluated ADCs in head and neck SCCs. Overexpression of tissue factor (TF)—a transmembrane protein activating pro-survival pathways [98]—has been associated with high metastatic potential and poor outcome in various solid tumors [99]. High TF expression levels are also associated with increased expression of VEGF, enhancing tumor angiogenesis [98]. Tisotumab vedotin, a MMAE-conjugated anti-TF ADC with a protease-cleavable linker ((A) in Table 5), demonstrated a 16% ORR (95% CI 10.2−22.5) in a phase I/II study with 147 patients with multiple solid tumors, including SCCs of the head and neck. The safety profile appeared manageable although 27% of patients had a treatment-emergent serious adverse event related to the drug, and one death from pneumonia possibly related to the treatment [100]. A phase II study is ongoing to determine the efficacy and safety of tisotumab vedotin after failure of first-line standard of care therapy, in solid tumors including head and neck SCCs (NCT03485209).

Table 5. Preclinical and clinical trials of ADCs in carcinoma. A. Squamous cell carcinoma. B. Merkel cell carcinoma.

Squamous Cell Carcinoma									
Commercial Name	**Target**	**Percentage of Positivity**	**Expression in Healthy Tissues**	**Antibody**	**Antibody Isotype**	**Bioconjugation Head (Antibody Amino Acid)**	**Linker**	**Drug**	**Phase**
Tisotumab vedotin	TF	75% (n = 20)	Brain, heart, intestine, kidney, lung, placenta, uterus	Tisotumab	Human IgG1	Maleimidocaproyl (Cysteine)	Cleavable/ proteolytic	MMAE	Phase I/II
Bivatuzumab mertansine	CD44v6	100% (n = 5)	Skin keratinocytes, cervix, cornea, tonsil	Bivatuzumab (or BIWA 4)	Humanized IgG1	Disulfide linker SPP (Lysine)	Cleavable/ hydrazone	DM1	Phase I (stopped)
Samrotamab vedotin (ABBV-085)	LRRC15	64% (n = 115)	Hair follicles, tonsil, stomach, spleen, osteoblasts	Ab1	Humanized IgG1	Maleimidocaproyl (Cysteine)	Cleavable/ proteolytic	MMAE	Phase I
Merkel Cell Carcinoma									
Commercial Name	**Target**	**Percentage of Positivity**	**Expression in Healthy Tissues**	**Antibody**	**Antibody Isotype**	**Bioconjugation Head (Antibody Amino Acid)**	**Linker**	**Drug**	**Phase**
Lorvotuzumab mertansine (IMGN901)	CD56	88% (n = 64)	NK cells, neuroendocrine cells, neurons	Lorvotuzumab (huN901)	Humanized IgG1	Disulfide linker SPP (Lysine)	Cleavable/ hydrazone	DM1	Phase I
Adcitmer®	CD56	66% (n = 90)	NK cells, neuroendocrine cells, neurons	m906	Human IgG1	Maleimidocaproyl (Cysteine)	Cleavable proteolytic (cathepsin B)	MMAE	Preclincal

TF: tissue factor; LRRC15: leucine-rich repeat containing 15.

Leucine-rich repeat containing 15 (LRRC15) is a marker of cancer-associated fibroblasts [101]. TGF-β, an immunosuppressive cytokine frequently expressed by tumor cells, induces LRRC15 expression in activated fibroblasts in the TME [102,103]. Targeting cancer-associated fibroblasts through LRRC15 in order to reduce immunosuppressive properties of the TME could overcome therapeutic resistances [104]. In line with the frequent LRRC15 expression in SCC cancer-associated fibroblasts (81% of cases) [101], an anti-LRRC15 MMAE-conjugated ADC, ABBV-085, triggered complete response in SCC xenograft (PDX) models [101]. Moreover, combination of this drug with an anti-PD1 antibody (mouse IgG2a, 17D2 clone) has shown potent activity in tumor models [101]. This ADC is currently being investigated in a phase I clinical trial including head and neck SCCs (NCT02001623) (Table 5A). Of note, LRRC15 is also expressed by melanoma tumor cells, which could constitute an additional application field for this ADC [101].

The major issue of ADCs targeting a molecule expressed by healthy tissues is illustrated by the outcome of the program development of bivatuzumab mertansine (Table 5A). This anti-CD44v6 DM1-conjugated ADC, CD44v6 being an aggressive variant of CD44, was assessed in four clinical trials including one enrolling head and neck SCC patients. After occurrence of a case of fatal toxic epidermal necrolysis together with other skin-related adverse events, clinical development of the ADC was discontinued [105]. This severe adverse event probably occurred due to CD44v6 expression by both SCC and normal squamous epithelium [105].

5.2. Merkel Cell Carcinoma

Merkel cell carcinoma (MCC) is an aggressive skin cancer induced either by UV light or the Merkel cell polyomavirus [106,107]. Until 2017, only cytotoxic chemotherapies, mostly platin salts and etoposide, were available for patients with advanced disease. In recent years, avelumab, an anti PD-L1 antibody, was shown to provide a 33% ORR in patients with metastatic disease, after failure of a first-line chemotherapy—most of these responses being durable. Avelumab was approved by the FDA and the EMA for treating patients with advanced metastatic MCCs [108]. Pembrolizumab was assessed in the first-line setting and was approved by the FDA in 2018 for treating advanced MCCs [109,110]. Although responses to ICI may be long-lasting, more than 50% of patients do not respond or develop secondary resistance [108].

CD56 has recently been suggested as a suitable target for antibody-dependent cellular cytotoxicity in MCCs, as it is expressed on the tumor cell surface by the majority of MCCs [111] (Figure 3C). Accordingly, an anti-CD56 DM1-conjugated ADC, lorvotuzumab mertansine (LM, also known as IMGN901), has been developed by covalently coupling the DM1 to the humanized anti-CD56 mAb, lorvotuzumab (huN901) (Table 5B). In a phase I clinical trial, this ADC demonstrated acceptable safety and tolerability in different CD56-expressing tumors [112,113]. Moreover, signs of clinical activity were especially detected in the four MCC-tested patients with two objective responses [112]. The following phase I/II clinical trial investigated the combination of carboplatine/etoposide/LM versus carboplatine/etoposide alone in patients with small-cell lung cancer [114]. This trial was first modified by a reduction in the dose of carboplatine/etoposide and finally discontinued because of safety concerns (NCT01237678) [114].

Recently, a new CD56-targeting MMAE-conjugated ADC, Adcitmer®, using a new bioconjugation approach has shown the control of MCC tumor growth in a mouse preclinical model [115].

Delta-like protein 3 (DLL3) is an inhibitory ligand of NOTCH receptors and is involved in neurogenesis during early embryonic development. DLL3 is upregulated in neuroendocrine tumor and has a minimal expression in normal tissues [116]. DLL3 expression was found in 91% of MCC patients [117] and high expression of DLL3 was associated with virus-positive MCCs [118]. Rovalpituzumab tesirine, an anti-DLL3 PBD-conjugated ADC [118], showed modest antitumor activity in patients with small-cell lung carcinoma treated in third lines [119] (NCT02674568), but such anti-DLL3 ADCs could represent a potential strategy for treating viro-positive MCCs.

6. Strengths and Weaknesses of ADCs: Challenges and Perspectives

When compared to other cancer therapies, notably conventional cytotoxic treatment, the use of ADCs is a vectorized therapy with low off-target toxicity. Nevertheless, ADCs could also have some drawbacks, due to recognition of the target on non-tumor cells, leading to "on-target off-tumor cytotoxicity", limiting the therapeutic window in which ADCs can be applied [14]. Moreover, unspecific linker cleavage can cause drug delivery without antibody recognition, resulting in systemic toxicity, which represents another important limitation of ADCs [120]. Improvements in the production process as well as modifications in ADC structures may counteract these constraints [14]. In order to obtain stable ADCs, engineering of the bioconjugation head might contribute to linker stability improvement [9]. As an example, maleimide residue either included in the linker or the bioconjugation head might react with free thiol of plasma, e.g., albumin, resulting in the so called "retro-Michael reaction". Hence, this retro-Michael reaction induces an unspecific release of the linker and drug in the plasma, which not only impacts the efficacy of the ADCs but also leads to off-target toxicity [120].

Several resistance mechanisms to ADCs have been described such as target antigen downregulation or mutations, and impairment of lysosomal degradation pathways. Moreover, ADC payloads can be rejected from tumor cells though multidrug resistance transporter efflux [121]. For instance, loss of CD30 expression can be observed in cutaneous

CD30-positive lymphoid neoplasm treated with brentuximab vedotin [58,122]. Trogocytosis, i.e., the extraction of the antibody–epitope complexes by monocytes may induce resistance to the ADC in CD30-positive lymphoma [122].

A wide number of ADC optimizations have been developed to improve ADC performance. A new class of therapy aiming to improve the tumor specificity of mAbs, named probodies, consists in the optimization of mAbs used for ADC technology [123]. These antibodies are modified in order to mask the Fab paratopes, thereby limiting their activity in healthy tissue. By contrast, in the TME, high protease activity removes the masking peptide, allowing paratope–epitope recognition. Enhancement of the therapeutic index by such probody-based therapy was demonstrated in an EGFR-overexpressing mouse model [123]. The use of probodies in ADCs to enhance tumor cell-specific delivery of drugs has recently been tested [124]. In this respect, an anti-CD166 probody–drug conjugate has shown on-target on-tumor specificity in a preclinical study on lung cancer [125].

ADC antitumoral effect could be optimized by the use of innovative payloads or a new bioconjugation strategy. As an example, trastuzumab emtansine is indicated as second-line treatment for HER2-positive metastatic breast cancer. Interestingly, a second anti-HER2 ADC, trastuzumab deruxtecan [126], with eight topoisomerase inhibitors conjugated to trastuzumab through a cleavable linker was recently approved by the FDA and the EMA—this ADC has shown higher efficacy than trastuzumab emtansine, demonstrating the involvement of a payload and bioconjugation strategy in ADC performance [126].

Among the challenges, ADC toxicity (i.e., thrombocytopenia, neutropenia or peripheral neuropathy) should be known and controlled, for example by dose modulation [127]. Moreover, unexpected combinatorial effects, in particular atypical ocular toxicities of bleomycin and brentuximab vedotin observed for Hodgkin's lymphoma, should be considered for future combination strategies [127].

7. Conclusions

Several targets in skin cancers are currently being investigated as candidates for ADC therapy, which allows the targeted delivery of a drug. ADC is a growing class of therapeutics in oncology, including one ADC approved for cutaneous T-cell lymphoma. In preclinical and clinical trials, further ADCs are currently evaluated for the treatment of CTCLs, melanoma, SCCs or MCCs. Various combinations of ADCs with other therapeutics are investigated to overcome limitations of these drugs due to intrinsic or acquired resistance. Increased knowledge on critical ADC features, such as target, antibody, linker and drug choice, allows a steady improvement in ADC design and better management of side effects.

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Abbreviations

ADC	antibody–drug conjugate
CTCL	cutaneous T-cell lymphoma
DAR	drug to antibody ratio
ET_BR	endothelin B receptor
FcRn	neonatal Fc receptor
FDA	food and drug administration
Gal-3BP	galectin-3-binding protein
gpNMB	glycoprotein-NMB
ICI	immune checkpoint inhibitors
LRRC15	leucine-rich repeat containing 15 mAb: monoclonal antibody
MCC	Merkel cell carcinoma
MF	mycosis fungoides
MMAE	monomethyl auristatin E
MMAF	monomethyl auristatin F
PBD	pyrrolobenzodiazepines
PDX	patient-derived xenograft
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma
SS	Sézary syndrome
TF	tissue factor
TME	tumor microenvironment

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Clara ESNAULT

Ciblage du CD56 comme option thérapeutique dans le carcinome à cellules de Merkel

Résumé :

Le carcinome à cellules de Merkel (CCM) est un cancer de la peau agressif. Bien que les inhibiteurs de point de contrôle immunitaire constituent une avancée majeure pour le traitement des patients avec un CCM métastatique, il reste nécessaire de développer de nouvelles options thérapeutiques pour les patients ne répondant pas à ces thérapies.

Le CD56 est un antigène membranaire présent à la surface des cellules tumorales du CCM et a été identifié comme une potentielle cible thérapeutique dans plusieurs cancers solides et hémopathies. Dans ce contexte, l'objectif de notre étude était de produire et d'évaluer les performances de l'Adcitmer®, un anticorps thérapeutique ciblant le CD56 bioconjugué à une molécule cytotoxique dans un modèle préclinique de CCM.

Pour ce faire, après avoir confirmé par immunohistochimie sur tissue microarray, l'expression fréquente du CD56 sur des tumeurs CCM issues de patients (66% de tumeurs positives), et démontré la fixation spécifique et l'internalisation du m906, un anticorps thérapeutique ciblant le CD56 par les lignées de CCM, l'Adcitmer® a été généré en couplant le m906 à une molécule cytotoxique (monomethyl auristatine E : MMAE) par le procédé de bioconjugaison innovant McSAF Inside®. L'homogénéité de l'Adcitmer® a été confirmée par chromatographie d'interaction hydrophobe. Les propriétés cytotoxiques de l'Adcitmer® médiées par l'expression du CD56 ont été démontrées *in vitro* sur des lignées de CCM. De plus, un effet bystander a pu être mis en évidence dans ce modèle. L'étude de l'interaction de l'Adcitmer® avec les cellules immunitaires *in vitro* a démontré que la non-reconnaissance de la partie Fc de l'anticorps par les effecteurs immuns, induite par la déglycosylation de l'anticorps, réduit la toxicité et améliore les performances de la molécule. Enfin l'Adcitmer® déglycosylé réduit significativement la croissance tumorale dans un modèle murin de CCM.

Notre étude suggère que l'Adcitmer® pourrait représenter une option thérapeutique chez les patients avec un CCM, utilisable en alternative ou en combinaison avec les inhibiteurs de point de contrôle immunitaire.

Mots clés : carcinome à cellules de Merkel, CD56, *antibody-drug conjugate*, immunothérapie

Summary:

Merkel cell carcinoma (MCC) is an aggressive skin cancer. While immune checkpoint inhibitors constitute a major advance in the treatment of patients with metastatic MCC, new therapeutic options are still urgently required.

CD56 is a membrane antigen expressed by MCC tumor cells and has been reported as an interesting target in solid tumors and hematological malignancies. In this context, our study objective was to evaluate the performances of Adcitmer®, a therapeutic antibody targeting CD56 conjugated to a cytotoxic molecule on a preclinical model of MCC.

To this end, after confirmation by immunohistochemistry on a tissue microarray of the frequent expression of CD56 by MCC tumors (66% of positives tumors) and demonstration of the specific fixation and internalization of m906 - a therapeutic antibody targeting CD56 by MCC cell lines -, Adcitmer® was generated by conjugating m906 to a cytotoxic molecule (monomethyl auristatin E: MMAE) through an innovative bioconjugation process, McSAF Inside®. The high homogeneity of Adcitmer® was confirmed by hydrophobic interaction chromatography. The CD56-mediated cytotoxicity of Adcitmer® was demonstrated *in vitro* in MCC cell lines. Moreover, a bystander effect was shown in this model. Investigation of Adcitmer® interaction with immune cells *in vitro* demonstrated that antibody deglycosylation, resulting in lack of Fc part recognition by immune cells, reduced the toxicity and improved performances of the molecule. Finally, deglycosylated Adcitmer® significantly reduced tumor growth in a MCC mouse model.

Our study suggests that Adcitmer® should be further assessed as a therapeutic option in patients with MCC, as an alternative therapy or combined with immune checkpoint inhibitors.

Keywords: merkel cell carcinoma, CD56, antibody-drug conjugate, immunotherapy