Effet du microbiote intestinal sur la pharmaco-onco-immunologie des statines

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Effet du microbiote intestinal sur la pharmaco-onco-immunologie des statines

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Liste des abréviations

AAA	Acide Aminé Aromatique
AhR	Aryl hydrocarbon Receptor
AOM	Azoxymethane
APC	Antigen Presentating Cell (Cellule présentatrice d'antigène)
BA	Acide Biliaire
BCR	B cell Receptor
BSH	Bile Salt Hydrolase
CA	Acide Cholique
CAR	Constitutive Androstane Receptor
CD	Cluster of Differentiation
CDCA	Acide Chénodésoxycholique
CHRM2	Cholinergic muscarinic 2 receptor
CMH	Complexe Majeur d'Histocompabilité
CR	Charles River Laboratories
CSF-1	Colony-Stimulating Factor 1
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
DC	Dendritic cell (Cellule dendritique)
DCA	Acide Désoxycholique
DMBA	7,12-Dimethylbenz[a]anthracene
DSS	Dextran Sodium Sulfate
EGFR	Epidermal Growth Factor Receptor
EMA	European Medical Association
FasL	Fas Ligand
FDA	Food and Drug Administration
FFAR	Free Fatty Acid Receptor
FoxP3	Forkhead box P3
FXR	Farnesoid X Receptor
GGPP	GéranylGéranyl-PyroPhosphate
GMCSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPR	G-Protein-coupled Receptor
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitor
HLA	Human Leucocyte Antigen
HMGCoA	Hydroxyméthylglutaryl-Coenzyme A
HMGCR	Hydroxyméthylglutaryl-Coenzyme A réductase
IDO	Indoleamine 2,3-dioxygénase
IFNg	Interferon g
IFNg-R1	Interferon g Receptor 1
IL	Interleukine
IO	Intra-osseuse

IP	Intrapéritonéale
IV	Intraveineuse
JL	Janvier Labs
KO	Knock Out
LCA	Acide Lithocholique
LDL	Low Density Lipoprotein
LDL-R	Low Density Lipoprotein Receptor
LPS	Lipopolysaccharide
MCA	Acide Muricholique
MCT-1	Monocarboxylate transporter 1
MDM2	Mouse double minute 2 homolog
NF-kB	Nuclear factor-kappa B
NK	Natural Killer
ORR	Overall Response Rate
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cell (cellules mononuclées du sang périphérique)
PD-1	Programmed cell death 1
PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PXR	Pregnane X receptor
RORgt	RAR-related orphan receptor gt
S1PR2	Sphingosine-1-phosphate receptor 2
SC	Sous-cutanée
SCFA	Short Chain Fatty Acid
SMCT1	Sodium-coupled monocarboxylate transporter 1
TAM	Tumor Associated Macrophage (Macrophage associé au tumeur)
T-bet	T-box expressed in T cells
TCD4	Lymphocyte T CD4
TCD8	Lymphocyte T CD8
TCR	T Cell Receptor
TDO	Tryptophan 2,3-dioxygenase
TGFb	Transforming growth factor b
TGR5	Takeda G protein-coupled receptor 5
Th1	Lymphocyte T helper 1
Th17	Lymphocyte T helper 17
Th2	Lymphocyte T helper 2
TMAO	Trimethylamine oxide
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNFa	Tumor Necrosis Factor a
Treg	Lymphocyte T régulateur
UDCA	Acide Ursodésoxycholique
VDR	vitamin D receptor
WT	Wild Type

Introduction

I. L’immunité tumorale

A. Généralités sur le cancer

Le cancer, est l’une des maladies les plus fréquentes dans le monde mais également l’une des plus meurtrières, avec près de 18 millions de nouveaux cas et plus de 9 millions de décès en 2018, et ce malgré l’amélioration de la prévention et de la précision des thérapies[1,2].

Les causes des cancers peuvent être intrinsèques ou extrinsèques. Les causes intrinsèques sont essentiellement liées à l’hérédité. En effet, le caractère héréditaire des cancers concerne près de 10% des cancers. Les facteurs extrinsèques concernent le tabagisme, les habitudes alimentaires, l’exposition aux produits chimiques et rayonnements, provoquant des mutations[2]. Ces mutations peuvent entrainer plusieurs dérèglements à l’origine de la carcinogenèse.

Il est généralement admis que le cancer est une maladie affectant l’expression des gènes (dérives génétiques et épigénétiques) et peut être décrit comme ayant 8 grandes caractéristiques lui sont attribuées (Figure 1). Parmi ces caractéristiques, on retrouve notamment la résistance à la mort cellulaire, la prolifération anarchique et l’échappement immunitaire[3,4].

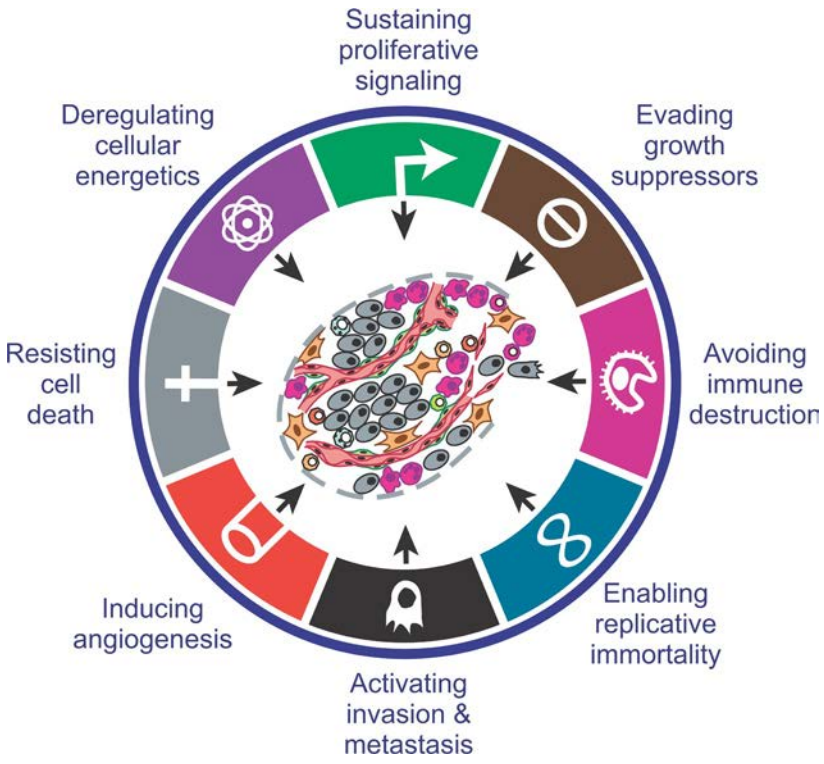

Figure 1 : Les caractéristiques du cancer, d'après Hanahan & Weinberg[2–4]

B. La progression tumorale d'un point de vue immunitaire

Depuis plusieurs décennies, l'importance du système immunitaire dans la progression tumorale est étudiée. Lorsque que le système immunitaire est déficient, l'incidence des cancers est plus grande[5,6]. Expérimentalement, les souris immunodéficientes sont plus susceptibles de développer des cancers et présentent une croissance tumorale augmentée par rapport à des souris immunocompétentes[7–10].

De plus, l'infiltration des tumeurs par le système immunitaire est également un facteur pronostique. En effet, les patients ayant des tumeurs dites « chaudes », c'est-à-dire, infiltrées par les cellules immunitaires, ont de meilleures chances de guérison et de survie que les patients ayant des tumeurs dites « froides », c'est-à-dire, non infiltrées[11,12].

Enfin, le type de cellules infiltrées dans la tumeur est également un facteur pronostique. Certains types de cellules immunitaires sont favorables à l'élimination de la tumeur et donc de bon pronostic pour le patient, d'autres au contraire sont en faveur de la progression de la tumeur et donc de mauvais pronostic pour le patient[12–15].

D'un point de vue immunitaire, le développement du cancer peut se décrire selon la règle des 3E dans lesquels les différentes populations immunitaires vont avoir une incidence soit pro-tumorale soit anti-tumorale. (Figure 2)

Les cellules « saines » sont constamment challengées par des facteurs intrinsèques et extrinsèques menant à leur dérèglement génétique et/ou fonctionnel. Ces cellules sont dites transformées. Dans la grande majorité des cas, les mécanismes de réparation et de morts programmées (apoptose, autophagie) permettent soit leur réparation ou leur destruction[3,4]. Les cellules tumorales ayant échappé à ces mécanismes peuvent être reconnues et éliminées par les cellules du système immunitaire grâce notamment à des signaux de danger par les cellules Natural Killer (NK) et par les macrophages de type M1. Les antigènes tumoraux sont captés et présentés par les cellules présentatrices d'antigènes (APC) aux lymphocytes T permettant leur activation. Les macrophages, les lymphocytes T helper 1 (Th1) et les lymphocytes T CD8 (TCD8) vont sécréter des cytokines pro-inflammatoires telles que l'IFNγ, le TNFα, la perforine et le granzyme, aboutissant à l'élimination des cellules tumorales.

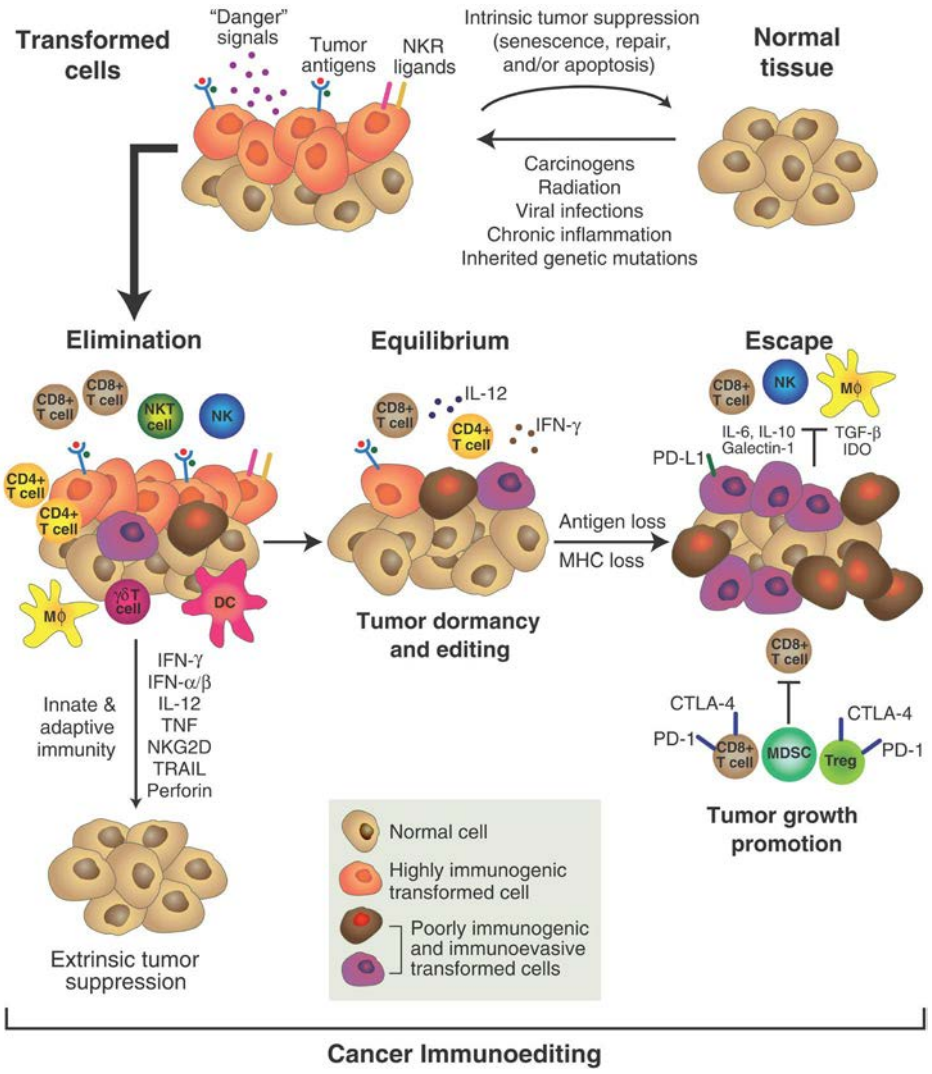

Figure 2 : L'immuno-édition du cancer[16]

Certains clones tumoraux peuvent résister à cette élimination et entrer en état de dormance. Un équilibre s'instaure alors entre la croissance tumorale et l'élimination par le système immunitaire. Une pression de sélection s'établit sur les différents clones tumoraux en faveur des clones résistants à l'élimination.

Cette émergence de clones immuno-résistants va rompre l'équilibre et conduire à l'échappement tumoral. Les cellules tumorales échappent au système immunitaire en devenant « invisibles », en n'exprimant plus d'antigènes tumoraux ou en n'apprêtant plus les antigènes à la surface des tumeurs, ou alors en créant un environnement immunitaire pro-tumoral. En effet, les cellules tumorales vont pouvoir changer le phénotype des macrophages qui deviennent des macrophages de type M2, promouvoir le recrutement et la prolifération des lymphocytes T régulateurs (Treg) et l'expression des molécules inhibitrices de checkpoints immunitaires, telles

que CTLA-4 ou PD-1. L'ensemble de ces actions va empêcher le système immunitaire d'éliminer les cellules tumorales[16].

Les cellules immunitaires participent ainsi à chaque étape de la cancérogénèse. Chacune d'entre elles sont des cibles thérapeutiques afin d'augmenter la réponse anti-tumorale ou inhiber la réponse immunosuppressive.

C. Les acteurs immunitaires dans les cancers

1. L'immunité innée

L'immunité innée constitue le premier mécanisme mis en place par le système immunitaire. Elle a pour avantage d'intervenir rapidement mais ne possède pas de mémoire immunitaire et contribue à l'établissement de l'immunité tumorale. Les acteurs cellulaires de cette immunité innée sont multiples incluant les cellules NK, les macrophages et les cellules dendritiques (DC). Ces dernières permettent de présenter les antigènes tumoraux dans le contexte tumoral aux cellules de l'immunité adaptative telles que les lymphocytes T[17].

a) Les cellules Natural Killer (NK)

Les cellules Natural Killer (NK) sont des cellules de l'immunité innée ayant des fonctions cytotoxiques. Dans le contexte tumoral, les NK peuvent ainsi contribuer à l'éradication des tumeurs. Chez la souris, les NK contrôlent la croissance des tumeurs dans plusieurs modèles tumoraux (prostate, fibrosarcome)[18–20]. Leur déplétion spécifique augmente l'incidence des tumeurs[21,22].

Chez l'Homme, les NK dans le microenvironnement tumoral sont plutôt de bon pronostic pour les patients dans les mélanomes, cancers du sein, de l'œsophage et de la tête et cou[23–26].

b) Les macrophages

Les macrophages modulent la réponse inflammatoire. Ils peuvent phagocyter les débris cellulaires et les pathogènes pour capter les antigènes et effectuer la présentation antigénique[27], ce qui fait des macrophages des APC comme les DC. Ils peuvent également éliminer les cellules mortes[28].

Ces cellules sont caractérisées par leur grande diversité, dépendant du tissu dans lequel elles résident, comme par exemple les cellules de Kupffer dans le foie, et dépendant du type d'inflammation, tel que les macrophages associés aux tumeurs (TAM) dans les cancers et les cellules spumeuses dans les lésions d'athérosclérose.

Selon les stimuli qu'ils reçoivent, les macrophages peuvent être polarisés pour avoir une réponse pro-inflammatoire (type M1) ou anti-inflammatoire (type M2)[29].

Les macrophages de type M1 répondent au lipopolysaccharide (LPS), en réponse aux produits bactériens et aux cellules endommagées, et à l'IFNγ produit par les lymphocytes Th1 (fonction détaillée dans un autre chapitre). Les macrophages de type M1 produisent et sécrètent de hauts niveaux de cytokines pro-inflammatoires telles que le TNFα, IL-1β et IL-6, permettant d'accentuer la réponse Th1[27].

Les macrophages de type M2 présentent 4 sous-ensembles répondant à des stimuli différents. De manière générale, ils sont polarisés par des cytokines anti-inflammatoires telles que l'IL-4 et l'IL-13 et sécrètent l'IL-10. Ils génèrent également l'arginase-1 déplétant la L-arginine et en conséquence perturbent la prolifération des lymphocytes T et la production d'IFNγ. L'ensemble accentue la réponse Th2[27].

Au début de la tumorigenèse, les TAM permettent les premières éliminations de cellules tumorales et relarguent les antigènes tumoraux capturés par les DC, et participent donc à l'éducation des lymphocytes T. Au cours de la tumorigenèse, ils se tournent de plus en plus vers un phénotype de type M2. Ces TAM sont aujourd'hui la cible de plusieurs stratégies anti-tumorales[30]. Leur rôle est de plus en plus décrit dans les stratégies anti-tumorales de checkpoints immunitaires[31,32].

L’ontogénie des macrophages est complexe. L’action des TAM et leur spécificité dépend du tissu dans lequel ils sont présents. En effet, selon la localisation du cancer, les macrophages peuvent être associés à un mauvais pronostic, ce qui est le cas de plus de 80% des cancers[33].

D’un point de vue expérimental, la déficience en macrophages associée à la déplétion du gène CSF-1 codant pour le GM-CSF nécessaire à la maturation des macrophages réduit la progression tumorale dans les modèles de cancers du sein et du colon[34–36]. La déplétion des macrophages par le clodronate permet également la réduction de la croissance tumorale de plusieurs types de cancers[37–40]. Bien que ces études soutiennent l’association des macrophages à un mauvais pronostic, d’autres études soutiennent l’association inverse [41].

2. L’immunité adaptative

L'immunité adaptative est une réponse immunitaire très précise et permanente jouant un rôle essentiel dans la distinction entre les antigènes du soi et du non-soi. L'immunité adaptative est principalement médiée par les lymphocytes T et B, qui reconnaissent précisément les antigènes grâce aux récepteurs spécifiques exprimés à leur surface, le récepteur des lymphocytes T (TCR) et le récepteur des lymphocytes B (BCR)[42].

Les lymphocytes B produisent des anticorps, agissent comme des APC et libèrent des cytokines. Les lymphocytes B sont classés en fonction de l'expression du marqueur de lignée cellulaire CD19, de diverses protéines de surface et intracellulaires, des BCR distincts et de leur production d'anticorps et de cytokines[42]. Bien qu’ils aient une importance dans l’immunité tumorale, leur rôle ne sera pas détaillé.

Les lymphocytes T sont classés en fonction de marqueurs membranaires et intracellulaires. Ils expriment le TCR αβ ou γδ, le CD3, et l'un des corécepteurs CD4 ou CD8. Le complexe TCR-CD3 reconnaît les antigènes présentés par une APC grâce aux molécules du complexe majeur d'histocompatibilité (CMH) ou antigène leucocytaire humain (HLA) chez l'Homme[43].

Les APC sont capables de présenter des antigènes aux lymphocytes T CD4 (TCD4) et TCD8 naïfs. Ces antigènes comprennent des antigènes du non soi et des auto-antigènes. Lors de leur activation, les TCD4 prolifèrent et se différencient en lymphocytes T helper (Th)

effecteurs spécialisés, tandis que les TCD8 activés prolifèrent et se différencient en TCD8 cytotoxiques (CTL)[43].

Les TCD4 jouent un rôle central dans l'immunité. Ils ont pour fonctions d'aider les lymphocytes B à produire des anticorps, augmenter et maintenir la réponse immunitaire des TCD8, et de réguler la réponse inflammatoire des macrophages. Ce sont également des médiateurs importants de la mémoire immunitaire[44].

Les TCD4 naïfs peuvent se différencier en divers sous-ensembles cellulaires, notamment les lymphocytes T effecteurs (T helper 1 (Th1), 2 (Th2) et 17 (Th17)) et les lymphocytes T régulateurs (Treg). La différenciation des lymphocytes T en sous-ensembles Th dépend du type d'antigène rencontré, de l'intensité du signal TCR et du milieu local des cytokines. Ces cellules se distinguent par l'expression de facteurs de transcription, de cytokines produites et des marqueurs à la surface de ces cellules[45].

Les différentes sous populations de lymphocytes T jouent un rôle dans la réponse immunitaire tumorale. Chez l'Homme, leur présence sur le pronostic dans les cancers est bien décrite (Tableau 1).

Tableau 1 : Association de lymphocytes T dans le pronostic des cancers chez l'Homme. (Liste non exhaustive)

Type de cancer	TCD8	Th1	Th2	Th17	Treg
Rein	Mauvais[46,47]	Bon[48]			Mauvais[49]
Foie	Bon[50–54]	Bon[55]		Mauvais[56]	Mauvais[54,57–60]
Poumon	Bon[61–67]	Bon[65,68]		Mauvais[69]	Mauvais[66,67,70]
Vessie	Bon[71–73]				Bon[74]
Prostate	Bon[75,76] Mauvais[77]				
Mélanome	Bon[63,78]	Bon[68]			Bon[79] Mauvais[80] Pas d'effet[81,82]
Ovaire	Bon[83–86]	Bon[87]		Bon[88]	Bon[89,90] Mauvais[91]
Sein	Bon[92–94]	Bon[95,96]	Bon[97]	Mauvais[98]	Mauvais[99,100] Pas d'effet[101]
Œsophage	Bon[102,103]	Bon[104]		Bon[105]	
Colon	Bon[15,106–108]	Bon[15,109,110]	Mauvais[15]	Mauvais[15,111]	Bon[15,112] Pas d'effet[108]
Pancréas	Bon[113–115]		Mauvais[116]		Mauvais[117]
Estomac	Bon[118,119]	Bon[120,121]	Mauvais[120]	Bon[122]	Bon[118,119] Mauvais[121]
Tête et cou	Bon[123–125] Mauvais[126]				Bon[123,126,127] Mauvais[128] Pas d'effet[125]

a) Les lymphocytes Th1

Les lymphocytes Th1 jouent un rôle essentiel dans la protection de l'organisme contre les agents pathogènes intracellulaires. Ils permettent d'augmenter la réponse pro-inflammatoire des macrophages, aident à la stimulation des TCD8 et servent également de médiateurs pour l'inflammation en produisant des cytokines spécifiques tels que l'IFNγ, l'IL-2 et le TNFα. Ils sont polarisés par l'IL-12, l'IL-18 et l'IFNγ, et sont également caractérisés par l'expression du facteur de transcription T-bet[44,45,129–131]

De par leurs fonctions immunitaires, les lymphocytes Th1 peuvent être considérés, dans l'ensemble, comme favorables à l'induction d'une réponse immunitaire anti-tumorale efficace et sont associés à un bon pronostic dans de nombreux types de cancers[13] notamment le mélanome, le cancer du sein, le cancer colorectal et le cancer des ovaires (Tableau 1).

T-bet est le facteur de transcription permettant la différenciation des lymphocytes Th1. Plusieurs études sur des souris déficientes en T-bet présentent par rapport aux souris WT une augmentation de la croissance tumorale[132] et de métastases[133,134] associée notamment avec une diminution des TCD8 produisant l'IFNγ[135]. De plus, une restauration de l'expression de T-bet dans ces souris permet une augmentation d'IFNγ et une baisse de la croissance tumorale et des métastases[134].

Par ailleurs, les lymphocytes Th1 sont induits par l'IL-12 exprimé par les DC. Les souris déficientes en IL-12 présentent une plus grande susceptibilité de développer des tumeurs et une croissance tumorale accélérée[136]. Inversement, l'apport exogène d'IL-12 permet une réduction de la croissance tumorale, associée à un niveau d'IFNγ plus élevé[137,138]. Bien que les études pré-cliniques montrent un effet anti-tumoral de l'IL-12, l'apport exogène de l'IL-12 chez les patients ne semblent pas être efficace[139].

Les lymphocytes Th1 sécrètent l'IFNγ tout comme les TCD8. Les effets de l'IFNγ sur le cancer seront détaillés dans le paragraphe portant sur les TCD8.

Ainsi, les réponses liées aux lymphocytes Th1 sont principalement bénéfiques en termes de mise en place d'une réponse immunitaire anti-tumorale efficace et sont donc associées à un bon résultat pour les patients dans de nombreux types de cancer.

b) Les lymphocytes Th2

Les lymphocytes Th2 interviennent dans l'élimination de certains agents pathogènes extracellulaires et de parasites. Ils sont polarisés par l'IL-2 et l'IL-4, produisent principalement l'IL-4, l'IL-5, l'IL-10 et l'IL-13 et sont également caractérisés par l'expression du facteur de transcription GATA-3. Ces cellules ont également la capacité d'inhiber la prolifération des lymphocytes Th1[44,45,129].

Dans un contexte tumoral, l'implication des Th2 est plutôt mitigée, décrit comme étant aussi bien pro-tumorale qu'anti-tumorale, aussi bien chez la souris que chez l'Homme.

Chez la souris, des cellules tumorales à potentiel métastatique différents ont été injectées[140]. Les TCD4 issus de souris métastasées présentent une production plus importante d'IL4 que les souris non métastasées, suggérant ainsi un lien entre l'agressivité du cancer et la production d'IL-4 par les Th2[141]. De plus, l'induction des tumeurs par AOM/DSS (Azomethane / Dextran Sodium Sulfate) sur des souris déficientes pour le récepteur de l'IL-4 est moins efficace, les souris présentant des tumeurs plus petites et moins nombreuses[142]. Enfin, l'administration d'anticorps bloquant IL-4[143] ou de son récepteur[142] diminue la croissance tumorale des souris. L'ensemble de ces études soutient un effet pro-tumoral des lymphocytes Th2.

Au contraire, d'autres études soutiennent un effet anti-tumoral des lymphocytes Th2. En effet, les souris déficientes en IL-4 présentent une mortalité plus importante suite à l'induction tumorale par TNBS (2,4,6-trinitrobenzene sulfonic acid)[144]. De plus, les lymphocytes Th2 spécifiques pour l'antigène OVA éliminent les tumeurs mais de manière moins efficace que les lymphocytes Th1. Cet effet est dépendant de l'action cytolytique des TCD8[145]. Enfin, une étude plus récente a montré que le transfert adoptif de lymphocytes Th2 permet l'éradication des tumeurs injectées, dû à la sécrétion d'IL-4, d'IL-5 et indépendamment des cellules NK, des lymphocytes Th1 ou TCD8 ou de la sécrétion d'IFNγ[146].

Chez l'Homme, les lymphocytes Th2 sont décrits comme ayant des effets bénéfiques (cancer du sein) ou délétères (cancer colorectal) (Tableau 1).

c) Les lymphocytes Th17

Les lymphocytes Th17 interviennent dans l'élimination de certaines bactéries extracellulaires et de champignons. Ils sont polarisés par le TGFβ et l'IL-6, produisent principalement l'IL-17 et sont également caractérisés par l'expression du facteur de transcription RORγt[44,45,129,147].

Le rôle des lymphocytes Th17 dans les cancers est moins connu. Le pronostic sur les cancers concernant leur présence est assez mitigé aussi bien chez l'Homme que dans les modèles in vivo[12,14,148–150]. In vivo, plusieurs études soutiennent un effet pro-tumoral des Th17. La greffe de cancer pulmonaire[151] ou l'induction de cancer colorectal par AOM/DSS[152] ou cutanée DMBA (7,12-Dimethylbenz[a]anthracene)[153] sur des souris déficientes en IL-17 diminue l'incidence, le nombre, la taille et la prolifération des tumeurs. Ces effets sont reproduits par le blocage de l'IL-17 suite à l'injection d'un anticorps bloquant anti-IL-17[151] et sur des souris déficientes pour le récepteur de l'IL-17 prédisposées à développer un cancer colorectal[154] ou greffées avec un mélanome[155]. A l'opposé, d'autres études montrent un effet anti-tumoral des lymphocytes Th17 sur des souris déficientes en IL-17. En effet, la greffe de mélanome ou de cancer colorectal augmente leur croissance sur ces souris[156,157].

Chez l'Homme, les lymphocytes Th17 peuvent être de bon pronostic dans les cancers ovariens mais de mauvais pronostic dans les cancers colorectaux (Tableau 1).

d) Les lymphocytes T régulateurs (Treg)

Les Treg jouent un rôle critique dans le maintien de la tolérance immunitaire et dans la régulation de la réponse immune. Ces cellules sont polarisées par le TGFβ et l'IL-2, produisent principalement l'IL-10 et le TGFβ et sont également caractérisées par une forte expression de CD25 et par l'expression du facteur de transcription FoxP3.

Les Treg exercent leur activité immunorégulatrice et suppressive par le biais de plusieurs mécanismes. L'un des principaux mécanismes est l'inhibition par contact cellules-cellules. Les Tregs expriment des molécules de checkpoints immunitaires CTLA-4 et PD-1. CTLA-4 régule la fonction des APC et l'activation des lymphocytes T en diminuant l'expression des CD80 et

CD86 dans l'APC et en bloquant l'interaction co-stimulatrice entre CD80/CD86 dans l'APC et CD28 dans le lymphocyte T[158]. Un autre mécanisme principal est la sécrétion de cytokines anti-inflammatoires telles que l'IL-10 et le TGFβ qui vont polariser les autres cellules immunitaires (lymphocytes T, macrophages, etc.) vers un phénotype plus tolérogène[159].

Dans le contexte tumoral, les Treg constituent une population particulière des lymphocytes T et ont un rôle important dans la cancérogenèse. Ils sont présents en quantité non négligeable dans les tumeurs. Chez les patients, l'implication des Treg dans le pronostic est plutôt mitigée, bien qu'il soit généralement admis que leur présence est de mauvais pronostic. En effet, selon le type de cancers ou d'étude, les Treg peuvent avoir un effet pro-tumoral, un effet anti-tumoral ou aucun effet (Tableau 1).

D'un point de vue expérimental, les Tregs ont un effet pro-tumoral. En effet, les premières études chez la souris ont permis de montrer que la déplétion des cellules $CD25^+$ par un anticorps bloquant permet un ralentissement de la progression tumorale[160,161]. De plus, le transfert adoptif de cellules $CD25^-$ permet une diminution de la croissance tumorale et également une meilleure survie chez ces souris[161]. FoxP3 étant exprimé quasi-exclusivement dans les Treg, des études plus récentes ont été menées sur des modèles murins avec une déplétion des Treg suite au traitement par la toxine diphtérique. Dans ces modèles murins, la déplétion de Treg a permis une diminution de la progression tumorale, voir une disparition complète des tumeurs, grâce à une meilleure action des TCD8[162,163] et des NK[164].

Très peu d'études expérimentales montrent un effet anti-tumoral des Treg. Dans un modèle murin d'induction de tumeur pancréatique, la déplétion des Treg accélère la carcinogenèse[165].

Exprimant les molécules de checkpoints immunitaires tels que CTLA-4 et PD-1, les Treg sont la cible des immunothérapies récentes visant à lever l'immunosuppression dans les tumeurs. Expérimentalement, l'administration d'anticorps bloquant anti-CTLA-4, anti-PD-1 ou anti-PD-L1 permet la résection des tumeurs[166–169]. Chez les patients, le blocage de ces voies permet de meilleures réponses thérapeutiques sur plusieurs types de cancers (poumon, mélanome, etc.)[12,170–172] (Figure 3).

e) Les lymphocytes T CD8 (TCD8)

Les lymphocytes T CD8 jouent un rôle prépondérant dans la défense contre les pathogènes intracellulaires. Ils reconnaissent l'antigène via la TCR dans le contexte des molécules du CMH de classe I exprimées de manière ubiquitaire (CMH-I ; HLA-A, B ou C chez l'Homme). Lors de la reconnaissance de l'antigène, les TCD8 naïfs sont activés, prolifèrent et se différencient en CTL. Les TCD8 jouent un rôle d'effecteur par la libération de cytokines pro-inflammatoires, telles que le TNF-α, qui peut induire l'apoptose, et l'IFNγ, qui favorise la régulation positive du CMH-I et la réponse inflammatoire. Les TCD8 favorisent l'apoptose médiée par l'expression de FasL et de son récepteur Fas dans la cellule cible et la lyse de la cellule cible par la libération de granzymes et de perforine[43,173].

Les TCD8 jouent un rôle majeur dans la réponse anti-tumorale. En effet, ces cellules cytotoxiques sécrètent des cytokines telles que l'IFNγ, la perforine ou le Granzyme B permettant l'élimination de la tumeur.

Leur présence dans le microenvironnement tumoral est un bon pronostic pour les patients (Tableau 1). En clinique, le ratio TCD8/Treg est souvent utilisé afin de déterminer un bon ou mauvais pronostic immunitaire chez les patients.

D'un point de vue expérimental, la présence des TCD8 est indispensable dans le contrôle des tumeurs. En effet, la déplétion des TCD8 par un anticorps bloquant augmente la croissance des tumeurs[174–176] ou annule l'effet des thérapies telles que la radiothérapie[177], la chimiothérapie, les immunothérapies ou une combinaison de ces thérapies[178].

Les TCD8 sécrètent la perforine permettant la régression tumorale. En effet, les souris déficientes en perforine présentent une incidence de cancer plus élevée[179], une croissance tumorale augmentée[180] et une survie diminuée[181].

Les TCD8 sécrètent également le granzyme B dont le rôle dans l'immunité anti-tumorale est plutôt controversé[182].

Les TCD8 sécrètent également l'IFNγ, tout comme les macrophages et les lymphocytes Th1. Les souris déficientes pour le récepteur de l'IFNγ (IFNγ-R1) présentent une incidence plus élevée de cancer suite à l'induction au methylcholanthrene[183]. De plus, des souris déficientes pour IFNγ-R1 et développant des tumeurs colorectales spontanées présentent une survie

diminuée et une augmentation en nombre et en taille de tumeurs[184]. Enfin, la sécrétion augmentée d'IFNγ par les TCD8 dans la tumeur suite au traitement par l'anti-PD-1[185] ou combiné avec l'anti-CTLA-4[186] permet une diminution, voir un résection totale des tumeurs. Ces effets sont annulés sur des souris déficientes pour IFNγ-R1[185,186]. Chez l'Homme, l'expression de l'IFNγ-R1 par les cellules tumorales permet une meilleure survie dans les cancers colorectaux notamment[187,188].

D. Les immunothérapies anti-cancéreuses

Différentes stratégies anti-cancéreuses ont été développées. Les thérapies les plus classiques ciblent directement la tumeur : la chirurgie, la radiothérapie et la chimiothérapie. Cependant, pour beaucoup de cancers, les stratégies anti-cancéreuses classiques ne permettent pas d'éradiquer les cancers. Ce n'est que très récemment que le rôle du système immunitaire a été pris en compte dans les stratégies thérapeutiques classiques. En effet, il a été montré que les cancers dits immunogènes sont les plus sujets à répondre aux thérapies classiques.

La prise en compte du système immunitaire dans le traitement des cancers n'est pas nouvelle. Cependant, malgré le grand nombre de tentatives, peu ont été fructueuses. Ce n'est qu'à partir des années 2010 que la stratégie dite des checkpoints immunitaires s'est développée avec les anti-CTLA4, puis avec les anti-PD1. Le principe est de supprimer l'inhibition immunitaire créée par la tumeur et ainsi permettre au système immunitaire d'éliminer les cellules tumorales. Cette stratégie a permis d'obtenir des réponses thérapeutiques satisfaisantes, notamment dans les mélanomes métastatiques de phase 4 avec une survie améliorée, voir une guérison totale alors qu'aucune autre thérapie n'avait fonctionné jusqu'alors. Malgré ces avancées majeures, le taux de réponse à ces immunothérapies reste faible, faisant ainsi développer les stratégies thérapeutiques combinatoires entre les immunothérapies et les thérapies classiques[171,189,190].

Afin d'améliorer la réponse thérapeutique, plusieurs études ont mis en évidence le rôle essentiel du microbiote intestinal sur la carcinogenèse, la réponse immunitaire anti-tumorale et l'efficacité thérapeutique des immunothérapies[191].

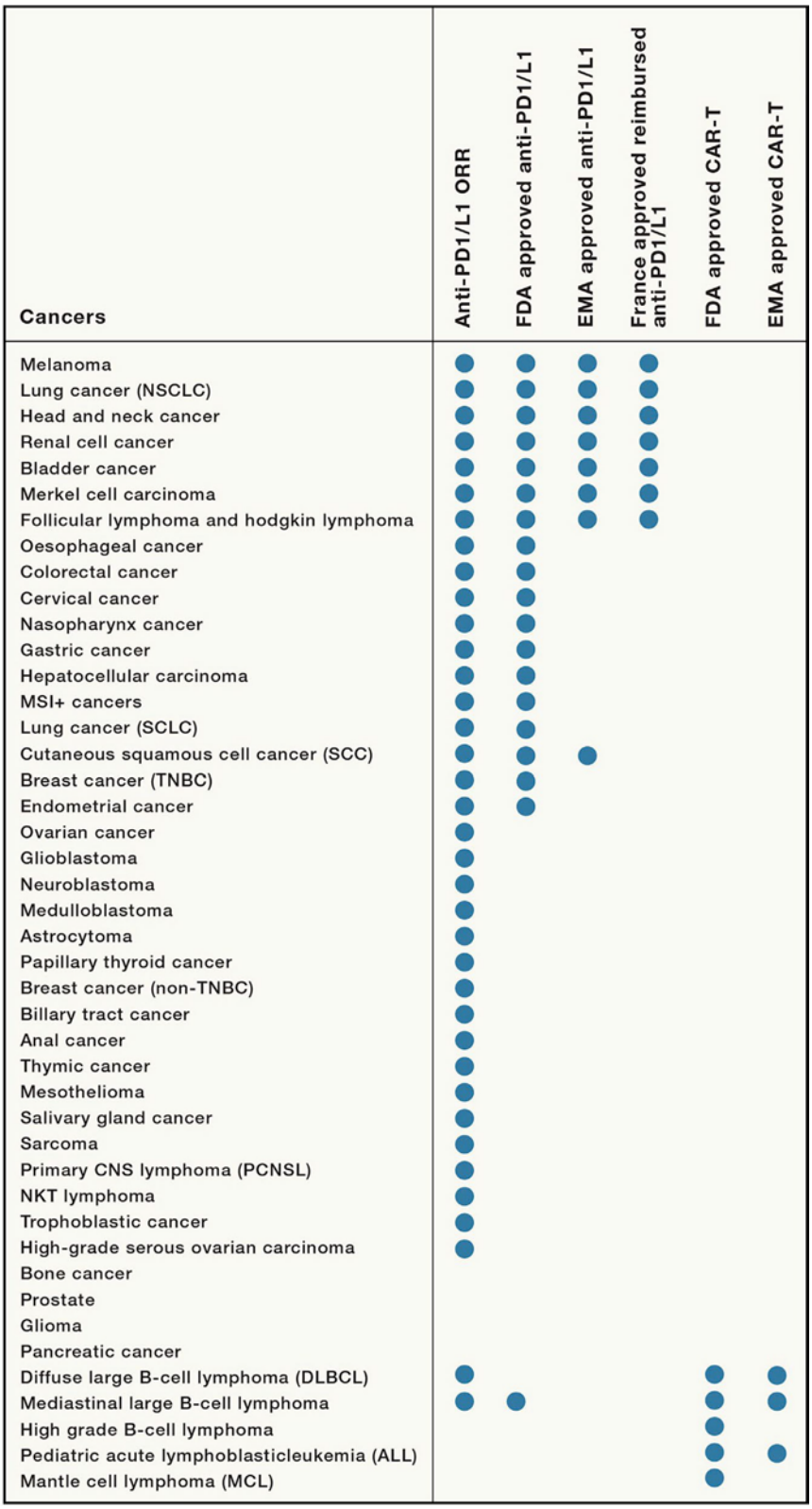

Figure 3 : Type de cancers répondant aux anti-PD1[12]. (ORR : Overall response rate, FDA : Food and Drug Administration, EMA : European medical association)

II. Le microbiote intestinal

A. Introduction

Le microbiote représente l'ensemble des communautés microbiennes vivants en symbiose avec l'hôte. Cet écosystème impacte plusieurs aspects de la physiologie de l'hôte : la capture de nutriments, le métabolisme des xénobiotiques, l'inflammation et le comportement. Il existe plusieurs types de microbiotes agissant d'abord au niveau local et puis plus largement sur tout l'organisme : le microbiote cutané, le microbiote pulmonaire, le microbiote vaginal et le microbiote intestinal[192]. Ici, nous allons nous intéresser au microbiote intestinal.

Les différentes espèces microbiennes vivent en équilibre. Leur diversité varie selon le mode de vie de l'hôte, à savoir le régime alimentaire, la prise de médicaments, le tabac, etc. La production équilibrée des métabolites microbiens est essentielle pour l'hôte et assure le maintien de l'homéostasie intestinale et de la physiologie de l'hôte[193] (Figure 4). *(voir en annexes deux revues auxquelles j'ai contribué qui montrent que les métabolites microbiens peuvent notamment réguler la cholestérolémie*[194] *(revue «Unraveling Host-Gut Microbiota Dialogue and Its Impact on Cholesterol Levels ») et une seconde revue qui évalue les perturbations de l'équilibre microbien susceptible d'apparaitre en cas de stress notamment lors des voyages dans l'espace*[195] *(revue «Gut Microbiome and Space Travelers' Health: State of the Art and Possible Pro/Prebiotic Strategies for Long-Term Space Missions»)).*

De manière intéressante, certaines pathologies peuvent se transmettre par le microbiote intestinal. Par exemple, des études chez la souris ont permis de montrer que les souris axéniques implantées avec le microbiote de souris obèses avaient une prise de poids plus importante par rapport à celles implantées avec du microbiote de souris maigres[196,197]. C'est également le cas avec le microbiote humain sur des souris axéniques[198,199].

Certaines espèces microbiennes peuvent être associées à différentes pathologies comme le cancer[200–202] ou les maladies cardiovasculaires[193,203]. Cela entraine des modifications de productions de métabolites bactériens et peut être aussi bien la cause que la conséquence de différentes maladies.

Par ailleurs, les métabolites microbiens sont également associés à des pathologies tels que le trimethylamine oxyde (TMAO) dans l'athérosclérose[194,204,205].

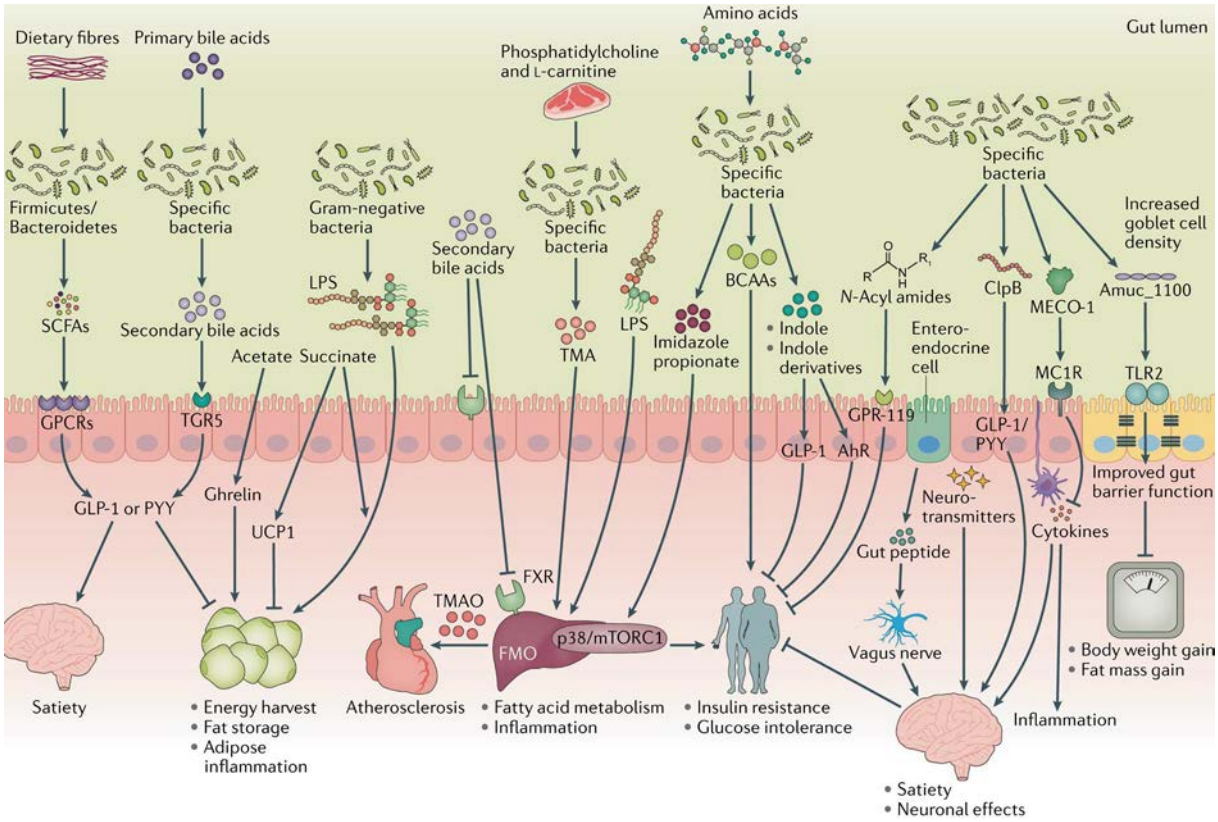

Figure 4 : Effets des métabolites microbiens sur le métabolisme de l'hôte[193]

Le microbiote intestinal module également le système immunitaire. En effet, des animaux sans microbiote présentent des déficits immunitaires importants et sont plus susceptibles aux infections[206]. Les métabolites microbiens permettent également de réguler le système immunitaire au niveau local et au niveau systémique[207,208].

Le microbiome et les métabolites microbiens font l'objet d'une particulière attention dans les cancers et les traitements antitumoraux.

B. Microbiote intestinal et cancer

Chez l'Homme, dans les cancers, il a été observé que les patients présentaient une composition microbienne différente par rapport aux personnes saines[209–213]. Plusieurs bactéries sont associées à la protection (exemple : Akkermansia muciniphila, Faecalibacterium prausnitzii) ou à une incidence (exemple : Fusobacterium nucleatum, Bacteroides fragilis) plus élevée de cancers[200,214,215].

La composition microbienne régule également la réponse immunitaire tumorale ainsi que les traitements cancéreux. Chez la souris, une étude japonaise a montré qu'un consortium de 11 bactéries permettaient d'augmenter la réponse des TCD8 par la sécrétion augmentée en IFNγ dans plusieurs tissus (rate, poumon, colon et sang). Cela était associé également à une meilleure réponse anti-tumorale. Enfin, les souris traitées avec le consortium et un anti-PD-1 ou un anti-CTLA-4 amélioraient cette réponse TCD8 IFNγ$^+$ déjà augmentée[216]. De plus, des souris génétiquement identiques mais provenant d'éleveurs différents présentent des croissances tumorales et une réponse immunitaire différentes. Cela est attribué au microbiote intestinal qui diffère selon l'élevage d'origine : la cohabitation annule les différences et la greffe fécale transfère les propriétés tumorales selon le donneur[217]. Enfin, les traitements antibiotiques ne sont pas sans conséquences sur le microbiote intestinal et dans les cancers. L'administration d'antibiotique chez la souris altère la réponse immunitaire anti-tumorale induite par les chimiothérapies[218]. Chez l'Homme, un traitement antibiotique avant l'administration d'inhibiteurs de checkpoints immunitaires chez des patients atteints de cancer pulmonaire diminue la survie des patients[219].

Par ailleurs, des microbiotes différents produisent des panels de métabolites microbiens différents. Dans les cancers, une différence de métabolites est retrouvée dans le sérum et dans les fèces de ces patients[211,220–224]. Plusieurs études montrent également que l'efficacité thérapeutique des immunothérapies telles que les anti-PD-1 peut être prédite sur la base des métabolites, notamment microbiens, retrouvés dans le sérum des patients[225].

Le microbiote intestinal ainsi que les métabolites produits ont une grande importance dans les cancers, la réponse immunitaire tumorale et les thérapies anti-cancéreuses. Ces métabolites microbiens sont principalement les Short-Chain Fatty Acids (SCFA), les acides biliaires secondaires et les dérivés du tryptophane. Ces métabolites sont retrouvés différentiellement dans les cancers chez les patients et modulent également la réponse immunitaire tumorale.

C. Short-Chain Fatty Acid (SCFA)

1. Production et métabolisme des SCFA

Les acides gras à chaines courtes ou Short-Chain Fatty Acids (SCFA) sont des molécules de chaines carbonées courtes produits à partir de fibres alimentaires échappant à la digestion par les enzymes de l'hôte dans l'intestin et métabolisés dans le caecum et le colon[226]. Les SCFA comprennent principalement l'acétate, le propionate et le butyrate avec un ratio d'environ 3/1/1. Leur rôle principal est de maintenir l'intégrité intestinale et l'homéostasie du système immunitaire intestinal. Elles sont essentiellement absorbées par les colonocytes de manière active, via les transporteurs MCT-1 et SMCT1 ou de manière passive[227]. Elles constituent leur principale source d'énergie et stimulent leur prolifération en entrant dans le cycle de Krebs[228,229]. Les SCFA non métabolisés rejoignent la circulation porte et sont utilisés pour les besoins énergétiques des hépatocytes. L'acétate et le butyrate peuvent y servir de précurseur pour la synthèse de cholestérol, le propionate pour la synthèse de glucose[194,230]. (Figure 5)

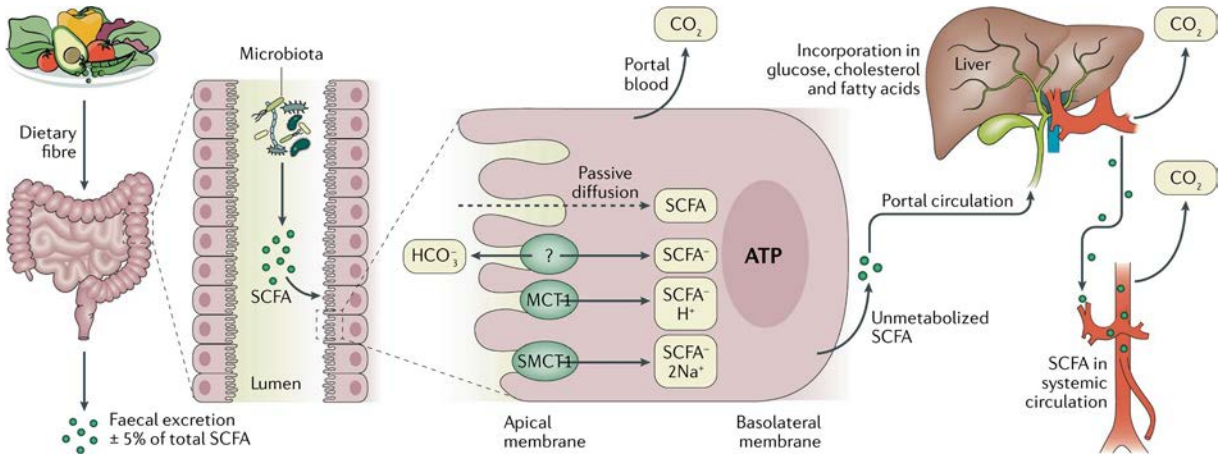

Figure 5 : Métabolisme des SCFA[231]

Seule une petite fraction des SCFA atteint la circulation sanguine pour étendre leur rayon d'action[231,232].

Les SCFA sont des ligands des récepteurs couplés aux protéines G : GPR41 (FFAR3), GRP43 (FFAR2), GRP109A et peuvent également rentrer dans la cellule par ces transporteurs ou par diffusion passive pour se lier aux histones désacétylases (HDAC) entrainant leur

inhibition. (Figure 6). Les récepteurs au SCFA sont retrouvés dans plusieurs types cellulaires, notamment immunitaires[233].

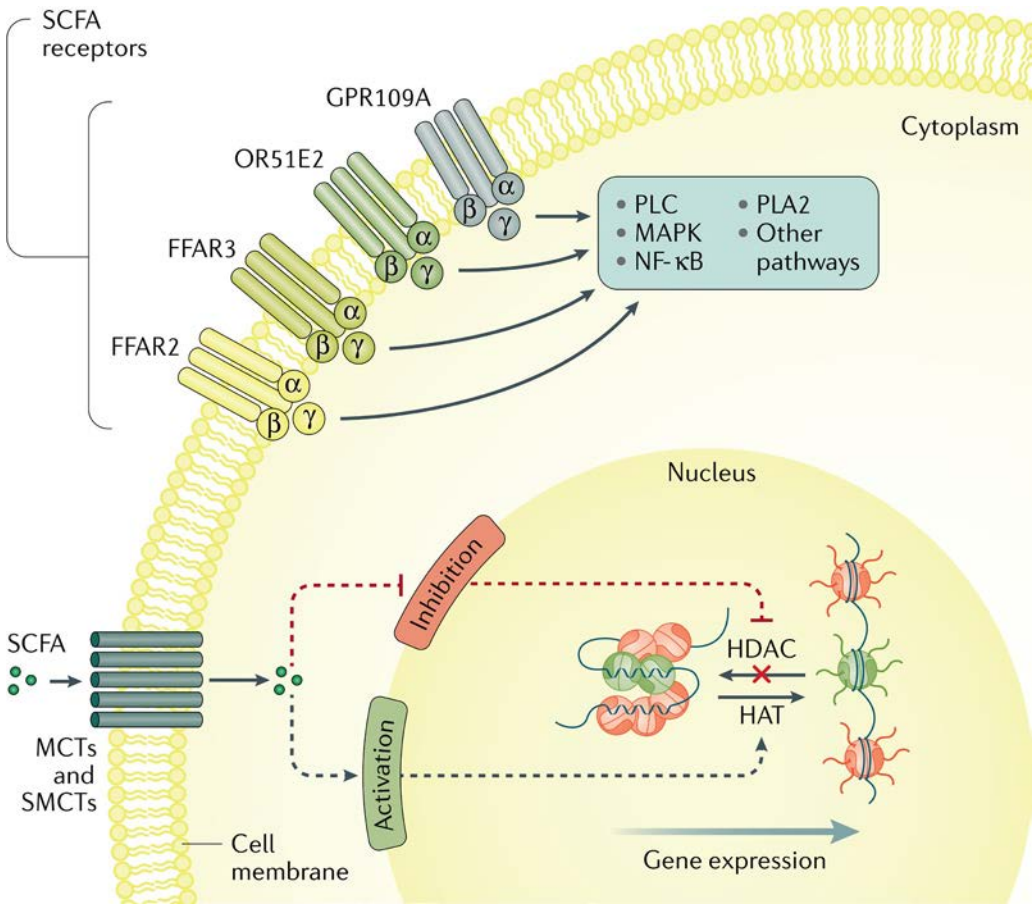

Figure 6 : Mécanismes d'actions des SCFA[231]

2. SCFA et cancer

L'effet des SCFA, surtout le butyrate, a surtout été décrit dans les cancers colorectaux.

Bien que le butyrate soit la première source d'énergie des colonocytes utilisée pour leur prolifération et leur survie[228], l'effet opposé est observé sur les cellules tumorales colorectales, à savoir des effets anti-prolifératifs et pro-apoptotiques[214,234].

Chez l'Homme, il est généralement admis que les fibres alimentaires protègent du cancer colorectal[235–237] et du cancer du poumon[238]. De manière analogue, un grand nombre d'étude montre que le niveau fécal de SCFA, particulièrement le butyrate, est abaissé mais également les bactéries qui les produisent chez les patients présentant un cancer colorectal[215,239–241].

L'expression des récepteurs des SCFA tels que le FFAR2 est réduite dans les cellules cancéreuses par rapport aux tissus sains[242]. D'autres études ne montrent en revanche pas d'association entre les niveaux de SCFA et les cancers colorectaux[243,244].

Un traitement AOM, DSS ou TNBS permet d'induire chimiquement des tumeurs intestinales et colorectales sur les modèles murins. Un régime riche en fibres permet une plus grande production des SCFA. Il a été observé que les souris et les rats traitées par AOM/DSS et nourris avec un régime riche en fibres présentaient moins de tumeurs par rapport à un régime normal[245,246] ou un régime sans fibres[247,248]. L'effet de ce régime riche en fibres est annulé sur les souris déficientes pour FFAR2 ou pour GRP109A[247,248] avec un effet dépendant du butyrate[245]. A régime équivalent, les souris déficientes pour FFAR2 ou GPR109A, dont les tumeurs sont induites par AOM/DSS ou sur des souris $APC^{min/+}$, présentent un plus grand nombre de tumeurs[247,249–251].

De même, une étude a été réalisée sur des souris $APC^{min/+}$. Ces souris développent des tumeurs colorectales et présentent une mutation inactivatrice hétérozygote pour le gène APC[252,253], inactivation majoritairement à l'origine des cancers colorectaux chez l'Homme[254,255]. Dans cette étude, ces souris ont été greffées avec le microbiote de patients atteints de cancer colorectal et ont développé plus de tumeurs par rapport aux souris contrôles greffées avec le microbiote de personnes saines. De plus, le niveau fécal en SCFA (acétate, propionate et butyrate) était diminué comme celui des bactéries productrices de SCFA[256]. Cette étude traduit ainsi la transmissibilité des phénotypes tumoraux par le microbiote intestinal.

3. SCFA et immunité tumorale

a) Effets des SCFA sur l'immunité

Les SCFA, en particulier le butyrate, sont généralement décrits comme ayant un rôle anti-inflammatoire.

Plusieurs études sur les macrophages dérivés de monocytes humains, dérivés de la moelle osseuse murine ou isolés à partir de colon murins montrent une altération de leur différenciation[257] et une inhibition de l'expression de cytokines pro-inflammatoires telles que l'IL6-et IL12[258] et donc une baisse de leur capacité phagocytaire[257] par le butyrate. Cependant, une étude plus récente soutient un effet pro-inflammatoire du butyrate sur les macrophages[259].

En effet, dans cette étude, le butyrate permet d'augmenter la capacité antimicrobienne des macrophages dérivés de monocytes humains. Malgré les différences de fonctions retrouvées dans les différentes études, l'action du butyrate sur les macrophages est indépendante des récepteurs FFAR2 et FFAR3. L'action est médiée par l'inhibition des HDAC (HDACi). Sur les DC, le butyrate altère leur maturation et la présentation antigénique aux les lymphocytes T[257].

Les effets des SCFA sur les lymphocytes T ont également été décrits, surtout sur les Treg. Les SCFA, en particulier le butyrate, augmentent la génération des Treg de la rate, des ganglions lymphatiques et du colon, aussi bien in vitro que in vivo, par l'activité HDACi[260,261] ou via le récepteur FFAR2[262]. Le transfert adoptif de Treg pré-incubés avec du butyrate ou un mélange de SCFA permet d'améliorer la colite des animaux[261,262].

De plus, le butyrate induit la sécrétion de cytokines anti-inflammatoires telles que l'IL-10 sur les lymphocytes Th1 in vivo. Cet effet est perdu sur des souris déficientes en FFAR2, suggérant que ce mécanisme est dépendant de FFAR2[263].

La plupart des études montrent des effets anti-inflammatoires. Cependant, sur les TCD8, l'effet des SCFA est plutôt pro-inflammatoire.

Le butyrate permet d'augmenter la sécrétion d'IFNγ et de granzyme B des TCD8 in vitro et in vivo, dépendant de l'activité HDACi[264]. Le butyrate promeut également le phénotype mémoire des TCD8 via les récepteurs FFAR2 et FFAR3[265].

En somme, l'effet des SCFA sur les lymphocytes T est aussi bien anti-inflammatoire par la promotion des Treg que pro-inflammatoire par la sécrétion cytokinique d'IFNγ et de granzyme B par les TCD8 à l'état basal. Leurs effets seront mieux déterminés en condition inflammatoire, notamment dans les cancers.

b) Rôle des SCFA dans la réponse immunitaire tumorale

A ce jour, rares sont les études explorant les effets des SCFA sur l'immunité tumorale ou sur l'efficacité des immunothérapies anti-tumorales tels que les anti-CTLA-4 et anti-PD-1.

Les études dans les modèles murins montrent essentiellement des effets protecteurs des fibres et des SCFA sur la colite.

Chez l'Homme, seules deux études ont été menées mais ne concordent pas.

Une étude japonaise publiée en 2020 a évalué la concentration des SCFA au niveau fécal et sérique sur des patients présentant des tumeurs solides (mélanomes, tête et cou, gastrointestinal et génito-urinaire) avant traitement par un anti-PD1. Les patients répondeurs au traitement anti-PD1 présentaient, avant traitement, une concentration fécale plus élevée d'acétate, propionate et butyrate par rapport aux patients non répondeurs[266]. Ainsi, l'évaluation des SCFA fécaux permettrait de prédire l'efficacité thérapeutique des anti-PD1. Le mécanisme probable serait la fonction HDACi des SCFA. En effet, plusieurs études chez la souris ont permis de montrer que le traitement par des inhibiteurs d'HDAC combinés avec un anti-PD1[267,268] ou un anti-CTLA-4[268,269] permet une baisse de la croissance tumorale et une meilleure survie. Ces effets sont associés à une meilleure infiltration tumorale des lymphocytes T activés, des macrophages de type M1 et des cellules NK et une meilleure sécrétion de cytokines pro-inflammatoires tels que IFNγ, TNFα, IL-6 et IL-12[268,269].

Une autre étude française publiée la même année a évalué la concentration des SCFA au niveau fécal et sérique chez des patients atteints de mélanomes métastatiques et traités par l'ipilimumab (anti-CTLA-4). Les patients ayant une concentration sérique basse en butyrate présentent une meilleure survie sans progression avec une concentration sanguine plus faible de Treg. La concentration sérique du butyrate est également corrélée à sa concentration fécale. De plus, cette étude a également montré chez la souris que le traitement par le butyrate réduit l'efficacité de l'anti-CTLA-4. Les souris présentent une moins bonne survie, une progression tumorale plus importante. Le mécanisme avancé est la réduction de la maturation des DC et de la présentation antigénique aux lymphocytes T par le butyrate[270]. Ce mécanisme est similaire à une étude chez la souris où le butyrate supprime la réponse immunitaire anti-tumoral engendrée par la radiothérapie[271].

D. Acides biliaires

1. Production et métabolisme des acides biliaires

La synthèse des acides biliaires est la voie métabolique prédominante pour le catabolisme du cholestérol dans le corps humain[194]. Ils sont nécessaires pour la digestion et l'absorption de lipides, de cholestérol exogène et de vitamines. La conversion du cholestérol en acides biliaires est un processus effectué par un ensemble d'enzymes hépatiques[272,273] (Figure 7).

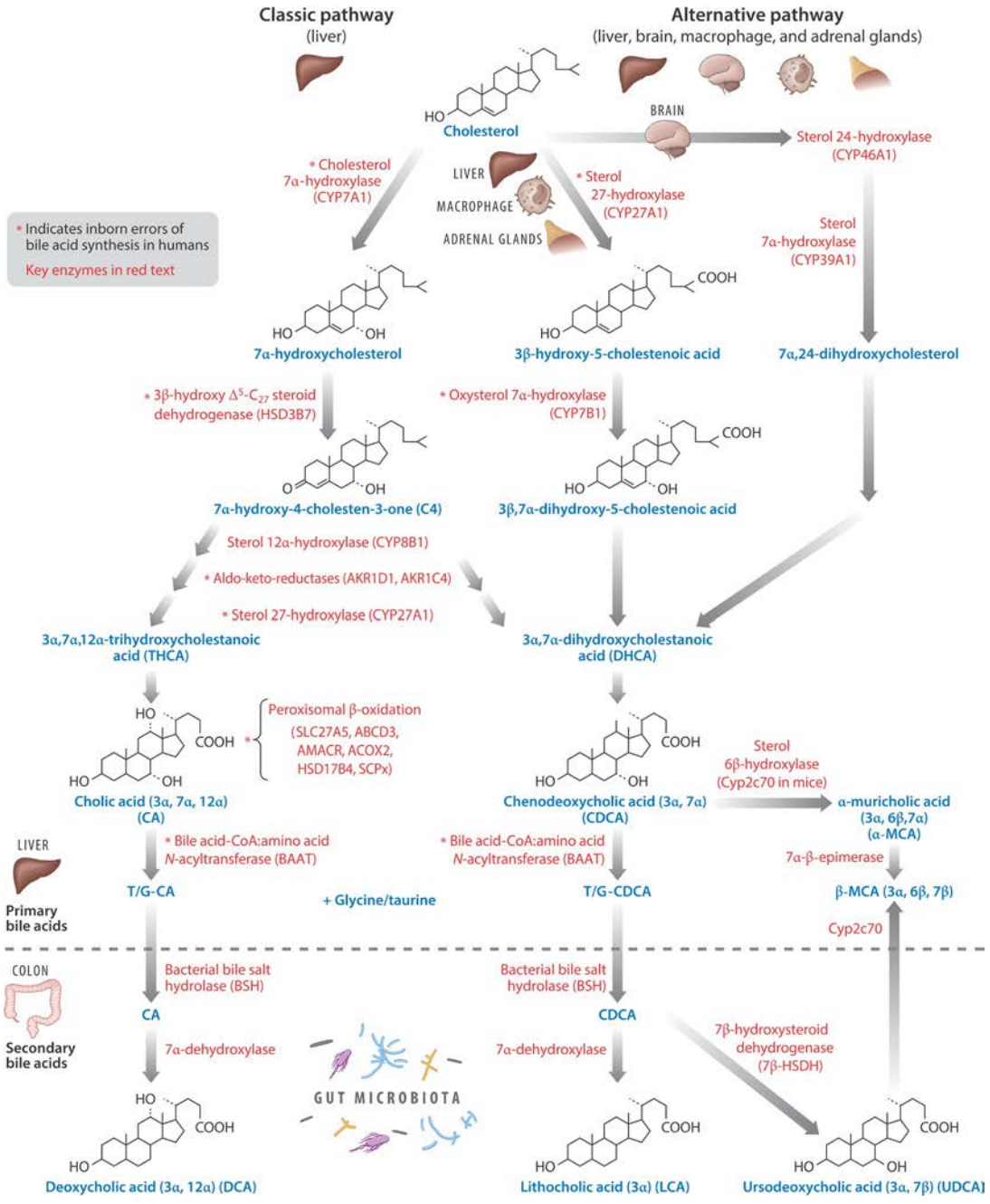

Figure 7 : Voies de synthèse des acides biliaires[272]

La synthèse des acides biliaires peut se faire par deux voies : la voie classique initiée par le CYP7A1 dans le foie, et la voie alternative initiée par le CYP27A1 dans le foie, les macrophages et les glandes surrénales, et par le CYP46A1 dans le cerveau. L'expression de CYP7A1 et de CYP27A1 est régulée par le microbiote intestinal[274]. Chez l'Homme, l'acide cholique (CA) et l'acide chénodésoxycholique (CDCA) sont les principaux acides biliaires primaires synthétisés dans le foie. Chez la souris, Cyp2c70 convertit le CDCA en acide α- et β-muricholique (α- et β-MCA). Les acides biliaires sont ensuite conjugués à la glycine ou à la taurine pour pouvoir être sécrétés. La synthèse de taurine est également régulée par le microbiote intestinal[274]. Près de 95% des acides biliaires seront réabsorbés dans l'iléum et reviennent dans le foie par le cycle entero-hépatique. Les acides biliaires non réabsorbés vont être déconjugués dans l'intestin par l'enzyme bactérienne BSH (bile salt hydrolase) puis le CA et le CDCA seront transformés en acides biliaires secondaires par l'enzyme bactérienne 7α-dehydroxylase en acide désoxycholique (DCA) et acide lithocholique (LCA) respectivement. Le CDCA peut également être transformé en acide ursodésoxycholique (UDCA) par l'enzyme bactérienne 7β-hydroxysteroid dehydrogenase et le β-MCA en ω-MCA chez la souris. Les acides biliaires non conjugués, primaires comme secondaires, peuvent subir d'autres transformation par le microbiote intestinal par épimérisation formant les iso-acides biliaires et les allo-acides biliaires et par oxydation formant les oxo-acides biliaires et les keto-acides biliaires (Tableau 2).

Acides biliaires primaires	CA, DCA, *α- MCA, β-MCA*
Acides biliaires secondaires	DCA, LCA, UDCA, *ω-MCA*, iso-BA, allo-BA, oxo-BA, keto-BA

Tableau 2 : Classification des acides biliaires. En italique : présent uniquement chez la souris.

L'ensemble de ces acides biliaires peuvent être excrétés dans les fèces ou revenir dans le foie où ils pourront également être conjugués et se retrouver dans la circulation sanguine et avoir un rayon d'action plus large[214,272–276].

Les acides biliaires sont également ligands de plusieurs récepteurs membranaires TGR5, S1PR2, CHRM2 et nucléaires FXR, PXR, VDR et CAR[277] (Tableau 3).

Récepteur	Ligands
FXR	CDCA > DCA > LCA >> CA > UDCA
TGR5	LCA > DCA > CDCA > CA > UDCA
PXR	LCA, 3-keto-LCA >> CDCA, DCA, CA
VDR	LCA, 3-keto-LCA
CAR	CA, 6-keto-LCA, 12-keto-LCA
S1PR2	Acides biliaires conjugués (GCA, TCA, GDCA, TDCA, TUDCA)
CHRM2	TCA

Tableau 3 : Ligands des récepteurs aux acides biliaires.

2. Acides biliaires secondaires et cancer

a) Acides biliaires et de leurs récepteurs sur le système immunitaire

Les acides biliaires influent également le système immunitaire. En effet, l'expression des récepteurs des acides biliaires, surtout FXR et TGR5 a été détectée principalement dans les cellules de l'immunité innée[278]. Leur expression dans les cellules de l'immunité adaptative est encore discutée[279].

Les acides biliaires ont un effet global anti-inflammatoire. En effet, les acides biliaires induisent la polarisation des macrophages de type M1 vers un phénotype M2[278–281] dépendant des récepteurs FXR et TGR5[278,279,282]. Les acides biliaires agissent également sur les DC. Plusieurs études montrent que leur fonction est altérée par les acides biliaires secondaires[278,283] et peut avoir un impact sur les cellules de l'immunité adaptative telles que les Treg[284,285]. Une étude a évalué l'effet d'un acide biliaire secondaire, l'isoDCA, sur la génération des Treg[284]. In vitro, l'isoDCA augmente la fréquence des Treg uniquement en présence de DC, sinon une baisse de cette fréquence est observée. Il s'avère que l'isoDCA diminue l'expression de gènes impliqués dans la présentation antigénique. Ce processus est dépendant de FXR exprimé par les DC. In vivo, les souris présentant un microbiote produisant plus d'isoDCA présentent une augmentation de Treg dans le colon[284]. Une autre étude a également évalué l'effet des acides biliaires sur la génération des Treg et des lymphocytes Th17[285]. In vitro, le 3-oxo-LCA inhibe la différenciation des lymphocytes Th17, sans affecter les lymphocytes Th1, Th2 et Treg. L'isoallo-LCA inhibe également la différenciation des lymphocytes Th17 in vitro et augmente

la génération de Treg, sans affecter les lymphocytes Th1 et Th2. L'ensemble de ces résultats a été confirmé in vivo[285].

Les acides biliaires ont donc une action directe ou indirecte sur les cellules immunitaires. Cette action globalement anti-inflammatoire des acides biliaires peut avoir un impact dans la réponse tumorale qui n'a pas été étudiée à ce jour. Cependant, les acides biliaires agiraient principalement sur les cellules tumorales.

b) Acides biliaires secondaires et cancer

(1) Cancer colorectal

Les régimes riches en gras, associés à l'obésité, augmentent la production d'acides biliaires et peuvent augmenter le risque de cancer colorectal[286–288]. De plus, l'obésité est un facteur de risque dans l'incidence dans plusieurs types de cancers : cancer du sein, pancréas ou colon. Cependant, la relation entre les acides biliaires et le cancer est surtout étudiée dans le cancer colorectal.

Chez ces patients, le niveau fécal des acides biliaires secondaires est augmenté[289]. En particulier, le niveau sérique de DCA est retrouvé plus élevé [290,291] et corrèle avec le niveau de prolifération sur les biopsies[292]. Ce taux de DCA sérique corrèle également avec le taux de DCA retrouvé dans l'eau fécale[293]. Plusieurs études chez la souris permettent de décrire les mécanismes mis en jeu.

L'apport supplémentaire de DCA augmente l'incidence de cancer colorectal sur des souris sauvages[294]. Dans le modèle de souris $APC^{min/+}$, l'apport supplémentaire de DCA augmente la colite, le nombre de tumeurs, leurs tailles et leurs prolifération[280,281,295]. L'intégrité intestinale se retrouve altérée[280]. La prolifération est augmentée par l'activation de la voie EGFR promouvant ainsi la croissance des cellules[295] combinée à la production de ROS engendrée par la DCA créant des dommages sur l'ADN et donc de l'instabilité génétique fréquemment retrouvée dans les cancers colorectaux[214,234,287,296,297]. De plus, l'inflammation est également induite sur ces souris, se traduisant par une augmentation d'expression des cytokines pro-inflammatoires IL-1β , IL-6 & TNFα et une polarisation des macrophages vers un phénotype M2[280,281]. Le phénotype induit est également transmissible. En effet, des souris $APC^{min/+}$ receveuses recevant une greffe fécale de souris $APC^{min/+}$ donneuses traitées par DCA

présentent un nombre de tumeurs plus important par rapport à la greffe de souris donneuses non traitées[281]. L'ensemble de ces études se base sur un apport direct d'un acide biliaire secondaire. Cela permet de déceler les mécanismes de toxicité induite par la DCA. Cependant cela ne permet pas de vérifier la capacité du microbiote intestinal à produire les acides biliaires secondaires. Une étude plus récente sur des souris $APC^{min/+}$ a analysé les effets d'un apport supplémentaire en CA, acide biliaire primaire précurseur du DCA, sur la tumorigenèse. Les souris traitées présentent une concentration fécale de DCA augmentée. Les effets produits par l'apport supplémentaire du CA sont identiques à ceux produits par l'apport du DCA, à savoir : une augmentation du nombre, de la taille et de la prolifération des tumeurs, une altération de l'intégrité intestinale et une augmentation des cytokines pro-inflammatoires IL-1β , IL-6 et TNFα[298]. Le traitement supplémentaire par antibiotique permet non seulement d'annuler les effets néfastes de l'apport de CA mais permet également une meilleure protection (moins de tumeurs et d'inflammation)[298]. De manière intéressante, cet apport de CA baisse significativement la concentration fécale en SCFA[298], coïncidant ainsi avec la perte de leurs effets protecteurs sur la colite (décrit précédemment).

L'expression des récepteurs aux acides biliaires est également altérée dans les cancers colorectaux. Chez l'Homme, l'expression de FXR est fortement diminuée dans les adénomes, et quasi nulle dans les cellules cancéreuses[299,300] et les patients ayant une forte expression de FXR ont une meilleure survie à long terme[187]. In vivo, les souris déficientes pour FXR présentent une augmentation de cancers colorectaux, accompagnée d'une augmentation de la prolifération des cellules cancéreuses et une augmentation de l'inflammation[301,302]. Inversement, l'activation de FXR par son ligand synthétique INT 747 protège les souris de la colite induite par un traitement DSS et TNBS[303].

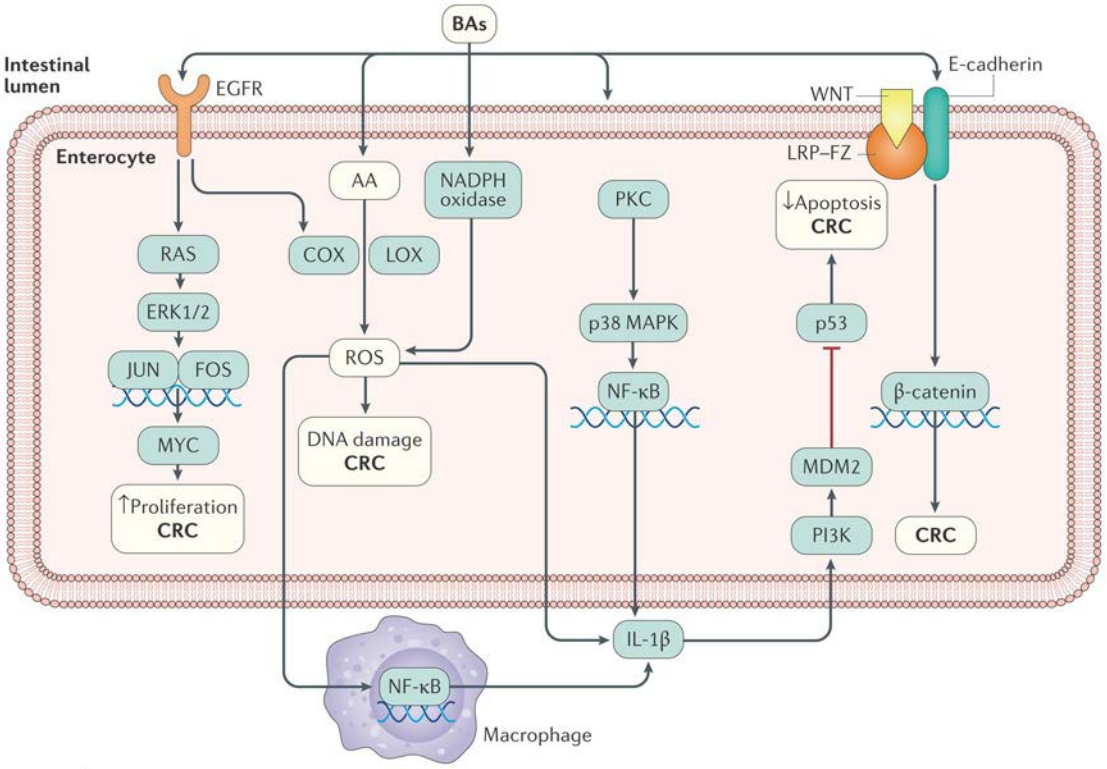

Figure 8 : Effets des acides biliaires secondaires (BAs), en particulier le DCA, sur l'induction du cancer colorectal[273].

FXR étant un des récepteurs pour le DCA, ces données sont contradictoires avec les effets pro-tumoraux du DCA. En réalité, le DCA peut agir à plusieurs niveaux. Lorsqu'il se lie à FXR, toute la signalisation FXR s'en suit, à savoir une protection de la barrière intestinale, inhibition de la prolifération et contrôle de l'inflammation locale. Sinon, le DCA promeut plusieurs voies pro-oncogènes dans les cancers colorectaux (Figure 8). Le DCA stimule EGFR activant ensuite la voie RAS, ERK, JUN & FOS et enfin MYC pour promouvoir la prolifération. Le DCA peut également perturber la membrane plasmique et entrer dans la cellule. Cela génère la production de ROS créant des dommages à l'ADN et donc de l'instabilité génétique. Le DCA peut également activer PKC puis la voie NF-kB. Cela active avec les ROS la stimulation d'IL-1β activant à son tour PI3K et MDM2, inhibant P53 et l'apoptose. Le DCA active également la voie Wnt/β-catenin impliquée dans les cancers colorectaux[273].

Le LCA est également impliqué dans les processus de tumorigenèse. De la même manière que le DCA, le LCA diminue l'apoptose et augmente la prolifération des colonocytes in vitro et in vivo[304].

(2) Autres types de cancers

L'implication des acides biliaires secondaires dans la tumorigenèse ne se cantonne pas uniquement au cancer colorectal : leur rôle dans les cancers hépatiques est également bien décrit. Les mécanismes moléculaires mis en jeu passent essentiellement par FXR. En effet, chez l'Homme, l'expression de FXR est diminuée dans les cancers hépatiques[305]. Les souris déficientes pour FXR développent des cancers hépatiques spontanés, accompagnés d'une augmentation de l'inflammation, des niveaux sériques des acides biliaires et de la prolifération des cellules cancéreuses[306]. Cependant, dans la réalité, l'expression de FXR est uniquement abaissée au niveau du foie dans les cancers hépatiques. Dans une autre étude, des souris déficientes pour FXR uniquement dans le foie ont été utilisées. En régime normale, ces souris ne développent pas de cancers hépatiques. Avec une supplémentation de CA, toutes ces souris développent des tumeurs hépatiques[307]. Enfin, l'induction de cancer hépatique par DMBA ou Streptozocin sur des souris nourries sous régime gras augmente les nodules tumoraux hépatiques, avec des niveaux sériques et fécaux de DCA augmentés[308,309]. L'ensemble de ces études suggère que dans les cancers hépatiques, deux conditions doivent être remplies : une expression diminuée de FXR hépatique et une production et/ou apport augmenté en acides biliaires[273].

La très grande majorité des effets des acides biliaires secondaires dans les cancers concerne les tissus où sont produits et où se trouvent en grande majorité les acides biliaires (foie et colon). Pour que les acides biliaires aient des effets potentiellement cancérigènes dans les autres tissus, les acides biliaires doivent ainsi se retrouver dans la circulation sanguine. Des modifications des acides biliaires sériques ont été rapportées à ce jour uniquement dans les cancers du sein. Le pool d'acides biliaires totaux sériques est significativement plus faible chez les patients. De plus, les niveaux de DCA et LCA sériques se retrouvent également diminués[310]. In vivo, la supplémentation du LCA sur des souris greffées de manière ectopique avec une tumeur mammaire 4T1 réduit l'agressivité du cancer par la diminution de métastases et une augmentation de lymphocytes infiltrant les tumeurs[310]. Le mécanisme d'action a pu être décrit in vitro. Le LCA interagit avec TGR5 et déclenche l'apoptose des cellules[310–312], ce qui est concordant du fait que l'expression de TGR5 est de bon pronostic chez les patients atteints de cancer mammaire[187]. Cette action apoptotique peut servir de stimulation antigénique supplémentaire pour le recrutement et l'activation des lymphocytes. De plus, la baisse des

niveaux sériques d'acides biliaires secondaires limiterait leur action anti-inflammatoire, favorable à une réponse anti-tumorale (décrit précédemment).

L'action des acides biliaires diverge selon le type de cancer. Dans les cancers colorectaux et hépatiques, les acides biliaires agissent principalement sur les colonocytes ou hépatocytes, créant de l'inflammation néfaste dans les cancers colorectaux. Dans les cancers mammaires, les effets des acides biliaires doivent être mieux étudiés afin de comprendre les mécanismes moléculaires mis en jeu.

E. Tryptophane et dérivés

Les acides aminés jouent un rôle dans la physiologie de l'hôte et sont également modulés par le microbiote intestinal. Les acides aminés essentiels, c'est-à-dire, non produit par l'hôte, doivent être apportés par l'alimentation. Les acides aminés aromatiques (AAA) tels que le tryptophane, la phénylalanine et la tyrosine peuvent être métabolisés par le microbiote intestinal et produire des métabolites microbiens ayant plusieurs fonctions chez l'hôte[313]. Le tryptophane et ses dérivés sont les plus étudiées en immunologie et en oncologie.

Le tryptophane est métabolisé à environ 95% vers la voie des kynurénines (indépendante du microbiote intestinal) par l'enzyme IDO-1, 5% vers la voie des indoles par le microbiote intestinal et le reste vers la voie de la sérotonine[314] (Figure 9). Ses dérivés présentent des fonctions importantes dans l'immunité, dans les cancers et également dans l'immunité tumorale[314,315].

Ces 3 voies sont interconnectées. En effet, les souris sans microbiote intestinal présentent une augmentation du niveau de tryptophane circulant[316–318]. De plus, les souris déficientes en IDO-1 présentent une augmentation de la production de dérivés microbiens du tryptophane[319]. Enfin, une insuffisance d'apport de tryptophane chez les souris entraine une altération de la composition du microbiote intestinal et de l'immunité intestinale[320]. Des déséquilibres du métabolisme du tryptophane peuvent mener à des pathologies chez l'Homme[321].

Le métabolisme du tryptophane par la voie des kynurénines est médié par l'enzyme limitante TDO dans le foie et IDO-1 dans les autres tissus. Cela mène à la production de ligands d'AhR (Aryl hydrocarbon Receptor) : la kynurénine et les métabolites en aval[322,323] tels que

l'acide kynurénique, l'acide anthranilique ou l'acide quinolinique[314]. AhR, particulièrement exprimé par les lymphocytes Th17, les macrophages et les DC, permet la régulation de la réponse immunitaire intestinale et le maintien de l'homéostasie intestinale[324,325].

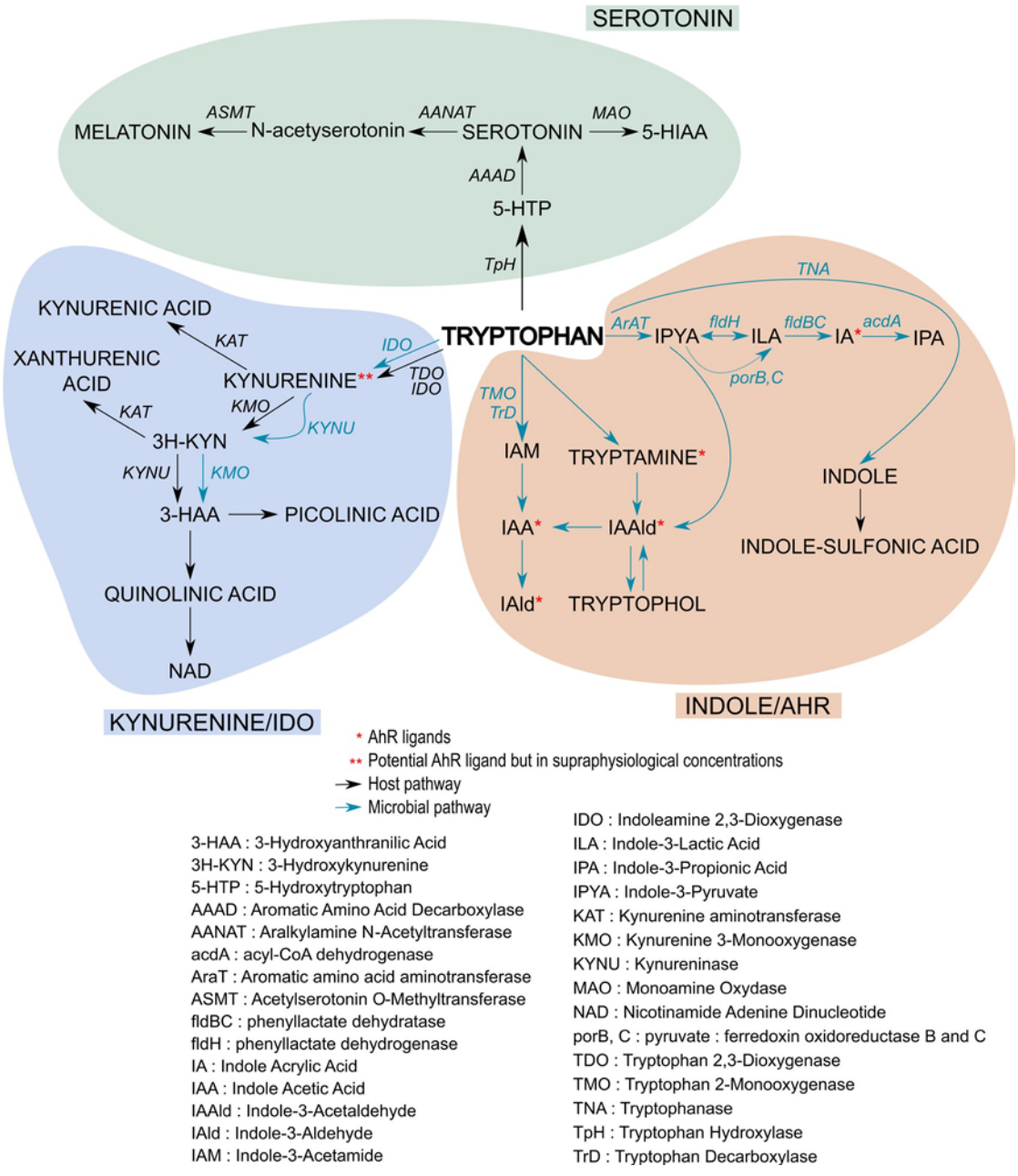

Figure 9 : Voies cataboliques du tryptophane[321].

IDO-1 est essentiellement exprimé dans les tissus inflammatoires (poumon, système digestif, placenta) et lymphoïdes (rate, ganglions lymphatiques) et par les cellules immunitaires, en particulier les DC et les macrophages[326]. Dans l'ensemble, l'expression d'IDO-1 a un effet

anti-inflammatoire et permet l'homéostasie et le maintien de la barrière intestinale[322]. De manière intéressante, l'expression d'IDO-1 est activée par la cytokine pro-inflammatoire IFNγ afin d'inhiber la réponse immunitaire mise en place. Plusieurs mécanismes sont mis en jeu et ne sont pas exclusifs. Le premier mécanisme concerne la déprivation du tryptophane. Le tryptophane est utilisé en tant qu'acide aminé permettant la synthèse protéique dans les cellules. Une déprivation du tryptophane entraine une accumulation d'ARNt non chargée en cet acide aminé provoquant l'apoptose des lymphocytes T[327–329]. Cette déprivation peut se faire de manière nutritionnelle ou par la consommation de tryptophane par les cellules environnantes telles que les DC ou les macrophages[328,329]. Le deuxième mécanisme concerne le rôle de la kynurénine. En effet, la consommation de tryptophane par les DC via IDO-1 entraine la formation de kynurénine, induisant l'apoptose des lymphocytes T[330], plus particulièrement les lymphocytes Th1 et pas d'effet sur les lymphocytes Th2[331], et également des cellules NK mais pas les DC[330]. De plus, l'activation d'AhR permet la génération et la différenciation des Treg[332,333], ce qui est également le cas de la kynurénine par l'activation d'AhR[334]. D'autres métabolites de la voie des kynurénines tels que l'acide anthranilique ou l'acide kynurénique, peuvent également activer AhR et reproduire les effets de la kynuréines[315,330,331].

La voie des indoles est exclusivement liée au microbiote intestinal. Les métabolites microbiens produits par cette voie peuvent également être des ligands du récepteur AhR. Cependant, leur rôle est encore assez peu étudié. Dans des modèles de cellules intestinales, il a été montré qu'in vitro que certains de ces métabolites peuvent être des agonistes (tryptamine, indole-3-acetate) ou des antagonistes (indole-3-aldehyde) d'AhR[335,336]. De manière globale, ces métabolites inhibent les lymphocytes Th17[333,337].

Les effets anti-inflammatoires d'IDO-1 et d'AhR peuvent mener à l'établissement d'un environnement immunosuppressif. En effet, IDO-1 est surexprimé dans plusieurs types de cancers (ex : foie, cerveau, mélanome)[314,315,322,326,338] mais son facteur pronostique dans les cancers est plutôt mitigé, avec des effets favorables (ovaire) ou délétères (rein)[187]. In vivo, l'expression d'IDO-1 ou de TDO par les cellules tumorales augmente la croissance tumorale avec une baisse de l'infiltration tumorale par les lymphocytes T, notamment les TCD8 sécrétant l'IFNγ[338,339]. AhR peut être anti-tumoral dans les cancers colorectaux mais également pro-tumoral dans les cancers prostatiques, pulmonaires, pancréatiques ou mammaires, pouvant être une cible dans les traitements anti-cancéreux[324,340]

III. Les statines

A. Généralités

1. Prescriptions médicales

Les statines représentent une classe de médicaments indiqués en prévention primaire et secondaire de maladies cardiovasculaires et sont consommées quotidiennement par plus de 200 millions de personnes dans le monde[341]. Elles inhibent la synthèse endogène du cholestérol.

La première statine, la Mevastatin, a été identifiée chez les champignons en 1976[341,342]. Structurellement, les statines peuvent être classées en deux types : type 1 ou type 2 (Figure 10).

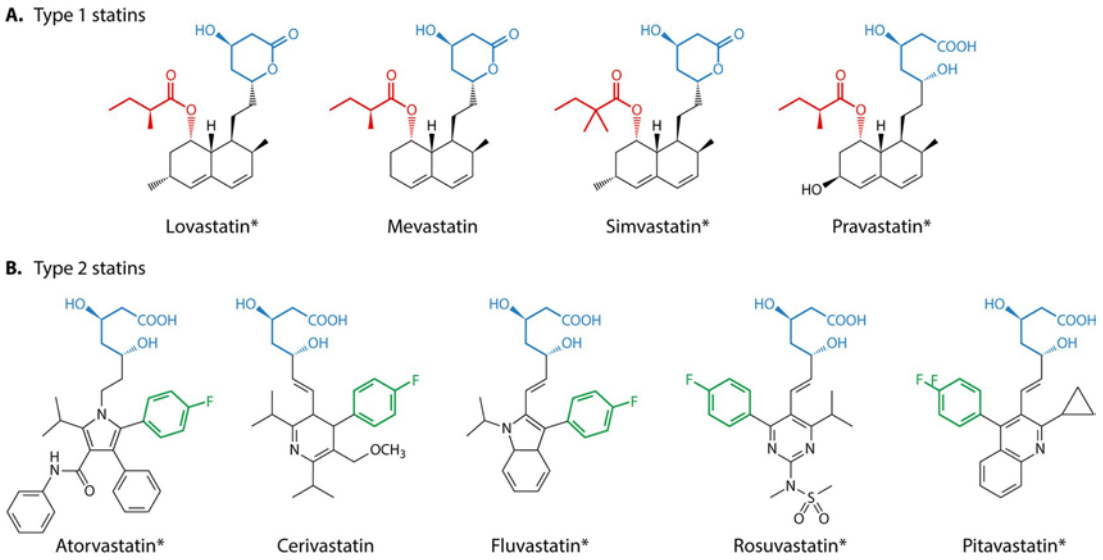

Figure 10 : Structures des statines[343]. Les statines possèdent un noyau lactone conservé entre toutes les statines. Les statines de type 1 sont caractérisées par une structure decahydronaphthalène (en noir) et d'une chaine butyryl (en rouge) différente pour chaque statine. Les statines de types 2 sont caractérisées par un groupe fluorophenyl (vert) à la place de la chaine butyryl et une chaine distincte plus longue à la place du groupe decahydronaphthalène.

Du fait de leur structure différente, les statines n'ont pas toutes les mêmes propriétés, en termes de solubilité, d'absorption hépatique, de demi-vie et de biodisponibilité[343–346] (Tableau 4). La plupart des statines sont lipophiles, ce qui s'accompagne, en général, par une faible biodisponibilité à cause d'un premier passage hépatique important. Cet effet est souhaité car la synthèse du cholestérol a lieu dans le foie. Les statines lipophiles peuvent également entrer de manière passive dans les cellules extra-hépatiques, engendrant de possibles effets pléiotropes.

Les statines hydrophiles ont besoin de transporteurs actifs pour traverser les cellules, ce qui les rendent plus hépato-sélectives[344–346].

Statine	Type	Origine	Pro-drogue	Solubilité	Absorp-tion (%)	Demi-vie (h)	Biodispo-nibilité (%)
Lovastatin	1	Naturel	Oui	Lipophile	31	2.5-3	<5
Simvastatin	1	Naturel	Oui	Lipophile	60-85	1.9-3	<5
Pravastatin	1	Naturel	Non	Hydrophile	37	0.8-3	18
Atorvastatin	2	Synthétique	Non	Lipophile	30	11-30	12
Cerivastatin	2	Synthétique	Non	Lipophile	98	2-3	60
Fluvastatin	2	Synthétique	Non	Lipophile	98	0.5-2.3	10-35
Rosuvastatin	2	Synthétique	Non	Hydrophile	50	20	20
Pitavastatin	2	Synthétique	Non	Lipophile	80	11	>60

Tableau 4 : Propriétés des statines[344–346].

2. Actions biologiques

Les statines bloquent la voie de synthèse endogène du cholestérol, aussi appelé la voie du mévalonate, en inhibant spécifiquement l'enzyme limitante de cette voie : l'HMGCoA-Réductase (HMGCR)[347].

La voie du mévalonate démarre à partir de l'Acétyl-CoA cytoplasmique. Ensuite, la voie se divise en deux branches. La première branche concerne la synthèse du cholestérol. Le cholestérol est le précurseur des acides biliaires, des hormones stéroïdiennes, de la vitamine D et représentent un composant essentiel des lipoprotéines. Il peut également réguler le niveau de cholestérol hépatique à deux niveaux. Le cholestérol régule la synthèse endogène du cholestérol en inhibant la transcription de l'HMGCR et de l'HMGCoA Synthase. Il régule également l'apport exogène de cholestérol en inhibant la transcription du récepteur au LDL, les LDL permettent le transport du cholestérol au sein de l'organisme[348].

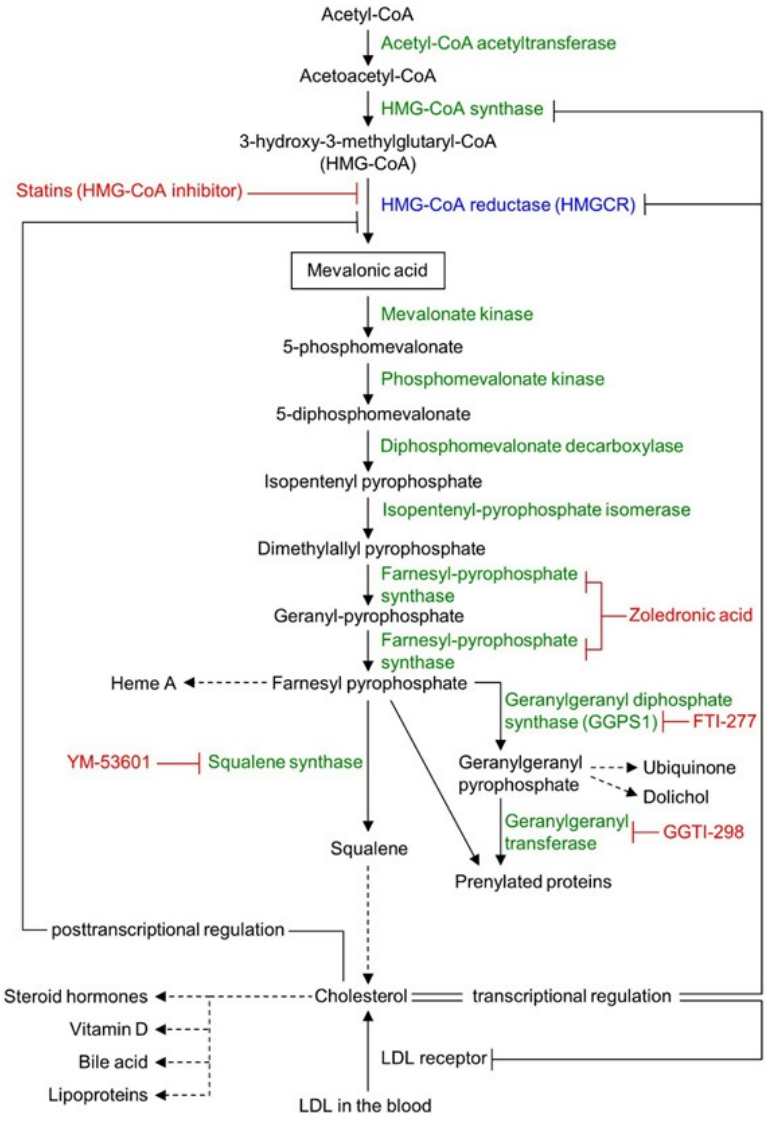

Figure 11 : Voie du mévalonate[348]

La deuxième branche de la voie va permettre la prénylation de plusieurs protéines. La prénylation est une modification post-traductionnelle des protéines leur ajoutant des composés lipidiques et permettant aux protéines d'être adressées à la membrane plasmique pour exercer leur fonction. Parmi ces protéines, on retrouve les protéines de la famille des GTP-ases comme la famille des protéines pro-oncogéniques Rho, Ras ou Rac, participant à la prolifération cellulaire[349]. Du fait de cette propriété, l'action des statines va impacter un plus grand nombre de types cellulaires[344].

B. Effets pléiotropes

1. Immunité

Il est généralement admis que les statines ont une action globalement anti-inflammatoire, passant par la voie du mévalonate.

Chez l'Homme, peu d'études ont été menées sur des volontaires sains afin d'évaluer l'effet des statines sur les cellules immunitaires. Une première étude a évalué l'effet de la Rosuvastatin sur des sujets sains normo-cholestérolémiques. La prise de Rosuvastatin a augmenté le taux de cytokines pro-inflammatoires (IFNγ, IL-1β, IL-6, TNFα) et la fréquence de neutrophiles. Une baisse significative des Treg activés a également été observé[350]. Une autre étude a observé une augmentation des Treg circulants suite au traitement par la Lovastatin ou par l'Atorvastatin chez des sujets sains normo-cholestérolémiques[351]. Enfin, une dernière étude a évalué la prise de statines (Atorvastatin, Pravastatin ou Rosuvastatin) par des sujets sains sur la polarisation des lymphocytes Th1, mais n'a observé aucune modification des niveaux des cytokines polarisant les lymphocytes Th1 (IL-12 et IL-18)[352].

Expérimentalement, de plus en plus d'études récentes mettent en cause les précédentes études sur le rôle de la voie du mévalonate et de son inhibition sur les cellules immunitaires, en particulier dans les lymphocytes.

Plusieurs études parues entre 2005 et 2008 montrent une polarisation de la réponse Th1 vers une réponse Th2. In vitro, les splénocytes traitées par l'Atorvastatin en culture présentent une baisse de sécrétions d'IFNγ et une augmentation d'IL-4, s'apparentant à une polarisation vers une réponse Th2[353]. La baisse d'IFNγ est également retrouvée sur des cellules mononuclées du sang périphérique (PBMC) humain circulant traitées par différentes statines (Atorvastatin, Lovastatin, ou Simvastatin)[354]. Cette polarisation de lymphocyte Th1 vers des lymphocytes Th2 est vérifiée in vivo sur des souris traitées par l'Atorvastatin[353] ou la Lovastatin[355], dépendant de l'inhibition de la voie de prénylation[353]. En 2013, une étude à partir de PBMC humains n'a pas permis d'observer d'effets de la Simvastatin sur les lymphocytes Th1 et Th2[356]. Enfin, une dernière étude parue en 2019 a évalué, in vitro, l'effet de l'Atorvastatin sur des PBMC humains. L'Atorvastatin et la Mevastatin mais pas la Pravastatin augmentent la sécrétion d'IFNγ et baissent la sécrétion d'IL-10 et d'IL-4 par les lymphocytes TCD4, indépendamment de la branche prénylation de la voie du mévalonate mais dépendamment de la branche cholestérol[357].

De la même manière, dans une étude parue en 2020, la Lovastatin inhibe la différenciation en lymphocytes Th2 in vitro chez la souris, mais dans ce cas la relation semble dépendre de la prénylation[358]. Inversement, une étude parue en 2019 également chez la souris a montré que le traitement par le mévalonate baisse la fréquence des lymphocytes Th1 in vivo, tout en n'observant aucun effet in vitro[359].

Cette divergence des études est également observée sur les lymphocytes Th17. Plusieurs études parues entre 2008 et 2013 montrent in vitro une baisse d'IL-17, de la fréquence des lymphocytes Th17 et une inhibition de leur différenciation chez l'Homme[356,360] et la souris[361] associés au traitement par la Simvastatin. En 2019, une augmentation d'IL-17 est observée sur les PBMC humains traitées in vitro par l'Atorvastatin[357]. Cela concorde avec les données in vivo, parues la même année, où le traitement par le mévalonate baisse la fréquence des Th17[359].

Les effets des statines et de la voie du mévalonate sont surtout étudiés dans les Treg. Les différentes études, parues entre 2008 et 2013, montrent que les traitements par les statines (Simvastatin, Lovastatin et Pravastatin) augmentent la fréquence des Treg in vitro chez l'Homme[356,362] et chez la souris[355,361,363]. Seule une étude n'observe aucune différence par le traitement à l'Atorvastatin ou la Pravastatin in vivo[362]. Une étude plus récente parue en 2019 montre un phénotype pro-inflammatoire des Treg in vivo sur des souris traitées par la Simvastatin[364]. En revanche, plus récemment dans une autre étude parue en 2019[359], l'augmentation de la fréquence des Treg a également été observée chez la souris, in vitro et in vivo, par un traitement au mévalonate. Enfin, une étude parue en 2017 a évalué le rôle de la voie du mévalonate dans les lymphocytes T[365]. Pour cela, des souris génétiquement déficientes pour l'HMGCR spécifiquement dans les lymphocytes T ou dans les Treg ont été utilisées, engendrant l'inhibition de la voie du mévalonate spécifiquement dans ces cellules. Les souris avec une inhibition de cette voie spécifiquement dans les lymphocytes T présentent une diminution de lymphocytes TCD4, TCD8 et Treg dans la rate, les ganglions périphériques et mésentériques. La déficience de la voie du mévalonate spécifiquement dans les Treg provoque un syndrome auto-immun sur les souris. Ces souris présentent une augmentation des lymphocytes TCD4 et TCD8, accompagnée par une sécrétion accrue d'IFNγ dans la rate et les ganglions périphériques. Ces souris présentent également une grande mortalité des Treg in vivo, avec un sauvetage de ces Treg in vitro par l'ajout de mévalonate ou de GGPP, montrant ainsi la dépendance des Treg de la branche prénylation de la voie du mévalonate[365].

L'ensemble de ces études montre expérimentalement une disparité des effets des statines sur les cellules immunitaires. En se basant sur les études les plus récentes, les statines auraient ainsi un effet pro-inflammatoire, concordant avec les études réalisées chez l'Homme. L'induction de cette réponse pro-inflammatoire serait ainsi bénéfique dans le traitement des cancers.

2. Cancer

Du fait de leur effet potentiellement anti-tumoral, les statines ont été massivement étudiées.

In vitro, sur des lignées tumorales murines[366–368] et humaines[366,367,377,378,369–376], les statines induisent la toxicité et la mort des cellules[366,367,380,368,371,372,374–377,379], l'inhibition de la croissance et de la prolifération[366,367,372–374,377], de la migration[366,369,372,375,379] et un arrêt du cycle cellulaire[368,370]. Ces effets s'appliquent sur une multitude de types de cancers : sein[367,369,371,373], foie[367,370], prostate[367], poumon[372], colon[375–378], cervicale[367], mélanome[366,368], ostéosarcome[367] et glioblastome[371,374]. Les différentes statines présentent des sensibilités variées sur les cellules, nécessitant ainsi d'adapter les doses pour retrouver les mêmes effets[371,373]. Les cibles moléculaires des statines dans le cancer sont détaillées en Figure 12[381].

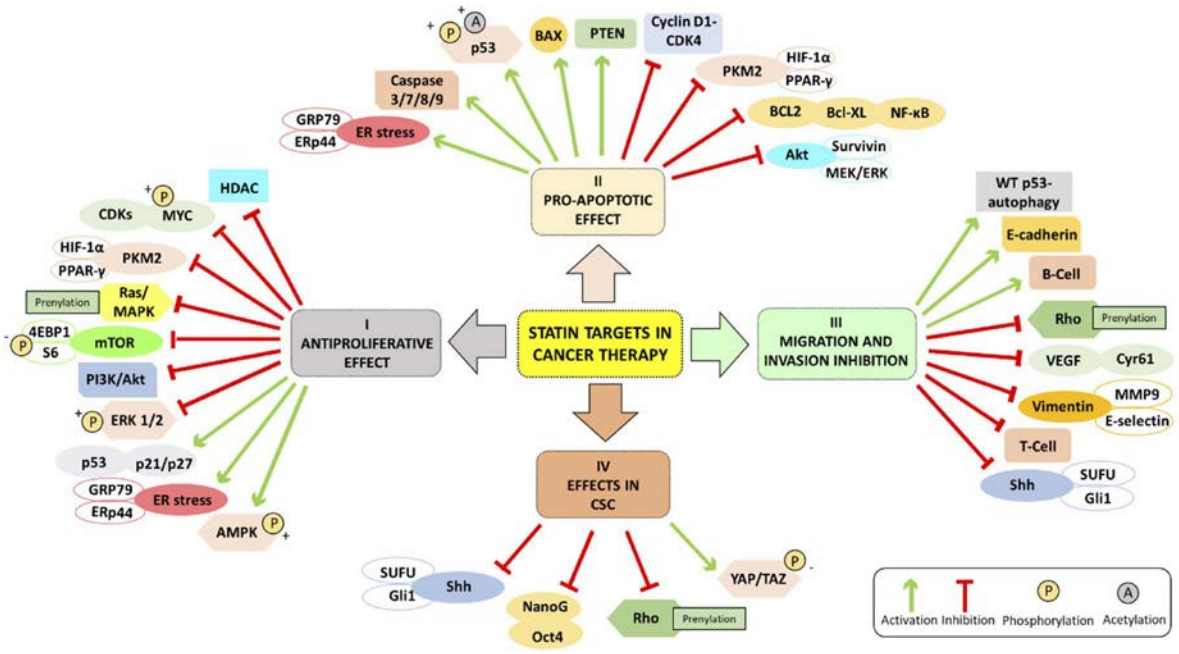

Figure 12 : Cibles moléculaires des statines dans les cancers[381]

In vivo, deux types d'études se distinguent selon le type de souris utilisées, présentant des intérêts différents : les souris immunodéficientes et les souris immunocompétentes. Les souris immunodéficientes permettent d'étudier l'effet direct des statines sur les cellules tumorales et limitent les potentiels effets pléiotropes des statines. Les souris immunocompétentes prennent en compte les effets pléiotropes des statines, notamment sur le système immunitaire, permettant ainsi d'évaluer de manière plus réalistes les effets des statines comme traitement potentiel des cancers.

Les souris immunodéficientes sont greffées avec des cellules tumorales humaines. Le traitement de ces souris par les statines engendre une baisse de la croissance tumorale, quelle que soit la voie d'administration : injection intrapéritonéale (IP)[370,371,382], intraveineuse (IV)[383] ou orale par gavage[371,375,380]. Une étude souligne que l'efficacité thérapeutique des statines diffère à dose équivalente. En effet, les souris traitées avec la Pitavastatin ou la Cerivastatin présentent une croissance tumorale réduite alors que celles traitées avec la Fluvastatin ne présentent pas d'effet sur la croissance[371]. Cette absence d'effet est également retrouvée dans une autre étude avec l'utilisation de l'Atorvastatin[374].

Les différentes études sur les souris immunocompétentes évaluent l'effet des statines sur la croissance tumorale et la survie des animaux. En revanche, la réponse immunitaire n'est pas étudiée. Malgré cela, ces études montrent un effet anti-tumoral des statines avec une diminution de la croissance tumorale[366–368,373,384,385], des métastases[384] et une amélioration de la survie[366,368,384,385].

Des variations peuvent cependant subsister selon les conditions expérimentales. Une des variations retrouvées est la voie d'administration des statines. Une étude sur des souris immunodéficientes a évalué l'effet de la voie d'administration (gavage ou IP) sur la réduction de la croissance tumorale[371]. L'administration de la Fluvastatin par voie IP a permis un plus fort ralentissement de la croissance tumorale par rapport à l'administration par gavage, malgré une dose plus élevée administrée par gavage (4-8mg/kg contre 1mg/kg en IP)[371]. Une autre étude sur des souris immunocompétentes a évalué l'effet de l'administration de la Simvastatin par voie oral, IP, sous-cutanée (SC), IV et intra-osseuse (IO) sur la croissance tumorale d'une tumeur mammaire murine greffée[385]. Sur ce modèle, les administrations orales, par IV ou IP ne présentent aucun effet. La croissance tumorale par SC a tendance à être plus élevée. Seule la voie IO a permis un effet anti-tumoral de la Simvastatin et une meilleure sensibilité aux chimiothérapies anti-cancéreuses[385].

De plus, une étude a évalué in vivo l'effet de la Simvastatin en tant qu'adjuvant vaccinal pour le traitement des cancers et la possible combinaison thérapeutique avec les anti-PD1[386]. Pour cela, des tumeurs exprimant l'antigène OVA ont été greffées sur les souris vaccinées avec la Simvastatin et l'antigène OVA administré en intra-musculaire. La croissance tumorale a pu être diminuée et la survie augmentée, associées à une réponse immunitaire spécifique des lymphocytes TCD8 et une augmentation de leurs sécrétions cytokiniques en IFNγ, TNFα et IL-6. La déplétion des lymphocytes TCD4 ou TCD8 par l'administration d'un anticorps bloquant sur ces souris ou l'utilisation de souris immunodéficientes annule cet effet thérapeutique. De plus, le traitement supplémentaire avec les anti-PD1 améliore drastiquement cet effet thérapeutique[386].

Enfin, les patients sous traitement par les statines et présentant une hypercholestérolémie sont fréquemment en surpoids, voir obèse, et donc présentent un métabolisme énergétique particulier. La majorité des études expérimentales, in vitro et in vivo, ne prend pas en compte l'ensemble de ces facteurs dans l'évaluation des effets des statines dans le traitement des cancers. Une étude observationnelle et préclinique a étudié l'effet des statines sur la croissance tumorale en contexte d'obésité[387]. Un modèle génétique de souris développant des tumeurs prostatiques spontanées a été utilisé. Les souris ont été nourries avec un régime obésogène (50% Carbohydrates, 21% Gras). Une fois l'obésité établie, la Simvastatin est ajoutée dans le régime à faible dose (12mg/kg de nourriture, dose équivalente à 40mg/jour chez l'Homme). De manière surprenante, ce traitement augmente la masse prostatique, l'incidence et la prolifération des cancers[387]. Les raisons sont inconnues. On peut cependant supposer que le régime alimentaire obésogène ait une influence dans la réponse tumorale à la Simvastatin. Le régime alimentaire peut influencer le microbiote intestinal dans la production de métabolites microbiens pouvant avoir des rôles dans l'immunité et les cancers. Cette hypothèse est vérifiée dans une étude in vivo où un régime obésogène, tel qu'un régime riche en cholestérol, augmente l'incidence des cancers hépatiques en modifiant la diversité microbienne et les métabolites microbiens produits[388].

Chez l'Homme, beaucoup d'études rétrospectives et observationnelles ont été réalisées afin d'évaluer le potentiel effet bénéfique des statines sur l'incidence et la protection des cancers (Tableau 5). Cependant, aucun consensus n'est établi sur l'effet protecteur, délétère ou nul des statines et ce quel que soit le type de cancer et de statines. De manière générale, la prise de statines est associée à une protection de l'apparition de cancers et une meilleure survie[389–395], dans les mélanomes[396,397], les cancers de la prostate[392], poumon[392,398,399], colon[392,398,408,409,400–

[407], reins[395,410], seins[411,412], foie[413–416], pancréas[417], estomac[418], œsophage[419–422], l'appareil reproducteur féminin[396,423] et les hémopathies malignes[396,424]. Certaines études ne montrent aucun effet des statines dans l'incidence ou dans l'amélioration thérapeutique des cancers[397,398,424–431], notamment dans les cancers pancréatiques[432], mammaires[433–435] et colorectaux[402–406,436–440]. Enfin, d'autres études montrent en revanche un effet délétère des statines dans l'incidence et la progression des cancers[441,442], notamment dans les cancers colorectaux[402,404,405,407,409,424,431,443,444], œsophagiens[442], pancréatiques[444] et mélanomes[442]. De manière intéressante, les effets des statines sur les cancers colorectaux sont très variés, peu importe la statine et la taille des études. Les cancers colorectaux étant les plus influencés par le microbiote intestinal, ces différents effets des statines peuvent être dus à des modifications de microbiote intestinal par les régimes alimentaires variés selon les pays et les habitudes alimentaires de chacun et par les statines elles-mêmes. Les statines peuvent d'elles-mêmes moduler le microbiote intestinal pouvant engendrer des réponses différentes dans les cancers.

Tableau 5 : Effets des statines sur les types de cancer chez l'Homme. (Liste non exhaustive)

Type de cancer	Effet bénéfique	Effet nul	Effet délétère
Tout type	REF[389–395]	REF[397,398,414–421]	REF[441,442]
Colon	REF[392,398,408,409,400–407]	REF[402–406,436–439,445]	REF[402,404,405,407,409,424,431,443,444]
Mélanome	REF[396,397]		REF[442]
Prostate	REF[392]		
Poumon	REF[392,398,399]		
Rein	REF[395,410]		
Sein	REF[411,412]	REF[433–435]	
Foie	REF[413–416]		
Pancréas	REF[417]	REF[432]	REF[444]
Estomac	REF[418]		
Œsophage	REF[419–422]		REF[442]
Appareil reproducteur féminin	REF[396,423]		
Hémopathies malignes	REF[396,424]		

3. Microbiote intestinal

Depuis quelques années, un nombre grandissant d'études montre que les médicaments qui ne sont pas des antibiotiques ont également des activités bactéricides et donc ont un impact sur le microbiote intestinal[446–450]. C'est également le cas des statines.

In vitro, les statines présentent toutes des effets bactéricides. Cependant, ces effets sur les différentes espèces bactériennes ne sont pas les mêmes selon les statines[341,449–451]. Ceci a pour conséquences une production différentielle de métabolites microbiens, y compris les SCFA[451]. De plus, le microbiote intestinal métabolise la Lovastatin en 4 métabolites[452], suggérant que des mécanismes similaires peuvent advenir avec d'autres statines.

Plusieurs études in vivo ont été menées afin d'étudier la relation entre le microbiote intestinal et les statines. Bien que les statines baissent le cholestérol sérique, les effets sur le microbiote intestinal diffèrent selon les études, indépendamment des statines utilisées (Simvastatin, Atorvastatin, Rosuvastatin, ou Pravastatin). En effet, il a été observé que les statines altèrent le microbiote intestinal [453–456], avec une baisse[457–459] ou une augmentation[459] de la diversité microbienne selon les études, indépendamment du modèle animal (souris[453,454,456–458] ou rat[455,459]) et du régime alimentaire (régime normal[455,456,459] ou régime gras[453,454,457–459]). De ce fait, les conséquences de cette diversité microbienne ne sont pas consensuelles entre les études. On peut tout de même noter une modulation de la production des acides biliaires primaires et secondaires[453,456,457] avec modification notamment de l'expression des gènes hépatiques Cyp7A1, Cyp7B1 et Cyp27A1[453,456,458]. Une modification de la production de SCFA[456] et de l'inflammation[454,457] est également observée.

Chez l'Homme, le très faible nombre d'études ne permet pas d'établir de consensus sur les conséquences de la modification du microbiote intestinal par les statines. En effet, une augmentation[460,461] ou une diminution[462,463] de la diversité est retrouvée.

Malgré les divergences entre les études, il est certain que les statines altèrent le microbiote intestinal. Par ce biais, elles peuvent avoir des effets pléiotropes plus larges, notamment dans l'immunité et dans les cancers.

IV. Hypothèses de travail

Le microbiote intestinal module plusieurs axes tels que la pharmacologie des médicaments, le système immunitaire et la croissance tumorale.

De très rares études se sont intéressées à étudier l'effet combiné de ces quatre acteurs. Pourtant, les patients cancéreux sont des sujets ayant des dérèglements microbiens, une modulation immunitaire affectée et très souvent d'autres maladies chroniques nécessitant des médications quotidiennes telles que la metformine, l'aspirine ou les statines.

En effet, plusieurs études montrent des effets bénéfiques de la metformine et de l'aspirine sur le cancer notamment via une meilleure réponse immunitaire anti-tumorale[464–466]. A titre d'exemple, la metformine peut moduler le microbiote intestinal[467,468], lui permettant d'induire une réponse inflammatoire[469]. En revanche, les effets bénéfiques de l'aspirine sur le cancer peuvent être atténués par le microbiote intestinal[470].

De plus en plus d'études relatent des effets bactéricides de médicaments non antibiotiques[447–449]. Ainsi, nous nous sommes intéressés aux statines pour plusieurs raisons. Tout d'abord, les statines représentent d'une des classes de médicaments les plus prescrits au monde[471]. Ensuite, les statines, en plus d'inhiber la synthèse endogène de cholestérol pouvant servir de nutriments aux cellules tumorales[472–475], sont également susceptibles d'inhiber la prénylation de plusieurs protéines pro-oncogéniques[349,476,477]. De plus, malgré le grand nombre d'études observationnelles ou rétrospectives chez l'Homme, aucun consensus sur l'effet pro-tumoral, nul ou anti-tumorale des statines ne ressort notamment dans le cancer colorectal (Tableau 5). Enfin, plusieurs études montrent des effets bactéricides des statines[341,447–451,478,479]. Parmi les statines, nous avons choisi la Simvastatin car c'est l'une des statines les plus prescrites[471] et les plus bactéricides[341] et pour sa faible biodisponibilité (Tableau 4) permettant un effet microbiote dominant dans notre étude.

Pour le choix de la lignée tumorale, nous avons choisi la lignée de cancer colorectal MC38. Les raisons de ce choix sont multiples. Tout d'abord, c'est une lignée tumorale qui, in vivo, est infiltrée par le système immunitaire qui contrôle sa croissance[166–169,480]. En effet, la quasi-totalité des études in vivo avec des statines ont été faites soit sur des souris immunodéficientes[370,371,374,375,380,382,383], soit sur des souris immunocompétentes mais greffées avec des souris peu infiltrées par les cellules immunitaires[366–368,384]. Ensuite, la croissance de la

MC38 est modulée par le microbiote intestinal. En effet, plusieurs études ont montré selon les espèces bactériennes colonisant les souris, avec ou sans immunothérapie, la réponse immunitaire anti-tumorale est modifiée, influençant la croissance de la MC38 in vivo[216,218,481]. Enfin, hormis une étude[386], aucune étude in vitro et in vivo n'a été réalisée sur l'effet des statines sur la MC38.

L'action conjointe des statines et du microbiote intestinal dans la réponse immunitaire des cancers n'a pas été étudiée.

Nous avons enfin choisi d'utiliser des souris génétiquement identiques, C57BL/6J, mais provenant d'élevage différents[482]. Ces souris présentent une composition microbienne différente, et donc potentiellement une production différentielle de métabolites microbiens pouvant influencer la réponse immunitaire tumorale[217,483].

Résultats

Article principal

Statin regulates microbial-derived metabolites to tune tumor growth through CD8 T cells refined response.

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Abstract

There is increasing evidence that statins display bactericidal and immunomodulatory functions. Recent evidences show that gut microbiome and microbial-derived metabolites can influence CD8 mediated cancer development. To understand the mechanisms by which the statin modulates gut microbiome cancer susceptibility, we examined mouse colonies from two providers that displayed distinct gut microbial-derived metabolites and communities and develop significantly different tumor growth using an ectopic tumorigenesis model of an immune-competent colorectal tumor cell line. Statin treatment had utterly opposite effect on tumor growth that was inversely correlated with tumoral IFNγ CD8 T cell frequency. Fecal microbiota transfer of the two microbial ecosystems in axenic recipient mice recapitulated the tumorigenesis and immune phenotypes. Notably, these responses were associated with an extensive alteration of cecal and plasma metabolome. Several microbial-derived metabolites were modulated, including SCFA, especially butyrate, bile acids, and tryptophan metabolism. These studies suggest that gut microbiota has a pivotal role in mediating statin effects on immune response to cancer.

Introduction

The last decade has provided compelling evidence that microbes that inhabit the gut are major determinants in health and disease [1,2]. Gut microbiota plays an essential role in host physiology through metabolic exchanges that are at the heart of the dialogue between hosts and symbiotic microbes [3–5]. The metabolic activities of the microbiota impact on multiple host functions in and outside of the gut. Microbes can activate a wide range of host signaling pathways either directly via the release of microbe-derived molecules (e.g., LPS, peptidoglycans, vitamins, DNA, RNA, ATP…) or indirectly via the transformation of food products (e.g., fiber-derived carbohydrates, trimethylamine…) or of host-derived metabolites (bile acids). Microbes can also deliver substrates, fueling key host metabolic pathways (e.g., amino acids, tryptophan, short-chain fatty acids).

A disruption of the microbial balance is associated with the epidemics of chronic metabolic and inflammatory diseases that accompany industrialization. Any change in the local metabolic environment induced by exogenous factors (e.g., antibiotics, diets) and or by endogenous host responses (e.g., host inflammatory response) can modify the structure of the microbial community, thus ascribing a significant role to the gut microbiota to neurodegenerative, metabolic, cardiac, infectious diseases and cancer.

Gut microbiota can metabolize pharmaceutical drugs and affect their pharmacodynamics and efficacy both positively and negatively [6–10]. Conversely, drugs can disrupt gut microbiota and behave like antibiotics [11]. Indeed, a growing list of drug classes displays microbicidal or microbiostatic properties *in vitro* and *in vivo* [7,11]. Additionally, drugs can impact the immune system that plays a crucial role in recognizing and controlling gut microbiota composition. These interconnected mechanisms may contribute to shifts in microbial ecosystems that can protect, attenuate, trigger, or perpetuate diseases as well as influence responses to treatments [7,12].

Among the pathologies associated with the gut microbiome, tumorigenesis is an area of active research. The link has been observed both with local gastrointestinal cancers as well as with other distal tumors [13]. Metabolomics and metagenomics studies highlighted the dual role of the gastrointestinal microbiome in cancer prevention, tumorigenesis, and anti-cancer therapy [14,15]. Gut-derived metabolites can either be tumor-suppressive or oncogenic [16,17]. To date, despite the progress made in conventional treatments (chemotherapy, radiotherapy, hormone therapies) and immunotherapies, or through the consideration of metabolic inhibitors prescribed in other pathologies (e.g., metformin, statins, etc.), a large number of patients suffer relapses and/or are non-responders.

Statins are a mainstay in cardiovascular pharmacotherapy, not only in patients with dyslipidemia but also in patients with coronary artery disease, acute coronary syndromes, diabetes, stroke, hypertension, and chronic kidney disease (with or without coexistent dyslipidemia). Despite its long-term efficacy [18], at the individual level, the patient's response to

statin therapy both in terms of LDL-Cholesterol lowering and side effects is heterogeneous, with good and poor responders to treatment and some patients being intolerant [19–21].

This heterogeneity of responses suggests that multiple mechanisms beyond inhibition of its primary target (HMG-CoA reductase) might play a significant role in the overall response and benefit of statin therapy. Several studies have explored and demonstrated that statins could directly influence the growth and virulence of bacterial pathogens and commensal as well as combat microbial infections, such as sepsis and pneumonia [8,22] . The first Statin "Mevastatin", a metabolic product of Penicillium citrinum, was initially characterized for its antibiotic properties and statin are now considered adjuvant antibiotic that can impact antimicrobial resistance [23]. Consequently, statins' role deserves to be explored beyond their traditionally established indications in light of their antimicrobial potential as a regulator of gastrointestinal microbiota [24]. Emerging studies indicate that gut microbiota is linked to the therapeutic response of a variety of drugs [6,25], including statins [26–28]. For example, gut microbiota can convert lovastatin to its bioactive metabolite [29,30]. Another study shows that the magnitude of simvastatin-induced LDL-C lowering effect is associated with specific secondary bacterial-derived-bile acids [26], thus indicating that microbial metabolism predicts simvastatin bioavailability or activity.
Our unpublished and published data, as well as other recent publications, have highlighted the role of the gut microbiota on the response to statins and reciprocally[23,31–34]

Apart from gut microbiota effects, the pleiotropic effects of statins on the immune system and cancer have been investigated. *In vitro* and *in vivo* studies report an overall immunosuppressive response to statins, particularly through Treg enrichment in mice models[35–37] and humans[38,39]. *In vitro*, statins display anti-tumoral effect[40]. *In vivo*, studies confirmed the anti-tumoral effect of statins immunodeficient mice [40] and some in immunocompetent mice mainly transplanted with B16 melanoma cell line[41–43]. However, in the absence of specific randomized controlled trials, statins' effects on human cancers are not established. Anti-tumor[44,45] and pro-tumor[46,47] effects have been reported as well as the absence of impact[48,49] on the prevalence of cancers, independent of the size of the studies. Such heterogeneous prevalence might be explained by the large variation of gut microbiota composition among countries of investigation[50–52].

To date, there is no evidence of the effect of the host microbiota on statin response to cancer, particularly colorectal cancer, which is most prone to the gut microbiota's effect.
Our study aims to determine the contribution of the gut microbiota on the effect of statins on colorectal cancer progression. We use a murine colorectal cancer cell MC38, on which effects of statins were not reported yet to our knowledge.

In addition, we took advantage of two independent and stable microbial ecosystems in equilibrium with their host (i.e., C57BL6J mice) distributed by two commercial providers (i.e., Charles River Laboratories and Janvier Labs)[53]. These mice with an identical genetic background were grafted with the MC38 colorectal cell line, frequently used for tumor immune and immunotherapy response[54–56] in which growth is modulated by the gut microbiota, through

modulation of anti-tumoral TCD8 lymphocytes (TCD8) expressing IFNγ[57–59] (also named Tc1 cells), a cytokine displaying anti-tumoral properties.

Material & Methods

Cell Line

Mouse colon adenocarcinoma MC38 were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2mM glutamine, 1mM sodium pyruvate, 10mM Hepes and Pen/Strep. Cells were maintained at 80% of confluency and split three times per week.

Statin in vitro toxic assay

Cells were seeded on a 12-well plate for MC38 and 96-well plate for PBMC in appropriate medium. The following day, the statin was added to the culture medium along with the other metabolites. After four days of culture, supernatant and trypsinized cells were harvested. Viability was assessed by cytometry with viability dye (eF506, Fixable Viability Dye, ThermoFisher).

Mice

Males and females C57Bl/6J mice, aged from 5 to 8 weeks, were obtained from Charles River Laboratories (CR) (Saint Germain Nuelles, France) and Janvier Labs (JL) (Le Genest St Isle, France). Six-week-old germ-free C57Bl/6J female mice were reared from germ-free breeding pairs at ANAXEM, the germ-free animal facilities of Micalis (Jouy-en-Josas, France). Mice were maintained on a 12 h light and dark cycle with *ad libitum* access to water and standard chow diet (no. 5058; Lab-Diet) under specific pathogen-free conditions in the mouse facility at Paris Sorbonne University. Mice were injected subcutaneously with 1.10^6 MC38 cells line in the right flank. Tumor growth was monitored with a caliper and volumes were determined using the following formula (length*width*height)/2. When tumor volume reaches 100mm3, mice were treated with simvastatin (ref sigma) by oral gavage at 5mg/kg daily until sacrifice. All procedures involving mice were carried out according to the Guide for the Care and Use of Laboratory Animals published by the European Commission Directive 86/609/EEC. All animal studies were approved by the regional veterinary services of the Paris police headquarters (agreements no. 75-751320) and by the ethical committee of Sorbonne University (authorization: APAFIS#20376-2019042514251937)

Fecal microbiota transfer

30-50 mg of feces from donor mice were diluted (1:10 w/vol) and homogenized in reduced sterile Ringer solution (VWR) containing 0.5 g/L L-Cysteine (Sigma) as a reducing agent. This solution was immediately administered to germ-free recipients by oral gavage. Six-weeks old germ-free mice were inoculated with donor fecal microbiota immediately after the opening of their sterile shipping container. During the experiment, mice were cohoused with correspondent donor commercial mice.

Phenotypic analysis by flow cytometry

Spleen and lymph nodes were smashed through a 70µM cell strainer with PBS + 3% FCS. Red blood cells from the spleen were lysed with Red blood cell lysis solution (ref). Tumors were cut into 1cm pieces and digested with 0.84mg/mL of collagenase IV and 10µg/mL DNAse I (Sigma Aldrich) for 1h at 37°C with intermediate flushing of the tissue. Cells were passed

through a 100µm-cell strainer and resuspended in PBS 3% FCS. TILS were isolated on an 80/40% Percoll gradient. Rings were collected, washed, and cell pellets were resuspended in PBS 3% FCS. Prior intracellular cytokine staining, cells were restimulated with PMA/Ionomycin + Golgi Stop according to vendor use for 5h in RPMI containing 10% FCS, 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and 100 U/ml of penicillin and streptomycin. Subsequently, cells were stained with viability dye (eF506, Fixable Viability Dye, ThermoFisher) for 15min at 4°C, Fc receptors were blocked with anti-CD16/32 (REF) for 10min. The following antibodies were added for 35min at 4°C: CD45 CD3 CD4 CD8 CD25. For intracellular staining, Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used, then the following intracellular antibodies were added for 45 min at 4°C: FOXP3, IFNγ, TNFα. Cells were washed with 1X PBS before acquisition on an X20 cytometer or LSR Fortessa (Becton Dickinson (BD), San Jose, CA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Chemical reagents

All Liquid Chromatography-Mass Spectrometry (LC-MS) grade reference solvents, acetonitrile (ACN), water (H2O), methanol (MeOH) and ammonium carbonate were purchased from VWR International (Plainview, NY). Analytical grade of ammonium hydroxide (NH4OH), sodium hydroxide (NaOH), propan-1-ol, pyridine, hexane and propylchloroformate (PCF) and formic acid were from Sigma-Aldrich (Saint Quentin Fallavier, France). Internal standards solutions of stable 13C and 15N isotope-labeled mix of amino acid for the untargeted approach and reference isotope-labeled compounds for SCFA analyses (Acetate, propionate, butyrate, isobutyrate, valerate and isovalerate, acetate-D3, propionate-D2, butyrate-13C2 and valerate-D9) were purchased as well from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water comes from a Milli-Q Elix system fitted with a LC-PaK and a MilliPak filter at 0.22µm (Merck Millipore, Guyancourt, France).

Sample preparation

Untargeted approach – Plasma samples

Eight volumes of frozen acetonitrile (-20°C) containing internal standards (labeled IS mix of amino acids at 10 µg/mL) were added to 100 µL plasma samples and vortexed. The resulting samples were then sonicated during 10 min and centrifuged during 2 min at 10 000 x g at 4°C. Supernatants were incubated at 4°C during 1 hour for slow protein precipitation process. Samples were centrifuged for 20 min at 20 000 × g at 4°C. Supernatants were dried and stored at -80°C prior to LC-MS analyses. Pellets were diluted 3-fold and reconstituted with 0.1% formic acid for PFPP column and in H2O/ACN (20/80) for HILIC column.

Untargeted approach – caecum and faeces samples

Prior to extraction, 30mg of caecum and faeces samples were weighted and homogenised in 100µl of a solution of 0.1% formic acid including internal standards (labelled IS mix of amino acids at 10 µg/ml). Caecum and faeces samples were supplemented with ceramic beads and homogenised 3x15sec at 6500rpm using a Precellys Evolution apparatus (Bertin Technologies,

Montigny-le-Bretonneux, France). Eight volumes of frozen acetonitrile (-20°C) containing internal standards (labeled mixture of amino acids at 12.5 µg/mL) were added to homogenized caecum and faeces samples and vortexed. Samples were sonicated during 10 minutes and centrifuged during 2 minutes at 10.000xg and at 4°C. Then, centrifuged samples were incubated at 4°C during 1 hour for slow protein precipitation. Samples were centrifuged at 20.000xg at 4°C. Supernatants were transferred to another series of tubes and then dried and frozen at -80°C until the LC-MS analyses. Samples were diluted 3-fold in 0.1% formic acid for PFPP column and in H2O/ACN (20/80) for HILIC column. Reconstituted samples were centrifuged and then, transferred to vials before LC-MS analyses

SCFA analysis - faeces samples

The protocol was modified based on method of Zheng et al.[60]. Extraction steps were performed at 4°C to avoid the loss of SCFA species. Approximately 30mg of mouse fecal samples were used and suspended in 1.5ml of NaOH solution at 0.005M containing internal standard mix and ceramic beads. Samples were homogenized at 6500 rpm, 3x20s using Prescellys® Evolution (Bertin Technologies, Montigny-le-Bretonneux, France). 300µl of supernatant were collected and transferred to 5ml glass tube. 500µl of propanol/pyridine mix (3:2 v/v) were added and then vortexed. 50µl of PCF were successively added twice to the solution and vortexed. 1.5ml of hexane were added to the glass tube. Mixture was sonicated and centrifuged at 2000xg, 4°C during 5min 200µl of organic phase were transferred to GC/MS vials before theirs injections

Data analyses of GC/MS and LC/MS

GC/MS analysis of SCFA

SCFAs in fecal samples were quantified by Gas Chromatographic/mass spectrometry using an ISQ LT™ equipped with a Triplus RSH (Thermo Fisher Scientific, Illkirch, France). A fused-silica capillary column with a (5%-phenyl)-methylpolysiloxane phase (DB-5ms, J&W Scientific, Agilent Technologies Inc., USA) of 50m x 0.25 mm i.d coated with 0.25 µm film thickness was used. Temperatures of the front inlet, MS transfer line, and electron impact ion source were set at 260°C, 290°C, and 230°C, respectively. Helium was supplied with carrier gas at a flow rate of 1 ml/min. Oven temperature was set initially at 50°C during 1.5min. Temperature was raised to 70°C at 8°C/min and to 85°C at 6°C/min. Then, temperature was successively elevated to 110°C at 22°C/min and to 120°C at 12°C/min. Oven temperature was finally set to 300°C at 125°C/min and held 3min. The run time was 15min in targeted SIM mode using mass list detailed in Table 1. Injected sample volume was set to 1µl in split mode with a 20:1 ratio. Data processing was performed using manufacturer software Xcalibur® (version 3.0, Thermofisher Scientific, Illkirch, France)

Table 1. GC/MS and quantitative parameters of SCFAs analysis. Linearity, calibration equation, limit of detection (LOD) and limit of quantification (LOQ)

SCFA species	*Mass (m/z)*	*Retention time (min)*	*Internal standards*	*Calibration equation (y=Ax+B)*	*R2*	*Linearity range (µM)*	*LOD (µM)**	*LOQ (µM)**
Acetate	43, 60	4.5	Acetate-D3	y = 0.008x + 0.003	0.993	2.3-500	2.3	2.39
Propionate	57, 75	5.9	Propionate-D2	y = 0.013x + 0.267	0.997	1.2-200	1.09	1.23
Isobutyrate	43, 71	6.9	Butyrate-13C2	y = 0.017x + 0.021	0.994	0.3-100	0.23	0.35
Butyrate	43, 71	7.7	Butyrate-13C2	y = 0.003x + 0.013	0.973	1.3-200	0.69	1.3
Isovalerate	73, 85	8.6	Valerate-D9	y = 0.035x + 0.001	0.998	0.2-100	0.15	0.26
Valerate	73, 85	9.2	Valerate-D9	y = 0.036x + 0.009	0.998	0.2-100	0.11	0.22

**LOD=Mb + 3xSDb; LOQ=Mblank+ 10xSD blank; where Mb is the mean concentration of the blank and SDb is the standard deviation of the blank*

LC-MS analyses and data processing of the global approach

The untargeted approach was performed using two kind of chromatographic columns, a HILIC, Sequant ZIC-pHILIC column 5µm, 2.1 × 150 mm at 15◦C (Merck, Darmstadt, Germany), and pFPP, Discovery HS F5-PFPP column, 5µm, 2.1 × 150 mm (Sigma, Saint Quentin Fallavier, France). Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA) were used and experimental settings for the global approach by LC-HRMS were carried out as detailed in Garali et al.[61].

Processing steps were carried out using the R software[62]. LC-MS raw data were first converted into mzXML format using MSconvert tool[63]. Peak detection, correction, alignment and integration were processed using XCMS R package with CentWave algorithm[64,65] and workflow4metabolomics platforms[66]. The resulted datasets were Log-10 normalized, filtered and cleaned based on quality control (QC) samples[67]. The remaining features were annotated based on their mass over charge ratio (m/z) and retention time using an “in house” database as described previously[68], and also putatively annotated based solely on their m/z using public databases such as the human metabolome database HMDB[69] and the Kyoto Encyclopedia of Genes and Genomes database, KEGG[70].

Statistical analysis

All statistical tests were performed with Prism software, Version 8.3.0 (Graph Pad Inc, La Jolla, CA, USA). To compare ranks between two groups, the p-value was calculated with a nonparametric two-tailed Mann-Whitney t-test. Statistical modeling of tumor growth was performed by linear and nonlinear regression using the exponential growth model. Results were considered statistically significant when $p < 0.05$.

Results :

Simvastatin reduces immunity on healthy mice.

Mice from different suppliers have distinct microbiomes and exhibit different pathological and therapeutic responses[28,53,71–73]. With this tool, we first evaluated the impact of the pharmacological response of statins on the immune system at a steady state. Mice from suppliers Janvier Labs (JL) and Charles River Laboratories (CR) were treated with simvastatin for three weeks by daily gavage at the therapeutic concentration used in Man (5 mg/kg).

At baseline, in the spleen, the secretory capacity of TCD8 is differing according to the supplier, despite a similar proportion of TCD8 (Supp Fig1A). Indeed, JL mice have a significantly higher secretory capacity of the pro-inflammatory cytokines IFNγ and TNFα than CR mice (Fig1B & Supp Fig1B). The level of Treg remains unchanged. (Fig 1A) The IFNγ and TNFα secretory capacity of Th1 lymphocytes are also higher in JL mice compared to CR mice (Supp Fig1C-E). These results indicate, at baseline, a stronger immunity in JL mice.

On statin treatment, an overall decrease of mice immunity is observed in the spleen. Indeed, statin treatment reduces the proportion of TCD8 regardless of the supplier (Supp Fig1A). However, only the IFNγ & TNFα secretory capacity of TCD8 and Th1 is significantly lowered in JL mice. Secretory capacity remains unchanged in CR mice under statin treatment (Fig 1B & Supp Fig1B, D-E). The proportion of Treg under statin treatment does not increase significantly (Fig 1A). The immunosuppressive effects are even more significant in the mesenteric lymph nodes close to the intestine (Fig 1C-D & Supp Fig1 F-J). Indeed, statin treatment reduces the secretory capacity of TCD8 and significantly increases Treg's proportion, independently of the supplier. Regardless of the organ, JL mice under statins have more Treg than CR mice. Moreover, IFNγ secretion by Th1 in also lowered under statin treatment. These data suggest that statins reduce mice immunity, with differences depending on mice suppliers.

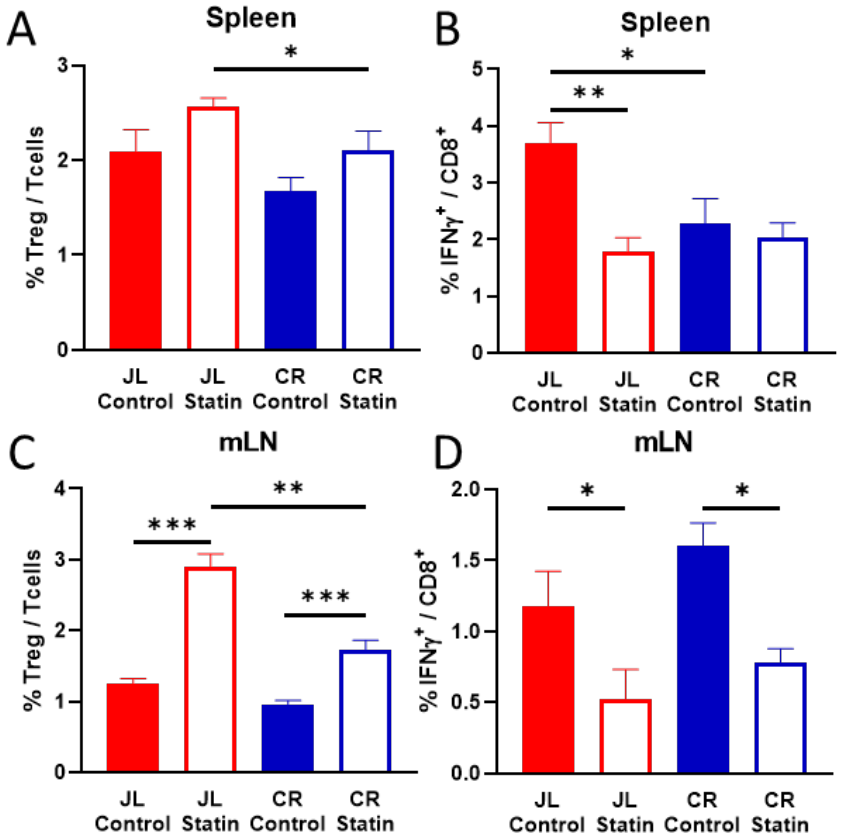

Figure 1: Statin immune effect at steady state in vivo (n=8 for each group).
6 weeks old female C57BL6J mice were treated with Simvastatin daily by oral gavage. Cells were stimulated 5h with PMA+ ionomycine, Brefeldine and monensime for cytokine detection.
(A-D): Frequencies of Treg (A&C) and IFNγ$^+$ CD8$^+$ cells (B&D) were analysed in the spleen (A&B) and mesenteric lymph node (mLN) (C&D).
Data were acquired by flow cytometry.
Data are presented as means ±SEM (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney test).

Commercial breeders imprint statin effect on colorectal cancer outcomes.

Since the pharmacological response of statins on the immune system globally did not differs between microbiota on healthy mice, we investigated these effects in an inflammatory model of grafted colorectal cancer (MC38). Suspecting a potential contribution of the gut microbiota on statin response, we did not use the DSS/AOM induction model since it was described as altering the gut microbial environment[74]. However, MC38 tumor growth has been reported to depend on the immune system and gut microbiota[57,59]. Furthermore, to our knowledge, no studies have been published on the effect of statins on the MC38 cell line *in vitro* nor *in vivo*, even less on the effect of gut microbiota on statin pharmacology in this model. Mice commercialized by JL and CR were grafted with MC38 and then treated with daily gavage of simvastatin. MC38 tumor growth in CR mice is increased after statin treatment compared to control mice (Fig 2B & D)), even though statin enhances the mortality of MC38 *in vitro* (Supp

Fig2B). On the contrary, tumor growth in JL mice is reduced after statin treatment (Fig 2A & C). Among the immune parameters analyzed, the only secretion of IFNγ by TCD8 was significantly affected by statin treatment and inversely correlated with tumor growth (Fig 2 & Supp Fig2 C-G). In CR mice, IFNγ secretion was reduced, whereas in JL mice, IFNγ secretion was increased (Fig 2F). Tregs were not affected by statin treatment (Fig 2E). These data revealed the opposite effect of statin treatment on tumor growth, controlled by IFNγ TCD8, depending on mice suppliers.

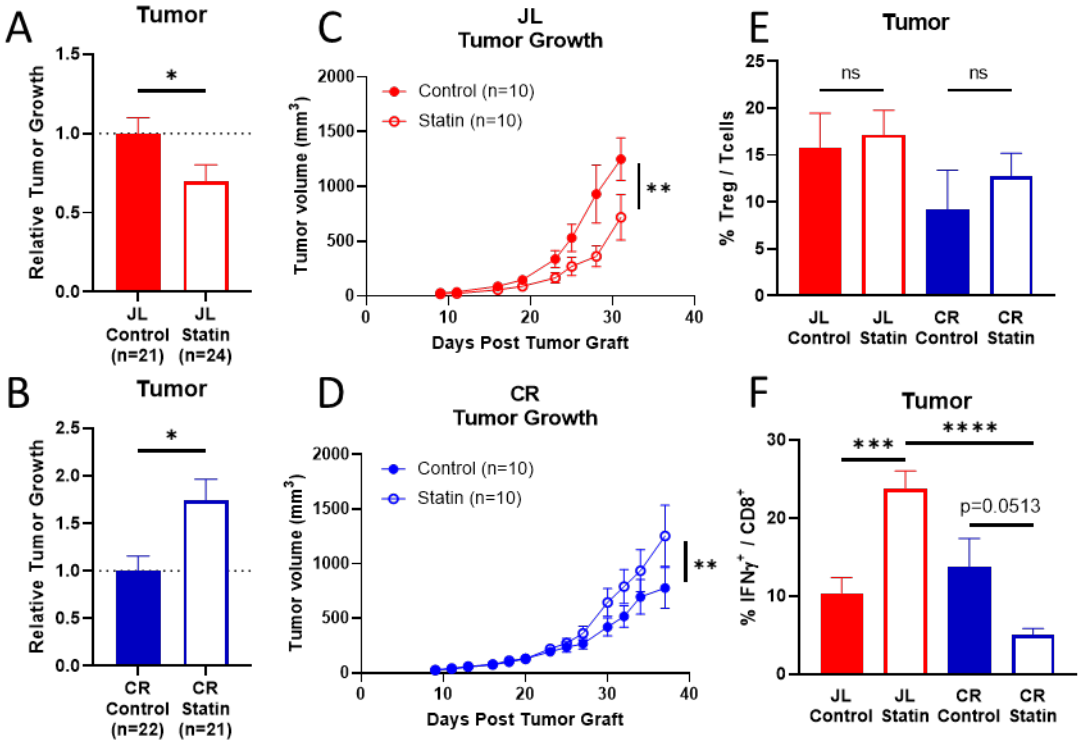

Figure 2: Providers determines statins effects on MC38 cancer tumor growth.
C57BL6J mice were grafted subcutaneously into the right flank with 1.10^6 MC38 tumor cells, then treated with simvastatin by oral gavage daily until sacrifice.
(A-B): Combined relative tumor growth from control group at the end of three independent experiments.
(C-F): Representative data from one experiment. (n=10 for each group)
(C-D): Tumor growth monitored in JL (C) and CR (D) mice.
(E-F): Frequencies of Treg (E) and $IFN\gamma^+CD8^+$ cells (F) infiltrated in the tumor. Cells were isolated from the tumor and stimulated for cytokine stimulation as described previously. Data were acquired by flow cytometry.
Data are presented as means ±SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by Mann–Whitney test).

Fecal microbiota transplantation into axenic mice replicated statins differential effects.

Inbred mice from different vendors display distinct microbial composition[53]. Despite theoretical genetics similarities, we cannot exclude genetic drifts of C57BL6J, which could underly our phenotypes and reproducibility of the generated data[75]. To confirm our findings, we, therefore,

used axenized C57BL6J mice that originated from CR and were produced by hysterectomy rederivation, and maintained in isolators under stringent handling. Mice were then transplanted with either CR or JL microbiota and were grafted with the MC38 tumor. Mice were subsequently treated to simvastatin by daily gavage as previously (Fig 3A). Similar to our previous findings, CR microbiota enhanced tumoral growth, in contrast to JL microbiota, which reduced tumoral growth upon statin treatment (Fig 3B-C). This phenotype also correlated with differential secretion of IFNγ by TCD8 (Fig 3D-E). The gut microbiota transfers experiments phenocopied results obtained with commercial mice. Altogether these data confirm that statins modulate graft colorectal tumor growth through modulation of TCD8 cells expressing IFNγ.

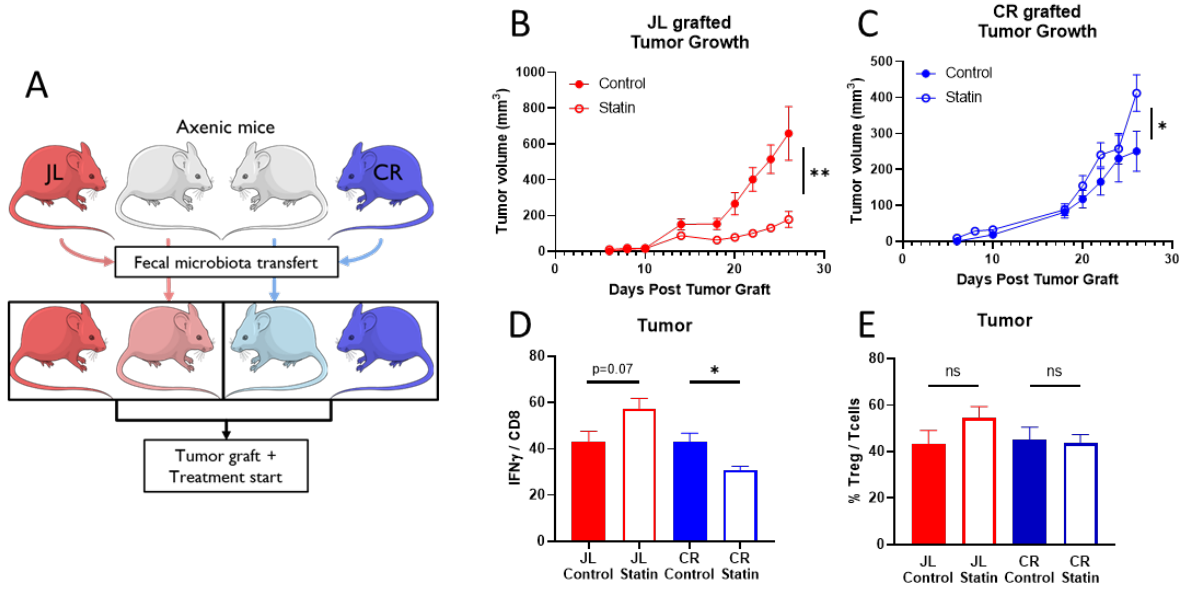

Figure 3: Fecal microbiota transfer reproduces providers effect of statins on MC38 cancer model.

(A): Axenized CR C57BL6J mice were transplanted with CR or JL microbiota, grafted subcutaneously into the right flank with 1.10^6 MC38 tumor cells then treated with simvastatin by oral gavage daily until sacrifice.

(B-C): Tumor growth monitored in JL (B) and CR (C) mice. (n=8 for each group)

(D-E): Frequencies of Treg (D) and $IFN\gamma^+CD8^+$ cells (E) infiltrated in the tumor. Cells were isolated from the tumor and stimulated for cytokine stimulation as described previously. Data were acquired by flow cytometry.

Data are presented as means ±SEM (*p<0.05, by Mann–Whitney test).

The original gut microbiome defines statin pharmacology and microbial metabolites production

Gut microbiota produces a wide range of microbial metabolites with various functions in the host, including on immune system and tumor cells. In order to better define the mechanisms related to this opposite effect of statins, we next looked at the potential culprit microbial metabolites by targeted analysis of fecal short-chain fatty acids (SCFAs) and bile acids (BAs). Indeed, SCFAs are generated by microbial fermentation of dietary polysaccharides in the gut, in particular acetate, propionate, and butyrate. Their actions are mediated through their receptor

GPR41, GPR43, GPR109A, or directly into the cell to exert their function as a histone deacetylase inhibitor (HDACi). The mechanisms of action in immune and cancer cells have already been described[4,76–78]. In control mice, the total concentration of SCFAs in feces is twice higher in JL mice compared to CR mice (Supp Fig 4A). Specifically, the three main SCFAs, acetate, butyrate, and propionate levels were lower by an average of half in CR mice compared to JL mice without treatment (Fig 4A-C). Statins' effect on acetate and butyrate production is antagonistic depending on the gut microbiota. Statin treatment significantly decreased acetate and butyrate concentrations in JL mice. In CR mice, statin treatment significantly increased butyrate concentration and tended to increase acetate levels (Fig 4A-C). Other SCFAs, isobutyrate, valerate, and isovalerate, were found at minor concentrations but were also affected (Supp Fig 4B-D).

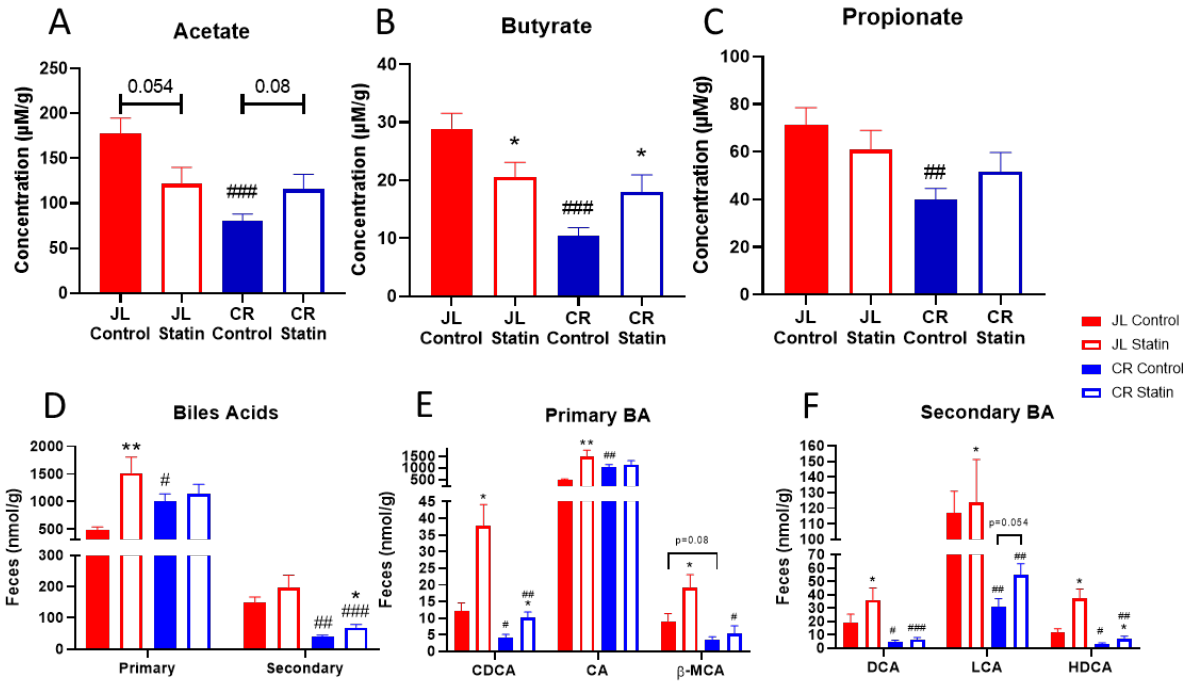

Figure 4: Microbial metabolites modulation between providers and statin treatment in supervised analysis.
Mice fecal sample were collected at the end of experiment.
(A-C): Short-Chain Fatty Acids concentrations of acetate (A), butyrate (B) and propionate (C) in feces.
(D-F): Biles acids concentrations in feces.
Data are presented as means ±SEM. * compared to control group, # compared to JL (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney test).

Bile acids (BAs) are endogenous molecules synthesized from cholesterol in the liver and are excreted into the gastrointestinal tract. BAs can then be metabolized into secondary BAs by gut microbes, which regulate the circulating bile acid composition and host metabolism. These molecules activate BAs receptors, such as TGR5, FXR, PXR, & VDR, in the gut and in the periphery[79,80]. BAs modulations have also been described in immunity and cancer[81,82]. In our study, we analyzed serum and fecal BAs at the end of the experiment. Fecal levels of primary

BAs were higher, while secondary BAs were lower in CR mice compared to JL mice. Statin treatment significantly increased primary BAs in JL mice and secondary BAs in CR mice (Fig 4D). Statin treatment increased individual concentrations of primary BAs (CDCA, CA & b-MCA) and secondary BAs (DCA, LCA & HDCA) in JL mice. However, in CR mice, statin treatment only increased CDCA for primary BAs and LCA and HDCA for secondary BAs (Fig 4E-F). The plasma analysis of primary BAs did not reveal any significant differences between the different groups (Supp Fig 4E-H). Altogether, these data show significant disparities between the two microbial ecosystems on fecal SCFAs and BAs levels before any statin treatment and profound alterations in microbial metabolites production as a result of statin treatment. These changes may contribute to the opposite outcome of the cancer burden under statin treatment.

SCFAs and BAs are not the only metabolites produced and/or regulated by the gut microbiota. Other pathways are also modulated directly or indirectly (tryptophan metabolism, aromatic amino acid metabolism, etc.)[83]. We performed an unsupervised analysis of fecal and plasma samples from the two mice providers at the end of the experimental period. Figure 5 shows the metabolites differentially detected in plasma, at least significantly in CR or JL mice after statin treatment. At the basal state, the general metabolomic profiles in JL and CR mice, is clearly distinct. Under statin treatment, these metabolomic plasma profiles are shifted in opposite directions according to the mice providers. The metabolomic profile of plasma of CR controls resembles those of JL mice under statin treatment, while JL controls resemble plasma of CR mice under statin treatment. Similarly, caecal metabolomic profiles were evaluated and are shown in supplemental Figure 5. The general trend is opposite to the plasma profiles, especially in CR mice, where significantly altered caecal metabolites are decreased upon statin treatment while they are increased in plasma. Metabolites that appear to be differentially altered upon statin treatment include bile acids and those belonging to the Krebs cycle, to tryptophan metabolism, to aromatic and other amino acids and nucleotide metabolism. Total aromatic amino acids (tAAA) levels, including phenylalanin, tyrosin and tryptophan signals, are altered both in plasma and caecal content. Plasma tAAA levels are higher in JL control mice than in CR control mice. Statins significantly increase the tAAA level in CR mice while a trend toward decreased is observed in JL mice. Interestingly kynurin/tryptophan ratio in plasma, which is an indicator of IDO activity, is higher in JL mice as compared to CR mice, although not altered by statin treatment.

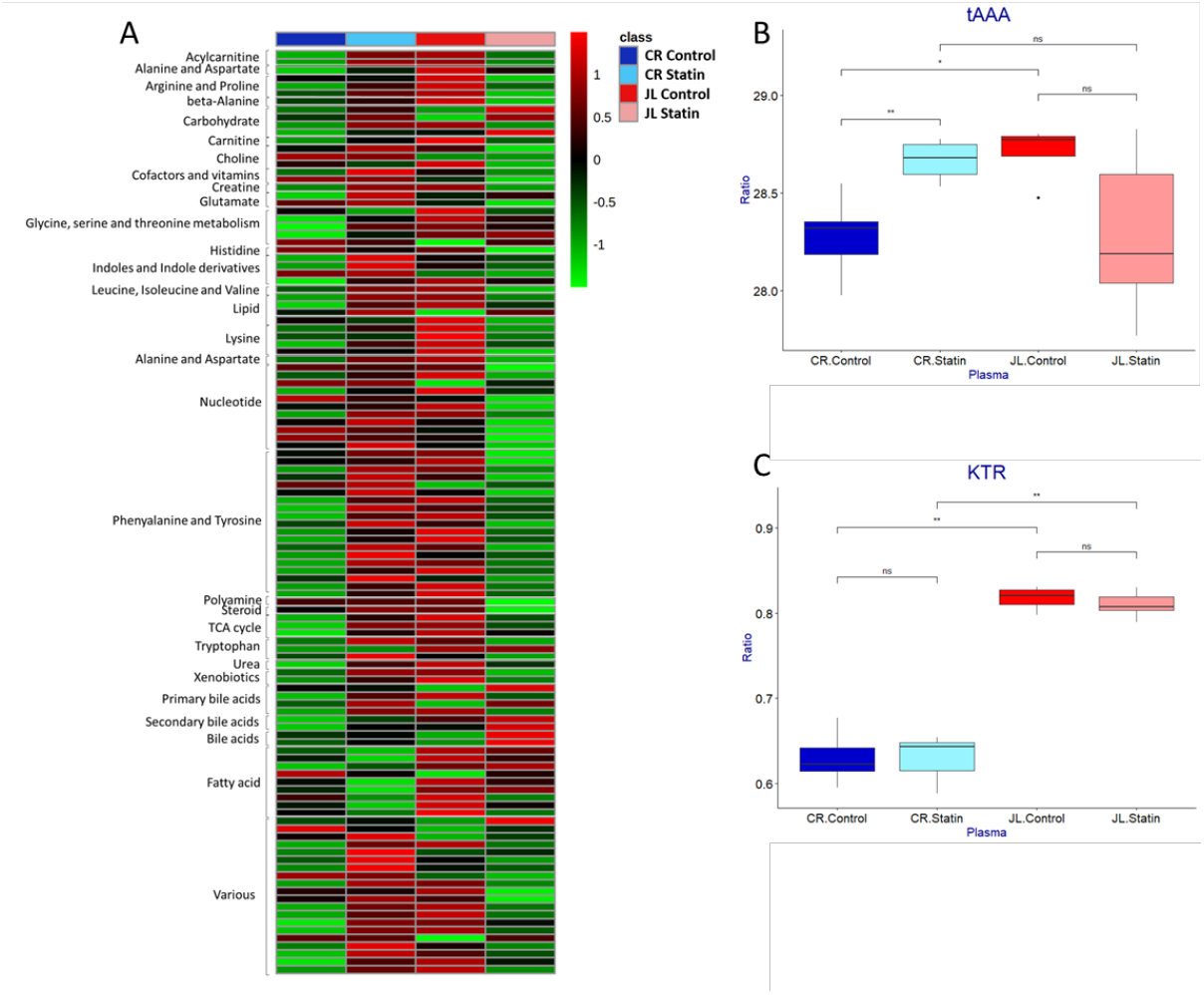

Figure 5: Plasma metabolites modulation between providers after statin treatment in untargeted analysis.

Mice plasma sample were collected at the end of experimental procedure. Plotted metabolites are significant in at least one of the comparisons (CR control versus CR statin or JL control versus JL statin)

(A) : Heatmap of metabolic pathway in different groups.

(B) : Total aromatic amino acids (tAAA): sum of phenylalanin, tyrosin and tryptophan signals

(C) : Kynurenin/Tryptophan ratio

Data are presented as means ±SEM. * compared to control group (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney test).

Discussion & Perspectives

The impact of gut microbiota on drug pharmacology affects their therapeutic efficacy in several diseases[7,11,84–86]. So does the therapeutic efficacy of statins[34,87,88]. However, these mechanisms have not yet been investigated.
In the present study, we used C57bl/6J mice from JL and CR suppliers harboring established and stable gut microbiota in equilibrium with their host immune systems. Both ecosystems displayed unique immune and metabolomic features. These experimental systems were then challenged for ectopic tumorigenesis of an immune-competent colorectal tumor cell line.

We first confirmed the immunosuppressive effect of statins regardless of the baseline microbiota. However, some differences were found in the secretion of the pro-inflammatory cytokines IFNγ and TNFα by Th1 and TCD8. These results were particularly noteworthy since the mice were genetically identical.

In inflammatory conditions (graft tumor model), the effect of statin on immune cells and cancer development were strikingly different. Indeed, the statins' effect on tumor growth is entirely opposite depending on whether the mice come from JL or CR. That is correlated with the differential secretion of IFNγ by intratumoral TCD8. These effects were also replicated by FMT, confirming the role of the gut microbiota on statin pharmacology. This antagonistic effect of statins on tumor growth mediated by the intestinal microbiota has never been described before. There is no consensus on the efficacy of statins in the prevention and treatment of cancer in humans, and no dedicated studies have been conducted. Statins have been described at their first use for their bactericidal effect. Other more recent studies have also highlighted the antimicrobial role of statins[23,30]. Since simvastatin's bioavailability is less than 5% [89], its intestinal concentration might be elevated, thus allowing a significant impact on gut microbiota composition. The selection of too different statin-resistant microbiota might lead to the production of two metabolites profiles with divergent effects on tumor growth.

We did not analyze and identify the differentiated bacterial species at steady-state and following statin treatment. However, we focused our analysis on host- and microbiota-derived metabolites such as SCFA and Bile Acids using targeted and quantitative approaches, as well as non-targeted methods. Thus, we were able to highlight differences in the production of SCFAs and BAs between the two microbiota at steady-state and following statin treatments.

Without treatment, tumor growth in JL mice is higher than in CR mice reflecting a constitutive effect of two independent ecosystems on cancer. Surprisingly the differences where not linked to changes in CD8 IFNγ frequency nor in Treg or other immune populations frequencies. As multiple mechanisms may underlies tumor growth, we can hypothesize that the observed differences could therefore be due to direct action of host- and microbial-derived metabolites on tumors cells growth rather than an effect on immune cells.
Under statin therapy, tumor growth is correlated with the fecal butyrate and acetate levels, which are all three are inversely correlated with IFNγ production by tumoral CD8 T cells.

Indeed, a decrease in butyrate and acetate levels would increase the cytotoxic potential of TCD8 by increasing the production of IFNγ in the tumor, consequently lowering tumor growth[90,91]
Yet we have not elucidated the underlying mechanisms.
However, consistent with earlier reports, SCFAs, and in particular butyrate, promote anti-inflammatory activity. Indeed, butyrate increases Treg differentiation directly through HDACi activity [92–94] or via FFAR2 (GPR43) [93,95], impact which was not found in our study, and also increases the secretion of anti-inflammatory cytokines such as IL-10 on Th1 cells via GPR43 [96]. Butyrate also alters macrophage [97–99] and dendritic cell [97,100,101] function, impairing their capacity to stimulate T lymphocytes [100,101].
As butyrate has been demonstrated to increases their IFNγ and Granzyme B secretory capacity of CD8T lymphocytes [102] and also to promotes their memory potential [103] the current scenario would therefore more likely involve a primary impact of butyrate on DC and macrophages.

We presently observed in our study that a decrease in fecal butyrate levels due to statin treatment in JL mice was correlated with increased IFNγ secretion by intratumoral TCD8.

However, we cannot determine whether a decrease in fecal butyrate level is causally associated with lower production by commensal bacteria, higher absorption into the systemic circulation and/or higher consumption by immune cells. Indeed, a lower production by commensal bacteria might lower differentiation of Tregs and improve DC maturation, thus ameliorates CD8 T cells cytotoxic functions, but a higher consumption by immune cells, which might imply preferential use of butyrate by CD8 T cells. These two mechanisms may coexist.

On the contrary, colorectal cancer patients have diminished butyrate-producing bacteria and fecal levels of SCFAs. Indeed, although butyrate is the primary source of energy for colonocytes [104] used for their proliferation and survival, the effect on colorectal cancers are the opposite: apoptotic and anti-proliferative effects [4,78]. Similarly, the consumption of dietary fibers reduce colorectal cancer risk by increasing SCFAs. The same effects are found in the genetically cancer-prone murine model (APCmin) or in the chemically-induced (DSS/AOM) colorectal cancers in the rat model [105,106].
Fecal butyrate variations found in the present study are conflicting with data from the scientific literature: a decrease in fecal butyrate results in a reduction of tumor outcome [107–110]. Several hypotheses can be proposed. (i) The effects on fecal levels of SCFAs in our study are due to statin treatment, not to dietary differences; (ii) statins have well-known bactericidal effects and can therefore affect SCFAs production ; (iii) in our study, we chose a tumor model (not induced) injected ectopically subcutaneously (and not in the colon), consequently, the fecal butyrate has no direct effect on tumor cells. Therefore the observed impact on tumor growth may pass through the systemic circulation, which is the case in patients with metastatic melanomas that display better survival associated with lower levels of serum butyrate which is also correlated with lower fecal levels [101].

Similarly, fecal acetate levels vary concomitantly with fecal butyrate levels. Acetate plays mainly a metabolic role by feeding the Krebs cycle[111]. We presently observed, a decrease in tumor growth that is associated with a reduction of fecal acetate. By considering that this decrease in fecal acetate results from reduction in acetate-producing bacteria, tumor cells may use less acetate as an energy source, thus limiting their growth [112,113]. By considering that the decrease in fecal acetate level results from increased consumption by immune cells, (ii) the acetate can be consumed by CD8 T cells, thereby activating the production and secretion of IFNγ and thus growing their anti-tumor capacity. If CD8 T cells preferentially consume acetate over tumor cells, then these two effects may coexist.
Moreover, the unsupervised analysis revealed that reduced cecal and plasma Krebs cycle metabolites were correlated with tumor growth. Similarly, the reduction of these metabolites may not serve for cancer proliferation, thus slow their growth.

Moreover, we presently observed variations in fecal concentrations of bile acids as function of either microbiota or statin treatment. Although it is generally recognized that bile acid levels increase colon carcinogenesis [115,116] or are increased in colorectal cancer at serum and fecal levels [82,117], we did not establish a correlation between bile acid levels and carcinogenesis in our study upon statin treatment. At the same time, the differences in BAs at the basal state in JL and CR mice are clearly associated with a prooncogenic effect. Among the many parameters that can influence bile acid levels: the microbiome is on the top list. It can impact levels of absorption and of conversions of primary BAs into secondary BAs [118]. Beside, BAs are derived from cholesterol, a direct target of statins thus indirectly altering the pool of BAs [26,119,120]. Furthermore, in the context of our study, we cannot exclude bile acids effects on immune cells. Indeed, bile acids have an overall anti-inflammatory effect by polarizing macrophages towards an M2 phenotype[78,121], promoting the generation and differentiation of Tregs[122,123] and inhibiting Th17 lymphocytes[123].Also, most of the described effects of BAs are prominently linked to their µmolar concentrations that can be easily achieved in gastrointestinal and livers cancers [81,124], while it is more unlikely for an ectopically injected cancer[125]. In fact, in breast cancer, BAs are associated in anti-tumoral effect[125–127].

We also used a non-targeted approach to detect fecal and serum metabolomic variations between different groups, showing variations in several metabolic pathways associated with or modified by the gut microbiota.
We noted that plasma tAAA levels, including phenylalanine, tyrosine and tryptophane signals, correlated with tumor growth. The gut microbiota can metabolize these amino acids into several metabolites[83,128]. Apart from tryptophan metabolites, their function is yet needed to be discovered. Tryptophan is one of the most studied amino acid in immunology and cancer. Tryptophan can be metabolized into several pathways, including the kynurenin pathway and the indoles pathway[129,130]. Indoles are produced by gut microbiota and can serve as AhR ligand[131]. The major tryptophan catabolism pathway, the kynurenin pathway, is initiated by IFNγ activated enzymes TDO in the liver and IDO immune and cancer cells. TDO/IDO activity can be measured by the kynurin/tryptophan ratio[129]. In our study, this ratio is higher in JL without alteration by statin treatment, revealing a higher TDO/IDO activity in these mice. Even if IDO activity is known to have an anti-inflammatory activity[132], its activation by IFNγ reveals

an established proinflammatory state, which can trigger the first immunogenic cancer death[133] in JL mice, contrary to CR mice. This may explain the later and better immune response upon statin treatment.

Other metabolic pathways are also altered in our study such as fatty acid, other amino acids or xenobiotics metabolism and may also play a role in our study. Therefore, no single metabolite or metabolic pathway is shown to be responsible for the observed effects. It is rather an entire metabolic system linked to the microbiota that is found to be modified, all converging towards different tumor immune responses.

To concluse, colorectal cancer is one of the most susceptible cancers to the gut microbiota. Some bacteria in humans are associated with a cancer incidence or, on the contrary, are protective against cancer. A non-negligible proportion of patients also present other comorbidity factors and therefore benefit from associated therapeutic management, including statins, in addition to anti-cancer therapy.
The various retrospective epidemiological studies in humans do not provide a consensus on whether statin use increases, decreases, or none at all the incidence of cancer. Since statins have bactericide properties, it was, therefore, necessary to establish the link between statins, gut microbiota, and cancer.
Our study on two different murine microbiota revealed opposite effects of statins on tumor growth, depending on the baseline microbiota. These results are inversely correlated with the secretion of IFNγ by intratumoral TCD8, and thus immune status.
In addition, our targeted and non-targeted metabolomics assays in serum and feces revealed different microbial metabolite signatures between control mice and following statin treatments. This highlights the importance of the gut microbiota in the reproducibility of pre-clinical studies.
Dedicated clinical studies are required to directly assess the benefits/risks of the host gut microbiota on statins' pharmacology and the potential incidence of cancers.
Serum and fecal microbial metabolite analysis may serve as tools to anticipate and evaluate the risks of cancer in patient receiving statin therapy and, in general, following any long-term treatment.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Designed the experiments: P.K. and P.L. Performed the experiments: P.K., F.I., E.G., S.L. and P.L. Performed the analysis: P.K., F.I., M.P., D.R., and P.L. Provided resources: G.M., M.G. and P.L., Drafted the manuscript: P.K. and P.L. Revised the manuscript :

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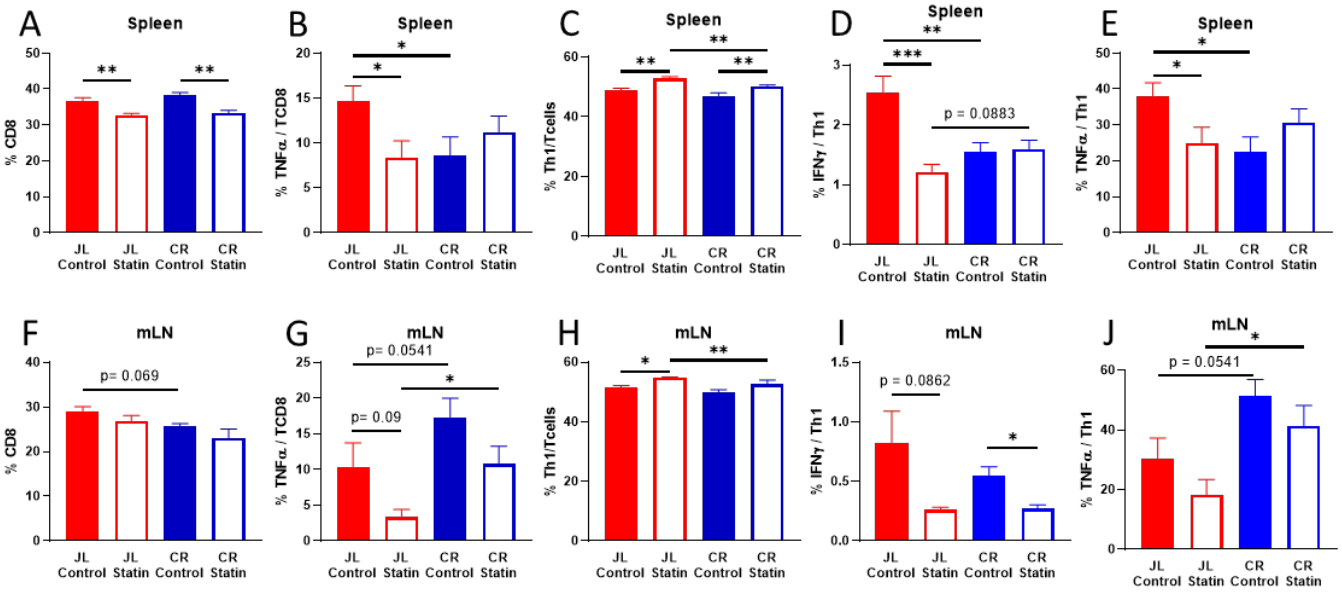
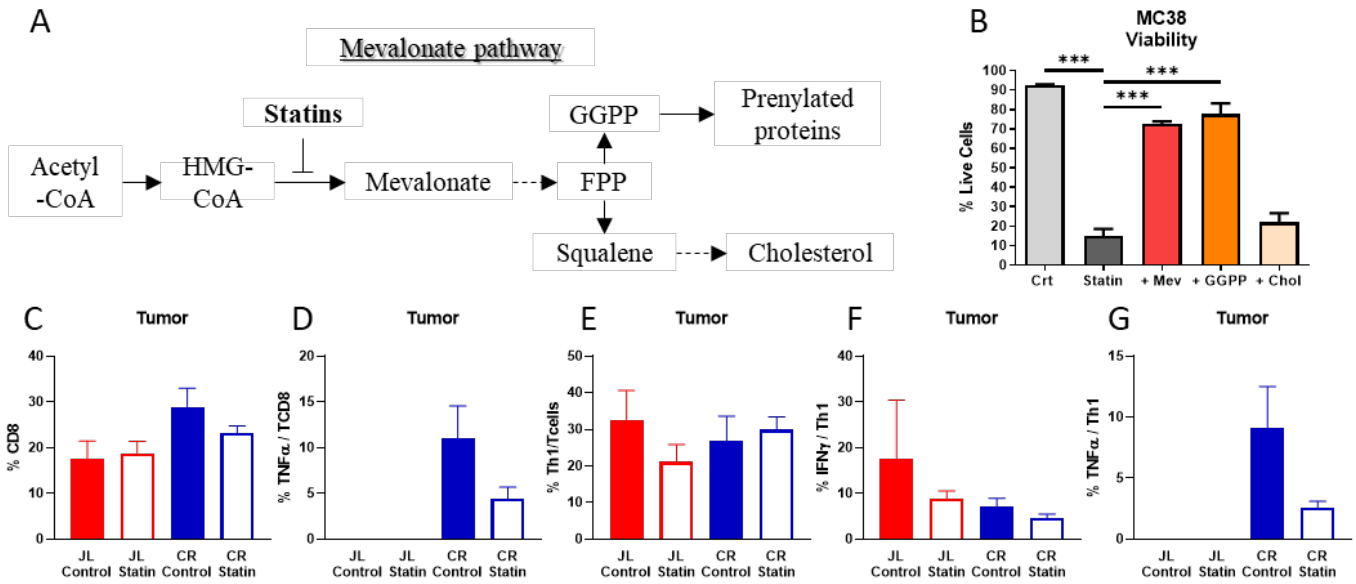
Supplemental Data:



Supplemental Figure 1: Statin immune effect at steady state in vivo (n=8 for each group).
Related to Figure 1
Frequencies of different immune population in the Spleen (A-E) and in mesenteric lymph node (F-J).
Data were acquired by flow cytometry.
Data are presented as means ±SEM (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney



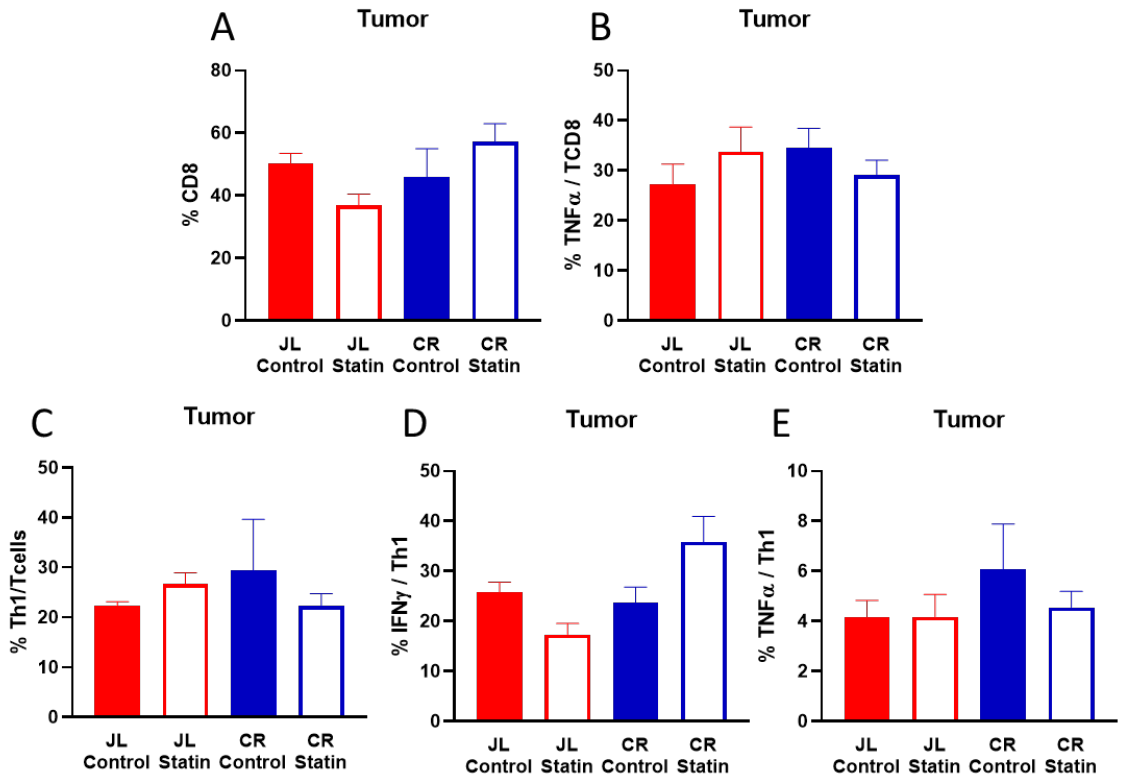
Supplemental Figure 2: Statin effect on cancer.

(A): Schematic representation of the mevalonate pathway.

(B): In vitro effect of statin. MC38 cells lines were incubated with statin alone or combined with mevalonate (Mev), GGPP or cholesterol (Chol). Crt: Control.

(C-G): *Related to Figure 2*. Frequencies of different immune population in the tumor. Data were acquired by flow cytometry.

Data are presented as means ±SEM (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney test).



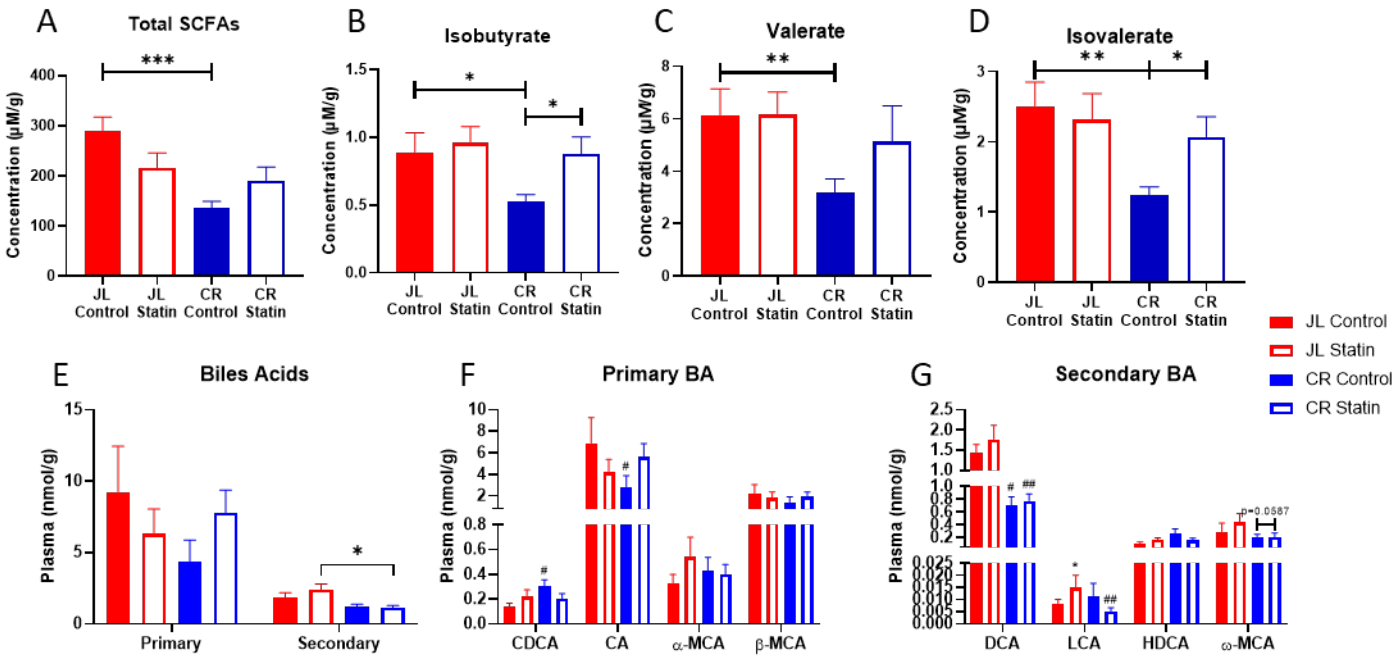
Supplemental Figure 3: Fecal microbiota transfer reproduces providers effect of statins on MC38 cancer model

Related to Figure 3

(A-E): Frequencies of different immune population in the tumor.

Data were acquired by flow cytometry.

Data are presented as means ±SEM (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney test).



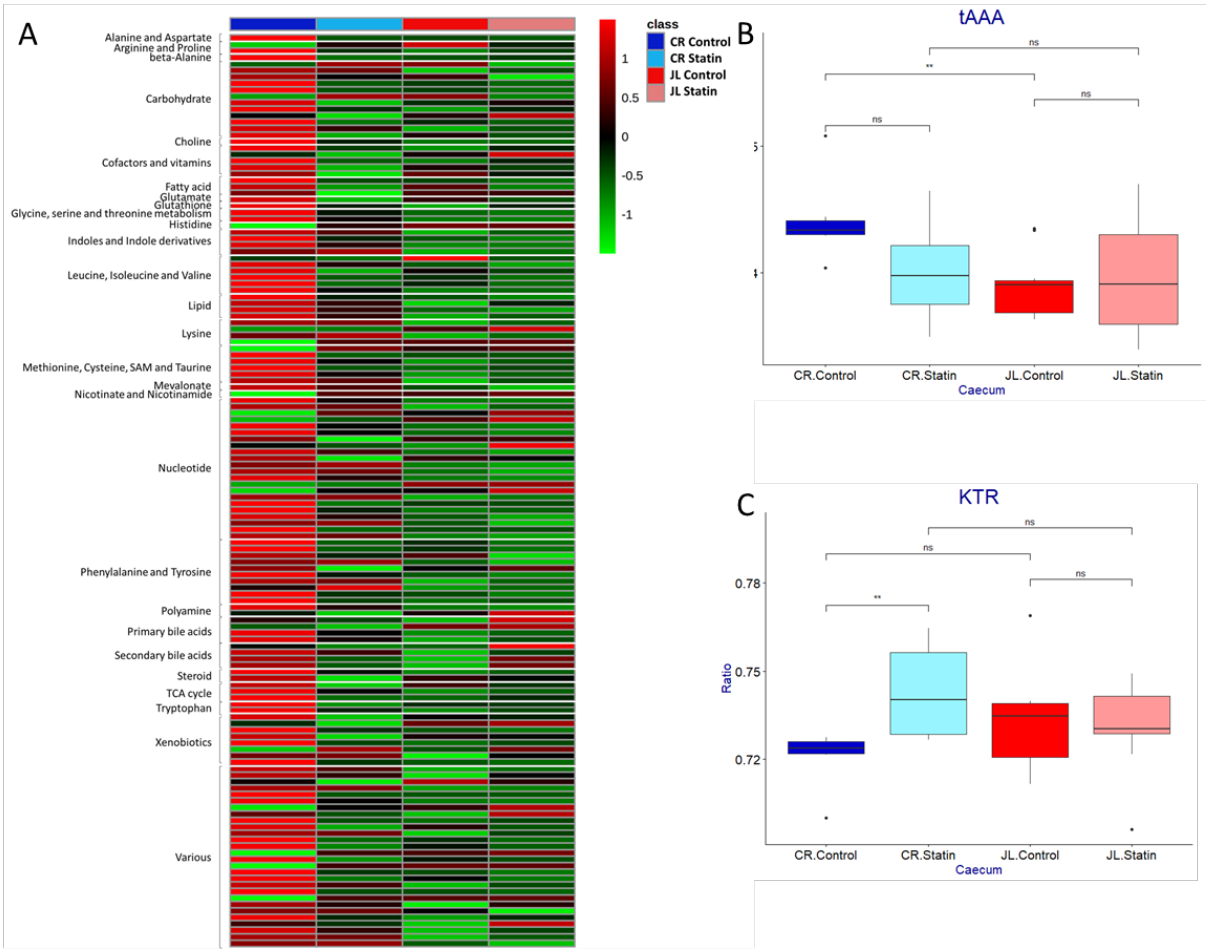
Supplemental Figure 4: Microbial metabolites modulation between providers and statin treatment in supervised analysis.

Related to Figure 4

(A-D): Total Short-Chain Fatty Acids (A), isobutyrate (B), valerate (C) & isovalerate (D) concentrations in feces.

(E-G): Biles acids concentrations in plasma

Data are presented as means ±SEM. * compared to control group, # compared to JL (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney test).



Supplemental Figure 5: Caecal metabolites modulation between providers after statin treatment in untargeted analysis.

Related to Figure 5

Mice caecal sample were collected at the end of experimental procedure. Plotted metabolites are significant in at least one of the comparisons (CR control versus CR statin or JL control versus JL statin)

(A) : Heatmap of metabolic pathway in different groups.

(B) : Total aromatic amino acids (tAAA): sum of phenylalanin, tyrosin and tryptophan signals

(C): Kynurenin/Tryptophan ratio

Data are presented as means ±SEM. * compared to control group (*p<0.05, **p<0.01)

Résultats complémentaires

Introduction

Les statines sont prescrites à des patients hypercholesterolémiques. Ces patients présentent des conditions métaboliques et microbiennes particulières et différents des personnes normocholesterolémiques. Cette physiologie particulière peut profiter à la progression et à l'extension des cellules tumorales[472,473]. En effet, le cholestérol, en tant que précurseur des acides biliaires, peut promouvoir la progression tumorale (mécanisme décrit en introduction). Le cholestérol peut également être utilisé par les cellules tumorales pour leur prolifération[474,475]. Les statines bloquant la synthèse du cholestérol, les cellules tumorales peuvent augmentent leur consommation de cholestérol exogène[474]. En effet, chez l'Homme, l'expression du récepteur au LDL (LDL-R), permettant l'import de cholestérol exogène dans les cellules, est augmentée sur les cellules tumorales, faisant du LDL-R un facteur de mauvais pronostic dans les cancers pancréatiques, urothéliales et rénales[187]. Enfin, le microbiote intestinal joue un rôle important dans la régulation des niveaux de cholestérol chez l'hôte[194].

Afin de comprendre l'influence de la cholestérolémie dans notre étude, nous avons reproduits nos expériences dans des souris déficiences pour le LDL-R (LDL-R KO). Les souris LDL-R KO sont des modèles murins couramment utilisées dans l'étude de l'athérosclérose. Ces souris sont également hypercholesterolémiques.

Matériel & Méthodes

Toutes les manipulations ont été effectuées de la même manière décrites dans l'article. Seule le modèle de souris change.

Des souris LDL-R KO mâles de 12-16 semaines ont subi une antibiothérapie dans l'eau de boisson (Néomycine, Métronidazole, Ampicilline : 10mg/mL; Vancomycine : 5mg/mL) avant le transfert fécale. Le transfert fécal a été réalisé dans les mêmes conditions décrites dans l'article.

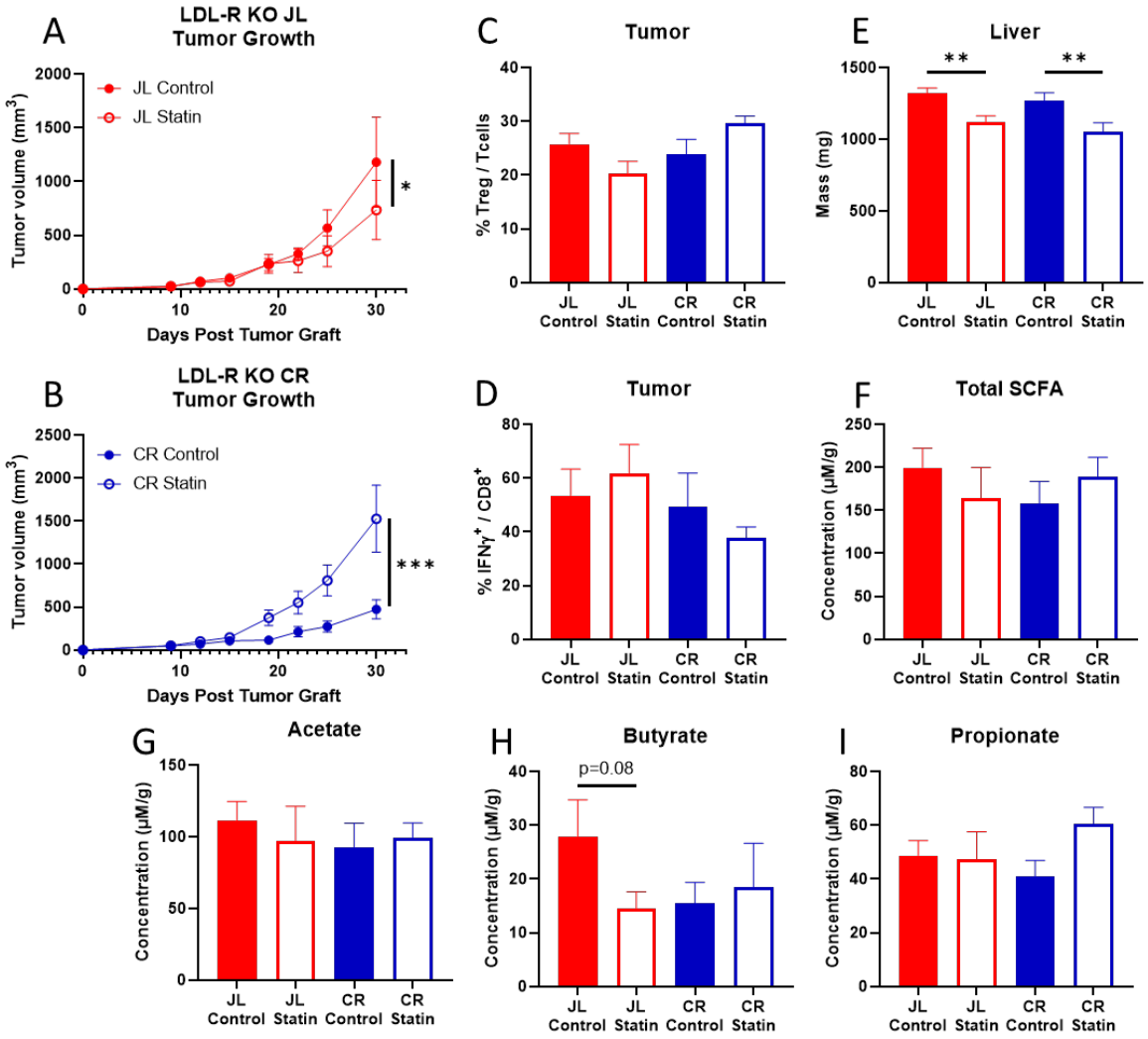


Figure : Effets de la Simvastatin sur la progression tumorale dans les souris LDL-R KO présentant un microbiote Janvier Labs (JL) ou Charles Rivers (CR).
(A-B) : Courbe de croissance tumorale.
(C-D) : Fréquences intratumorales de Tregs (C) et d'IFNg TCD8 (D).
(E) : Poids hépatiques au sacrifice
(F-I) : Concentrations fécales de SCFA totaux (F), d'acétate (G), de butyrate (H) et de propionate (I)
Data are presented as means ±SEM (*p<0.05, **p<0.01, ***p<0.001, by Mann–Whitney test).

Les différences de croissance tumorale sont reproduites dans les souris LDL-R KO (Figure A-B).

A l'état basal, la croissance tumorale dans les souris avec le microbiote de Janvier Labs (JL) est plus élevée que dans les souris avec le microbiote de Charles River Laboratories (CR). Sous traitement par la Simvastatin, la croissance tumorale des souris JL est diminuée, celle des souris CR augmentée (Figure A-B).

Au niveau immunitaire, aucune différence significative n'a été retrouvée sur les TCD8 sécrétant l'IFNγ, ni les Treg intratumoraux, bien que les tendances aient été reproduites (Figure C-D).

Enfin, les niveaux de SCFA fécaux ne diffèrent pas significativement selon les microbiotes, ni selon le traitement par les statines (F-I). On observe toutefois une tendance à la baisse du butyrate sous statine dans les souris JL (H).

Les niveaux d'acides biliaires et l'analyse non ciblée n'ont pas encore été réalisée.

Discussion

Les effets différentiels des statines sur la croissance tumorale ont pu être reproduits sur les souris LDL-R KO, tandis que les effets immunitaires et sur les SCFA semblent concordants mais pas de manière statistique. En effet, cette étude préliminaire n'a été réalisée qu'une seule fois, sur un nombre limité de souris (5 souris par groupe). Il est nécessaire de reproduire les expériences afin de confirmer ou infirmer ces résultats et surtout valider le degré d'implantation du microbiote JL et CR (analyse en cours) L'analyse concomitante des lésions d'athérosclérose sera également réalisée.

Discussion / Conclusion

A. Discussion article

L'impact du microbiote intestinal sur la pharmacologie des médicaments affecte leur efficacité thérapeutique dans plusieurs maladies[446–448,450]. Il en va de même pour l'efficacité thérapeutique des statines[460,462,463]. Cependant, ces mécanismes n'ont pas encore été étudiés.

Dans notre étude, nous avons utilisé des souris C57BL/6J de fournisseurs JL et CR abritant un microbiote intestinal bien établi et stabilisé en équilibre avec le système immunitaire de leur hôte[482]. Les deux écosystèmes présentaient des caractéristiques immunitaires et métabolomiques uniques. Nous avons greffé la lignée de cancer colorectal MC38 de manière ectopique sur les souris. Plusieurs arguments nous ont permis de choisir cette lignée tumorale. La lignée MC38 est infiltrée in vivo par les cellules immunitaires. De ce fait, elle est couramment utilisée dans les études sur la réponse immunitaire tumorale[166–169,480]. De plus, plusieurs études montrent que la croissance tumorale de ce cancer est modulée par le microbiote intestinal[216,218,481]. Enfin, la lignée MC38 est l'une des seules lignées murines tumorales à ne pas avoir été étudiée pour les effets des statines, hormis une étude publiée en 2019[386]. En somme, aucune étude n'a été réalisée sur les possibles effets des statines et du microbiote intestinal dans la réponse immunitaire tumorale. Nous avons choisi d'utiliser la Simvastatin dans notre étude pour plusieurs raisons. La Simvastatin est l'une des drogues et l'une des statines les plus consommées dans le monde[471], celle ayant le plus d'effet bactéricide[341] et sa biodisponibilité est relativement faible (<5%) (cf Introduction : Tableau 4) suggérant un fort effet potentiel sur le microbiote intestinal.

Nous avons d'abord confirmé au niveau basal l'effet immunosuppresseur de la Simvastatin indépendamment du microbiote d'origine. Cependant, certaines différences ont été constatées dans la sécrétion des cytokines pro-inflammatoires IFNγ et TNFα par lymphocytes Th1 et TCD8. Ces résultats sont particulièrement intéressants puisque les souris sont génétiquement identiques.

Les différences entre les deux microbiotes étaient plus flagrantes dans le modèle cancer.

Sans traitement, la croissance tumorale chez les souris JL est plus élevée par rapport à celle des souris CR. Les niveaux d'IFNγ$^+$ TCD8, de Treg et d'autres populations immunitaires ne sont pas différents entre les types de microbiomes. Les effets observés seraient donc dû à une action des métabolites microbiens sur les tumeurs directement.

En revanche, l'effet de la Simvastatin sur la croissance des tumeurs est diamétralement opposé selon que les souris proviennent de JL ou de CR. Cela est corrélé avec la sécrétion différentielle d'IFNγ par les TCD8 intratumoraux. Ces effets ont également été reproduits par la greffe fécale des microbiotes JL ou CR sur des souris axéniques, ce qui confirme le rôle du microbiote intestinal dans notre étude. Cet effet antagoniste des statines sur la croissance des tumeurs en fonction du microbiote intestinal n'a jamais été décrit auparavant. Chez l'Homme, il n'y a pas de consensus sur l'efficacité des statines dans la prévention et le traitement du cancer (cf Introduction : Tableau 5). Plusieurs études ont souligné le rôle antimicrobien des statines[341,449–451,478,479]. La biodisponibilité de la Simvastatin étant inférieure à 5%, son impact sur le microbiote intestinal serait prédominant dans notre étude. Ces effets bactériens des statines pourraient être à l'origine des différences observées : le déséquilibre généré pourrait entraîner la production de différents métabolites bactériens, influençant la croissance des tumeurs.

Nous n'avons pas analysé ni identifié les espèces bactériennes à l'état basal et après le traitement par les statines. Cependant, nous avons concentré notre analyse sur les métabolites microbiens tels que les SCFA et les acides biliaires dans des approches ciblées quantitatives et sur d'autres métabolites produits directement ou influencés par le microbiote intestinal dans des méthodes non ciblées.

Ainsi, nous avons pu mettre en évidence des différences de niveaux de SCFA fécaux et d'acides biliaires entre les deux microbiotes avec et sans traitement par la Simvastatin.

Sans traitement, les souris JL présentent une croissance tumorale plus élevée par rapport aux souris CR. Cela est corrélé aux taux fécaux d'acétate, de propionate et de butyrate. Sous traitement par statine, la croissance tumorale est corrélée aux taux fécaux de butyrate et d'acétate, qui sont tous les deux inversement corrélés à la production d'IFNγ par les TCD8 intratumoraux.

Les différentes études in vivo ont montré au contraire un effet protecteur des SCFA sur la colite et les cancers colorectaux[235–237,245–248,256]. Or, dans toutes ces études, les tumeurs se situaient dans le colon et non greffées ectopiquement comme dans notre étude. En effet, le butyrate a une action pro-apoptotique en agissant directement sur les cellules tumorales dans le colon[214,234]. Une étude chez l'Homme a permis de montrer que les patients atteints de mélanomes métastatiques présentaient une meilleure survie avec des niveaux de butyrate fécale plus faible[270]. Pour avoir des effets sur les mélanomes, les SCFA doivent entrer dans la

circulation systémique, ce qui est le cas dans cette étude, le butyrate sérique étant également diminué. Dans cette étude et dans la nôtre, les effets des SCFA sur la progression tumorale ne seraient pas liés à l'action directe des SCFA sur les cellules tumorales mais plutôt liée à l'action des SCFA sur le système immunitaire. Plusieurs hypothèses sur le mécanisme d'action peuvent être envisagées. D'une part, le butyrate est décrit, in vitro et in vivo, ayant une action anti-inflammatoire, en générant notamment des Treg[260–262]. Or dans notre étude, nous n'observons aucun changement sur la fréquence des Treg, sans pour autant exclure des effets sur leurs fonctions. D'autre part, d'autres études décrivent une action pro-inflammatoire du butyrate, en augmentant la capacité sécrétoire en IFNγ par les TCD8[264,265]. Cependant, dans notre étude, le butyrate est inversement corrélé à la production d'IFNγ par les TCD8 intratumoraux. On peut émettre l'hypothèse que le butyrate est consommé par les TCD8, diminuant ainsi la concentration fécale de butyrate que nous observons.

Nous avons également constaté dans notre étude des variations dans les concentrations fécales des acides biliaires. Bien qu'il soit généralement reconnu que les niveaux d'acides biliaires sériques et fécaux augmentent la cancérogenèse ou qu'ils augmentent les cancers colorectaux[280,281,298,304,289–292,294–297], nous n'avons pas établi de corrélation entre les niveaux d'acides biliaires et la cancérogenèse dans notre étude par le traitement par la Simvastatin, alors que les différences d'acides biliaires à l'état basal chez les souris JL et CR peuvent être associées à un caractère pro-tumoral. Parmi les nombreux paramètres qui peuvent influencer les niveaux d'acides biliaires : le microbiote intestinal se trouve majoritaire. Il peut influer sur les niveaux d'absorption et de conversion des acides biliaires primaires en acides biliaires secondaires[194]. De plus, les acides biliaires sont dérivés du cholestérol, une cible directe des statines, ce qui modifie indirectement le pool d'acides biliaires[484–486]. En outre, dans le cadre de notre étude, nous ne pouvons pas exclure les effets des acides biliaires sur les cellules immunitaires. En effet, il est décrit que les acides biliaires secondaires ont un effet anti-inflammatoire en induisant la génération et la différenciation des Treg[284,285], en inhibant la différenciation des lymphocytes Th17[285] et en polarisant les macrophages vers un phénotype M2[278–282]. Dans notre étude, nous n'avons pas identifié de changements sur les Treg. N'ayant pas analysé les lymphocytes Th17 et les macrophages dans notre étude, il se peut que ces cellules soient affectées, et jouent un rôle sur les phénotypes tumoraux. Enfin, la plupart des effets décrits des acides biliaires sont liés à leurs concentrations au niveau micromolaire qui peuvent être facilement atteintes dans les cancers gastro-intestinaux et hépatiques[273,487]. Cette concentration

est peu probable dans les autres cancers, où les niveaux d'acides biliaires sont plus faibles dans les cancers mammaires[310–312] et donc pour un cancer injecté par voie ectopique.

L'analyse non ciblée dans notre étude a permis de détecter des variations métabolomiques dans le sérum et dans le caecum sur les différents groupes expérimentaux. A l'état basal, le profil général des souris JL et CR est bien distinct. Le traitement par la Simvastatin modifie ces profils. Dans le sérum, la Simvastatin semble orienter le profil métabolomique des souris CR vers celui des souris JL contrôle et le profil des souris JL vers celui des souris CR contrôle. Les métabolites altérés par le traitement par la Simvastatin appartiennent au cycle de Krebs, au métabolisme des acides biliaires, du tryptophane, des acides aminés notamment aromatiques et des nucléotides.

Dans notre étude, la diminution des métabolites caecaux du cycle de Krebs, incluant l'acétate, est associée à la diminution de la croissance tumorale. Si l'on considère que cette diminution est le résultat d'une réduction des bactéries productrices d'acétate, les cellules tumorales peuvent être privées d'acétate comme source d'énergie et, par conséquent, peuvent ralentir leur croissance[488,489]. Si l'on considère que la diminution d'acétate résulte d'une consommation accrue par les cellules immunitaires, l'acétate peut être consommé par les TCD8, activant ainsi la production et la sécrétion d'IFNg[490] et augmentant ainsi leur capacité anti-tumorale. Ces deux hypothèses peuvent coexister si les TCD8 consomment l'acétate de préférence aux cellules tumorales.

Nous avons constaté que les niveaux d'acides aminés aromatiques (AAA) dans le plasma, comprenant la phénylalanine, la tyrosine et le tryptophane, étaient corrélés avec la croissance de la tumeur. Ces acides aminés peuvent être métabolisés par le microbiote intestinal en plusieurs métabolites[313,491]. En dehors des métabolites du tryptophane, leur fonction reste à être découverte. Le tryptophane est l'un des acides aminés les plus étudiés en immunologie et en cancérologie[314,315,321]. Le tryptophane peut être métabolisé en plusieurs voies, telles que la voie des kynurénines et la voie des indoles[314,321]. Les indoles sont produits par le microbiote intestinal et peuvent servir de ligand d'AhR[315,335,492,493]. La principale voie de catabolisme du tryptophane, la voie des kynurénines, est initiée par les enzymes TDO activées par l'IFNg dans le foie et les cellules immunitaires et cancéreuses IDO. L'activité TDO/IDO peut être mesurée par le rapport kynurine/tryptophane[314]. Dans notre étude, ce rapport n'est pas modifié par le traitement des statines. De manière générale, celui-ci est plus élevé dans les souris JL, ce qui révèle une activité TDO/IDO plus élevée chez ces souris. IDO est connue pour avoir une activité

anti-inflammatoire[323], son activation par l'IFNg révèle un état pro-inflammatoire établi, qui peut déclencher la mort immunogénique des cellules tumorales[494] chez les souris JL, contrairement aux souris CR. Cela peut expliquer une meilleure réponse immunitaire lors du traitement par les statines.

B. Discussion résultats complémentaires

Le cholestérol peut profiter à la progression et à l'extension des cellules tumorales[472–475]. De plus, les statines bloquant la synthèse du cholestérol, les cellules tumorales peuvent augmenter leur consommation de cholestérol exogène[474], notamment en augmentant leur expression de LDL-R[187]. Enfin, le microbiote intestinal joue un rôle important dans la régulation des niveaux de cholestérol chez l'hôte[194]. Les statines sont prescrites à des patients hypercholesterolémiques. Nous avons utilisé un modèle de souris hypercholesterolémiques LDL-R KO afin de vérifier nos précédents résultats dans un contexte d'hypercholestérolémie. Les effets différentiels des statines sur la croissance tumorale ont pu être reproduits significativement sur les souris LDL-R KO. Nous avons également retrouvé les mêmes tendances sur les effets immunitaires et des niveaux de SCFA fécaux.

Nous avons retrouvé les mêmes tendances sur la sécrétion d'IFNγ par les TCD8 intratumoraux, suggérant que les mécanismes immunitaires seraient identiques. Nous n'avons pas analysé les cellules de l'immunité innée telles que les macrophages, les DC et les NK. Ces cellules peuvent être déterminantes dans la progression tumorale et peuvent être également moduler par le microbiote intestinal. Dans le contexte de l'hypercholestérolémie, le système immunitaire est également altéré[495–497], pouvant influencé la réponse immunitaire tumorale[498].

Ce contexte particulier peut également altérer la production de métabolites microbiens. Dans notre étude, les niveaux fécaux de SCFA fluctuent de la même manière que dans les souris normocholestérolémiques, notamment avec une tendance à la baisse du niveau fécale de butyrate dans les souris JL. Il est ainsi nécessaire de reproduire ces expériences pour confirmer ces résultats préliminaires.

La modulation des acides biliaires pourrait avoir un impact plus important sur la progression tumorale à la vue de l'hypercholestérolémie mais devra être vérifiée. Enfin, tout comme dans les souris normocholestérolémiques, un seul type de métabolite ne serait pas

responsable de tout un phénotype. Il est également important de rappeler que la biodisponibilité de la Simvastatin est de moins de 5%, (cf Introduction Tableau 4) suggérant que les effets microbiens sont dominants. C'est ainsi tout un système microbien et métabolique qui se retrouve modifié, l'ensemble convergeant vers des réponses immunitaires et tumorales différentes.

De plus, l'effet pharmacologique des statines concerne uniquement l'inhibition de la synthèse du cholestérol endogène. Les cellules des souris LDL-R KO n'ont pas la capacité de métaboliser le cholestérol exogène, entrainant ainsi l'hypercholestérolémie. Il se peut que la lignée tumorale injectée, MC38, exprime le LDL-R, les privilégiant de métaboliser ce cholestérol pour des fins énergétiques et prolifératives. Cependant, nous ne savons pas si la lignée MC38 exprime le LDL-R. En revanche, nos études in vitro ont permis de montrer que cette lignée n'a pas besoin de cholestérol pour proliférer, excluant ainsi cette hypothèse.

Enfin, afin d'éviter tout biais nutritif, les souris LDL-R KO ont été nourries avec un régime normal. Sur ce modèle murin, l'apparition de l'athérosclérose se fait tardivement sauf lors des régimes gras ou hypercholestérolémiques[499]. Notre étude se faisant sur des souris assez jeunes, il se peut que l'hypercholestérolémie de ce modèle murin à cet âge soit très faible, ne permettant donc pas de voir des effets différentiels sur les SCFA. De plus, chez l'Homme, très peu de personnes sont prédisposées génétiquement à l'hypercholestérolémie[500], les patients deviennent hypercholérolémiques à cause en grande partie de facteurs environnementaux, incluant les habitudes alimentaires[501]. Il serait intéressant d'étudier l'effet du régime alimentaire sur la modification des phénotypes microbiens et tumoraux dans notre étude.

C. Perspectives générales

Dans notre étude, nous avons essentiellement analysé les lymphocytes Th1, Treg et TCD8. Les autres populations immunitaires peuvent également avoir un rôle important sur notre phénotype tumoral. Les macrophages, les DC et les lymphocytes Th17 participent à la réponse immunitaire tumorale[17,30–33,150] et leurs fonctions sont également modulés par les métabolites microbiens[207,208,502,503] et les statines[356,361,504].

Nous avons choisi d'utiliser la lignée de cancer colorectal MC38 du fait de sa susceptibilité à une modulation par le système immunitaire[166–169,480] et le microbiote intestinal[216,218,481]. Pour généraliser les effets des statines sur la modulation des métabolites

microbiens, et la tumorigénèse, il sera nécessaire de répéter ce type d'étude avec d'autres lignées tumorales et également avec des microbiotes d'origine humaines caractérisées pour leurs propriétés modulatrices de la réponse TCD8.

Nous avons validé que les effets observés sont dus à des écosystèmes microbiens intestinaux différents selon les élevages d'origine (CR et JL) grâce à la reproduction de nos résultats suite à la greffe fécale sur les souris axéniques. Il serait intéressant d'observer si les effets pro- ou anti-tumoraux des statines sont perdus lorsque les souris sont sous traitement antibiotiques. De plus, la Simvastatin étant donné en gavage, il serait intéressant d'observer sur les souris CR si, en administrant la Simvastatin par IP, on retrouve cette fois-ci un effet anti-tumoral. Enfin, nous n'avons pas analysé les populations microbiennes qui seraient responsables de nos différents phénotypes.

Le cancer colorectal est le type de cancer le plus influencé par le microbiote intestinal[505,506] du fait de sa localisation. Dans le cadre de notre étude, nous avons utilisé une lignée de cancer colorectal injectée de manière ectopique. Pour observer un effet du microbiote intestinal, les métabolites produits ou modifiés par le microbiote et susceptibles d'influencer la progression des tumeurs doivent agir à distance et circuler de manière systémique, ce qui n'est pas forcément nécessaire pour les cancers de la voie digestive. Ainsi, il serait intéressant d'étudier, dans nos conditions expérimentales, les effets des statines et du microbiote intestinal dans un modèle murin de cancer colorectal tel que les souris $APC^{min/+}$. Ce modèle se rapproche des conditions humaines de cancer colorectal et pourrait également expliquer les différents effets des statines sur les cancers colorectaux retrouvés chez l'Homme.

Nous avons effectué notre étude sur la Simvastatin exclusivement. D'autres statines ont également des effets sur le microbiote intestinal mais ne ciblent pas les mêmes espèces bactériennes[341,449–451]. De plus, les biodisponibilités entre les statines sont également différentes (Tableau 4). Des études similaires à la nôtre avec d'autres statines permettraient de déceler les effets propres aux statines en générale et les effets spécifiques de chaque statine sur le microbiote intestinal et la réponse immunitaire tumorale. Ainsi, des métabolites microbiens présents dans les fèces et/ou dans la circulation sanguine pourraient être des marqueurs prédictifs de l'incidence de cancers ou d'une réponse immunitaire tumorale efficace par les traitements aux statines. Cela pourrait également orienter les cliniciens à prescrire d'autres types de statines en fonction de chaque patient. Ce genre d'étude peut également s'appliquer à tout traitement de longue durée.

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Résumé

Les statines, prescrites dans les maladies cardiovasculaires, inhibent la synthèse endogène de cholestérol. De plus en plus de données leurs attribuent des propriétés bactéricides, immunomodulatrices et une potentielle implication dans les cancers. Cependant, chez l'Homme, aucun consensus n'est établi sur les potentiels effets bénéfiques, délétères ou l'innocuité des statines dans le cancer colorectal. Des études récentes montrent que le microbiote intestinal et les métabolites qu'il produit, influencent le développement des cancers, en particulier le cancer colorectal. Pour comprendre les mécanismes par lesquels les statines modulent le microbiote intestinal et la tumorigènèse, nous avons utilisé deux types de souris génétiquement identiques mais présentant des communautés microbiennes intestinales distinctes. Ces souris, greffées avec la lignée de cancer colorectale MC38, développent des tumeurs très différentes. Le traitement aux statines a eu des effets opposés sur la croissance des tumeurs qui était inversement corrélée avec la réponse IFNγ portée par les lymphocytes TCD8 intratumoraux. Le transfert de microbiote fécal des deux écosystèmes microbiens sur des souris axéniques a reproduit les phénotypes tumoraux et immunitaires. Ces réponses sont notamment associées à une altération importante du métabolome caecal et sérique. Plusieurs métabolites sont modulés, notamment les acides gras à chaines courtes, en particulier le butyrate, les acides biliaires et les métabolites du tryptophane. Ces études suggèrent que le microbiote intestinal joue un rôle essentiel dans la médiation des effets des statines sur la réponse immunitaire au cancer.

Statins, prescribed for cardiovascular disease, inhibit endogenous cholesterol synthesis. There is growing evidence that statins have bactericidal, immunomodulatory and cancer properties. However, in humans, no consensus has been established on the potential beneficial or harmful effects or on the innocuity of statins in colorectal cancer. Recent studies show that the gut microbiota and its metabolites influence cancers' development, particularly colorectal cancer. To understand the mechanisms by which statins modulate the gut microbiota on cancer predisposition, we used two types of genetically identical mice that carried distinct gut microbial communities and metabolites. These mice, grafted with the MC38 colorectal cancer cell line, develop very different tumors. Statin treatment had an opposite effect on tumor growth, which was inversely correlated with the IFNγ response by intratumoral TCD8 lymphocytes. Fecal microbiota transfer from both microbial ecosystems to axenic recipient mice replicated both tumor and immune phenotypes. In particular, these responses were associated with significant alterations in caecal and serum metabolome. Several bacterial metabolites were modulated, including SCFA, particularly butyrate, bile acids, and tryptophan-derived metabolites. These studies suggest that the gut microbiota plays a critical role in mediating the effects of statins on the immune response to cancer.

Liste des publications

KC P, Ichou F, Ponnaiah M, Rainteau D, Gauliard E, Lecocq S, Marodon G, Guérin M, Lesnik P. **Statin regulates microbial-derived metabolites to tune tumor growth through CD8 T cells refined response**. En préparation

Lamaziere A, Rainteau D, **KC P**, Humbert L, Gauliard E, Ichou F, Ponnaiah M , Bouby N, Salem J-E , Mallet J-M, Guerin M, Lesnik P. **Distinct postprandial bile acids responses to a high-calorie diet in Men volunteers underscore metabolically healthy and unhealthy phenotypes**. Nutrients. En revision

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Villette R, **KC P**, Beliard S, Salas Tapia MF, Rainteau D, Guerin M, Lesnik P. **Unraveling Host-Gut Microbiota Dialogue and Its Impact on Cholesterol Levels**. Front Pharmacol. 2020 Apr 3;11:278. doi: 10.3389/fphar.2020.00278. PMID: 32308619; PMCID: PMC7145900.

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Annexes

frontiers in Physiology

REVIEW
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Gut Microbiome and Space Travelers' Health: State of the Art and Possible Pro/Prebiotic Strategies for Long-Term Space Missions

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The upcoming exploration missions will imply a much longer duration than any of the missions flown so far. In these missions, physiological adaptation to the new environment leads to changes in different body systems, such as the cardiovascular and musculoskeletal systems, metabolic and neurobehavioral health and immune function. To keep space travelers healthy on their trip to Moon, Mars and beyond and their return to Earth, a variety of countermeasures need to be provided to maintain body functionality. From research on the International Space Station (ISS) we know today, that for instance prescribing an adequate training regime for each individual with the devices available in the respective spacecraft is still a challenge. Nutrient supply is not yet optimal and must be optimized in exploration missions. Food intake is intrinsically linked to changes in the gut microbiome composition. Most of the microbes that inhabit our body supply ecosystem benefit to the host-microbe system, including production of important resources, bioconversion of nutrients, and protection against pathogenic microbes. The gut microbiome has also the ability to signal the host, regulating the processes of energy storage and appetite perception, and influencing immune and neurobehavioral function. The composition and functionality of the microbiome most likely changes during spaceflight. Supporting a healthy microbiome by respective measures in space travelers might maintain their health during the mission but also support rehabilitation when being back on Earth. In this review we are summarizing the changes in the gut microbiome observed in spaceflight and analog models, focusing particularly on the effects on metabolism, the musculoskeletal and immune systems and neurobehavioral disorders. Since space travelers are healthy volunteers, we focus on the potential of countermeasures based on pre- and probiotics supplements.

Keywords: gut microbiota, spaceflight, metabolic health, musculoskeletal system, immune system, short-chain fatty acids, astronauts, circadian rhythms

INTRODUCTION

When entering microgravity the bodies system starts to adapt to the new environment. Fluid shifts from the lower into the upper part of the body and might alongside other factors, cause changes in gastrointestinal function. In combination with reduced fluid intake often seen in space travelers, this might cause reduced gastrointestinal motility. Gastrointestinal transit time has not been systematically studied during flight, but results from analog studies [rats hindlimb suspension (HU) and human short-term bed rest] show that the transit time was significantly longer than during ambulatory control periods (Lane et al., 1993; Shi et al., 2017). Lowering of mechanical loading leads to muscle breakdown and loss of bone mass (Smith et al., 2005; LeBlanc et al., 2007; Grimm et al., 2016). Data from spaceflights has also shown that most space travelers do not achieve their required energy intake through the on-board rations and typically consume about 75–80% of their daily requirements (Zwart et al., 2014). This is associated with a variety of effects on space travelers ranging from a decrease in cognitive ability to general microgravity-induced physiological responses, such as impaired cardiovascular performance, exacerbated muscle atrophy and diminished immune function (Smith et al., 2014). For shorter spaceflights up to 6 months, these effects could be reversed but when moving on to exploration missions of 1 year and longer, the effects may be much more serious and lead to mission failure, i.e., early return because of malnourishment and consequent diseases or even survival of space travelers as has been seen with some pioneering expeditions on Earth.

Inadequate energy intake has been confirmed by short-term missions (8–14 days) where total energy expenditure (TEE) was analyzed by the double-labeled water method (Lane et al., 1997). While TEE did not change in spaceflight, energy intake of these space travelers decreased leading to negative energy balances and loss in body mass (Lane et al., 1997; Stein et al., 1999b). A common cause of reduced dietary intake during the first days of a mission seems to be space motion sickness (Seddon et al., 1994; Reschke et al., 1998; Lackner and DiZio, 2006). The effects of space motion sickness typically pass after the first several days of flight, but the decreased dietary intake can extend well beyond the first week (Stein et al., 1999b). Inadequate energy intake is associated not only with loss of fat tissue, but also with decreased protein synthesis (in flight), increased protein catabolism (in bed rest), and subsequent loss of lean tissue mass (Stein et al., 1999a). Existing data suggest that systems such as muscle, bone and cardiovascular systems are adversely affected by inadequate energy intake. Studies show that undernutrition, depending on severity, exacerbates the negative effects of bed rest/spaceflight on muscle mass and strength (Biolo et al., 2007), bone mass (Ihle and Loucks, 2004), motor and cognitive function (Phillips, 1994), and the cardiovascular system (Smith et al., 2009; Florian et al., 2015).

Adaptive mechanisms in microgravity lead to an optimal state for the microgravity environment but the intention is that space travelers who fly to Moon or Mars with some level of gravity, are immediately fit for duty. They won't stay in this environment forever, they will get back to Earth and should be healthy and able – after a short recovery - to live in the 1 G environment. Many countermeasures, mainly different training regimes and modification in nutrient supply, have been tested so far but none of them fully maintains the physiological condition in a 1 G environment.

Food intake is intrinsically linked to the composition and function of the gut microbiome. Recent research provides a growing body of evidence demonstrating that host appetite and food intake are linked to the gut microbiome (Alcock et al., 2014; Fetissov, 2017). Perhaps this is due to the fact that most of the microbes that inhabit our body provide benefits to the entire host-microbe system, including production of important resources, bioconversion of nutrients, and protection against pathogenic microbes (Turroni et al., 2018). The gut microbiome is also acknowledged to be critical in maintaining immunological and neurological homeostasis. However, there may be altered (i.e., dysbiotic) gut microbial patterns, which promote intestinal inflammation and systemic low-grade inflammation. Both in turn may promote the development of several disorders, such as type 2 diabetes. Reduced insulin sensitivity has been demonstrated in various short- and longer-term space missions (Leach and Alexander, 1975; Leach and Rambaut, 1977; Stein et al., 1994; Hughson et al., 2016). Since diet is recognized as a pivotal determinant of gut microbiome composition and function (Zmora et al., 2019), changing general food habits on Earth to space food or the respective space travelers' selection might – beside other environmental factors – deeply affect the gut microbiome structure and functionality with repercussions on the space traveler's health. Extending the countermeasure portfolio by supplementing pre- and or probiotics might be of interest to support health maintenance of space travelers on exploration missions.

In this review, we aim to summarize the effects of changes in the gut microbiome seen on Earth and in microgravity, which might affect the health of space travelers during exploration missions. Our main focus is –with respect to the physiological system- changes in the gastrointestinal tract, energy intake imbalance, altered metabolism and satiety impairment, effects on the musculoskeletal and immune system and neurobehavioral health. For potential measures we review the supplementation of pre- and probiotics, since space travelers are usually healthy individuals and other strategies such as fecal microbiota transfer or application of bacterial phages, although both with great future potential in clinical practice and beyond, seem to be out of scope for the present review.

GUT MICROBIOME CHANGES IN SPACEFLIGHT AND ANALOG STUDIES

Space travels are typically associated with several stressors, including microgravity, fluid shifts, galactic cosmic radiation (beyond the Earth's Van Allen Belt), sleep deprivation with alteration of circadian rhythms, sleep quality and performance proficiency and, in general, stressful conditions due to prolonged isolation and confinement, collectively referred to as "the space exposome" (Crucian et al., 2018). Specifically, for a space traveler, the exposome is recognized to include endogenous processes

(i.e., neurohumoral regulation, aging processes and changes in metabolism and immune responses), external exposures related to spaceflight (i.e., radiation, microgravity, pathogens, dietary constraints, overloads during launch and landing, constant noise, hypodynamia and hypomagnetic fields), and the extensive and inevitable social and psychological issues. Since the early 1960s, some of these stressors have been shown in both animal and human studies, to promote gut microbiota dysbiosis, which may drive gastrointestinal disease and metabolic imbalances, as well as changes in bacterial physiology in the spaceflight environment and ground-based analog studies (Nickerson et al., 2004; Wilson et al., 2007, 2008; Barrila et al., 2010; Bailey et al., 2011; Castro et al., 2011; Crabbe et al., 2011).

Among the medical events that occurred from 1981 to 1998 on space shuttle flights, gastrointestinal problems accounted for 8% and rank third after space adaptation syndrome (42%) and neurosensory alterations (17%) (Hamm et al., 2000). The incidence of the types of illness seen during spaceflights is comparable to that observed during extended submarine missions with high rates of occurrence of gastrointestinal diseases and infections. Literature reporting effects of long-duration spaceflight on the gastrointestinal system is still limited, although space motion sickness is associated with transient decrease in normal gastric myoelectrical activity and delayed gastric emptying (Muth, 2006). Diarrhea related to overmedication for constipation has also been reported and treated according to current practices on Earth (Hamm et al., 2000). However, problems to which the gastrointestinal tract is particularly prone are infection and inflammation (Brown, 2015). In this context, the gut microbiome may play a critical role being able to exert a barrier effect against potential enteropathogens, promote the integrity of the epithelial barrier and influence immune function. Also in light of its additional extra-intestinal effects, such as those involved in metabolic and neurological homeostasis (through the gut-brain axis), the numerous disturbances in spaceflight have a strong potential to impair not only gastrointestinal homeostasis but all related symptoms (illness, immune decline, organ malfunction, muscular dystrophy, response to medications and stress).

The gut microbiome is permanently under the influence of endogenous and exogenous variables. Specific spaceflight cues such as exposure to radiation, changes in circadian rhythms and light–dark cycles, drug intake, confinement, intense exercise and microgravity, might impact the composition and functions of the microbiome as a result of continual exposure, as suggested by ground-based literature in both animals and humans (Green et al., 2008; Clark and Mach, 2017; Kaczmarek et al., 2017; Doestzada et al., 2018; Gerassy-Vainberg et al., 2018; Rothschild et al., 2018; Dong et al., 2019). Preserving eubiosis requires a definition of a healthy microbiome, which is the topic of intense research (Backhed et al., 2012; Lloyd-Price et al., 2016; He et al., 2018). High microbiome richness and diversity are generally considered as a recurrent pattern of a healthy gut ecosystem, and, consequently, a marker of stability and resilience to perturbation (Backhed et al., 2012) but there is still no consensus on the actual health-related values (Proctor, 2019) (richness is defined as the total number of bacterial species in a gut microbiome; diversity refers to the number of different species and how evenly they are distributed in a given microbiome). In many cases, decreased microbial richness will be accompanied by metabolic shifts that might be a more suitable read-out of impaired homeostasis (or intervention success) than richness or diversity. Indeed, neither microbial taxonomy (as obtained by 16S rRNA gene-based sequencing) nor the repertoire of microbial genes (as profiled by shotgun metagenomics) actually provide direct insight into active microbial functions, which will impact on the host physiology (Dorrestein et al., 2014). The identification of a core set of microbiome-produced or derived metabolites universally present in healthy individuals who lack overt disease phenotypes, under the hypothesis that alterations in their levels would indicate dysbiosis, would help rationalize preventive/therapeutic personalized countermeasures to strengthen/restore microbiome resilience to deep space exploration.

The gut microbiome of space travelers has been monitored since the early 1970s, although with different techniques over the years, mostly culture-dependent at the beginning while based on advanced omics technologies in recent years. Even the perspectives have changed over time, from the monitoring of microbial health hazards to the detection of microbiota dysbiosis and early testing of manipulation strategies toward a health-promoting layout. The progressive development of sequencing technologies has allowed researchers, since the 2000s, to explore in depth the compositional and functional structure of the gut microbiome, the possible exchange of microbiota within the crew or with the environments and its dynamics during space missions. However, it should be emphasized that to date only a few studies on the gut microbiome from spaceflight have been published so caution must be taken when interpreting the findings discussed below. Data on real missions are available from experiments in mice flown for 37 days on the ISS (Jiang et al., 2019) as well as 13 days aboard Space Shuttle Atlantis STS-135, and confirmed a higher abundance of Clostridiales and fewer Lactobacillales, in line with previous findings from Lencner et al. (1984). Mice flown for 37 days onboard ISS also demonstrated "unchanged richness of microbial community, an altered community structure and an elevated Firmicutes-to-Bacteroidetes ratio" (Jiang et al., 2019). These observations in mice are comparable with the data from a recent study carried out in twin astronauts (Garrett-Bakelman et al., 2019).

More recently, the Astronauts' Microbiome project has been specifically designed to study the impact of long-term space travel with all its relevant aspects (in terms of microgravity, g-forces, radiation and anxiety) on the microbiome of crew members and surrounding ISS environment, and the consequences on human health. Skin, saliva, nostril and fecal samples were collected from 9 astronauts prior to launch, during and after 6-month and 1-year missions, along with ISS surface swabs taken from module locations used every day, such as sleeping quarters, exercise equipment and handled microphone. In parallel, innate and adaptive immune responses were evaluated by sampling saliva and blood, and astronauts were asked to fill in an Environmental Health and Hygiene survey to recover metadata on subject health and hygiene as well as environmental factors, such as temperature and humidity.

The data demonstrates that the microbiome composition of the gastrointestinal tract, skin, nose and tongue changed in microgravity and became more similar between astronauts (Voorhies et al., 2019). However, as the authors state, it is not clear whether these microbiota alterations represent a risk to the health of astronauts. With specific regard to the gut microbiota, they report space-associated increases in the relative abundance of the beneficial butyrate producer *Faecalibacterium*, but also of *Parasutterella*, which has previously been associated with chronic intestinal inflammation. Furthermore, they found reduced proportions of genera with anti-inflammatory properties, such as *Akkermansia*, possibly contributing to the moderate increase in the inflammatory immune response observed in the crew during spaceflight. The authors therefore suggest the implementation in space of prebiotics or next-generation probiotics, such as *Akkermansia*, to reduce the risk of diseases associated with chronic inflammatory responses.

By sampling twin astronauts, one of whom stayed on the ISS for 1 year while the other on Earth, the Twins Study provided a unique opportunity to understand the health impact of long-duration spaceflight while controlling for genetics (Garrett-Bakelman et al., 2019). Through multidimensional, longitudinal assays, changes in physiological, telomeric, transcriptomic, epigenetic, proteomic, metabolomic, immune, microbiomic, cardiovascular, vision-related and cognitive parameters were assessed. Most of the biological and human health variables returned to baseline after mission but some changes persisted, including gene expression levels, increased DNA damage and number of short telomeres, and attenuated cognitive function. With specific regard to the gut microbiome, notwithstanding individual features and personalized temporal variations, more changes in microbial community composition and function were found during the flight period, with a spaceflight-specific increase in the Firmicutes-to-Bacteroidetes ratio, not persisting upon return to Earth. While in space, changes in small-molecule markers of microbial metabolism were also observed, with particularly low levels of metabolites with anti-inflammatory activity (such as 3-indole propionic acid). On the other hand, as anticipated above, the microbiome diversity remained substantially unchanged. A marked impact on the composition and functionality of the gut microbiome, without compromising individual specificity, has also recently been observed in the short term (15 and 35 days) in two spaceflight missions successfully completed from China (Liu Z. et al., 2020). In particular, according to the authors, *Bacteroides* abundance increased, consistent with simulated space environment tests, while that of the probiotic taxa *Lactobacillus* and *Bifidobacterium* decreased, possibly affecting host immune function. Furthermore, there were fluctuations in antibiotic resistance genes, mobile genetic elements, virulence genes and genes related to biofilm formation worthy of further attention, as they seem to suggest increased virulence potential and possibility of infection by opportunistic pathogens or pathobionts of the gut microbiota in space missions. Such mechanisms may parallel viral activation and infection by opportunistic pathogens as shown through the shedding of viral DNA in the body fluids of astronauts associated to the duration of spaceflight (Rooney et al., 2019).

In recent years, a number of papers have been published on space simulations, involving both animal models and human subjects (Casero et al., 2017; Turroni et al., 2017; Hao et al., 2018; Dong et al., 2019; Jiang et al., 2019). For instance, by using a mouse model for exposure to high linear energy transfer ionizing radiation (^{16}O), Casero et al. (2017) reported a pro-inflammatory dysbiotic profile (including decreased proportions of *Bifidobacterium*, *Lactobacillus* and *Clostridiaceae* members) with increased levels of metabolites mechanistically linked to gut epithelial loss (e.g., *N*-acetyl-L-citrulline) that persisted at least 30 days after a single exposure to radiation. However, it should be pointed out that ^{16}O exposures were performed at high dose rates, not actually reflecting the continuous low dose rate exposure occurring in space.

In the framework of ground-based analog studies, such as MARS500, a 520-day simulation study conducted at the Institute of Biomedical Problems of the Russian Academy of Sciences in Moscow (Russia), Turroni et al. (2017) explored the temporal dynamics of the gut microbiota of six crew members across the entire duration of the mission, including the period before entering isolation modules and after the return to regular life, up to 6 months later. Probably the most interesting fact is that some microbiota components followed similar trajectories (i.e., increased relative abundance of *Bacteroides* spp. in the very first stage of the mission and decreased proportions of some short-chain fatty acid (SCFA) producers, especially *Faecalibacterium prausnitzii*, around about 1 year of confinement), regardless of the baseline profile, that paralleled major alterations at psychological (dominance of negative feelings and increased salivary cortisol), intestinal health (positivity to the calprotectin test), and immune function level (higher lymphocyte numbers and immune responses), thus potentially serving as red flags for the space traveler's health, to identify early warning periods and promptly adopt the necessary countermeasures. A parallel experiment, MICHA (MIcrobial ecology of Confined Habitats and humAn health), has instead drawn attention to the microbiology of the environments where space travelers dwell, identifying areas with human activity as hotspots for dispersal and accumulation of crew's microorganisms, especially of potential pathogenic, stress-tolerant or mobile element-bearing microbes (Schwendner et al., 2017). More recently, ground-based space simulations have provided intriguing (although not entirely unequivocal) insights into the possibility of maintaining a eubiotic gut microbiome layout (poor in potential pathobionts while rich in health-promoting SCFA producers) through a bioregenerative life-support system (BLSS), i.e., a confined, self-sustained artificial ecosystem to biologically regenerate O_2, food, water and other basic living necessities (Hao et al., 2018; Chen et al., 2020). In short, the crewmembers followed a fixed schedule that included contact with plants for several hours a day and a high-plant high-fiber diet. Although with a certain individuality and some conflicting data, probably related to the different duration of cohabitation (60 vs. 105 days), both studies have highlighted an impact on the gut microbiome, which varies from an increase in richness and diversity, to an increased relative abundance of some SCFA producers and reduced proportions of potential pathogens. Despite the difficulties in

translating this approach into real space missions, these studies are worthwhile as they stress the importance of dietary guidance, with high fiber intake, as a potential means of balancing the gut microbiome and maintaining the space traveler's health in the long term.

GUT MICROBIOME AND METABOLIC HEALTH

Low-caloric intake with inadequate intake of micronutrients is generally associated with increased inflammation and oxidative stress, and could have possible repercussions on the functioning of the immune system (Bergouignan et al., 2016; Crucian et al., 2018). Although the space travelers' diet cannot yet be defined as optimized, considerable progress has been made since then, with the average caloric intake having been significantly increasing in recent years. However, it remains a fact that during spaceflights astronauts and cosmonauts eat less than on Earth, probably for several reasons, including but not limited to cultural habits (but the international coordination imposed by ISS is changing this aspect), the palatability of foods (still not comparable to what is available on Earth), space motion sickness, changes in light-dark cycle and appetite-regulating hormones, and, in general, stress (Laurens et al., 2019). Though the reasons are not entirely clear, impaired glucose and lipid metabolism, with insulin resistance and glucose intolerance, are also frequently observed in both spaceflights and ground-based microgravity analogs, representing a serious concern for the general health of space travelers (Tobin et al., 2002; Hughson et al., 2016; Wang Y. et al., 2019).

As a countermeasure, providing the crewmembers with balanced diets, optimized to reduce nutrient deficiency, along with functional foods/bioactive compounds might help improve energy supply and prevent nutritional imbalances, counteracting the potential downstream dysregulation of the immune system. Such diets should be rich in fibers, possibly delivered through Biological Life Support Systems (BLSSs), as non-digestible carbohydrates are well known to exert multiple benefits on human health, mediated by the gut microbiome fermentation in SCFAs (Kolodziejczyk et al., 2019). Acting as signaling molecules (e.g., through G protein-coupled receptor binding or inhibition of histone deacetylase), these microbial byproducts are recognized to be variously involved in energy extraction and storage or, more generally, in maintaining metabolic homeostasis, with some of them, especially butyrate, being potent immune modulators (Koh et al., 2016; Makki et al., 2018). For example, they have been shown to improve glucose and lipid metabolism, by inducing intestinal gluconeogenesis and production of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), and regulating lipolysis/lipid incorporation (Cani et al., 2009). Specifically, butyrate and propionate have been reported, in rats, to trigger intestinal gluconeogenesis gene expression through complementary mechanisms, i.e., by increasing the cAMP concentration in colonocytes for butyrate, and through a gut-brain neural circuit involving the GPR41 receptor for propionate, which can itself be converted into glucose. Propionate along with acetate is also a potent activator of GPR43, resulting in the secretion of GLP-1 and PYY. Acetate has been found to be responsible for the anti-lipolytic properties of SCFAs. Acting as a preferred energy source for colonocytes, butyrate is fundamental to preserve the integrity of the epithelium and maintain anaerobiosis in the gut lumen, thus limiting aerobic expansion of opportunistic pathogens. Similarly to acetate and propionate, it retains the potential to control distant organs by activating hormonal and nervous systems, and probably represents the SCFA to which most of the beneficial effects are attributed (please see also the following sections). SCFAs have also been shown to control the production of the anorexigenic hormone leptin in adipocytes, which is well known to play a central role in human basal metabolism, regulating glucose homeostasis, insulin and GLP-1 secretion, and appetite (see for a review Turroni et al., 2018). With specific regard to appetite control, a microbiome–host integrative homeostatic model has recently been proposed, according to which gut microbes may regulate intestinal release of satiety hormones and directly activate central appetite pathways mainly through molecular mimicry of microbial antigens (e.g., caseinolytic peptidase B from *Escherichia coli*) that cross-react with hunger and satiety hormones (Fetissov, 2017). Other plausible biological mechanisms involved in microbiome control of eating behavior include manipulation of reward pathways, production of mood-altering toxins, changes to taste receptors and hijacking of neurotransmission via the vagus nerve (see for a review Alcock et al., 2014).

A healthy-like gut microbiome profile, capable of producing SCFAs, especially butyrate, while low in pathobionts, could therefore be decisive in ensuring a fine regulation of host energy metabolism, by maintaining a balance between orexigenic and anorexigenic signals, especially in long-term missions, when energy deficits are no longer tolerable. It should also be remembered that microbial metabolism of fiber has additional, SCFA-independent beneficial effects, ranging from increased availability of ferulic acid and macro/micronutrients released when fibers are metabolized, to the regulation of bile acids levels (Makki et al., 2018). Of note, the use of fibers for preventive, therapeutic application has shown variable results in human intervention studies (Martinez et al., 2010; Davis et al., 2011). Such conflicting phenotypes may result from both the nature of the fibers as well as the individual basal composition in microbial enzymes supporting fibers digestion (Kovatcheva-Datchary et al., 2015; Chen T. et al., 2017). Thus, personalized nutritional approaches that evaluate space travelers' responses will have to be anticipated on the ground, based on enterotype/metabolotype identifications. In a blinded randomized controlled dietary intervention study, such an approach successfully identified microbiome-based features underlying glycemic responses (Zeevi et al., 2015).

In addition to the design of balanced diets enriched in prebiotics, probiotics-based countermeasures could also be taken into consideration. For example, in a recent spaceflight analog study based on HU mouse model, Wang Y. et al. (2019)

have shown that the supplementation of *Bifidobacterium* spp. suppressed endotoxemia and liver inflammation, and improved glucose tolerance. It is also worth noting that the relative abundance of *Akkermansia muciniphila* [found on Earth to be associated with improved metabolic health (Everard et al., 2013) and whose supplementation was recently demonstrated to improve several metabolic parameters in the first human proof-of-concept exploratory study (Depommier et al., 2019)] was significantly reduced in HU mice over time, thus paving the way for its possible use in space missions as well. As for appetite, most of the literature is consistent in reporting positive associations between probiotics (mainly lactobacilli and bifidobacteria) and increased satiety (Falcinelli et al., 2018), while one of the main goals is to have space travelers eating more with adequate macro- and micronutrient supply. Beside that, many reports, based on both analog and spaceflight studies, suggest reduced levels of SCFAs (Turroni et al., 2017; Voorhies et al., 2019). Without prejudice to the usefulness of traditional probiotics in improving several aspects of host physiology (as discussed in other sections of the present review), the available data also support the possible administration to space travelers of SCFA-producing next-generation probiotics (also called live biotherapeutics – O'Toole et al., 2017), such as *Faecalibacterium*, *Roseburia*, etc. However, it should be stressed that many of these novel probiotic candidates are still in the very early stages of the mechanistic investigation and are currently not available on the market.

Several space missions have evidenced relationships between sleep quality, circadian rhythm stability, and performance proficiency in both ground-based simulations and space mission studies. Transcriptomic profiling studies have shown that about 10 percent of our genes are under circadian control (Ueda et al., 2004). With specific regard to issues related to alterations of circadian rhythms in space travelers, the gut microbiome has recently been proposed as an endogenous circadian organizer, capable of influencing epigenetic, transcriptional and metabolic programming in the whole body, thereby impacting diurnal fluctuations of host physiology and disease susceptibility (Thaiss et al., 2016). While well-scheduled sleep, wake rhythms and meal times can serve as synchronizers (Yamamoto et al., 2015), probiotics or other microbiome-modulating approaches might help mitigate the cumulative effects of sleep and circadian disruption and enhance operational performance. For example, heat-killed *Lactobacillus brevis* SBC8803 has been shown to modulate circadian locomotion and sleep rhythms in rodents, through enhanced intestinal release of serotonin (5-HT) and efferent vagal nerve activity mediated by 5-HT3 receptors, which also resulted in increased appetite (Miyazaki et al., 2014). The research in this field is still in its beginning, largely based on animal models and therefore of limited transferability to the human system, and there is a need to better appreciate the molecular mechanisms of probiotics action if they are to be integrated into spaceflight clinical practice. Of course, one of the main challenges would be the standardization of probiotics used in a universally accepted measurement-based approach that considers personal sensibility (Suez et al., 2019).

GUT MICROBIOME AND THE MUSCULOSKELETAL SYSTEM

The most important changes caused by microgravity after long-term stay in space are bone loss and muscle atrophy that occur mostly in weight-bearing bones and their associated skeletal muscles. Regarding bones, the main deleterious mechanism appeared to be an increased bone resorption activity during spaceflight (Smith et al., 2012; Bloomfield et al., 2016). Recent evidence suggested that the gut microbiome might be a novel actor to consider in the regulation of bone physiology in health and disease. The consequences of gut dysbiosis on bone tissue involve complex mechanisms including alteration in minerals and vitamin intestinal absorption and, importantly, modulation of immunity and inflammation. It has recently been demonstrated that activation of inflammation and innate immunity by gut microbiota components increases the production of TNFα and the osteoclastogenic factor RANKL (receptor activator of nuclear factor kappa-B ligand) in bone, and as a consequence, promotes bone loss that can be estimated by a reduction in cortical bone thickness (Ohlsson et al., 2017; Ibanez et al., 2019). This effect of the microbiome on bone is supposed to be dependent on bacterial peptidoglycan sensing by the NOD receptors NOD1 and NOD2 (Ohlsson et al., 2017). Some other potential mechanisms linking the gut microbiome and bone physiology have recently emerged from studies using germ-free mice supplemented with specific bacterial strains. While not representing a model of bone loss, germ-free mice are characterized by reduced body and bone growth when compared to conventional counterparts. Fascinatingly, gut colonization of germ-free infant mice with a specific strain of *Lactobacillus plantarum* (*L. plantarum* WJL) was able to recapitulate juvenile growth, including radial and longitudinal bone growth (Schwarzer et al., 2016). The proposed mechanism for this effect is a strain-dependent stimulation of the somatotrophic axis and the production of IGF-1. The authors also discussed that optimization of enterocyte nutrient uptake and SCFA production may explain the modulation of serum IGF-1 levels (Poinsot et al., 2018). The potential effects of these bacterial strains on osteoporosis or protection against bone loss have not been reported so far.

Considering these relationships between the gut microbiome and bone homeostasis, probiotics are now suggested as an attractive strategy to protect against bone loss (Pacifici, 2018). Supplementing probiotics has been tested as a potential measure to improve the musculoskeletal system. Probiotics, for instance, can modulate the synthesis of vitamins and co-enzymes that are required for matrix formation and bone growth including vitamin D, K, C and folate. Furthermore, by producing SCFAs, they reduce intestinal tract pH and consequently increase mineral absorption (Collins et al., 2017). Accordingly, an increased production of SCFAs in the gut has been correlated with increased calcium absorption and increased bone density and strength in animal models (Chen Y. C. et al., 2017). Finally, the effects of probiotics in enhancing the epithelial barrier function, associated with the regulation of the immune response, have also been

suggested as possible contributors to their beneficial effects on bone health (Pacifici, 2018).

Studies in healthy mice and those with mild inflammation suggest that the oral administration of *Lactobacillus reuteri* ATCC PTA 6475 may, in a gender-dependent manner and with different time response, lead to significant increases in femoral and vertebral trabecular bone density, trabecular number, trabecular thickness, mineral apposition rate, bone mineral content and bone mineral density (BMD) (McCabe et al., 2013; Collins et al., 2017). Similar results have been shown in ovariectomized mice, i.e., the classical model of bone loss and osteoporosis due to estrogen deficiency (Zhao, 2013). Hence, supplementation with either *L. reuteri* ATCC PTA 6475 or the commercially available VSL#3 preparation (including *Bifidobacterium*, *Lactobacillus* and *Streptococcus* strains) decreased osteoclastogenesis and bone resorption (Britton et al., 2014; Li et al., 2016). Considering that the response to probiotic supplementation might involve a possible inhibition of inflammation, the findings indicate that in a pro-inflammatory state, probiotics reduce bone resorption and potentiate bone formation, two processes that are classically affected by inflammation (Pacifici, 2018).

Very few clinical studies on the effects of probiotic supplementation focusing on bone loss prevention in humans have been published to date. Nilsson et al. (2018) performed a double-blind, placebo-controlled study involving 70 women (75–80 years old) with low BMD supplementing *L. reuteri* or placebo. After 12 months, women in the *L. reuteri* group showed a lower loss of volumetric BMD (vBMD) compared to placebo. However, none of the secondary bone variable outcomes (BMD measured at the hip and spine; trabecular bone volume fraction; cortical vBMD and cortical thickness) was significantly affected although there was a trend for a beneficial effect for each of them. Biomarkers of bone turnover or inflammation status were unchanged (Nilsson et al., 2018).

Muscles and bones together the "forces" and "rods" of the articulation levers, are the mechanical pillars of mobility. Their development and homeostasis are intimately coordinated by the so-called mechanostat, which couples muscle activity to bone (re)modeling (Frost, 1998). Fascinatingly, it has been demonstrated in the past 10 years that skeletal muscles and bones communicate with each other through the release of hormones called myokines and osteokines, respectively (Brotto and Bonewald, 2015). In many pathophysiological situations, such as aging, immobilization, estrogen deficiency and also microgravity, there is a parallel loss of bones and muscles, suggesting common deleterious mechanisms. While this is clearly demonstrated for inflammation, even subclinical, with TNFα and several pro-inflammatory cytokines (such as IL-17) able to induce both bone loss and skeletal muscle atrophy, additional mechanisms are more specifically involved in the reduction of skeletal muscle mass and strength, such as reduced contraction activity, insulin resistance and low availability of energy fuel substrate or low level of FGF-1 (fibroblast growth factor-1) (Haran et al., 2012; Deutz et al., 2014). As summarized above for bones, there is a growing number of publications pointing to a relationship between gut microbiome and skeletal muscle physiology.

The first evidence arose from observations of changes in the microbiome composition with physical activity, both in animal models and in humans. Several studies in rodents have shown that exercise is associated with higher microbiome diversity and regulation of intestinal integrity and inflammation (Campbell et al., 2016). Some reports also showed exercise-induced changes in the gut microbiome in humans (Pedersini et al., 2020). Bressa et al. (2017) found that several health-promoting bacterial taxa (such as the SCFA producers *Faecalibacterium* and *Roseburia* and the mucin degrader *Akkermansia*) were significantly over-represented in fecal samples of women with an active lifestyle when compared to sedentary age-matched women. Until now, there are very little studies reporting the association between the gut microbiome composition and muscle in situation of muscle atrophy or sarcopenia, while it is well described that the classical consequences of gut microbiome dysbiosis, such as increased circulating levels of lipopolysaccharides, TNFα or other pro-inflammatory cytokines, are able to affect muscle protein synthesis, mitochondrial function in myotubes and skeletal muscle metabolism (Ticinesi et al., 2017; Grosicki et al., 2018).

Interestingly, several compounds and metabolites produced or modified by intestinal bacteria can enter the systemic circulation and affect skeletal muscle biology and function, such as vitamin B_{12}, folate or amino acids (like tryptophan), representing critical factors or substrates for muscle protein anabolism (LeBlanc et al., 2013). Other important gut microbiome-derived compounds able to affect skeletal muscles are SCFAs. It has been demonstrated that SCFAs can directly act on skeletal muscle cells, modulating glucose uptake and metabolism, promoting insulin sensitivity (Kimura et al., 2014) and potentially affecting mitochondrial biogenesis through activation of the NAD-dependent deacetylase sirtuin-1 (SIRT1) pathway (Ticinesi et al., 2017). Among SCFAs, butyrate was shown to increase ATP production and improve the metabolic efficiency of myofibers (Leonel and Alvarez-Leite, 2012). In aged mice, the administration of butyrate prevents muscle mass loss (Walsh et al., 2015).

Few studies have evaluated the effects of probiotics and the modulation of the gut microbiome on muscle mass and function. One of the first studies in rodents was in a leukemic mouse model in which Bindels et al. (2012) found a marked gut dysbiosis characterized by selective reduction of *Lactobacillus* spp. associated with muscle cachexia. To restore *Lactobacillus* levels, the authors treated the mice with a probiotic combination of *L. reuteri* 100-23 and *Lactobacillus gasseri* 311476, added to the drinking water for 2 weeks. This treatment was associated with increased tibialis anterior muscle mass and decreased expression of atrogenes in the muscle (MuRF1 and Atrogin-1), as well as decreased serum levels of inflammatory markers (Bindels et al., 2012). More recently, it has also been found in different mouse models of cancer that the administration of *L. reuteri* ATCC PTA 6475 in drinking water can prevent the development of cachexia (Varian et al., 2016). Interestingly, in this study, the authors demonstrated that probiotic supplementation can also protect wild-type mice from age-associated sarcopenia, through a mechanism dependent on the transcription factor Forkhead Box N1 (Varian et al., 2016). Another strain of *Lactobacillus*,

L. plantarum TWK10, has recently been demonstrated to increase lean mass and improve muscle function (grip strength and swim time tests) in healthy young mice, after oral administration for 6 weeks (Chen et al., 2016). Taken together, these different studies, although in murine model, suggest a possible link between *Lactobacillus* species and skeletal muscle mass and strength that would support further investigation in humans.

To our knowledge, there is no published clinical trial to date testing directly the effects of probiotics supplementation on muscle parameters in humans with muscle atrophy or cachexia. A recent interesting intervention study with older patients involved the administration for 13 weeks of a prebiotic formulation containing fructooligosaccharides and inulin in a randomized controlled trial with 60 volunteers (Buigues et al., 2016). In the treatment group, the subjects experienced a significant improvement in muscle function as estimated by exhaustion and handgrip strength tests (Buigues et al., 2016), supporting the concept that the modulation of the gut microbiome could affect muscle function, muscle strength and possibly muscle mass.

GUT MICROBIOME AND NEUROBEHAVIORAL DISORDERS: POTENTIAL USE OF PRO/PSYCHOBIOTICS

Another well-known threat to the success of space missions is the degradation of psychomotor functions and neurocognitive performance, occurring as a result of a multitude of mission-related environmental and psychosocial stressors (De la Torre et al., 2012; De la Torre, 2014). In light of the well-established bidirectional interactions between the gut microbiome and the brain (i.e., the gut-brain axis) (Palma et al., 2020), strategies aimed at maintaining a healthy microbiome might also be helpful in mitigating unwanted neurobehavioral effects. The gut microbiome has indeed been reported to influence, among others, stress physiology and psychology, mood, cognition, and behavior. The bidirectional gut/brain communication occurs directly and indirectly via the central and enteric nervous systems, the vagus nerve, the endocrine and immunoinflammatory systems, and through the modulation of neurotransmitters (Mazzoli and Pessione, 2016; Mittal et al., 2017; Baj et al., 2019). Moreover, gut microbes can themselves produce neuroactive compounds, such as SCFAs and tryptophan metabolites, neurotransmitters (e.g., gamma-aminobutyric acid – GABA, and nitric oxide), hormones or neurotoxic metabolites (i.e., D-lactic acid and ammonia) (Galland, 2014).

Although most of the research concerning intestinal microbiome and mental health is based on rodent studies, human studies have provided preliminary evidence that orally administered probiotics may support mental health (reviewed in Romijn and Rucklidge, 2015; Reis et al., 2018). However, since not all probiotics may be beneficial in all conditions and for all individuals (Romijn and Rucklidge, 2015; Reis et al., 2018; Suez et al., 2018; Zmora et al., 2018), selection of appropriate strains based on the baseline microbiome features and the desired clinical outcome is essential. Specifically, it has been demonstrated that probiotics can modulate the production and release of neuroactive substances. For instance, *Lactobacillus* and *Bifidobacterium* species secrete GABA, *Bifidobacterium infantis* may increase levels of tryptophan (a 5-HT precursor), and *Lactobacillus acidophilus* may modulate the expression of cannabinoid receptors (Romijn and Rucklidge, 2015; Suez et al., 2018; Zmora et al., 2018). In randomized, double-blind, placebo-controlled studies, it has also been shown that: (i) *Bifidobacterium* spp. modulate resting neural activity that correlates with enhanced vitality and reduced mental fatigue in healthy volunteers during social stress (Wang H. et al., 2019); (ii) *L. plantarum* decreases kynurenine concentration and improves cognitive functions in patients with major depression (Rudzki et al., 2019); and (iii) *L. plantarum* alleviates stress and anxiety in stressed adults through enhancement of the 5-HT pathway, as established by lower expression of plasma dopamine β-hydroxylase, tyrosine hydroxylase, indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase associated with increased expression of tryptophan hydroxylase-2 and 5-HT6 receptor (Chong et al., 2019). Interestingly, by a large-scale metagenomic study of independent microbiome population cohorts (Flemish Gut Flora Project, n = 1,054 and Dutch LifeLines DEEP cohort, n = 1,070), Valles-Colomer et al. (2019) assembled the first catalog of the neuroactive potential of the gut microbiome and evaluated its role in quality of life and depression. According to their findings, the butyrate producers *Faecalibacterium* and *Coprococcus* are consistently associated with higher quality of life indicators probably through the production of butyrate as well as of the dopamine metabolite 3,4-dihydroxyphenylacetic acid. *Coprococcus* and *Dialister* spp. were also found to be depleted in depression, even after correcting for the confounding effects of antidepressants. The authors also indicated a potential role of microbial GABA production in depression. The glutamate degradation pathway I (to crotonyl-coenzyme A and acetate) and the GABA synthesis pathway III (GABA shunt pathway), tended to be respectively depleted and increased in participants with depression, thus representing future population-based knowledge and rationally based objective for probiotic choice in clinical studies (Valles-Colomer et al., 2019). SCFAs (acetate, propionate, and butyrate) might also influence psychological functioning via interactions with G protein-coupled receptors or inhibition of histone deacetylases, and exert their effects on the brain via direct humoral effects, indirect hormonal and immune pathways and neural routes (Dalile et al., 2019). For instance, SCFAs maintain intestinal barrier integrity and protect from intestinal inflammation (Lewis et al., 2010). Butyrate, in particular, can enhance intestinal barrier function by regulating the expression of tight junction proteins, mediated by the activation of AMP-activated protein kinase (Peng et al., 2009) and downregulation of claudin 2 expression (Daly and Shirazi-Beechey, 2006). In a rodent model, *Bifidobacterium* alleviates symptom of depression and related microbiota dysbiosis, with improvement of serotonin levels and brain-derived neurotrophic factor (BDNF) concentration in brain (BDNF is essential for neuronal development and survival, synaptic plasticity, and

cognitive function), and reduced serum corticosterone level and increased cecal butyrate level, which were significantly and positively correlated with depression-related indexes (Tian et al., 2019). The health effects that bifidobacteria exert can be the result of interactions with the resident gut microbiota (Cani and Van Hul, 2015), as a result of cross-feeding interactions between bifidobacteria and butyrate-producing colon bacteria, such as *F. prausnitzii* (clostridial cluster IV), and *Roseburia* species (clostridial cluster XIVa) (Rivière et al., 2016). Hence, all these taxa (i.e., *Dialister*, *Coprococcus*, *Bifidobacterium*, *Faecalibacterium,* and *Roseburia*) could represent potential leads for psychobiotics, i.e., probiotics capable of conferring mental health benefits (Dinan et al., 2013), and whose utility in space missions deserves dedicated research.

GUT MICROBIOME AND IMMUNE SYSTEM DECLINE

The effects of spaceflight on the immune system have been reported for several decades (Cogoli, 1993; Konstantinova et al., 1995; Sonnenfeld, 1998; Stowe et al., 1999; Sonnenfeld et al., 2003). Overall, all immune populations are affected in number, proportion, generation, and/or function. In general, a decrease in immunity is observed during spaceflight. This results, in particular, in the reactivation of latent herpesviruses such as Varicella-zoster virus (VZV), Epstein-Barr Virus (EBV), and cytomegalovirus (CMV) (Mehta et al., 2013, 2017; Rooney et al., 2019). The causes of these immune changes can be directly related to spaceflight (microgravity, radiation, etc.) or indirectly (microbiome, bone metabolism, nutrition, anxiety, depression, infections, etc.), and can hardly be dissociated in the studies carried out. Most common alterations, such as an increase in white blood cells, granulocytes, and a decrease in NK cells, are generally found in both humans (Stowe et al., 1999; Crucian et al., 2000, 2015) and mouse models (Crucian et al., 2008; Gridley et al., 2009), regardless of flight time. The phagocytic function of these cells is also reduced (Konstantinova et al., 1995; Simpson et al., 2016). The effects of spaceflight on lymphocytes are less clear. The humoral response mediated by B lymphocytes is not well studied. These cells appear to be minimally involved during spaceflight, since their frequency does not change during flight but seems to be reduced on return to Earth (Tascher et al., 2019). No changes in the immunoglobulin repertoire were observed in mice (Ward et al., 2018) and space travelers studies (Stowe et al., 1999; Rykova et al., 2008). The variations observed in T lymphocytes are more complex. Although there is a decrease in their generation (Benjamin et al., 2016), their numbers remain stable (Crucian et al., 2013, 2015). However, several alterations on these cells have been observed: a decrease in intracellular trafficking (Hashemi et al., 1999; Hatton et al., 2002; Tauber et al., 2015), proliferation (Cogoli et al., 1984; Pippia et al., 1996) and function (Hashemi et al., 1999; Crucian et al., 2008; Bradley et al., 2017). Although EBV-specific T lymphocytes increased, their function is reduced (Mehta et al., 2014, 2017; Crucian et al., 2015). In addition, CD8 T lymphocytes have a more mature phenotype (Crucian et al., 2015). This suggests an unsuccessful attempt by the immune system to eliminate the reactivation of latent viruses. Immune cells secrete cytokines to regulate the immune response by activating, inhibiting and recruiting immune sub-populations. The study of cytokines varies considerably depending on the duration of the flight, cell culture systems used and mitogens added to stimulate cytokine secretion. Indeed, the different mitogens used (Concanavalin A, LPS, PMA-ionomycin, anti-CD3) differentially stimulate cytokines in peripheral blood mononuclear cells (PBMCs) (Crucian et al., 2013). In general, the pro-inflammatory cytokine IFNγ, secreted by CD4 and CD8 T lymphocytes, is decreased during and after flight (Crucian et al., 2000, 2008, 2015). Although the level of the anti-inflammatory cytokine IL-10 varies between studies, the IFNγ/IL-10 ratio remains decreased (Crucian et al., 2008), suggesting a shift in favor of the Th1/Th2 response (Mehta et al., 2013). However, the observations made following the stimulation of PBMCs are opposite to the cytokine assays directly on astronauts' plasma (Crucian et al., 2013; Mehta et al., 2013; Garrett-Bakelman et al., 2019). Interestingly, the production of IL-17, secreted by Th17 cells associated with the gut microbiota, is also altered (Crucian et al., 2013, 2015; Garrett-Bakelman et al., 2019). It is, therefore, necessary to differentiate between the secretory capacity of cytokines by immune cells and the presence of cytokines in plasma. The immune system and the intestinal microbiome are strongly linked and have been widely studied both as a consequence and as a cause of several immunosuppressed human pathologies such as cancer. Indeed, the modulation of the intestinal microbiome enhances immune response of immunotherapies in the anti-cancer response (Vetizou et al., 2015; Routy et al., 2018; Schramm, 2018). During space travel, the intestinal microbiome is also altered, and it is, therefore, difficult to assess whether it is the cause or the consequence of the observed immune changes. These close links must also receive special attention when using pre- and probiotics. Space travel alters the intestinal microbiota and thus the associated metabolic and immune functions. For example, astronauts' fiber intake is low, which may lead to a decrease in metabolites associated with the intestinal microbiota such as SCFAs. SCFAs play multiple roles in the immune system, acting directly on their target cells, which mainly carry their receptors FFAR2 (GPR43), FFAR3 (GPR41) and GPR109a, and also having histone deacetylase inhibitor (HDACi) activity. Indeed, butyrate, which can also act as a HDACi, has been shown to inhibit pro-inflammatory cytokine expression in both monocytes and macrophages while simultaneously inducing the expression of IL-10. This has been suggested via a mechanism involving the inhibition of NFκB activation (Kim, 2018). SCFAs are also able to control T cells, especially butyrate. For example, butyrate promotes the generation of regulatory T lymphocytes (Tregs) via the FFAR2 receptor (Smith et al., 2013) and HDACi activity (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013), thus shifting the immune system to a more tolerogenic phenotype. More recently, several studies support the pro-inflammatory effect of SCFAs. Indeed, acetate, propionate, and especially butyrate increase the IFNγ and Granzyme B secretory activity of CD8 T lymphocytes (Balmer et al., 2016; Luu et al., 2018) and reduce IL-17 secretion by Th17 (Luu et al., 2018) via FFAR2&3

and HDACi activity. Butyrate also allows the differentiation of activated CD8 T lymphocytes toward a memory phenotype (Bachem et al., 2019).

It is accepted that intake of fruit and vegetables-derived dietary fiber in astronauts is rather low (Crucian et al., 2018; Makki et al., 2018). Their low consumption on the ISS would result in low SCFA production. Butyrate supplementation increases the secretion of IFNγ by CD8 T lymphocytes (Luu et al., 2018). In humans, it is commonly accepted that a healthy and balanced diet, with a regular intake of dietary fiber through fruits and vegetables, allows the prevention of several diseases with immune deficiencies such as cancer (Wang et al., 2012; Aune et al., 2016), and a better immune response against pathogens (Desai et al., 2016). In view of the effects on immunity, the use of prebiotics and probiotics to stimulate the production of SCFAs would thus increase nutrient and metabolic resources and the eliminatory capacity of lymphocytes, which may limit the re-emission of latent viruses. The twin study revealed modulations of other microbial metabolites belonging to the indole family, aromatic amino acids and secondary bile acids (Garrett-Bakelman et al., 2019), which are also associated with modulation of the immune system. Indoles are synthesized by commensal bacteria from tryptophan, an essential amino acid. Indoles are non-exclusive ligands of AhR receptors expressed by immune cells, and regulate inflammation genes such as FoxP3, IL-10, IL-6, etc., allowing the preservation of intestinal homeostasis (Gao et al., 2018; Kim, 2018). Tryptophan can be metabolized by the microbiota-dependent indole pathway, the partially microbiota-dependent kynurenin pathway and the microbiota-independent serotonin pathway. The metabolism of tryptophan into these three pathways is balanced. A disruption of this balance in one of the pathways is frequently observed in several diseases (Agus et al., 2018). Synthesized by the gut microbiota from primary bile acids, secondary bile acids bind to their receptors TGR5, FXR and PXR and are implicated in several diseases (Schaap et al., 2014; Zhang et al., 2019). Secondary bile acids also play a role on immune cells by interacting with macrophages, CD4 T lymphocytes, T helper (Th1 and Th17) and Tregs, neutrophils, and NK cells, and control the production of pro-inflammatory cytokines, IFNγ, TNFα, IL-17, IL-6 (Cao et al., 2017; Van den Bossche et al., 2017; Fiorucci et al., 2018; Hang et al., 2019; Song et al., 2020). With the exception of tryptophan, the role of aromatic amino acid metabolites is less known on the immune system but they are found to be deregulated in several immune and other diseases (Liu Y. et al., 2020). All these microbial metabolites offer wide approaches in the modulation of the immune system and must be taken into account in the design of future pre- and probiotics. For example, dietary enrichment with fiber/SCFAs, tryptophan or other microbial metabolites has been shown to improve clinical outcomes in several mouse models of diseases (Matt et al., 2018) such as colitis (Islam et al., 2017; Wang et al., 2020). Interestingly, in one of the rare randomized controlled trials (RCTs) that evaluated the effect of 6-week treatment with a prebiotic/probiotic/synbiotic on immune markers in 45 healthy young individuals, the authors reported a reduction in C-reactive protein, IL-6, IL-1β, and TNFα with a more pronounced reduction in the synbiotic group (Rajkumar et al., 2015). Another RCT that evaluated prebiotic/probiotic/synbiotic effects on vaccine responses to influenza vaccination did report enhanced antibodies titers, albeit with substantial heterogeneity (Yeh et al., 2018) thus holding promise for targeting immune response through such strategies.

LIMITATIONS AND NEXT STEPS

As discussed in the sections above, there are many current shortcomings on the usability of probiotics in space. Despite encouraging data on their survival and stability in microgravity environments, the studies available on Earth do not actually allow to draw definitive conclusions on their effects on health/reconstitution of microbiomes. The sample size is sometimes inadequate, only a few strains belonging to a few genera are usually used, different methodologies are employed for sequencing and analysis of microbiome data, mechanistic information is often missing and conflicting data are sometimes reported. Furthermore, host and microbiome baseline information is very often not taken into consideration in strain selection but a one-size-fits-all approach is generally pursued (Suez et al., 2019). While the knowledge of the human gut microbiome, accelerated by next-generation sequencing, has extended the range of microorganisms with suggested health benefits (i.e., next-generation probiotics or live biotherapeutics), many of these are still at the very early stage of mechanistic investigation and only proof-of-concept exploratory studies are currently available. Future directions should therefore include changes at different levels, such as conception, research methodology and approach, which should be a precision mechanism-based one, taking into account host and microbiome features (to identify permissive vs. resistant phenotypes toward probiotics colonization, be it transient or persistent) (Suez et al., 2019), including diet.

Similarly, despite the well-recognized benefits of prebiotics on Earth, particularly those resulting from the promotion of SCFA producers (as “ecosystem service providers”), there are still several issues to deal with, such as the complexity of the mutualistic and competitive interactions that are established in the intestine, the microbiome resilience and individuality in the response to the diet, and from a practical point of view, the definition of the exact dose of fiber to be administered to obtain a certain effect and its tolerability. In this regard, a very recent study suggests that discrete dietary fiber structures may be used for precise and predictable manipulation of the gut microbiome and its metabolic functions relevant to health, by specifically directing changes in the SCFA outputs (Deehan et al., 2020). Rational, machine learning or artificial intelligence approaches are strongly advocated by the literature, to predict the effect of a specific dietary component on physiology, by addressing complex datasets of microbiome and host features (Kolodziejczyk et al., 2019).

Based on the recognized benefits of probiotics on gut microbiome and globally on health, their use either added to food or as supplements during spaceflights might be a

promising alternative to counteract the dysregulation and health outcomes encountered by space travelers. However, there are still some questions regarding the persistence of the efficacy of pro (or pre-)biotics under microgravity conditions. To test this, Castro-Wallace et al. (2017) have assessed the behavior of the probiotic strain *L. acidophilus* ATCC 4356 in a microgravity environment. They did not observe differences in growth, survival in simulated gastric or small intestinal juices, or in bacterial gene expression in comparison to control cultures, suggesting that the strain will behave similarly during spaceflight and consequently will maintain its beneficial properties (Castro-Wallace et al., 2017). Recently, Sakai et al. (2018) specifically developed a freeze-dried probiotic product for space experiments using the *Lactobacillus casei* Shirota probiotic strain, and tested its stability over 1 month of storage on the ISS. For the study, a SpaceX/Dragon spacecraft for the 8th commercial resupply mission (SpX-8) was used for the launch to the ISS and return of probiotic samples. The absorbed dose rate of the flight sample was 0.26 mGy/day and the dose equivalent rate was 0.52 mSv/day. The authors did not observe differences between the probiotic flight samples and ground controls regarding the profiles of randomly amplified polymorphic DNA, the sequence variant frequency, the carbohydrate fermentative patterns, the reactivity to strain-specific antibody, and the cytokine-inducing ability of *L. casei* Shirota. Evaluation of survival after 6 months showed that the number of viable cells in the probiotic flight samples was around 11 log CFU/g, a value comparable to that of ground controls (Sakai et al., 2018). Although these results are very encouraging, additional mechanistic studies under microgravity and simulated space environment are still needed, especially to directly test the health benefits of probiotics in space. Once the best bacterial strains will be identified and selected, clinical trials or intervention studies in space travelers should be rapidly carried out to validate their potential during long-term stay in space.

CONCLUSION

Studies available to date show that the space exposome can strongly influence the gut microbiota of space travelers, with the potential impairment of the homeostatic relationship with the host. In light of the crucial role of intestinal microbes in maintaining metabolic, immunological and neurological health, as well as of muscles and bones, strategies aimed at recovering and preserving a eubiotic microbiota profile might help mitigate the unwanted effects on the space traveler's body, thus contributing to the success of long-term missions. This could be achieved by optimizing diets to ensure adequate energy and fiber supply for SCFA production, while avoiding nutritional imbalances, as well as by integrating them with prebiotics, bioactive compounds and probiotics for potentially synergistic effects. Aside from prebiotics and bioactive compounds, probiotics, both traditional and next-generation ones, during spaceflights can be postulated as a non-invasive alternative –given that safety is assured- to protect space travelers against altered metabolism, satiety impairment, immune dysregulation, circadian rhythm changes, bone and muscle loss, as well as neurobehavioral disorders. Additional mechanistic studies under microgravity and simulated space environment, but also intervention studies and clinical trials directly in space travelers are needed to support current evidence on pre-, probiotics or combined strategies on Earth, before these microbiota manipulation tools can be integrated into spaceflight clinical practice. The use of prebiotics for the production of SCFAs is currently being investigated for space travel (Matsuda et al., 2019; Akiyama et al., 2020).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Unraveling Host-Gut Microbiota Dialogue and Its Impact on Cholesterol Levels

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Disruption in cholesterol metabolism, particularly hypercholesterolemia, is a significant cause of atherosclerotic cardiovascular disease. Large interindividual variations in plasma cholesterol levels are traditionally related to genetic factors, and the remaining portion of their variance is accredited to environmental factors. In recent years, the essential role played by intestinal microbiota in human health and diseases has emerged. The gut microbiota is currently viewed as a fundamental regulator of host metabolism and of innate and adaptive immunity. Its bacterial composition but also the synthesis of multiple molecules resulting from bacterial metabolism vary according to diet, antibiotics, drugs used, and exposure to pollutants and infectious agents. Microbiota modifications induced by recent changes in the human environment thus seem to be a major factor in the current epidemic of metabolic/inflammatory diseases (diabetes mellitus, liver diseases, inflammatory bowel disease, obesity, and dyslipidemia). Epidemiological and preclinical studies report associations between bacterial communities and cholesterolemia. However, such an association remains poorly investigated and characterized. The objectives of this review are to present the current knowledge on and potential mechanisms underlying the host-microbiota dialogue for a better understanding of the contribution of microbial communities to the regulation of cholesterol homeostasis.

Keywords: gut microbiota, microbiome, gut metabolites, cholesterol, LDL-cholesterol, cholesterolemia, dyslipidemia and cardiovascular disease

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INTRODUCTION

Atherosclerosis is the underlying cause of the majority of cardiovascular disease (CVD) events, the complications of which can be fatal (myocardial infarction, sudden death, and ischemic cerebral accidents). According to WHO projections, exposure to multiple genetic and environmental risk factors and the growing number of dysmetabolic conditions (metabolic syndrome, type 2 diabetes mellitus, obesity, non-alcoholic fatty liver diseases) will contribute to making atherosclerotic cardiovascular disease (ACVD) a leading cause of death in the world by 2030 (Kaptoge et al., 2019). Among the etiological factors of this multifactorial pathology, circulating levels of total

cholesterol (TC) or LDL-Cholesterol (LDL-C) represent major risk factors for ACVD. Consistent evidence from numerous epidemiological, clinical, and genetic studies unequivocally establishes a causal role of LDL-C in ACVD (Ference et al., 2017). A major instigating event is the recognition of oxidized-LDL-C by immune cells due to molecular mimicry with foreign antigens, thus promoting chronic inflammatory and self-perpetuating responses (Wolf and Ley, 2019).

Genetics plays an important role in regulating the levels of TC and associated lipoproteins (van Dongen et al., 2013; Hoffmann et al., 2018), yet genetic variation may account for 20% of plasma cholesterol levels (Surakka et al., 2015; Hoffmann et al., 2018). Environmental factors such as the amount and composition of the diet (Mente et al., 2017) as well as dietary cholesterol intake (Griffin and Lichtenstein, 2013) are well-established contributors; however, the individual-level contribution of intestinal microbiota to cholesterol homeostasis and the relevant pathways through which microbiota may exert their actions need to be documented and characterized. The gut microbiota functions as an endocrine system, which communicates with distal organs through metabolic pathways (Cani and Knauf, 2016). Additionally, modifications in the gut microbial ecosystem induced by external factors may cause radical changes in the symbiotic relationship between the microbiota and the host, and thus contribute to the low-grade inflammation that is constitutive of metabolic diseases (Cani et al., 2007; Fändriks, 2017). As a consequence, these modifications may account for a substantial proportion of the variation of plasma lipids, including cholesterol levels (Fu et al., 2015). In this context, the objectives of this review are to explore pre-clinical and clinical evidence and mechanisms linking gut microbiota and host-cholesterol metabolism in conditions of normal or altered homeostasis.

CLASSICAL RISK FACTORS ASSOCIATED WITH CHOLESTEROLEMIA

Genome-wide association studies (GWAS) have identified multiple human genetic variants contributing to plasma LDL-C and TC concentrations (Willer et al., 2013; Surakka et al., 2015; Liu et al., 2017; Hoffmann et al., 2018). These latter studies identified 289 and 189 independent variants significantly associated with circulating levels of TC and LDL-C, respectively (Willer et al., 2013; Liu et al., 2017). These genetic polymorphisms collectively account for the phenotypic variance of nearly 20% of TC and LDL-C (Surakka et al., 2015; Hoffmann et al., 2018). Among them, only 1.7 to 2.5% of subjects with elevated LDL-C levels were carrying the known genetic variants identified from familial hypercholesterolemia (LDLR, APOB, and PCSK9) (Abul-Husn et al., 2016; Khera et al., 2016). The results of both twin and family studies estimated a heritability of 46-57% for TC and LDL-C (Yu et al., 2005; Goode et al., 2007; van Dongen et al., 2013). In this respect, we can estimate that environmental/lifestyle factors may account for not less than 50% and up to 80% of the complementary fluctuations of TC and LDL-C. Moreover, development of atherosclerosis and regulation of plasma TC and LDL-C levels are also closely linked to consumption of dietary fatty acids, dietary fibers, carbohydrates, and alcohol, as well as to obesity, tobacco use, and level of physical activity (Yusuf et al., 2004); most of these CV risk factors are correlated with significant changes in the gut microbial ecosystem (Cerdá et al., 2016; Costantini et al., 2017; Savin et al., 2018; Medina et al., 2019; Sarin et al., 2019). Among the best known, saturated-fatty acids, trans-fatty acids, and fibers are the nutritional factors that have the most significant impact on LDL-C (Fernandez, 2001; Riccioni et al., 2012).

Reduction in body weight in severely obese subjects has a modest influence on TC and LDL-C, with each kilogram lost associated with a decrease of ~0.8 mg/dL in LDL-C. When weight reduction is even higher (e.g., bariatric surgery), the cholesterol-lowering effect is even more pronounced (Benetti et al., 2013). LDL-C can be reduced by regular physical activity (Leon and Sanchez, 2001), as suggested by new genetic variants interacting with physical activity and associated with cholesterol levels (Kilpeläinen et al., 2019). Additionally, other common causes of elevated LDL-C such as biliary obstruction, nephrotic syndrome, hypothyroidism, and pregnancy (Stone et al., 2014) have been connected to adverse effects on the gut microbiota composition (Ejtahed et al., 1969; Tsuji et al., 2018; Lv et al., 2019; Vieira-Silva et al., 2019).

THE NEW PLAYER: COMMENSAL GUT MICROBIOTA

In the last decade, research developments have positioned the commensal gut microbiota at the interface between living organisms and the environment and demonstrated its considerable influence on optimum metabolic functioning (Sekirov et al., 2010). One of the contributions of gut bacteria to host biology is the circulating pool of bacteria-derived metabolites (Nicholson et al., 2012), which can reach or exceed concentrations achieved by a typical drug dose (*m*M to mM). In many cases, these co-metabolites signal through specific receptors and impact multiple metabolic pathways and host biology. Nearly half of the circulating metabolites are believed to come from bacterial metabolism (Wikoff et al., 2009; Sridharan et al., 2014). In the symbiotic relationships established between resident microorganisms and the host, bacteria benefit from a stable environment (nutrients, temperature, pH, osmolarity, oxygen pressure), and the biological functions of the microbiota are increasingly seen as essential to health: maturation of the immune system, metabolic and nutritional functions, and protection against pathogens. A growing number of pathologies are associated with combined quantitative and qualitative dysbiotic changes in the intestinal microbiota composition and function: diabetes, obesity, cancer, inflammatory bowel disease, autoimmune and allergic diseases, autism spectrum disorders, anxiety, and depression (Lynch and Pedersen, 2016). The microbiota thus appears to be a critical player at the crossroads of physiology and multiple pathologies. It is also emerging as a

powerful transmission channel of environmental changes linked to diet and exposure to drugs, antibiotics, pollutants, and infectious agents. Microbiota modifications induced by recent changes in the human environment thus seem to be a determining factor in the current epidemic of chronic metabolic and inflammatory diseases.

More than 65 million years of mammalian-microbe co-evolution has led to an interdependence. The diversity of bacterial genes allows a wide variety of metabolic activities, such as energy extraction (5–10% of the daily energy requirements of the host) by digesting macromolecular complexes (polysaccharides, glycosaminoglycans, glycoproteins) that are not easily digestible by humans. Bacterial genes also allow the synthesis of vitamins (Yoshii et al., 2019), neurotransmitters (Onaolapo et al., 2020), and metabolites derived from tryptophan (Agus et al., 2018); they can also provide substrates that can feed critical metabolic pathways of the host (short-chain fatty acids) (Sun et al., 2017); they metabolize steroids such as cholesterol (Allayee and Hazen, 2015) or its derivatives, for instance, bile acids (Ridlon et al., 2014) and can thus influence the metabolism of lipids and cholesterol of the host. They can also contribute to or suppress the detoxification of xenobiotics and the biological activities of drugs (Koppel et al., 2017). In this ecosystem, eukaryotic and prokaryotic genes will constitute a reservoir of metabolic response that can be mobilized as a function of nutritional and xenobiotic intakes (Foster et al., 2017).

However, the complexity of intestinal microbial communities and their dialogue with the host's metabolic pathways make functional connections complicated to disentangle in these pathologies. The fundamental challenge now is to understand the causal dimension of these relationships.

OVERVIEW OF THE EPIDEMIOLOGY OF THE GUT MICROBIOTA-CHOLESTEROLEMIA RELATIONSHIP

Recent data from epidemiological studies report associations between phylum, bacterial taxa, and cholesterolemia (Koren et al., 2011; Karlsson et al., 2013; Le Chatelier et al., 2013; Fu et al., 2015) (Table 1). These data are based on microbial taxonomy derived from 16S rRNA gene sequencing (Karlsson et al., 2013; Fu et al., 2015) or whole-genome shotgun sequencing of microbial genes collectively present in feces (Karlsson et al., 2013; Le Chatelier et al., 2013), methods that reflect the current state of the art. However, these repertoires of genes or bacterial species do not make it possible to directly report microbial functions, which can vary considerably from one strain to another within the same species. Besides, the repertoire of genes identified at the bacterial DNA level does not necessarily reflect the repertoire of functions that can or will be expressed in the host. Nevertheless, cross-validation analysis on fecal taxonomy and on circulating lipid and lipoprotein levels from 893 individuals of the general Dutch population support a contribution of the microbiome to 1.5% of the variance in TC and 0.7% in LDL-C regardless of age, gender, and genetics, with the family of *Clostridiaceae/Lachnospiracease families* being specifically associated with LDL-C (Fu et al., 2015). Comparable results are found by whole-genome analysis approaches on the same cohort enlarged up to 1135 individuals (Zhernakova et al., 2016). Of note, this population is primarily composed of normolipidemic subjects displaying a mean TC and LDL-C of 1.97 ± 0.39g/L and 1.24 ± 0.36g/L, respectively. These convergent data indicate that circulating concentrations of TC and LDL-C are correlated with changes in microbiota composition, and a recent study conducted on the LifeLines-DEEP cohort (1293 subjects) supports this hypothesis. In this study, 92 plasma proteins associated with CV risk were quantified. Among them, the variance in the concentration of circulating LDL receptor is explained by microbial factors for 5%, while only 0.1% is explained by genetic factors (quantitative trait locus) (Zhernakova et al., 2018).

In patients with metabolic syndrome, interindividual variations in circulating TC and LDL-C are associated with microbial gene richness and diversity (Le Chatelier et al., 2013). The correction of diversity loss after nutritional intervention in dysmetabolic patients corrects hypercholesterolemia (Cotillard et al., 2013) and is associated with a higher abundance of *Akkermansia muciniphila* (Dao et al., 2016) (Table 1). When hypercholesterolemia coexists with obesity, hypertension, and glucose intolerance, it should be taken into account that multiple mechanisms can contribute to the

TABLE 1 | List of major clinical evidence.

Evidence	Cohort	Correlation Association	References
Epidemiological	268 healthy subjects (16S)	Enterotypes/ Cholesterol	(de Moraes et al., 2017)
	896 healthy subjects (16S)	Phylum/ Cholesterol	(Fu et al., 2015)
	1135 healthy subjects (MGS)	Taxa/ Cholesterol	(Zhernakova et al., 2016)
	Metabolic Syndrome (MGS)	Fecal microbial gene richness and diversity/Cholesterol	(Karlsson et al., 2013) (Le Chatelier et al., 2013)
	Dyslipidemic cohort	nd	nd
Dietary intervention	49 overweight/ obese adults (MGS)	Fecal microbial gene richness/ LDL-C	(Cotillard et al., 2013) (Dao et al., 2016)

nd, not done.

regulation of cholesterolemia, including pathways through which these pathologies are associated collectively (metabolic syndrome) or individually with dysbiosis (Ussar et al., 2015; Lim et al., 2017; Hoyles et al., 2018). Finally, a study in patients displaying clinical features of atherosclerosis in comparison with control subjects found associations between TC, LDL-C, and the oral abundance of some bacterial species (Koren et al., 2011).

Interestingly, studies performed in pigs, which have a metabolism and microbiome much closer to humans than rodent models, showed a significant contribution of the caecal microbiome of 5.6% to TC and of 2.8% to LDL-C (Huang et al., 2017). Additionally, most microbial taxa positively associated with TC and LDL-C belong to the pathogenic bacteria. These data are consistent with the known relationship between inflammation and serum cholesterol (Khovidhunkit et al., 2004), which needs to be further explored.

Although obtained in general populations, the influence of the gut microbiota on cholesterol levels would undoubtedly benefit from an investigation in a dyslipidemic cohort where reciprocal effects of hypercholesterolemia on microbiota functions may amplify dysbiosis and its consequences on host metabolism. Indeed, such correlative data do not establish a causal link. A disease may modify the gut microbiota, and conversely, the gut microbiota may trigger or aggravate a condition. Additionally, the bacterial species distribution is not homogeneous along the digestive tract, and fecal microbiota mostly reflects colonic species. Thus, feces analysis neglects the potential involvement of commensal species of the small intestine in dysbiosis, though this represents an essential site for the metabolism of cholesterol. Therefore, evaluation of the contribution of the microbiota to cholesterol levels is not optimal. In a recent study, albeit in a small cohort, the authors show that in hyperlipidemic patients, the higher prevalence of small intestinal bacterial overgrowth (SIBO) is positively associated with LDL-C levels (Kvit et al., 2019).

MODULATION OF THE MICROBIOTA AND ITS IMPACT ON CHOLESTEROLEMIA IN HUMANS

After birth primo-colonization of the digestive tract, the gut microbiota becomes richer and more diversified all through life as a result of environmental challenges such as those from nutritional status, cultural habits, and drug treatments (Rothschild et al., 2018). Thus, the effect of changes in diet composition, eating patterns, on cholesterolemia is likely related to the benefits of prebiotics (Beserra et al., 2015) or a Mediterranean (Filippis et al., 2016) or vegan (versus omnivorous) (Wu et al., 2016) diet, which correlate with variations in microbiota composition. Likewise, lipids are strongly modified at birth and at weaning, two periods associated with major changes in microbial composition (Nuriel-Ohayon et al., 2016) and bile acid (BA) metabolism (Jönsson et al., 1995), which can influence circulating lipid and lipoprotein concentrations (Joyce et al., 2014). When administered orally, antibiotics induce a reduction in circulating cholesterol, which is strongly associated with changes in the composition of microbiota-derived secondary BAs (Samuel and Whithe, 1961; Samuel et al., 1973; Miettinen, 1979).

GUT MICROBIOTA AND CHOLESTEROL TRAITS IN PRECLINICAL MODELS

Numerous studies that specifically evaluated the potential role of the microbiota in the regulation of cholesterol homeostasis have been conducted by using conventional approaches to eradicate the microbiota by either antibiotic therapy or an axenization procedure. These latter studies revealed that the absence of microbiota significantly influences cholesterolemia (Table 2). However, these associations are not all consistent in the normolipidemic context. Some teams show decreases in TC (Rabot et al., 2010) (Velagapudi et al., 2010; Joyce et al., 2014; Zhong et al., 2015), while others find no effect (Danielsson and Gustafsson, 1959; Sayin et al., 2013; Out et al., 2015; Caesar et al., 2016; Mistry et al., 2017; Zarrinpar et al., 2018) or even an increase (Danielsson and Gustafsson, 1959; Caesar et al., 2016). The potential underlying explanations of such variability involve (i) differences in microbiota composition between the animal facilities, (ii) normolipidemic wild type mice carry the majority of plasma cholesterol in HDL, (iii) reduced penetrance of the influence of the microbiota in a homeostatic context, as observed in the general human population (Fu et al., 2015). By contrast, in a commonly used mouse model of dyslipidemia (apolipoprotein-E and Ldl-r deficient mice), almost all studies show increased cholesterol levels in the absence of gut-microbiota (Table 2) (Wright et al., 2000; Stepankova et al., 2010; Chen et al., 2016; Kasahara et al., 2017; Lindskog Jonsson et al., 2018; Kiouptsi et al., 2019; Le Roy et al., 2019). The lipid-rich environment is associated with an impoverishment of gut microbiota diversity and richness (Martínez et al., 2013; Bo et al., 2017; Tran et al., 2019), increased intestinal barrier permeability, and endotoxemia (Netto Candido et al., 2018; Schoeler and Caesar, 2019; Wisniewski et al., 2019). Thus, in this inflammatory context, the influence of microbiota on cholesterolemia is revealed, as suggested by data obtained in the pig model (Huang et al., 2017). These findings are in agreement with data obtained in rodent models in which hypercholesterolemia associated with acute activation of innate immune receptors by endotoxin/lipopolysaccharide (LPS) is connected with an increase hepatic cholesterol synthesis and VLDL production and decreased VLDL and LDL clearance (also termed the lipemia of sepsis) (Harris et al., 2000). The underlying molecular mechanisms involve decreased nuclear receptor signaling of peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR), and retinoid X receptor (RXR) (Khovidhunkit et al., 2004). It additionally involves inhibition of reverse cholesterol transport (RCT) at multiple points, including decreased hepatic production of apolipoprotein A1, cholesterol ester transfer protein (CETP),

TABLE 2 | List of major pre-clinical evidence.

Model	Diet	Phenotype in germ-free	References
Axenic Normolipidemic Rat	CD	Chol ↗	(Danielsson and Gustafsson, 1959)
	CD with 0.5% cholesterol	Chol →	(Danielsson and Gustafsson, 1959)
Axenic Normolipidemic Mice	HFD 0.03% cholesterol	Chol ↘	(Rabot et al., 2010)
	CD*	Chol ↘	(Velagapudi et al., 2010)
	CD	Chol →	(Sayin et al., 2013)
	WD with 0.2% cholesterol	Chol ↘	(Zhong et al., 2015)
	CD source of fat: lard	Chol ↗	(Caesar et al., 2016)
	CD source of fat: fish oil	Chol →	(Caesar et al., 2016)
	CD	Chol →	(Mistry et al., 2017)
Normolipidemic Mice (axenization by a mixture of antibiotics)	CD*	Chol ↘	(Joyce et al., 2014)
	CD	Chol →	(Out et al., 2015)
	CD	Chol →	(Zarrinpar et al., 2018)
	CD	Chol →	Personal observations
Axenic Dyslipidemic Mice	Apoe$^{-/-}$ 0.15% cholesterol diet	Chol →	Wright et al., JEM 2000
	Apoe$^{-/-}$ CD	Chol ↗	(Stepankova et al., 2010)
	Apoe$^{-/-}$ 2% cholesterol	Chol ↗	(Stepankova et al., 2010)
	Apoe$^{-/-}$ CD	Chol ↗	(Kasahara et al., 2017)
	Apoe$^{-/-}$ CD	Chol ↗	(Lindskog Jonsson et al., 2018)
	Apoe$^{-/-}$ WD	Chol →	(Lindskog Jonsson et al., 2018)
	Ldlr$^{-/-}$ CD	Chol ↗	(Kiouptsi et al., 2019)
	Ldlr$^{-/-}$ 0.2% cholesterol diet	Chol →	(Kiouptsi et al., 2019)
	Apoe$^{-/-}$ CD	Chol ↗	(Chen et al., 2016)
Dyslipidemic Mice (axenization by a mixture of antibiotics)	Apoe$^{-/-}$ 0.15% cholesterol diet	Chol ↗	(Chen et al., 2016)
	Apoe$^{-/-}$ CD	Chol ↗	(Le Roy et al., 2019)
	Ldlr$^{-/-}$ CD	Chol ↗	(Le Roy et al., 2019)
Dyslipidemic Mice FMT	Apoe$^{-/-}$ CD	Cholesterol levels transmitted**	(Le Roy et al., 2019)

*All studies involved mice of C57Bl6 genetic background, except for two studies that used Swiss Webster mice (indicated by *). CD, chow diet; HFD, high-fat diet; WD, western diet; FMT, fecal material transfer.*
***Phenotype in recipient mice.*
↗ Increased; ↘ decreased; → no changes.

ATP binding cassette transporters ABCG5 and ABCG8, and Cyp7a1. These findings are consistent with the association found of the lipopolysaccharide receptor Toll-like receptor 4 (TLR4) and NIMA-related kinase 7 (NEK7) polymorphisms with LDL-C in human (Zhu et al., 2015; Gomes Torres et al., 2019). NEK7 is a serine/threonine kinase required for NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome assembly. However, this relationship should be tempered as chronic TLR-signaling deficiency in MyD88$^{-/-}$/Apoe$^{-/-}$ (Björkbacka et al., 2004) (Michelsen et al., 2004), TLR4$^{-/-}$/Ldl-r$^{-/-}$, (Ferreira et al., 2015), TLR2$^{-/-}$/Apoe$^{-/-}$, and TLR4$^{-/-}$/Apoe$^{-/-}$ (Higashimori et al., 2011) mice is not associated with changes in cholesterol levels as compared to control mice. It should also be noted that the evidence for the role of microbiota in genetically modified mice is difficult to interpret, as numerous studies in the literature do not report the experimental conditions (production of experimental groups, use of littermates, housing conditions). Indeed, fecal microbiota is partially normalized by extended co-housing conditions, due to coprophagic and grooming behaviors, thus abrogating microbiota-genotype dependent phenotype.

Finally, using a standardized method, our recent work (Le Roy et al., 2019) demonstrates the microbiota-dependent transmissibility of a significant proportion of the cholesterol level (around 15-20%). Indeed, transplantation of the microbiota from hypercholesterolemic (without known genetic cause) human donors into recipient mice is sufficient to transfer the phenotype compared to the same experiment performed with normolipidemic donors. The more hypercholesterolemic phenotype is associated with "low hepatic cholesterol synthesis" and "high intestinal cholesterol absorption" traits in recipient mice. Several bacterial phylotypes affiliated with *Beta-proteobacteria phylum*, *Alistipes genus, and Barnesiella genus* were enriched in hypercholesterolemic mouse recipients. Similarly, *Alistipes* were recently associated with TC and LDL-C in HFD-fed hamsters treated with a chitin9-derived polysaccharide (chitosan) (Tong et al., 2019).

It is also of particular clinical interest to show the influence of the intestinal microbiota on the balance between absorption and cholesterol synthesis (Le Roy et al., 2019) since it has been observed in human cohorts that "high absorption" and "low synthesis" patterns are associated with higher LDL-C levels and are predictive of cardiovascular events (Matthan et al., 2009; Silbernagel et al., 2010; Weingärtner et al., 2011). Thus, inter-individual evaluation of microbiota diversity or dysbiosis opened up new opportunities for better therapeutic decision-making in ASCVD.

GUT BACTERIAL METABOLITES, THE NEW FRONTIER FOR DEFINING PATHOLOGICAL METABOLOTYPES

Primary Bile Acids

Bile acid (BA) biosynthesis is the predominant metabolic pathway for cholesterol catabolism in the human body. The

conversion of cholesterol to bile acids is a process performed by a set of hepatic enzymes necessary for the conversion of the steroid nucleus of cholesterol, the elimination of the side chain, amidation on the side chain with either glycine or taurine (GCA, GCDA, TCA, TCDCA) and eventually sulfonation or glucuronidation in the steroid backbone (Hofmann and Hagey, 2014). Another critical aspect of BA physiology is their circulation in the enterohepatic cycle, a finely tuned and orchestrated system, in which BAs synthesized in the liver are actively transported in the bile ducts, stored in the gallbladder, then secreted in the duodenum, absorbed again in the ileum, and recaptured by the liver *via* the portal circulation. Each stage of this enterohepatic cycle is influenced by diet, hormonal cross-regulation, and bacterial activities that maintain a functional and non-toxic supply of bile acids in circulation. Indeed, BAs have a pro-inflammatory and cytotoxic potential when they are not regulated, due to their detergent activity and destabilization of membranes, as shown in cholestatic liver diseases (Figure 1).

In the post-prandial period, primary BAs are released into the intestinal lumen. Due to their amphiphilic properties, primary BAs adsorb at an oil–water interface to form mixed micelles with other bile lipids (cholesterol, phosphatidylcholine), fat-soluble vitamins (such as vitamins A, D, E, and K) and lipolysis products (free fatty acids, 2-monoglycerides). In the absence of bile secretion, fat absorption is impaired (Hofmann, 1999; Dawson and Karpen, 2015). In the ileum, a highly efficient transporter system allows active reabsorption of conjugated-BAs redirected to the liver through mesenteric and hepatic portal veins (Dawson and Karpen, 2015). At each enterohepatic cycle (4-5 cycles per day), about 5% of the non-absorbed BAs are released into the colon, modified by bacteria, and then excreted. This represents around 600 mg per day, the loss of which is compensated for by an equivalent synthesis from hepatic cholesterol. The co-excretion of fecal sterols and BAs in a 2:1 ratio in humans therefore represents a significant pathway for regulating cholesterol homeostasis (Groen et al., 2014).

Secondary Bile Acids

Another complexity in BA metabolism is the modification of the BA structure by intestinal bacteria (Ridlon et al., 2014). BAs that are not reabsorbed encounter anaerobic resident bacteria in the colon (Figure 1). Microbial enzymes such as bile salt hydrolases (BSH) deconjugate conjugated -BAs, bacterial 7 *a*-dehydroxylases and 7 *b*-dehydroxylases convert CA and CDCA to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Bacterial 7*b*-isomerization of the 7*a*-hydroxyl group of CDCA forms ursodeoxycholic acid (UDCA). Sulfated and glucuronidated BAs formed during hepatic detoxification to facilitate their urinary and fecal excretion can be hydrolyzed of their ester linkage by microbial enzymes (Takikawa et al., 1983; Gérard, 2013), ultimately leading to the presence of a vast repertoire of secondary BAs. In the colon, unconjugated BAs produced by microbial metabolism can diffuse passively over the intestinal border and can eventually be captured by the liver through multispecific organic anion-transporting polypeptide (OATP) transporters that can vehicle unconjugated BAs

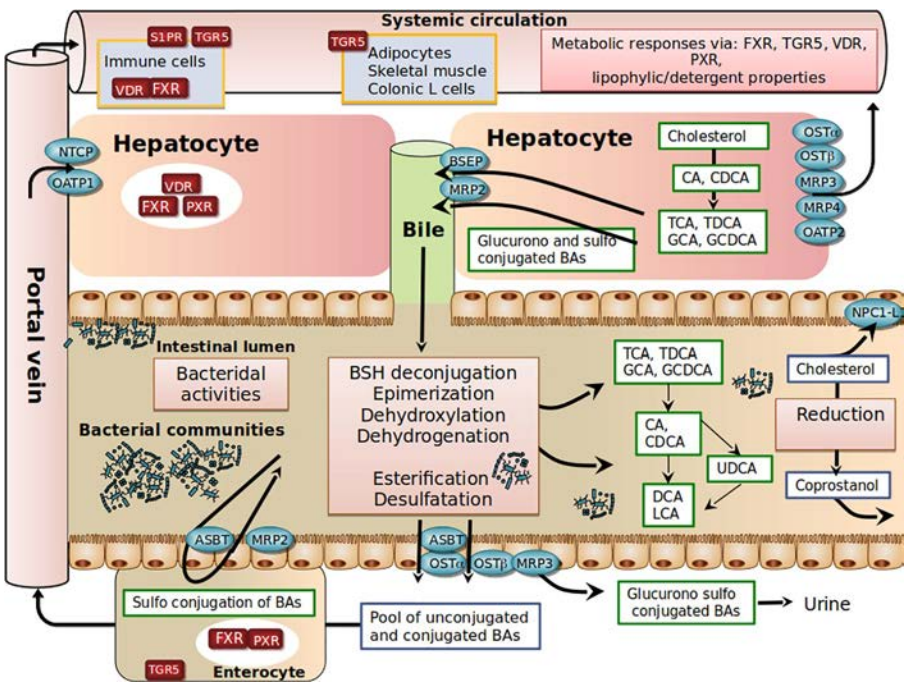

FIGURE 1 | Schematic view of host-gut microbial co-metabolism of bile acids and cholesterol in enterohepatic circulation.

(Unconj-BA) and sulfated BAs (Dawson and Karpen, 2015). In the liver, conjugated BAs are more efficiently recycled from portal blood at the hepatic basolateral membrane by the high-affinity sodium-dependent taurocholate cotransporting polypeptide (NTCP) than are unconjugated BAs (Angelin et al., 1982; Hofmann and Hagey, 2014; Eggink et al., 2018). The estimated hepatic fractional uptake of total BAs ranges from 50 to 90% depending on the bile acid structure (Angelin et al., 1982) and is reflected by differences in systemic blood concentration versus portal blood concentration (Eggink et al., 2018). During the completion of the enterohepatic cycle, unamidated BAs can be conjugated again in the liver, leading to the formation of their glycine or taurine conjugates (GDCA, GLCA, GUDCA, TDCA, TLCA, and TUDCA), while CDCA and LCA can be 6α-hydroxylated to form hyocholic acid (HCA) and hyodeoxycholic acid (HDCA), respectively (Bodin et al., 2005). UDCA represents about 4% of total fecal BAs, and a cholesterol-lowering effect has been reported in patients with primary biliary cirrhosis (Poupon et al., 1993) or hypercholesterolemia (Cabezas Gelabert, 2004). Other exclusively microbial activities, including esterification, oxidation, and desulfation, contribute to the high chemical diversity and changes in hydrophobicity (Ridlon et al., 2014; Devlin and Fischbach, 2015; Watanabe et al., 2017) and bile acid signaling activities (Thomas et al., 2008; de Boer et al., 2018) (Figure 1).

It should be noted that molecular species of BAs are involved not only in lipid metabolism but also in carbohydrate metabolism, energy homeostasis, and host immune responses through their agonistic or antagonistic activities on diverse receptors, the best characterized being the farnesoid X nuclear receptor (FXR) and the TGR5 membrane receptor (Thomas et al., 2008; de Boer et al., 2018); these aspects have been extensively reviewed elsewhere (Houten et al., 2006; Thomas et al., 2008; Lefebvre et al., 2009). Basically, FXR impacts cholesterolemia through the repression of CYP7A1, the rate-limiting enzyme that catabolizes conversion of cholesterol into BAs, resulting in decreased hepatic cholesterol content, followed by upregulation of the LDL-receptor expression and activity, which consequently reduces plasma LDL-C levels. This mechanism underlies the hypocholesterolemic effect of BAs sequestrants (Spinelli et al., 2016). Administration of obeticholic acid (FXR agonist) to chow-fed mice elevates liver LDL receptor expression by mRNA stabilization and reduces plasma LDL-C in mice (Singh et al., 2018). Of note, a recent study discovered a novel association of a variant in human NR1H4 gene (encoding the BA receptor FXR) with levels of TC and LDL-C (Deaton et al., 2018), thus highlighting the role of FXR in the regulation of plasma cholesterol levels in humans.

In the context of cholesterol reduction following antibiotic treatment, in humans (Jenkins et al., 2005), the prevailing hypothesis is that inhibition of the conversion of primary BAs to secondary BAs reduces their hydrophobicity, which results in poorer reabsorption by passive diffusion through the colonic epithelium. Similarly, this decrease in hydrophobicity of BAs is associated with a poorer micellization of cholesterol, which would, therefore, be less efficiently absorbed. These joint activities contribute to the outflow of BAs and cholesterol in the stool and therefore to a decreased sterol pool of the whole body. However, in humans, other molecular mechanisms must coexist to the extent that treatments with primary bile acids (CDCA) or secondary bile acid (LCA) only slightly alter the absorption of cholesterol and the serum concentrations of LDL-C (Wang et al., 2006). Other putative mechanisms qualitatively and quantitatively modulating the pool of bile acids may be at work; a pool of depleted BAs will be associated with the proliferation of pro-inflammatory microbes (Kakiyama et al., 2013) and intestinal barrier dysfunction (Kang et al., 2017), and a pool reconstituted after transfer of fecal material or liver transplantation will correct endotoxemia (Kang et al., 2017; Bajaj et al., 2018) and lipidemia (Kang et al., 2017).

Interestingly, the treatment of 51 naive type-2 diabetic patients with an antidiabetic (acarbose: a tetra-saccharide inhibiting hydrolysis of carbohydrates in the upper intestine and thus reducing glucose absorption) led to improvements of glycemia and cholesterolemia (Gu et al., 2017). These changes were correlated with variations in plasma BA profiles. The primary-BA/secondary-BA ratio and UDCA and T-DCA concentrations were negatively correlated with plasma cholesterol. Accordingly, metagenomics analysis confirmed a lower capacity for 7α/β dehydroxylation of BAs after acarbose treatment. The relative abundances of baiE (rate-limiting enzyme for 7α-dehydroxylation) and baiI (7β-dehydratase) were significantly decreased after acarbose treatment. Phylogenic analysis established a strong inverse correlation between *Lactobacillus rhamnosus* and plasma cholesterol and LDL-C levels. Interestingly, decreases in plasma cholesterol levels associated with acarbose treatment were associated with a decline in *allistipes* spp., in accordance with recent studies (Le Roy et al., 2019; Tong et al., 2019).

In conclusion, the mechanisms underlying bile acid-cholesterol-lowering relationships remain largely undefined. The specific roles of bile acids *in vivo* remain difficult to disentangle, due to the large number of compounds and biological properties involved, including detergent and bactericidal activities and FXR signaling potential (Figure 1). The picture is even more complex if we consider the newly involved receptors such as pregnane X receptor (PXR), vitamin D3 receptor (VDR), muscarinic acetylcholine receptors, and sphingosine 1-phosphate receptor (S1PR). In addition to qualitative parameters, it is also necessary to consider all the poorly known circadian and post-prandial quantitative variations of BAs that will need to be well-defined to better understand the impact of BAs on cholesterol homeostasis (Han et al., 2015).

Short-Chain Fatty Acids (SCFAs)

SCFAs are the main end-product produced by the bacterial fermentation of non-digestible dietary fibers in the caecum and proximal colon. Consumption of dietary fibers such as inulin, oat bran, and pectin is effectively associated with lower plasma cholesterol levels, with reductions in cholesterol level ranging from 0.5% to 2% per gram of intake (Ripsin et al., 1992). Fibers

reduce both TC and LDL-C (Anderson and Chen, 1979) through increased BA excretion and decreased hepatic synthesis of cholesterol (Vahouny et al., 1980; Jenkins et al., 2010). Other potential mechanisms are related to the microbiota-dependent formation of SCFAs (acetate, propionate, butyrate) that are produced and can be used as a macronutrient source of energy. Alternatively, SCFAs can act as hormone-like signaling, entering the portal circulation to ultimately bind to G-protein-coupled receptors (GPR) in numerous cells (Maslowski et al., 2009) and inhibit the histone deacetylase (HDAC), resulting in numerous epigenetic modifications in targeted cells (Riggs et al., 1977; Candido, 1978).

Several studies have described the role of SCFAs on immunity (Aguilar et al., 2014) and its protective effects against cardiovascular disease (Bazzano et al., 2003; Fernandez et al., 2019), yet few of them have individually examined the effects on cholesterol levels. *Ex vivo* and *in vivo* studies have shown that acetate and butyrate (but not propionate) are potential precursors of cholesterol synthesis that can be incorporated into the endogenous cholesterol synthesis pathway (Wolever et al., 1991; Demigné et al., 1995; den Besten et al., 2013). By contrast, earlier studies reported inhibition of cholesterol synthesis by propionate through decreased expression of the HMGCS and HMGCR genes (Bush and Milligan, 1971; Rodwell et al., 1976; Demigné et al., 1995), white more recent studies have not confirmed such effects (Zhao et al., 2017). Accordingly, in healthy subjects, the oral administration of propionate does not lower plasma cholesterol but does increase HDL and triglycerides levels (Venter et al., 1990). In vivo, supplementation of a cholesterol-rich diet with acetate in rats resulted in a lower increase of TC levels associated with lower hepatic HMGCS and increased conversion of cholesterol into BAs due to the upregulation of Cyp7a (Fushimi et al., 2006). A similar rise in Cyp7a was observed in Apoe-deficient mice fed with a cholesterol-rich diet supplemented with butyrate, with additional beneficial effects on the "reverse cholesterol transport" (RCT) (Du et al., 2019). The connection between SCFAs and BAs metabolism was further reported in Syrian hamsters (Zhao et al., 2017). The addition of acetate, propionate, or butyrate to a cholesterol-rich diet resulted in decreased CT levels and LDL-C/HDL-C ratio and to increased fecal excretion of BAs (LCA, DCA, CDCA, CA). Expression of the SREBP2, LDLR, and CYP7A1 genes was also involved (Zhao et al., 2017). The effects of SCFAs were more specifically addressed in mice deficient for FFAR2/GPR43, one of the SCFA receptors (Bjursell et al., 2011). High-fat fed GPR43-deficient mice displayed lower CT levels than control mice.

Collectively, the role of SCFAs in cholesterol levels is poorly defined. SCFAs may be used as precursors of cholesterol synthesis, but the overall hypocholesterolemic effect seems to be associated with the conversion of cholesterol into BAs. Translation studies into humans will be critical to move forward (Figure 2).

Trimethylamine-oxide (TMA-O)

The contributing role of intestinal microbiota to ACV diseases through the production of trimethylamine-N-oxide (TMA-O,

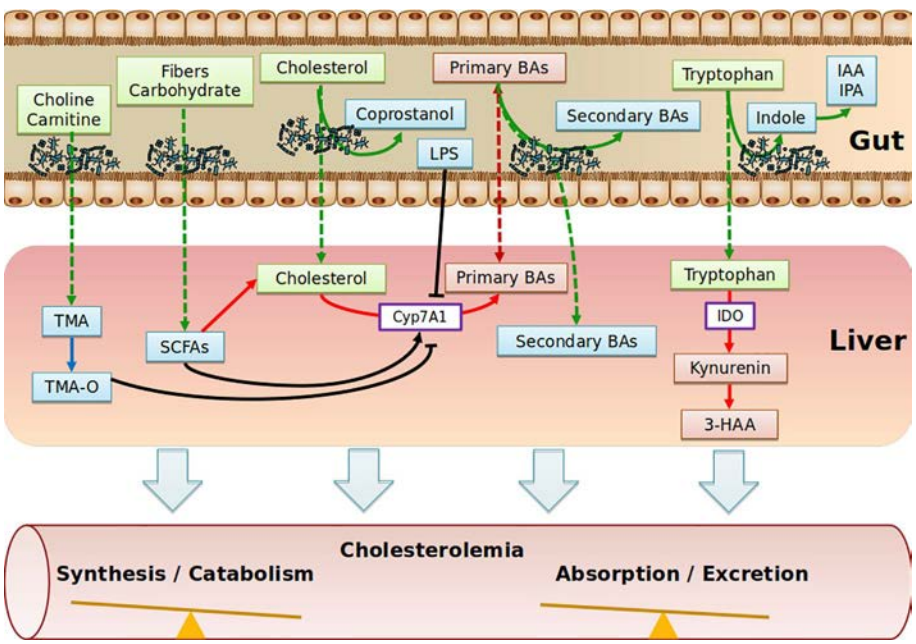

FIGURE 2 | Schematic depicting the range of putative pathways through which the gut metabolites impact on cholesterol metabolism. Diet nutriments (green boxes) are metabolized and transformed into microbial metabolites (blue boxes) by gut microbiota. Purple boxes represent enzymes. Red boxes represent host metabolites. TMA, Trimethylamine; TMA-O, Trimethylamine-Oxide; SCFAs, Short Chain Fatty Acids; BAs, Bile Acids; IAA, Indole-3-acetic acid; IPA, Indole-3-propionic acid; IDO, indoleamine2,3-dioxygenase; 3-HAA, 3-hydroxyanthranilic; LPS, lipopolysaccharides.

has been recently demonstrated (Randrianarisoa et al., 2016; Tang et al., 2017; Yang et al., 2019) and has been covered in recent reviews (Brown and Hazen, 2018; Yang et al., 2019). Essentially, trimethylamine-containing dietary nutrients (choline, phosphatidylcholine, *g*-butyrobetaine, and carnitine) are metabolized by microbes, leading to the production of trimethylamine (TMA), which is rapidly converted by host hepatic flavin monooxygenase 3 (FMO3) into trimethylamine N-oxide (TMA-O). Interestingly, in dyslipidemic mouse models, TMA-O affects cholesterol homeostasis mainly by suppressing reverse cholesterol transport (RCT) (Koeth et al., 2013) and impacting the BA metabolic pathways at multiple levels (decreased expression of hepatic BAs synthetic enzymes (Cyp7a1 and Cyp27a1) and hepatic BA transporters (Oatp1, Oatp4, Mrp2, and Ntcp) (Koeth et al., 2013). Additionally, TMA-O appears to promote cholesterol uptake by macrophages by inducing scavenger receptors CD36 and SRA1, both of which are involved in the intracellular accumulation of modified lipoproteins (Wang et al., 2011). Likewise, under normal dietary conditions, TMA-O did not impact plasma cholesterol levels in mice deficient for FMO genes (Veeravalli et al., 2018). Identification of TMA-O receptors would be of particular interest to substantiate a potential association of TMA-O with cholesterol levels, albeit that no significant correlations between TMA-O and TC, LDL-C, even when excluding individuals taking cholesterol-lowering medications, have been observed (Li et al., 2017). This might explain why TMA-O has been demonstrated to be a prognostic marker for ACV diseases beyond traditional risk factors (Manor et al., 2018) (Figure 2).

Diet-Associated Tryptophan (Trp) Derivatives

Tryptophan is an essential amino acid that is degraded through the kynurenine pathway, leading to the generation of several biologically active compounds. Endogenous kynurenine metabolites contribute to the initiation of ACV disease. In human atherosclerotic plaques, Trp metabolites were found to be associated with unstable plaque phenotype (Taleb, 2019). Tryptophan is processed 95% by the kynurenine pathway (gut-microbiota independent) and 5% by the indole pathway (gut microbiota-dependent). Regarding the kynurenin pathway, supplementation of 3-hydroxyanthranilic (3-HAA), a tryptophan-derivative metabolite from the kynurenine pathway has anti-atherosclerotic effects, associated with lower plasma cholesterol levels in Ldl-r-deficient mice fed an HFD regime (Zhang et al., 2012) ora western diet (Berg et al., 2019). Correspondingly, indoleamine 2,3-dioxygenase (IDO) inhibition showed the exact opposite phenotype in Apoe-deficient mice fed an HFD (Polyzos et al., 2015; Liang et al., 2019), while no effect was reported in chow-diet-fed double-deficient mice for Apoe and IDO (Cole et al., 2015). IDO enzymes are involved in the catabolism of tryptophan, and the ratio of kynurenine to tryptophan (kyn/trp) can be used to reflect IDO activity. In clinical investigations, IDO activity has been reported to be positively correlated with a range of atherosclerosis risk factors in the female population, including LDL-C (Pertovaara et al., 2007). Concerning the indole pathway, tryptophan, indole-3-propionic acid, and indole-3-aldehyde were shown to be decreased in atherosclerotic patients, while kynurenine/tryptophan ratios were increased (Cason et al., 2018); still, no independent correlation with cholesterol has yet been reported. In conclusion, the scarcity of studies does not allow the indole pathway to be implicated in regulation of cholesterolemia (Figure 2).

CONVERSION OF CHOLESTEROL INTO COPROSTANOL

Cholesterol from the diet, bile, or intestinal cells is actively metabolized by intestinal bacteria, mainly in coprostanol (Gérard, 2013). Unlike cholesterol, coprostanol is very poorly absorbed by the intestine (Bhattacharyya, 1986). In a singular way, the rate of conversion of microbial cholesterol to coprostanol in the general human population appears to be multimodal, with an average of 65% of high converters (80% to 100% of luminal cholesterol is converted to coprostanol in the colon), 21% of intermediate converters and 14% of non-converters (Wilkins and Hackman, 1974; Midtvedt et al., 1990; Veiga et al., 2005; Benno et al., 2005). It was also demonstrated that this phenotypic characteristic was maintained in axenic rodents (without germs) colonized with a high-converter or non-converter human microbiota (Gérard et al., 2004). Finally, several clinical and preclinical studies support the hypothesis that the conversion of cholesterol in coprostanol could influence the bioavailability of cholesterol, leading to modulation of plasma cholesterol levels (Sekimoto et al., 1983; Li et al., 1995; Li et al., 1998). Larger studies are needed to validate this relationship. Notably, the disconnection between the major cholesterol uptake site (small intestine *via* the Niemann-Pick C1-Like 1 transporter (NPC1L1)) and the site of cholesterol conversion to coprostanol (colon) does not plead for a causal relationship. Nevertheless, normolipidemic subjects treated with Ezetimibe (NPC1L1 inhibitor) show residual absorption of cholesterol, which suggests as yet unidentified additional absorption mechanisms (Jakulj et al., 2016) (Figures 1 and 2).

IMPACT OF THE MICROBIOTA ON HYPOCHOLESTEROLEMIC DRUG EFFICACY

The gut microbiota has been shown to impact, negatively or positively, drug efficacy. This effect has been shown to result either from modifications in pharmacokinetic or pharmacodynamic properties or by synergistic/antagonistic effect of microbiota toward drugs. As a matter of fact, the impact of the microbiota on drugs is not restrained to oral drug intake, as studies have shown modifications in monoclonal antibody efficacy (Sivan et al., 2015; Doherty et al., 2018). The interaction of gut microbiota with drug efficacy/toxicity has recently been exhaustively reported upon (Spanogiannopoulos et al., 2016; Wilson and Nicholson, 2017; Clarke et al., 2019).

Direct links between the gut microbiota and hypocholesterolemic drugs are still thin. Statins, which are the leading pharmaceutical class

in hyperlipemia therapeutic care, are ineffective for almost 20% of patients treated and are sometimes even deleterious (Stroes et al., 2015). Several studies have explored and demonstrated that statins can directly influence the growth and virulence of bacterial pathogens and commensal bacteria as well as combating microbial infections, such as in sepsis and pneumonia (Hennessy et al., 2016; Zimmermann et al., 2019). The first statin, "Mevastatin," which is a metabolic product of Penicillium citrinum, was initially characterized for its antibiotic properties, and statins are now considered as adjuvant antibiotics that can impact antimicrobial resistance (Ko et al., 2017). Consequently, the role of statins deserves to be explored beyond their traditionally established indications in light of their antimicrobial potential as a regulator of gastrointestinal microbiota (Nolan et al., 2017). Only a few studies have shown the impact of the microbiota on statin efficacy. Still, these studies suggested that the microbiota participated in statin's effect (He et al., 2017) and was responsible for statin metabolization (Yoo et al., 2014) and that the microbiota from patients unresponsive to statin was different from that of responsive patients (Sun et al., 2018).

Additional studies are required in this context and should, therefore, also be conducted on other therapeutic classes of hypolipemic drugs. Understanding the impact of the microbiota on drug efficacy/toxicity should bring us closer to personalized medicine and should result in an improvement in therapeutic care.

CONCLUDING REMARKS

LDL-C is the primary target for the management of atherogenic dyslipidemia and the reduction of cardiovascular events. New actors such as the microbiota introduce more complexity into this multifactorial disease but allow new insight into pathogenicity and the development of new prevention and prophylaxis approaches. In addition to the usual pharmacological approaches (statins, ezetimibe, fibrates, resins, proprotein convertase subtilisin-kexin 9 (PCSK9) inhibitors), new biotherapies targeting the microbiota are possible. Indeed, the data in the literature support the notion that the microbiota has a causal contribution to the metabolism of lipoproteins and host cholesterol. The mechanisms of this reciprocal influence need to be clarified, and the advent of functional analyses of the microbiota, the development of new technologies allowing the culture of anaerobic microbes, and the advent of more and more better-performing technologies will make it possible to specify the dynamics of the relationship of the intestinal microbiota with cholesterol metabolism.

AUTHOR CONTRIBUTIONS

RV, PK, SB, MS, DR, MG, and PL originally conceived and wrote the manuscript. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH

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A novel combination of chemotherapy and immunotherapy controls tumor growth in mice with a human immune system

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ABSTRACT

Mice reconstituted with a human immune system and bearing human tumors represent a promising model for developing novel cancer immunotherapies. Here, we used mass cytometry and multiparametric flow cytometry to characterize human leukocytes infiltrating a human breast cancer tumor model in immunocompromised NOD.SCID.γc-null mice reconstituted with a human immune system and compared it to samples of breast cancer patients. We observed highly activated human CD4$^+$ and CD8$^+$ T cells in the tumor, as well as minor subsets of innate immune cells in both settings. We also report that ICOS$^+$ CD4$^+$ regulatory T cells (Treg) were enriched in the tumor relative to the periphery in humanized mice and patients, providing a target to affect Treg and tumor growth. Indeed, administration of a neutralizing mAb to human ICOS reduced Treg proportions and numbers and improved CD4 + T cell proliferation in humanized mice. Moreover, a combination of the anti-ICOS mAb with cyclophosphamide reduced tumor growth, and that was associated with an improved CD8 to Treg ratio. Depletion of human CD8$^+$ T cells or of murine myeloid cells marginally affected the effect of the combination therapy. Altogether, our results indicate that a combination of anti-ICOS mAb and chemotherapy controls tumor growth in humanized mice, opening new perspectives for the treatment of breast cancer.

One sentence summary: Targeting ICOS in combination with chemotherapy is a promising strategy to improve tumor immunity in humans.

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Introduction

Preventing immune suppression in tumors is the "next frontier" in immuno-oncology research. Despite recent success of anti-PD-1 mAbs for metastatic melanomas and other cancers, it seems reasonable to propose that combination therapy will be key to the success of cancer immunotherapy, as suggested in patients receiving a combination of anti-PD-1 and anti-CTLA-4.[1] Likely, the most efficient combinations will emerge from the ever-growing list of mechanisms that prevent an efficient immune response to the tumor. Recently, a murine study combining a tumor-targeting mAb, a recombinant cytokine, an anti-PD-1 mAb, and a T cell vaccine achieved a remarkable efficacy at clearing large established syngeneic tumors,[2] illustrating the power of combinations for T-cell and innate cell-mediated tumor control. However, studies purely performed in syngeneic models are only useful to gain biological insights but are not relevant to validate mAbs or drugs selectively targeting human cells. Therefore, a mouse model in which the impact of human-specific mAbs on tumor control could be tested would be most invaluable.

It has been shown that human breast tumor morphology and metastatic properties were conserved in xenografted immunodeficient mice.[3,4] This was also shown for ovarian cancers[5] and later confirmed for melanomas,[6] suggesting that a large panel of human tumors engrafts efficiently in immunodeficient mice and reproduces features of clinical progression. Xenograft models might be useful to target human tumors, but these immunodeficient models are not relevant to test drugs that target the human immune system, the very definition of immunotherapy. Mice carrying a human immune system (HuMice) and human tumors could, therefore, represent a relevant model for cancer immunotherapy research, at the interface of mice and humans.[7] Most of the literature regarding the use of HuMice for immuno-oncology relies on the direct transfer of total PBMC into immunodeficient mice of various genetic backgrounds. Therapeutic efficacy of a combination of an anti-PD-1 and an anti-4-1-BB mAbs has been demonstrated in a model in which the tumor and the PBMC were from the same patient.[8] However, PBMC-implanted mice invariably and rapidly suffer from

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Graft vs Host Disease (GVHD), which complicate the interpretation of the results and limits the duration of the experiments. To preclude this problem, NOD.SCID.gamma-c-null (NSG) immunodeficient mice can be reconstituted with human CD34$^+$ hematopoietic progenitors,[9] most often isolated from cord blood. Because we demonstrated that a pool of human T cells with a diverse repertoire was then generated,[10] it became feasible to assess combination therapy targeting human tumors and/or human T cells *in vivo*, generating results in a setting closer to human physiology than syngeneic murine models. However, there is still a lack of information on the composition and the function of human leukocytes infiltrating human tumors in CD34-reconstituted HuMice. Indeed, very few studies have evaluated the nature of human leukocytes in these models.[11-15] Activated T cells in the tumor were observed in a peculiar model of tumor implantation at birth in NSG HuMice that did not lead to tumor rejection.[11] A more advanced model of HuMice (MISTRG) was used to decipher the contribution of the innate immune system to tumor control, but the presence of T cells in the tumor was not reported.[13] Very few T cells were detected in a Patient-Derived Xenograft (PDX) HuMice model of head and neck squamous cell carcinomas.[14] Likewise, very few T cells were detected in breast cancer-bearing NSG HuMice with myeloid cells being the subset the most represented in the tumor.[12] Finally, a recent study described the presence of Treg in the tumor of NSG HuMice but a precise quantification of T cells and their associated phenotype was not reported.[15] Thus, the presence of human T cells in tumors of HuMice is still open to debate. This is a crucial question to be answered if one wants to use the model in a reliable and relevant way. To document this question, we used cytometry Time-of-flight (CyTOF) (or mass cytometry) allowing quantification of the expression of 30 to 40 proteins at the same time at the single cell level, a very useful feature when analyzing samples containing a limited number of cells. When compared to the immune landscape of breast cancer patients, several conserved features were observed. Among those, the Inducible COStimulatory molecule (ICOS) emerged in both patients and HuMice as an eligible marker to target Tregs, a crucial T cell subset dampening the anti-tumoral immune response. Here, we provide the first proof-of-principle that a combination of chemotherapy and neutralizing anti-ICOS mAb leads to reduced tumor growth in HuMice, opening the possibility to translate this combination into the clinics.

Results

T cell infiltrate in the tumors of HuMice

The general design of our study is represented in Figure 1A. Irradiated NSG newborns were injected in the liver with cord blood CD34$^+$ cells. After 16 to 20 weeks, mice were grafted s.c with MDA-MB-231 human breast cancer cells. After 30 to 40 days of tumor growth follow-up, mice were euthanized and the spleen and tumors were analyzed by mass cytometry. The CyTOF panel was designed to include the most common lineage markers for murine and human leukocytes in order to get a global picture of the immune infiltrate and also incorporated several activations and proliferation markers of human T lymphocytes (Table S1).

We first compared the frequencies of human and murine cells of the hematopoietic lineage (CD45$^+$) in the spleen and in the tumor in four independent experiments, totalizing 15 CD34-reconstituted NSG mice. Within human cells, a classical 2D analysis of the CyTOF data revealed the presence of CD20$^+$ B cells and CD3$^+$ T cells (CD4$^+$ and CD8$^+$) in the spleen and the tumor, although B cells tended to disappear from the tumor (Fig. S1A). To get a deeper insight into the nature of immune cells present in the tumor, we performed unsupervised clustering of the data using the tSNE algorithm. This algorithm reduces the multidimensional nature of mass cytometry data to a 2D representation, in which each dot is a cell, clustered according to the level of expression of chosen markers.[16] We first focused our analysis on manually gated hCD45$^+$ and ran the tSNE algorithm based on lineage and activation markers. Clusters of CD3$^+$, CD4$^+$, and CD8$^+$ cells were readily detected with that method (Figure 1B). To compare the distribution of human leukocytes in HuMice to human samples, we determined the distribution of human leukocytes in the blood and tumors of nine breast cancer patients by regular flow cytometry (Figure 1C). The mean frequencies of CD3$^+$ T cells were similar in HuMice and patients in the tumor, representing around 50-60% of the total CD45$^+$ cells. In contrast, the frequencies of CD4$^+$ T cells were higher and the frequencies of CD8 + T cells were lower in the tumor of HuMice compared to patients. Thus, the MDA-MB-231 cell line was indeed infiltrated by human T cells in CD34-reconstituted HuMice, albeit the ratio of CD4 to CD8 T cells was different than in patients.

Innate immune cells in the tumors of HuMice

We next investigated the nature of non-CD19, non-CD3-expressing human cells in the same t-SNE analysis. A distinct cluster of CD33$^+$ HLA-DR$^+$ cells was observed in the spleen and tumor of HuMice (Figure 2A). Zooming on CD33$^+$ cells, two distinct populations of CD14$^+$HLA-DR^{lo} and CD123$^+$HLA-DR^{hi} cells further segregated, most likely representing monocytes and plasmacytoid dendritic cells (pDCs) precursors, since pDC precursors and mature pDCs differ by CD33 expression[17] (Figure 2B). A subset of CD33$^+$ cells not expressing CD14 nor CD123, and heterogeneous for HLA-DR were enriched in the tumor, likely including classical DCs (cDC). In addition, two clusters of Granzyme-B-expressing cells (Grz-B$^+$) were observed in the spleen and the tumor (Figure 2C): one expressed CD8 and CD3 (Figure 1B) and thus represented CTL, while the others expressed CD335 (NKp46) (Figure2C) but not CD3 (Figure 1B) and thus represented NK cells. In this later cluster, NKp46 expression was lower in the tumor than in the spleen. Thus, in addition to CD4$^+$ and CD8$^+$ T cells, the immune landscape of tumors in CD34-HuMice also included monocytes, pDC, cDC, and NK cells, albeit in small proportions and not in all HuMice. A similar analysis of human patients was not available.

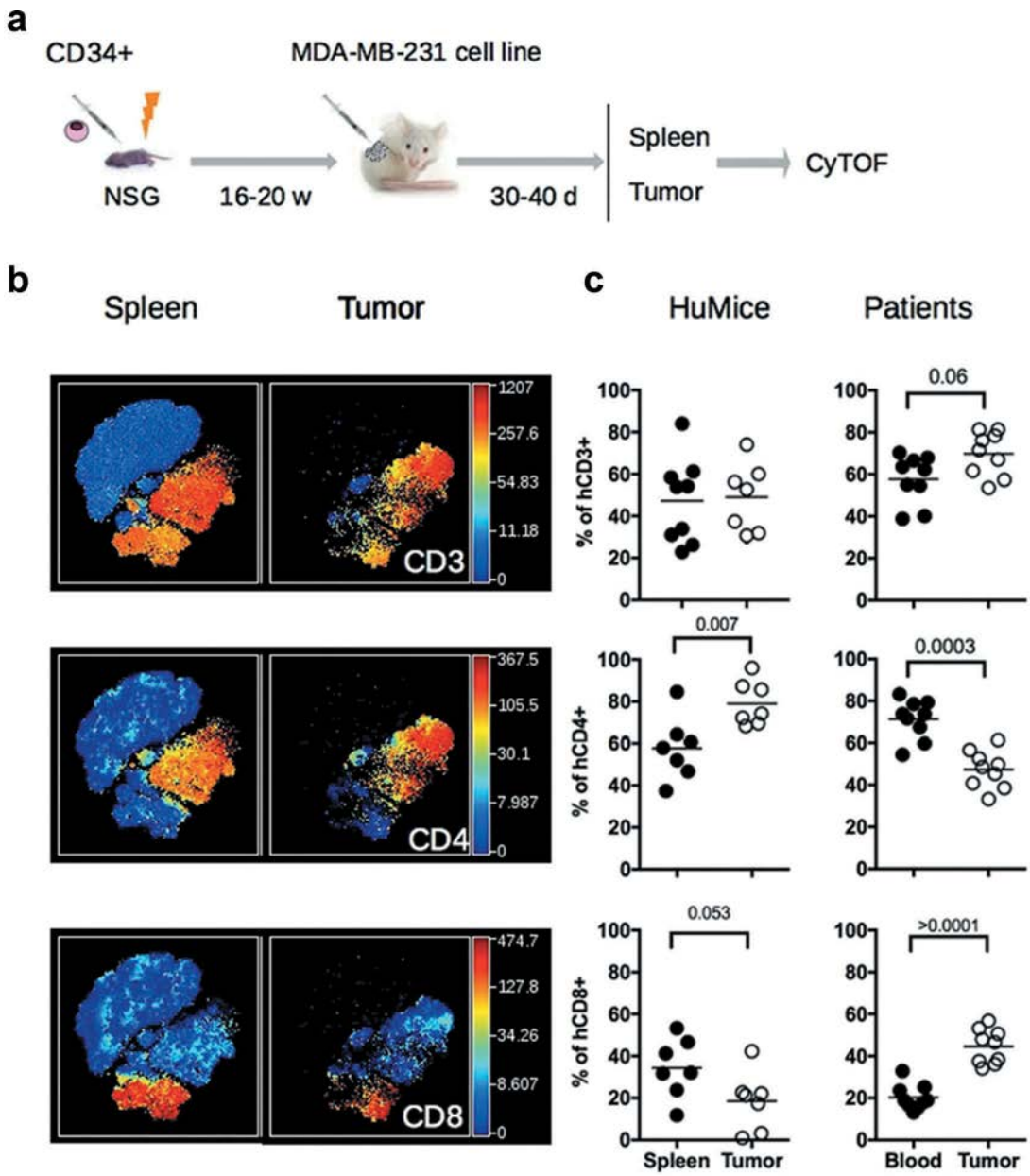

Figure 1. Distribution of human and murine leukocytes in HuMice and in breast cancer patients. (a) Experimental scheme of the study, as detailed in the text. (b) viSNE representation of gated human CD45$^+$ cells in the spleen and the tumor of HuMice from a representative experiment. The viSNE plot was generated according to NKp46, CD38, CD33, CD45RO, PD-1, CD4, CD8, CD20, CD25, Grz-B, and HLA-DR expression using proportional sampling with 54422 events in the spleen and 10274 events in the tumor. The level of expression of each of the indicated markers is represented by a color scale on the right. (c) Frequencies of the indicated subsets were determined in HuMice by supervised 2D-gating from CyTOF data and in patients by multi-parametric flow cytometry. Each dot represents a mouse from four independent experiments or a patient in individual experiments. The horizontal line represents the mean value. The p-values indicated in the figures are from non-parametric two-tailed Mann−Whitney t-test.

To gain information on the murine infiltrate, the same unsupervised representation of the data was performed on gated mCD45$^+$ cells (Fig. S1B). As expected from NSG mice, which are deprived of all lymphoid lineages, most mCD45$^+$ cells expressed CD11b in the spleen and the tumor (Fig. S1C). Clusters of Ly-6C$^+$ monocytes, Ly6-G$^+$ neutrophils, and CD11c$^+$ DC were readily observed in the spleen and the tumor (Fig. S1C). Overall, myeloid cells of NSG mice were similarly represented in the spleen and the tumor, with the exception of Ly6G$^+$ neutrophils which were less abundant in the tumor (Fig. S1D).

Activated/memory T cells in tumors of HuMice

Having established unambiguously that tumors in HuMice contained human T cells, we next investigated the activation status of those cells. We performed a t-SNE analysis on manually gated human CD3$^+$ T cells, allowing visualization of two main clusters of CD4$^+$ and CD8$^+$ T cells (Figure 3A). When activation/memory and functional markers such as CD45RO, HLA-DR, PD-1, CD25 or Grz-B were considered, we noticed a general increase in the frequencies of cells positive for those markers in the tumor relative

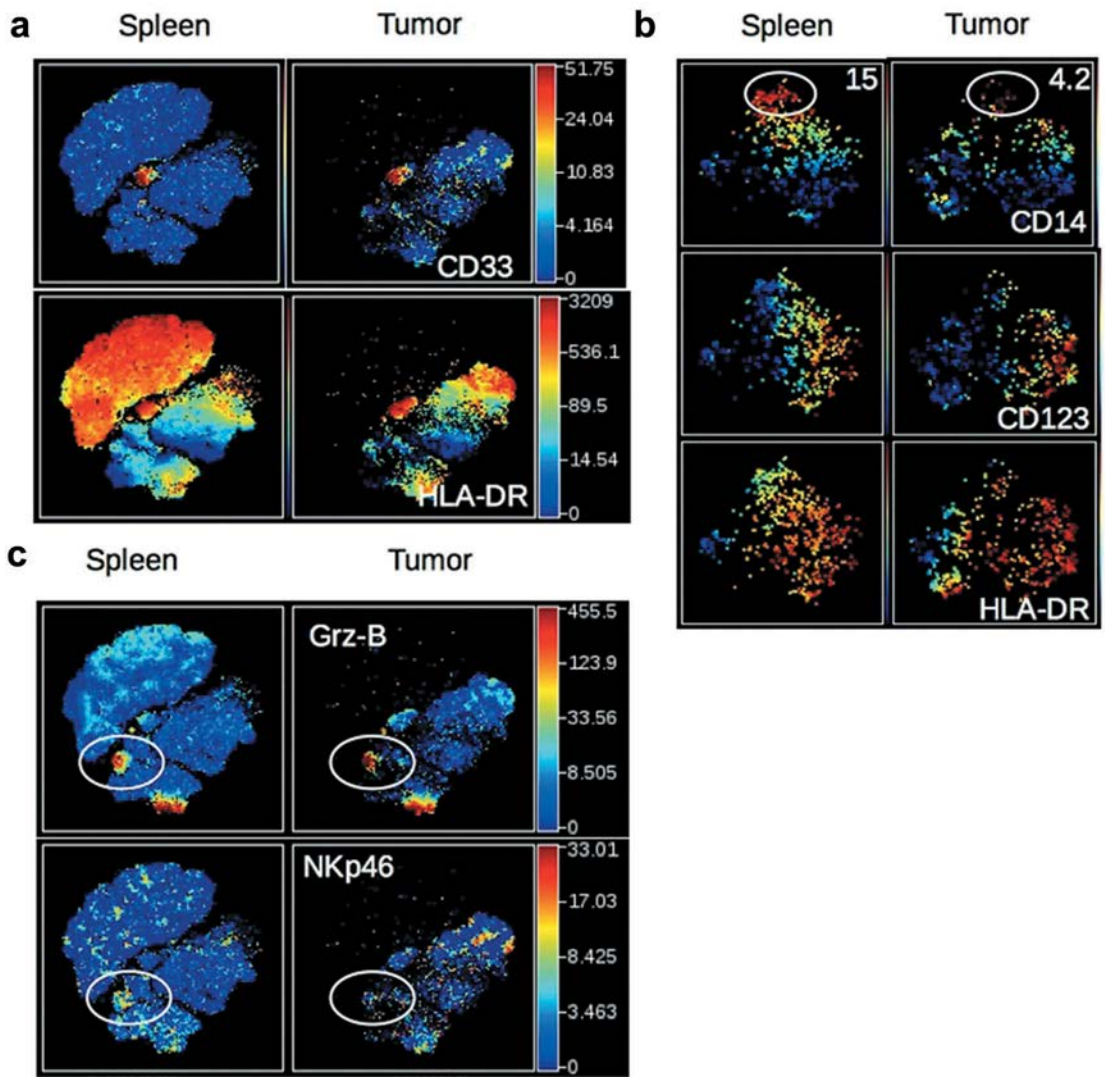

Figure 2. Detection of human myeloid cells and NK cells in HuMice. (a) The viSNE plot was generated as in Figure 1B and represents the expression of CD33 and HLA-DR in hCD45+ cells of the spleen and tumor. (b) The viSNE plots were generated in gated hCD45+CD33+ cells according to CD14, CD45RO, CD123, CD4, CD11b and HLA-DR expression using proportional sampling with 535 cells in the spleen and 476 events in the tumors. Indicated on the plots are the frequencies of CD14-expressing cells in the CD33+ cluster. (c) The visNE plots were generated as in Figure 1B and represent the expression of Granzyme-B (Grz-B) and NKp46 in the spleen and the tumor. Gates highlight the localization of putative NK cells.

to the spleen for both $CD4^+$ and $CD8^+$ cells, a first indication that T cells were activated in the tumor environment. Up-regulation of CD45RO and HLA-DR expression was observed in both $CD4^+$ and $CD8^+$ subsets, whereas higher PD-1 and CD25 expression was noted among $CD4^+$ T cells. As expected, up-regulation of Grz-B expression was restricted to $CD8^+$ T cells (Fig. S2A). To determine the activation status of human T cells more precisely, we performed a boolean analysis calculating the frequencies of cells expressing 0, 1, any combination of two or all three above-mentioned activation markers in $CD4^+$ and $CD8^+$ T cells. Results depicted in Figure 3B show that the frequencies of $CD4^+$ or $CD8^+$ cells co-expressing two or three activation markers were vastly increased in the tumor, while frequencies of T cells expressing none of the activation markers were drastically reduced. Despite this massive activation, the frequencies of proliferating Ki-67^+ cells co-expressing three activation markers were lower in tumors relative to the spleen (Fig. S2B), although this did not reach statistical significance by a two-way ANOVA. Importantly, a similar enrichment for activated/memory T cells was observed in the tumor relative to the blood in breast cancer patients, assessed by the co-expression of two or three activation/memory markers (Figure 3C). Thus, tumor-infiltrating T cells were of an activated/memory phenotype in both HuMice and breast cancer patients. Of note, PD-1 expression was correlated with a higher proliferation of $CD4^+$ and $CD8^+$ T cells in the spleen of HuMice. In contrast, it was inversely correlated with the proliferation of $CD4^+$ but surprisingly not of $CD8^+$ T cells in the tumor (Fig S2C-E), appearing as an exhaustion marker only for $CD4^+$ T cells.

Increased expression of ICOS on regulatory T cells in tumors

Effector Treg is defined by high expression of the transcription factor FOXP3 and the IL-2R-alpha chain CD25, and by the lack of CD45RA expression.[18] They are important actors of immunosuppression in the tumors of human patients. It has also been shown

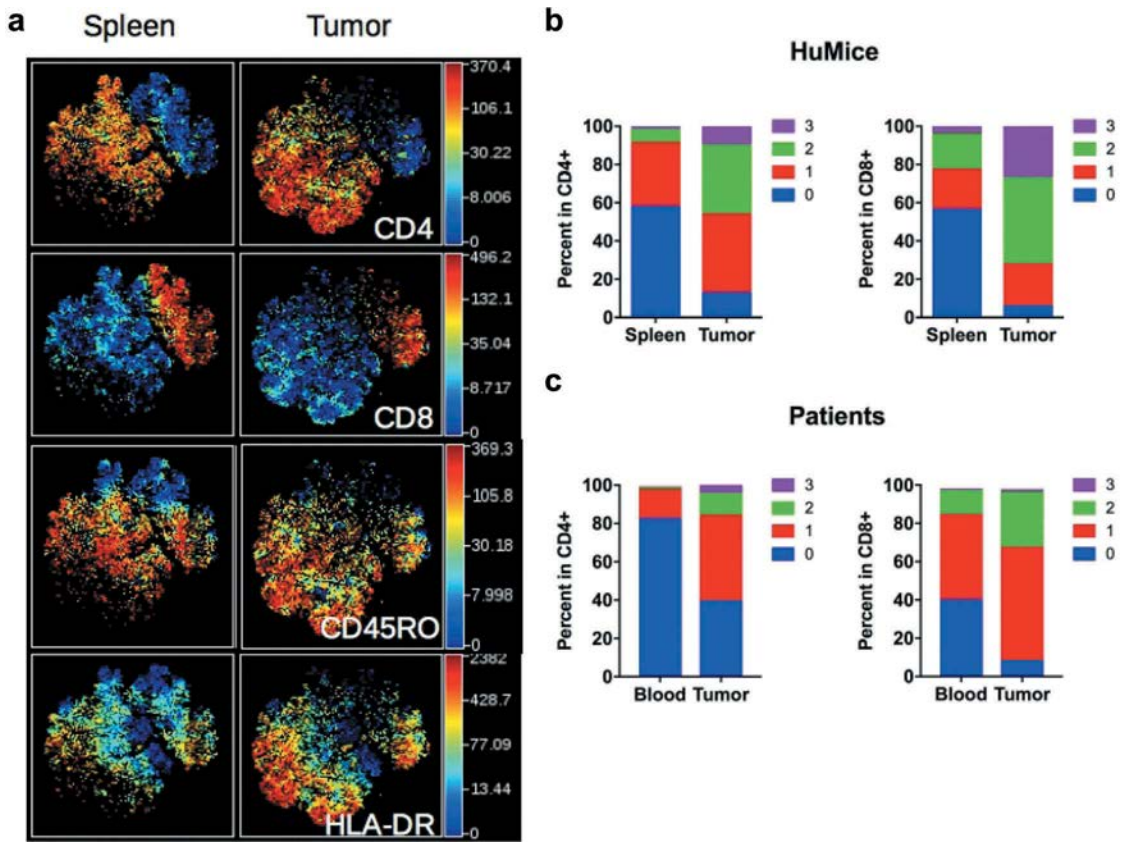

Figure 3. Activation status of Tumor-infiltrating T cells of HuMice and breast cancer patients. (a) hCD45$^+$CD3$^+$ from HuMice were gated in Cytobank and a viSNE plot was generated according to CD4, CD8, CD45RO, HLA-DR, PD-1, CD25, Ki-67 and GrzB expression using equal sampling with 8574 events in the spleen and tumor. (b) Mean frequencies of cells expressing 0, 1, any combination of two or all three activation/memory markers are shown in the indicated tissue. Activation/memory markers used for boolean analysis were CD25, HLA-DR, and PD-1 for CD4$^+$ T cells and HLA-DR, Grz-B, and CD45RO for CD8$^+$ T cells. Results are from one experiment out of three. (c) Same analysis for T cells from breast cancer patients. Results are mean frequencies in nine breast cancer patients in nine independent experiments in the blood and the tumor determined by flow cytometry. The activation markers used for boolean analysis were HLA-DR, ICOS, and PD-1 for CD4$^+$ T cells and CD45RA, Grz-B and HLA-DR for CD8$^+$ T cells (negativity for CD45RA was considered as an activated/memory phenotype).

that ICOS/ICOS-L interaction can regulate Treg functions in humans[19] and that ICOS expression is linked to Treg-mediated immunosuppression in breast cancer patients.[20,21] Furthermore, ICOS expression was associated with poor prognosis in breast cancer patients due to its promoting action on Treg.[21,22] A distinct cluster of CD4$^+$ cells expressing FOXP3, high levels of ICOS and negative for CD45RA, representing effector Treg was clearly visible in the tumor whereas it was absent in the blood in a patient (Figure 4A). This cluster was represented at higher frequencies in breast tumors compared to blood in nine patients (Figure 4B). It has been shown in humans that CD4$^+$FOXP3$^+$CD45RA^{neg} cells might include activated effector T cells with lower FOXP3 expression than effector Treg.[18] We observed that the frequencies of ICOS$^+$ cells were higher in FOXP3^{hi} cells than in FOXP3^{lo} cells in the tumor (Figure 4C), suggesting that CD4$^+$CD45RA^{neg}FOXP3^{hi}ICOS$^+$ cells represented *bona fide* effector Treg. The higher expression of ICOS on Treg of the tumor was also documented by an increase in the MFI of ICOS in FOXP3$^+$ vs. FOXP3^{neg} cells (Figure 4D). Importantly, a similar increase was observed in the tumors of HuMice (Figure 4D), showing that overexpression of ICOS by Treg in human tumors was recapitulated in HuMice. We thus surmise that ICOS could represent a suitable target to affect Treg, with a possible impact on tumor growth, a testable hypothesis in HuMice.

Impact of anti-ICOS on Treg in HuMice

To test the hypothesis that ICOS/ICOS-L neutralization might affect Treg *in vivo*, we injected once an anti-hICOS mAb, reported as neutralizing *in vitro*[21] in tumor-bearing HuMice and determined the frequencies of Treg in the spleen and the tumor 30 days after (Figure 5A). Regular flow cytometry was performed to determine frequencies of FOXP3$^+$ICOS$^+$ and FOXP3$^+$CD25$^+$ cells following treatment. There was a statistically significant effect of the treatment on the proportions of FOXP3$^+$ICOS$^+$ cells ($p = 0.0027$) and of FOXP3$^+$CD25$^+$ cells ($p = 0.0039$) in the tissues using two-way ANOVA. Taking into account multiple comparisons with corrected p-values, the treatment led to a statistically significant reduction in the frequencies of FOXP3$^+$ cells expressing CD25 or ICOS in the spleen of HuMice and a similar tendency was observed in the tumor (Figure 5A). Moreover, the anti-ICOS mAb treatment was associated with the increased proliferation of total CD4$^+$ but not CD8$^+$ T cells in the spleen (Figure 5B). The treatment also affected the absolute numbers of total T cells in the spleen but that did not reach statistical significance. In contrast, Treg counts were significantly reduced by the treatment with numbers dropping almost 20-fold (Figure 5C). Thus, the anti-ICOS mAb led to a significant reduction of FOXP3$^+$ cells in treated animals and also improved the proliferation of CD4$^+$ T cells.

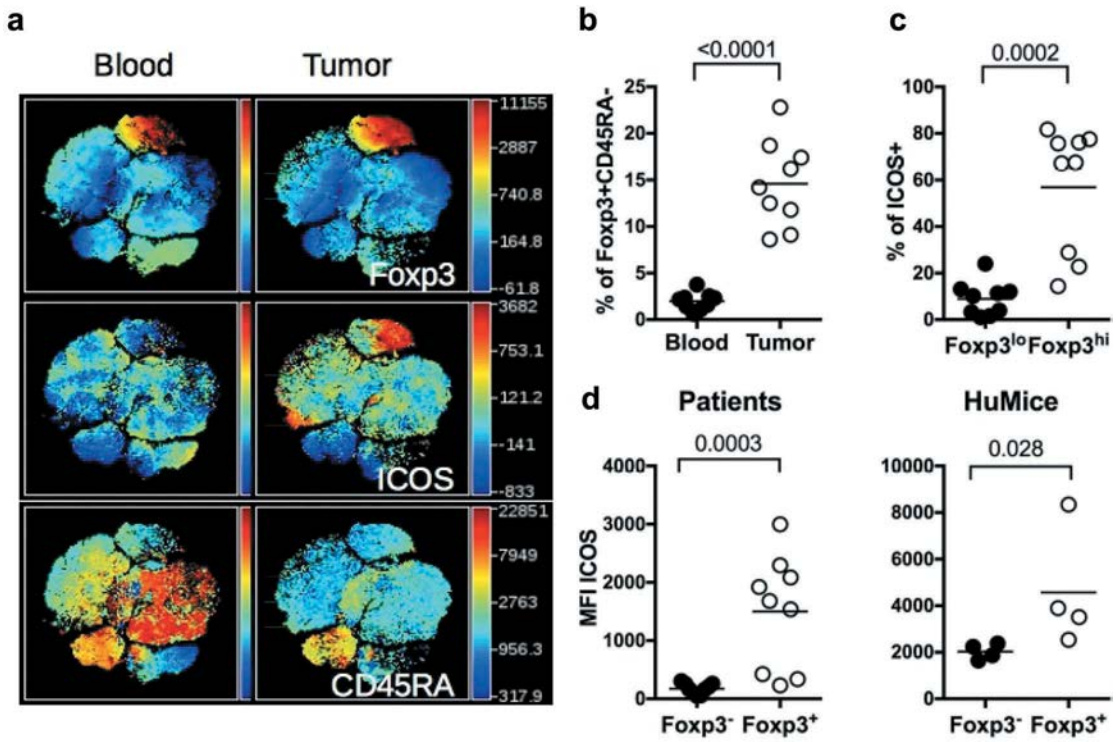

Figure 4. ICOS expression by Treg in HuMice and breast cancer patients. (a) viSNE plot of hCD45+ from a representative breast cancer patient showing FOXP3, ICOS and CD45RA expression in the blood and the tumor, determined by flow cytometry. (b) Frequencies of FOXP3+CD45RA^{neg} cells in the indicated tissue from breast cancer patients among CD4+CD3+ cells. (c) Frequencies of ICOS+ cells in FOXP3^{lo} or FOXP3^{hi} CD4+CD45RA^{neg} in the tumor of breast cancer patients. (d) MFI of ICOS in CD3+CD4+Foxp3− or CD3+CD4+Foxp3+ in the tumor of breast cancer patients or in the tumor of HuMice. The horizontal line represents the mean value. Each dot represents a patient or a mouse. The p values indicated on the graphs are from non-parametric two-tailed Mann−Whitney t-test.

Combination of chemotherapy and anti-ICOS to control tumor growth in HuMice

To evaluate the impact of the mAb on tumor growth, we dispatched HuMice with similar T cell reconstitution prior the initiation of the experiments (Fig. S3) into a pre-clinical-like trial with random assignments into groups, blinded evaluation, mixed sex ratio, and sufficient number of animals to detect the observed effect with enough statistical power (the experimental scheme is summarized in Figure 6A). Despite the clear reduction in Treg and CD4+ T cells reported above, the anti-ICOS mAb injected alone at day 7 post-tumor implantation had no effect on tumor growth (Figure 6B). We thus reasoned that combining the anti-ICOS mAb with a known inducer of immunogenic cell death (ICD) might be more efficient, as shown in syngeneic murine models with anti-CTLA-4 or anti-PD-1 mAbs.[23] Cyclophosphamide (CTX) is widely used as a chemotherapy for treatment of breast cancer for its cytostatic properties, and has also been described as a potent inducer of ICD and may affect Treg as well.[24] Indeed, CTX at a dose of 3 mg per mouse completely abolished tumor growth in non-humanized NSG mice, whereas a dose of 1.5 mg per mouse only moderately affected tumor growth in non-humanized (Fig. S4A) and HuMice alike (Figure 6B), associated to reduced proportions of Treg in treated animals (Fig. S4B). The combination of the anti-ICOS mAb and CTX injected at day 7 post tumor implantation profoundly reduced tumor growth in HuMice compared to single treatments (Figure 6B). Thus, a combination of CTX with neutralizing anti-ICOS mAb efficiently controlled tumor growth in CD34-reconstituted HuMice. As expected from the results reported above, the proportion of Treg was lower and the CD8 to Treg ratio was higher in the tumor of the combo group relative to CTX alone (Figure 6C), showing that the combined effect of CTX and anti-ICOS was associated to a favorable CD8 to Treg ratio.

Role of human CD8 T cells and murine myeloid cells in the control of tumor growth by the combination of chemotherapy and anti-ICOS mAb

Frequencies of human CD45+CD3+ T cells in the tumor were similar in all groups (Fig. S5), indicating that better tumor control in the combo group was not associated to a quantitative increase of human T cells. We thus investigated the role of human CD8+ T cells on tumor control in the combo group. For that, we injected HuMice with a chimeric CD8-depleting recombinant Ig after tumor implantation and before the combination of treatments. As already noted in HuMice,[25] efficient CD8+ T cell depletion was observed in the spleen and tumors of euthanized animals (Fig. S6A) that was quantified by a large increase in the CD4 to CD8 ratio (Fig. S6B). Although there was a statistical difference between the two curves, tumor growth was marginally affected by the absence of CD8+ T cells (Figure 7A), showing that these were not entirely responsible for

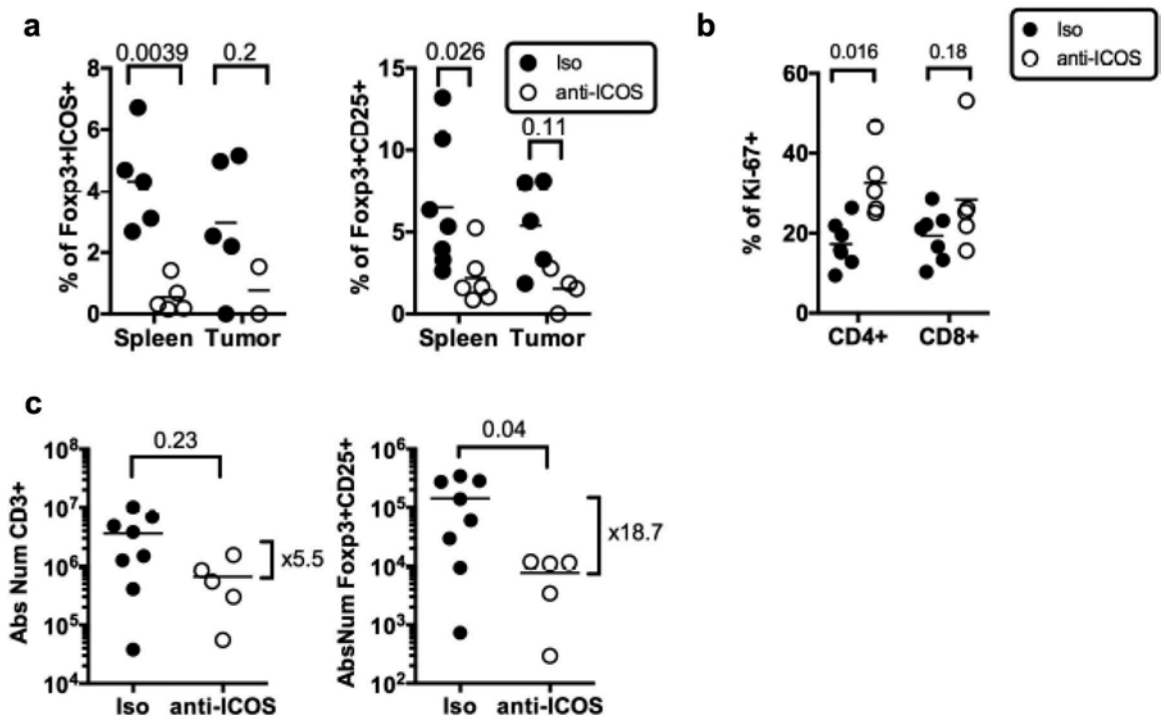

Figure 5. Impact of anti-ICOS mAb on Treg in humanized mice. (a) Frequencies of FOXP3$^+$ICOS$^+$ or FOXP3$^+$CD25$^+$ cells in CD4$^+$CD3$^+$ T cells and of (b) Ki-67+ cells in CD3$^+$ T cells in the spleen and tumor of HuMice injected with isotype control (Iso) or anti-ICOS mAb (50µg/mouse). Results shown are cumulative of at least three independent experiments. Only high expressing cells were gated. The p-values reported on the graph are from a two-way ANOVA with multiple comparisons test corrected by the Sidak method. (c) Absolute numbers of total T cells (CD3$^+$) or Treg (FOXP3$^+$CD25$^+$) were based on absolute counts of the spleen of HuMice treated with isotype control (Iso) or anti-ICOS mAb. Each dot represents a mouse. Fold change in mean numbers is indicated on the right. The p-values indicated on the graphs are from an unpaired two-tailed non-parametric Kolgomorov–Smirnov t-test. Data are cumulative of at least three independent experiments. The horizontal line represents the mean value.

better tumor control in the combo group. It was not possible to use a CD4-depleting reagent in HuMice to determine the role of CD4 T cells on tumor control since it would have depleted both Treg and effector CD4 T cells, preventing any firm conclusions to be drawn. We thus investigated the role of murine myeloid cells on the effect of the combination treatment. We treated a novel set of combo-treated HuMice with the anti-Gr1 mAb that depletes Ly6C$^+$ and Ly6G$^+$ cells, mostly monocytes and neutrophils. Like for human CD8$^+$ T cells, tumors grew marginally better in the anti-Gr1-treated group compared to isotype controls (Figure 7B), indicating that murine myeloid cells also participated in tumor control evoked by the combination of CTX and anti-ICOS mAb.

Discussion

In the present study, we provide the first demonstration that CD34-reconstituted HuMice can be used as a platform for the discovery and the validation of novel combinations of chemotherapy and immunotherapy, potentially applicable to patients. This demonstration originates from several similarities between HuMice and patients that we reveal here using multiparametric fluorescent and mass cytometry. A first similarity that we uncovered here was the composition of the tumor immune landscape. Within total human hematopoietic cells, it is remarkable to note that within CD45$^+$ cells, a similar proportion of CD3$^+$ T cells infiltrated the tumors in patients and in HuMice. Thus, HuMice models might be useful to decipher the molecules and mechanisms at play that attracts T cells within the tumor environment. We also observed similar infiltration of memory/activated CD4$^+$ and CD8$^+$ T cells that expressed a combination of activation markers. However, this activation status did not translate into increased proliferation in the tumor of HuMice, suggesting that the tumor microenvironment of this triple negative (TN) PR^{neg}ER^{neg}HER-2^{neg} cell line might be immunosuppressive *in vivo*. The immunosuppressive status of the tumor microenvironment in breast cancer patients varies according to the nature of the tumor (ER+, HER2+, TN) that may condition T cell infiltrate and T cell activation status.[26] Among breast tumor entities, TNs are the most infiltrated by T cells,[27] CD8$^+$ and FOXP3$^+$ cells alike, suggesting that TN might be an immunosuppressive-prone microenvironment. Here, we did not compare the proliferative status of T cells from patients with HuMice since none were carrying TN tumors. Further studies should confirm whether the immunosuppressive environment of the TN cell line observed here in HuMice is also observed in patients.

The enrichment for ICOS-expressing Treg is a remarkably conserved feature in HuMice and patients and led us to investigate the therapeutic potential of targeting ICOS for cancer immunotherapy. ICOS, a member of the Ig superfamily, is an essential co-stimulatory molecule for T cell activation and function. Although originally thought of as a co-stimulatory molecule for effective T helper cell response, the

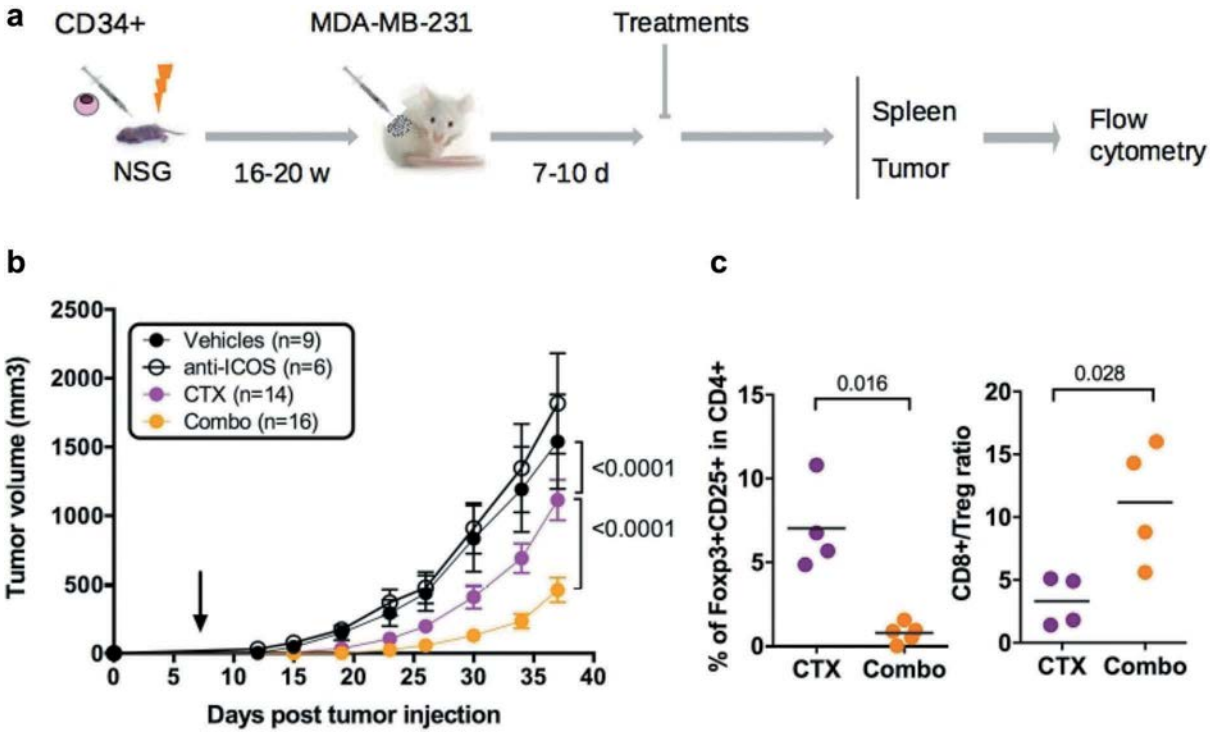

Figure 6. Impact of anti-ICOS mAb on tumor growth in HuMice. (a) Experimental design of the study, as detailed in the text. (b) Tumor growth was determined in four independent experiments in the indicated number of mice treated by PBS and isotype control (Vehicles), anti-ICOS and PBS (anti-ICOS; 50 μg/mouse), cyclophosphamide and isotype control (CTX; 1.5 mg/mouse) or a combination of CTX and anti-ICOS (Combo). Error bars are SEM. The arrow indicates the day the anti-ICOS, and the CTX treatment was performed. (c) Frequencies of $Foxp3^+CD25^+$ cells in $CD4^+CD3^+$ cells of the tumor in the indicated conditions. The CD8 to Treg ratio was obtained by dividing the frequencies of $CD8^+$ T cells by the frequencies of $Foxp3^+CD25^+$ cells in $CD3^+$ cells of the tumor in the indicated conditions. Each dot is a mouse and results are cumulative of two experiments.

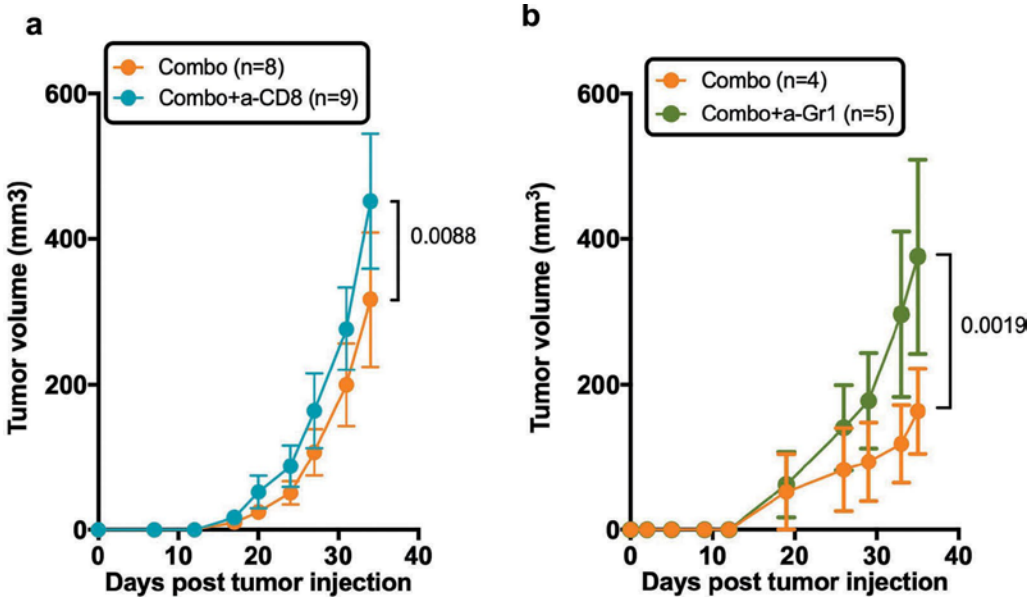

Figure 7. Role of human T cells and murine myeloid cells in the prevention of tumor growth by the combination of chemotherapy and anti-ICOS mAb. (a) Tumor growth in the absence of $CD8^+$ T cells in the combo group. Neonatal CD34-reconstituted NSG mice were grafted s.c with the MDA-MB-231 cell line, injected with cyclophosphamide (CTX; 1.5 mg/mouse) and the anti-ICOS mAb (50 μg/mouse) with (Combo+a-CD8) or without (Combo) the MT807R1 recombinant Ig (10mg/kg) (b) Tumor growth was followed in HuMice treated with the combination of CTX and anti-ICOS mAb and an isotype control (Combo+Iso) or an anti-Gr1 mAb (200μg/mouse i.p). The first injection began 2 days before the combo treatment, followed by five injections, twice a week for 3 weeks. In this experiment, NSG female mice were humanized at the age of 3 weeks, then grafted as mentioned with the MDA breast cancer cell line. Results shown are cumulative of two independent experiments. The total number of mice is indicated in brackets. Error bars are SEM.

biology of ICOS now extends well beyond this narrowed view.[28] Relevant to the present study, we and others have shown that the survival, the expansion, and the suppressive functions of human Treg were dependent on ICOS

signaling.[19,21,29] Thus, we evaluated whether an anti-ICOS mAb could affect human Treg *in vivo*, with possible impact on tumor growth. The reduction in the absolute numbers of total T cells and Treg after anti-ICOS mAb treatment that we report here is in line with an important role for ICOS/ICOS-L on T cells survival. An alternative possibility is that Treg would be physically depleted by the mAb, even though the murine IgG1 isotype used herein is not recognized as a strong inducer of ADCC. This physical depletion is unlikely to occur by complement-dependent cytotoxicity since NSG mice, like their NOD relatives, bears a 2-bp deletion in the C5 gene.[30] The murine anti-ICOS mAb might have engaged activating FcRs expressed by myeloid cells, such as FcgRIII, leading to the active killing of ICOS-expressing cells, including Treg. Indeed, Treg depletion mediated by myeloid cells has been proposed as a mechanism to explain slower tumor growth following administration of a murine IgG2a isotype to GITR in mice.[31] As put forward in the study of Bulliard et al.,[31] much remains to be done to decipher the mechanisms responsible for mAb-mediated T cell depletion in mice. Whatever the precise mechanisms that remain to be uncovered, Treg depletion is now considered a major mechanism for therapeutic efficacy of anti-CTLA-4 or anti-OX40 mAbs in mice[32–34] and possibly of Ipilimumab in humans.[35] Additionally, the engagement of the ICOS/ICOS-L pathway is required for anti-CTLA-4 efficacy in some models.[36] Several mAbs targeting Treg are in development or already approved, including but not limited to anti-CCR4,[37] anti-OX-40,[38] anti-GITR[39] and anti-CD25 mAb.[40] In addition, the chemokine receptor CCR8 was recently proposed as an attractive target to affect Treg in breast cancer, although this was not directly demonstrated.[41] Our results indicate that ICOS might be a useful addition to this growing list of Treg targets.

In line with published results in murine models,[42] we confirm here that blocking ICOS alone was not sufficient to impact tumor growth. However, a combination of chemotherapy and anti-ICOS mAb significantly impacted tumor growth in CD34-HuMice. This result supports the notion that the reduction in Treg is associated with better tumor control if ICD is simultaneously induced by chemotherapy. However, the results of *in vivo* depletion experiments did not support a major role for human $CD8^+$ T cells nor murine myeloid cells on tumor control in the combination group. Thus, we surmise that $CD4^+$ T cells might be central for tumor control following the combined treatment. Releasing Treg-mediated immunosuppression may allow a better CD4-mediated response directly against the tumor and/or indirectly through improved CD4-help to various immune subsets, notably myeloid cells. Supporting this hypothesis, it was recently demonstrated in syngeneic models that combination immunotherapy leads to polarization of monocytes to macrophages.[43] This is also remnant of a comprehensive study showing that the proportion of circulating classical monocytes is a good indicator of successful anti-PD-1 treatment for melanoma.[44]

In addition to human $CD3^+$ T cells, our CyTOF analysis showed that the tumors of HuMice could also contain rare populations of immune cells known to play important roles during the anti-tumor immune response, such as monocytes or pDCs. The pDC subset that we describe here for the first time in the tumor of HuMice might have played a role in the amplification of $ICOS^+$ Treg, as described in breast cancer patients.[21] We also revealed the presence of NK cells with low expression of NKp46 in the tumor, suggesting impaired function, in agreement with clinical observations.[45] Nevertheless, innate human subsets were rare and not always present in HuMice, casting doubts on their effective role during the anti-tumor immune response in regular HuMice. It will be important in the future to monitor the human myeloid compartment in HuMice models optimized for innate human cells development, since those cells can positively[46] and/or negatively affect the local anti-tumor immune response.[47]

To our knowledge, our study represents the first demonstration that ICOS represents a target to impact tumor growth in the context of chemotherapy. The presence of $ICOS^+$ Treg has been described in breast,[20] ovarian,[48] and gastric[49] carcinomas, melanomas[50,51] and more recently follicular lymphomas,[52] suggesting that ICOS-based cancer immunotherapy might be applicable to a wide range of cancers.

Methods

Mice and humanization

NOD.SCID.gc-null mice (stock ≠005557) were originally purchased from the Jackson Laboratory and were bred in our own animal facility under a 14-10-h light cycle with ad libitum food and water. Mice were given Baytril in their water every other week. Newborns NSG mice were reconstituted by intrahepatic injection of 5. 10^4 to 10^5 magnetically purified $CD34^+$ cord blood cells according to the manufacturer's instructions (Miltenyi, Paris, France) or were purchased from ABCell Bio (Paris, France). Reconstitution was followed over time in the blood by multicolor flow cytometry using the following markers in various combinations of fluorescent dyes: mCD45, hCD45, hCD3, hCD20, hCD4, and hCD8. Validated males and females humanized mice of 16 to 20 weeks old were grafted s.c in the right flank with $1.5.10^6$ MDA-MB-231 breast cancer cells. Mice were euthanized when the tumor reached 3000 mm3 in control groups or 4 to 5 weeks after tumor implantation. All protocols were approved by the French National Ethical Committee on Animal Experimentation (Ce5/2012/025). To assess the effects of the various treatments on tumor growth, a total of four experiments are presented. Not every experiment included all conditions. Males and females NSG were randomly dispatched into the various experimental groups to avoid sex-linked effects. Experimental groups were dispatched in different cages to avoid cage-related effects. Tumor growth was monitored in a blinded fashion. The number of mice used in each condition is indicated in the figure legend.

Cell line

The triple-negative ($PR^{neg}ER^{neg}HER\text{-}2^{neg}$) MDA-MB-231 cell line was grown in DMEM media supplemented with 10% FCS, L-glutamine and antibiotics (Penicillin/Streptomycin) (all from Thermo) in tissue culture flasks. Cells were transduced with a lentiviral vector co-expressing GFP and Luciferase to follow efficient engraftment *in vivo* using luciferase and to allow the exclusion of tumor cells from the

analysis based on GFP expression. Cells were confirmed of being mycoplasma-free by a standard Hoechst-dye coloration on indicator Vero cells before injection into mice. A genetic profiling was established and confirmed the identity of the cell line (Eurofins Forensic Department, Ebersberg, Germany).

Reagents preparation and injection

The 314.8 mAb (mouse IgG1 anti-human ICOS) has been described before.[21] Isotype controls (mouse IgG1, MOPC-1; rat IgG2b, LTF-2) and anti-Gr1 mAb (rat IgG2b, RB6-8C5) were purchased from BioXcell (West Lebanon, NH, USA). The MT807R1 recombinant Ig consisting of rhesus IgG1k constant regions and CDRs derived from the anti-human CD8 antibody M-T807 grafted into rhesus variable framework regions and was provided by the Nonhuman Primate Reagent Resource (NIH contract HHSN272200900037C and grant RR016001). The antibody was expressed *in vitro* using serum-free medium and purified by protein-A affinity chromatography. Endotoxin was <1EU/mg. Cyclophosphamide (CTX, Sigma Aldrich) was prepared extemporaneously according to supplier technical data sheet, i.e to 20 mg/ml of injectable water. All reagents were injected intraperitoneally.

Phenotypic analysis of leukocytes in the spleens and tumors of humanized mice

Splenocytes and tumors were digested with 1.6 mg/ml of collagenase IV and 10 µg/ml of DNAse I (Sigma Aldrich) for 2 h at 37° with regular flushing of the tissue. Cells were passed through a 40 µm-cell strainer and resuspended in PBS 3% SVF. To eliminate dead cells and debris, tumor cell suspensions were isolated on a Ficoll gradient. Rings were collected, washed, and cell pellets were resuspended in PBS 3% SVF before counting with Trypan blue exclusion. Subsequently, 3 to 6. 10^6 cells live cells were stained with corresponding antibodies (lanthanide labeled mAbs for CyTOF or fluorochrome-labeled mAbs for FACS analysis).
The details of each panel (each one corresponding to one experiment) can be found in Table S1. For CyTOF, 1 to 3 µl of each lanthanide-labeled mAbs was added in 1.5 ml Eppendorf tubes in a final volume of 50 µl of Max Par Staining Buffer (Fluidigm, San Francisco, USA), according to manufacturer protocol. Intracellular staining was performed with FOXP3 staining kit (eBioscience, Courtaboeuf, France). Cell events were acquired on the CyTOF-2 mass cytometer and CyTOF software version 6.0.626 (Fluidigm) at the Cytometry Pitié-Salpétrière core (CyPS). Dual count calibration, noise reduction, cell length threshold between 10 and 150 pushes, and a lower convolution threshold equal to 10 were applied during acquisition. Data files produced by the CyTOF-2 were normalized with the MatLab Compiler software normalizer using the signal from the 4-Element EQ beads (Fluidigm) as recommended by the software developers. GFP exclusion was performed to remove tumor cells from the analysis. To normalize the variability between mice for supervised (i.e. 2D plots) and unsupervised (i.e. tSNE) analysis, samples from tumors and spleen individually acquired on the CyTOF were aggregated in an individual file in each organ and in each experiment.

Clinical samples

The main clinical characteristics of the patients are summarized in Table 1. Luminal breast tumors were collected from nine untreated cancer patients undergoing standard surgery at Institut Curie Hospital, in accordance with institutional ethical guidelines and approved by the ethical (CPP ref: 99-15) and medical (ANSM ref: 2015-A00824-45) committees. Flow cytometry data in those patients were collected prior to the initiation of the HuMice study; hence, no CyTOF data were collected from human patients.

Tumor samples from patients and cell isolation

Samples were cut into small fragments, digested with 0.1 mg/ml Liberase TL in the presence of 0.1 mg/ml DNase (Roche, Meylan, France) and incubated for 30 min at 37°C in 5% of CO2 incubator. Subsequently, cells were filtered on a 40-µm cell strainer (BD Biosciences, Le Pont-de-Claix, France), washed and submitted to staining with specific antibodies. Peripheral blood from breast cancer patients was collected in tubes containing EDTA, washed in PBS and submitted to staining with specific fluorescent-labeled antibodies.

Phenotypic analysis of leukocytes in whole blood and tumors in human breast cancer patients

Tumor cell suspension and whole blood were stained with LIVE/DEAD Fixable Aqua (Life Technologies, Courtaboeuf, France) for 10 min at RT. Then, cells were washed and stained with Aqua BV500 dead cells exclusion dye (Life

Table 1. Clinical characteristics of the primary breast tumors samples. Age = age of the patient at the time of surgery/Meno. status = Menopausal status/Histo. = Histologic type/Grad. = SBR Grade (histo-prognostic grade based on "Scarff-Bloom-Richardson")/pTNM = tumor classification based on Tumor-Nodes-Metastasis score/ER = % of estrogen receptor positivity/PR = % of progesterone receptor positivity/Ki67% = cellular marker of proliferation/TILs = % of Tumor-infiltrated Lymphocytes.

Code Pat.	Age (yes)	Meno status	Histo.	Grad.	Size (mm)	pTNM	ER (%)	PR (%)	HER2	Ki67 (%)	TIL (%)
808541	32	no	Ductal	2	26 + 17 (bi-focal)	pT2N2M0	95 et 100	95 et 95	neg	10 et 40	20
809206	52	pre-meno	Ductal	3	90*60	pT4N3a	80	90	neg	30	10
809113	60	yes	Ductal	2	28	pT2N1M0	100	30	pos	17	10
809090	40	no	Ductal	3	25	pT2N2M0	90	90	neg	40	30
809846	54	no	Ductal	2	23	pT2N2a	80	50	neg	10	15
809797	89	no	Lobular	2	50	pT2N2M0	90	<5	neg	10	10
BR914	73	yes	Ductal	3	23	pT2N2M0	100	0	neg	40	30
810227	41	no	Ductal	2	15	pT1cN2aM0	100	40	neg	10	10
AB3160	54	no	Lobular	2	55 + 5 + 6	pT3mN3aM0	40	5	neg	13	20

Technologies); anti-CD3 (clone OKT3, BV650), anti-CD4 (clone OKT4, BV785), anti-PD-1 (EH122.2H7, BV711), anti-CD27 (O323, BV605) from Biolegend (London, UK); anti-CD 45 (clone 2D1, APC Cy7), anti-CD8 (clone RPA-T8, BUV395), anti-CD19 (clone HIB 19, Alexa 700) anti-CD56 (NCAM16.2, BUV737), anti-HLA-DR (G46.6, PECy5), anti-PD-L1 (MIHI, PE-CF594) from BD Biosciences; anti-EpCAM (1B7, eFluor660), anti-CD14 (2G5, FITC), anti-CD45RA (HI100, PECy7), and anti-ICOS (ISA3, PERCPe710) from eBioscience for 20 min at 4°C. After incubation, cells were washed and permeabilized for 16 h according to manufacturer's instructions for staining with anti-FOXP3 (236A/E7, PE, eBioscience) and anti-Granzyme-B (GB11, BV421, BD Biosciences). Cells were then fixed for subsequent analysis on a Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo Version v10 (FlowJO LCC, Ashland, USA), or Cytobank (www.cytobank.org).

Mass cytometry data analysis

For CyTOF data analysis, 10 healthy cord blood donors were used in four independent experiments, each experiment including mice reconstituted with different donors. A total of 15 mice were analyzed at the steady state by mass cytometry for human and murine cell content in the spleen and the tumor. Due to the paucity of cells in tissues of some HuMice, and to increase the resolution of the analysis, tissue samples from 2 to 3 of those mice were pooled before staining and CyTOF analysis. The frequency of $hCD45^+$ determined by flow cytometry was used to normalize the representation of each mouse within the pool. In case where a sufficient number of cells were collected, tissue samples from individual mice were run into CyTOF. To normalize the analysis from these various conditions, concatenation of individual mice from the same tissue in the same experiment was performed using FlowJo v10. Concatenated samples were exported in Cytobank for unsupervised viSNE analysis or were analyzed using FlowJo v10 for supervised analysis. For viSNE analysis, the default settings were applied: 1000 iterations, perplexity of 30 and a theta factor of 0.5.

Statistical analysis

Statistical analyses were performed using Prism v6.0h for Mac (GraphPad) with statistical models indicated in the figure legends. The outliers detection method is reported in the figure legends when applicable. All tests were performed with $\alpha < 0.05$ (probability of detecting a difference in the means by chance below 5%). No a-priori sample size estimation to fit statistical power with the observed effect on tumor growth was used. However, a reverse analysis of our data (G-Power; gpower.hhu.de) showed that given the number of mice included in the study, the observed difference in the means at the end of the experiment and the standard deviations in both groups, the β power was >95%, hence validating the rejection of the null hypothesis by statistical modeling of the data. For statistical analysis of tumor growth, the null hypothesis stating that one curve fits all the data in the compared groups was rejected if the p-value was inferior to 0.05, determined by nonlinear regression modeling of the data using the exponential growth equation.

Abbreviations

ICOS	Inducible Costimulatory
NSG	NOD.SCID.gc-null
Treg	regulatory T cells
CTX	cyclophosphamide
HuMice	humanized mice
CyTOF	cytometry time-of-flight
tSNE	t-distributed stochastic neighbor embedding
pDCs	plasmacytoid dendritic cells
DC	dendritic cells
ICD	immunogenic cell death

Author's contributions

Conceptualization, AB, CC, and GM; Methodology, AB and GM; Formal Analysis, AB, RR, PKC, KS, AC, and GM; Investigation, AB, RR, PKC, KS, and AC; Resources, EP, DO, and CMC; Writing-Original Draft, AB and GM; Writing-Review and editing, AB, RR, EP, PKC, CMC, CC, and GM; Visualization, AC, GM; Supervision, GM; Funding acquisition, CMC, CC, DO, and GM.

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Competing interests

The authors declare no competing interests. GSK had neither implication in the design of the experiments nor in the interpretation of the results.

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