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**Effet des radiations ionisantes sur le système immunitaire de l'hôte et de la tumeur dans un modèle murin d'allogreffe et des cancers du sein localement avancés.**

Nhu Hanh To

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UNIVERSITÉ PARIS-EST

École Doctorale Sciences de la Vie et de la Santé

**Thèse de doctorat**

Pathologie et Recherche Clinique

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**Effets des radiations ionisantes sur le système immunitaire  
de l'hôte et de la tumeur dans un modèle murin d'allogreffe  
et des cancers du sein localement avancés**

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**Nhu Hanh TO**

Soutenue le 08 novembre 2022

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## Résumé

La radiothérapie (RT) fait partie des principales armes thérapeutiques dans le traitement des cancers. Actuellement en France, on estime à environ 200.000 le nombre de patients qui reçoivent une RT à divers stades de leur maladie chaque année.

L'impact de la RT sur le système immunitaire est reconnu pour son effet « myéloablatif » depuis plusieurs décennies chez les malades candidats aux greffes mais aussi chez les victimes des accidents nucléaires et des explosions aux bombes atomiques ayant induit une insuffisance médullaire mortelle. Au plan thérapeutique, des effets immunostimulateurs de la RT ont été rapportés sous le terme « effet abscopal » correspondant à des réponses antitumorales après la RT dans des sites à distance non irradiés des tumeurs solides.

Mes travaux dans laboratoire (équipe I-BIOT de l'IMRB) se sont focalisés sur la modulation de la réponse immunitaire appliquée aux allogreffes et au cancer dans les deux modèles suivants :

1. Modèle murin d'allogreffe : la maîtrise des paramètres de l'irradiation corporelle totale (ICT) est capitale pour la reproductibilité et la fiabilité des résultats expérimentaux dans le modèle murin. Les données de la littérature ont montré une variation considérable dans la reproduction des résultats d'une expérience à l'autre en fonction du dispositif utilisé pour délivrer l'ICT avant allogreffe de moelle osseuse. L'objectif de ce travail était de comparer 2 types d'appareils d'irradiation utilisant différents niveaux d'énergie de photons en termes de résultats post-allogreffe (chimérisme, réaction du greffon contre l'hôte et du contrôle tumoral post-greffe). Nous avons démontré que l'ICT avec des photons de basses énergies induit une

inflammation post-ICT plus importante que les photons de hautes énergies et par conséquent une alloréactivité plus importante post-greffe. Cette inflammation peut être modulée par un retard à l'injection de la moelle osseuse programmée en dehors du pic inflammatoire pour réduire ainsi l'alloréactivité post-greffe. Ces données sont à prendre en considération pour l'interprétation des résultats obtenus selon le type d'appareil utilisé dans le modèle expérimental.

2. Modèle clinique de cancer du sein : la deuxième partie s'inscrit dans le cadre d'une étude translationnelle d'une recherche clinique prospective évaluant la place d'une radiothérapie (RT) préopératoire dans le cancer du sein triple négatif et luminal B. Ces derniers sont connus pour leur agressivité et leur immunogénicité particulière. Dans ce modèle la RT pré opératoire avait pour objectif d'augmenter les réponses tumorales en agissant, entre autres, sur l'immunité tumorale. Les biomarqueurs immunitaires dérivés du microenvironnement tumoral et du sang périphérique ont été analysés. Sur une analyse préliminaire de 42 patientes, nous avons identifié différents biomarqueurs prédictifs de la réponse pathologique complète selon le traitement pré-opératoire administré (avec ou sans RT). Ces résultats permettent de mieux sélectionner les patientes pour une meilleure personnalisation de cette nouvelle approche thérapeutique.

## Abstract

Radiotherapy (RT) is one of the main therapies for cancer treatment. Currently, in France, it is estimated that approximately 200,000 patients receive RT each year for different stages of the disease.

The impact of RT on the immune system has been recognized for several decades for its "myeloablative" effect in patients who are candidates for bone marrow transplant (BMT), but also in victims of nuclear accidents and atomic bomb explosions who suffered from fatal bone marrow failure. Regarding therapeutic potentials, immunostimulatory effects of RT have been reported under the term "abscopal effect," corresponding to antitumor responses after RT in non-irradiated sites distant from solid tumors.

My work in the laboratory (I-BIOT team of the IMRB) focused on the modulation of the immune response applied to allografts and cancer in the two following models:

1. Allograft mouse model: understanding the parameters of total body irradiation (TBI) is essential for the reproducibility and reliability of experimental results in mouse models. Data from the literature have shown considerable variation in the reproduction of the results between experiments depending on the device used to deliver the ICT before BMT. The objective of this work was to compare two types of irradiation devices using different energy levels regarding post-allograft results (chimerism, graft-versus-host disease, and post-transplant tumor control). We demonstrated that TBI with low-energy photons induced more post-TBI inflammation than high-energy photons and, therefore, more post-BMT alloreactivity. This inflammation could be modulated by delaying the graft outside of the inflammatory peak and thus reducing post-BMT



alloreactivity. These data should be taken into consideration for the interpretation of the results obtained according to the type of device used in the experimental model.

2. Clinical model of breast cancer: the 2nd work is part of a translational study of a prospective clinical trial evaluating the place of preoperative radiotherapy (RT) in triple-negative and luminal B breast cancer. These subtypes of breast cancer are well known for their aggressiveness and their immunogenicity. In this model, the preoperative RT aimed to increase tumor responses by acting on tumor immunity. Immunity-related biomarkers derived from tumor microenvironment and peripheral blood were analyzed. In a preliminary analysis of 42 patients, we identified different potential biomarkers associated with pathologic complete response according to the preoperative therapy administered (with or without RT). These results might help to better select patients individually for this new therapeutic approach.

## **Mots clés**

- Radiothérapie
- Immuno-modulation
- Hôte
- Tumeur
- Allogreffe
- Cancer du sein

## **Keywords**

- Radiotherapy
- Immuno-modulation
- Host
- Tumor
- Allograft
- Breast cancer

## **Laboratoire d'accueil**

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## Abréviations

AC	: protocole de chimiothérapie à base de doxorubicine et de cyclophosphamide
AI	: aromatase inhibitor (inhibiteurs d'aromatase)
AL	: accélérateurs linéaires
ALC	: absolute lymphocyte count (nombre absolu de lymphocytes)
APBI	: accelerated partial breast irradiation (irradiation mammaire partielle et accélérée)
APC	: antigen-presenting cell (cellule présentatrice d'antigène)
ATP	: adénosine triphosphate
CCL2	: motif chémokine ligand 2
CDB	: cassure double-brin
CMH	: complexe majeur d'histocompatibilité
CRTNA	: chimio-radiothérapie néoadjuvante
CSB	: cassure simple-brin
CSH	: cellules souches hématopoïétiques
CTA	: chimiothérapie adjuvante
CTL	: cytotoxic T lymphocyte (lymphocytes T cytotoxiques)
CTLA-4	: cytotoxic T lymphocyte associated protein 4
CTNA	: chimiothérapie néoadjuvante
DAMP	: damage associated molecular pattern (motif moléculaire associé aux dégâts)
DC	: dendritic cell (cellule dendritique)
DFS	: disease-free survival (survie sans maladie)
EFS	: event-free survival (survie sans évènement)
GVHD	: graft-versus-host disease (maladie du greffon contre l'hôte)
GVHDa	: GVHD aiguë
GVHDc	: GVHD chronique

GVL	: graft-versus-leukemia (effet du greffon contre la leucémie)
Hb	: hémoglobine
HIF-1 $\alpha$	: hypoxia inducible factor 1 subunit alpha
HLA	: human leukocyte antigen (antigènes des leucocytes humains)
HMGB1	: high mobility group box 1
HSCT	: hematopoietic stem cells transplantation (transplantation de cellules souches hématopoïétiques)
HTNA	: hormonothérapie néoadjuvante
I-BIOT	: équipe d'Immunorégulation et Biothérapie
ICD	: immunogenic cell death (mort cellulaire immunogène)
ICI	: immune checkpoint inhibitors (inhibiteurs des points de contrôles immunitaires)
ICT	: irradiation corporelle totale
IFN	: interféron
IHC	: immunohistochimie
IL	: interleukine
IMC	: indice de masse corporelle
IMRB	: Institut Mondor de Recherche Médicale
kV	: kilovolts
LAL	: leucémie aiguë lymphoblastique
LAM	: leucémie aiguë myéloblastique
LA	: luminal A
LB	: luminal B
LINAC	: linear accelerator (accélérateurs linéaires)
LMR	: lymphocyte-to-monocyte ratio (rapport entre lymphocytes et monocytes)

LPBC	: lymphocyte-predominant breast cancer (cancer du sein à prédominance lymphocytaire)
LyB	: lymphocyte B
LyT	: lymphocyte T
mAb	: anticorps monoclonaux
MAC	: myeloablative conditioning (conditionnement myéloablatif)
miR	: micro-ARN
NA	: néoadjuvant
NLR	: neutrophil-to-lymphocyte ratio (rapport entre neutrophiles et lymphocytes)
NMA	: non-myeloablative conditioning (conditionnement non-myéloablatif)
MO	: moelle osseuse
MV	: méga-volts
NK	: natural killer (cellule killeuse naturelle)
NREx	: non réponse exceptionnelle
OS	: overall survival (survie globale)
pCR	: pathologic complete response (réponse complète pathologique)
PD-1/PD-L1	: Programmed cell Death protein 1 and its Ligand
PLR	: platelet-to-lymphocyte ratio (rapport entre plaquettes et lymphocytes)
PNN	: polynuclear neutrophil (neutrophile polynucléaire)
RC	: rémission complète
REx	: réponse exceptionnelle
RI	: rayonnements ionisants
RIC	: reduced-intensity conditioning (conditionnement à intensité réduite)
RNS	: reactive nitrogen species (espèces réactives de l'azote)
RO	: récepteur d'œstrogène

RP	: récepteur de progestérone
RT	: radiothérapie
RTNA	: radiothérapie néoadjuvante
RxBE	: rayons X de basses énergies
RxHE	: rayons X de hautes énergies
SBRT	: radiothérapie stéréotaxique
TAM	: tumor-associated macrophages (macrophages associés à la tumeur)
TCR	: T cell receptor (récepteur des lymphocytes T)
Teff	: lymphocyte T effecteur
TFG- $\beta$	: transforming growth factor beta (facteur de croissance transformant bêta)
Th1	: T helper 1 (lymphocyte T auxiliaire de type 1)
TIL	: tumor-infiltrating lymphocytes (lymphocytes infiltrant dans la tumeur)
TME	: tumor microenvironment (microenvironnement tumoral)
TN	: triple-négatif
TNF	: tumor necrosis factor (facteur de nécrose tumorale)
TNM	: classification de stadification basée sur Tumeur-Node-Métastase
cTNM	: classification TNM clinique
pTNM	: classification TNM pathologique
Treg	: lymphocytes T régulateurs

## AVANT-PROPOS

La radiothérapie (RT) fait partie des principales armes thérapeutiques dans le traitement des cancers. Actuellement en France, on estime qu'environ 200.000 patients bénéficient d'une RT chaque année (Defossez et al., 2019).

Le but carcinologique de la RT est de contrôler l'évolution tumorale en inhibant la prolifération cellulaire par (i) effet direct par un dépôt d'énergie des rayonnements ionisants (RI) dans les molécules à l'intérieur des cellules ou en entraînant des dommages de l'ADN par des cassures de simple- ou de double-brin (CSB ou CDB) ou (ii) un effet indirect par la production des radicaux libres oxygénés hautement réactifs (Bailey et al., 2016; Hall, 2018). L'accumulation des dommages dépassant la capacité des mécanismes de réparation de l'ADN conduit finalement à la mort cellulaire (Kroemer et al., 2009) ou à la sénescence qui se caractérise par un arrêt permanent du cycle cellulaire (Collado and Serrano, 2010).

En dehors de l'effet antitumoral, l'impact de la RT sur le système immunitaire a été aussi connu pour son effet « myéloablatif ». En effet, certaines victimes des accidents nucléaires et les explosions de bombes atomiques au milieu du XX<sup>e</sup> siècle mourraient des suites d'insuffisance médullaire (Jacobson and Marks, 1949; Mathe et al., 1959). C'est dans ce contexte qu'est née la transplantation des cellules souches dérivées de la moelle osseuse après des conditionnements associant les irradiations corporelles totales à la chimiothérapie dans le cadre des hémopathies malignes et non malignes (Thomas et al., 1957).

A la même période, des effets immunostimulateurs de la RT ont été rapportés, sous le terme « effet abscopal » (Mole, 1953) correspondant à des réponses immunitaires



antitumorales après la RT des sites à distance non irradiés des tumeurs solides. Cependant, l'effet abscopal restait très rare après une RT conventionnelle seule. Les progrès récents de l'immunothérapie et des techniques d'irradiation nous offriront des opportunités de mettre en lumière ce phénomène. En effet, la RT, notamment quand elle est délivrée à des hautes doses par fraction, peut induire un autre type de mort cellulaire dite « immunogène », et provoquer une réponse immunitaire pro-inflammatoire antitumorale, favorisée par son adjonction aux inhibiteurs des points de contrôle immunitaire (Reynders et al., 2015). La modulation du système immunitaire permet de réduire en partie les séquelles thérapeutiques après RT alors que le statut immunitaire de la tumeur avant traitement peut être déterminant pour la sensibilité tumorale à la RT et offre des possibilités d'associations thérapeutiques personnalisées plus efficaces.

Dans ce contexte d'évolution de la radio-immunothérapie, je me suis intéressée dans ma pratique et mes recherches à l'impact de la RT sur le système immunitaire. J'ai eu ainsi l'opportunité de rejoindre l'équipe d'Immunorégulation et Biothérapie (I-BIOT) de l'Institut Mondor de Recherche Médicale (IMRB) qui travaille sur la modulation de la réponse immunitaire appliquée aux allogreffes et au cancer. Mes travaux de thèse se sont intéressés à l'impact de l'irradiation sur la réponse immunitaire impliquée dans deux pathologies :

1. Le premier travail vise à étudier l'impact de l'irradiation corporelle totale dans les modèles murins d'allogreffe ;
2. La deuxième partie constitue l'étude translationnelle d'un essai clinique étudiant l'impact d'une RT préopératoire de sous-types de cancers du sein caractérisés par une grande immunogénicité, les cancers triples négatifs (TN) et luminaux B (LB).

Ainsi, mon mémoire de thèse s'articule de la façon suivante :

- La première partie introduit les notions et concepts de la radiobiologie et les effets des radiations ionisantes sur le système immunitaire.
- La deuxième partie présente les effets de l'irradiation corporelle totale sur le système immunitaire, dans les modèles murins de greffe de cellules souches hématopoïétiques.
- La troisième partie présente les potentiels biomarqueurs prédictifs à la réponse thérapeutique de l'association chimio-radiothérapie néoadjuvante (CRTNA) dans un modèle de tumeurs solides, les cancers du sein des sous-types moléculaires TN et LB.
- Le manuscrit s'achève sur une conclusion générale et des perspectives.



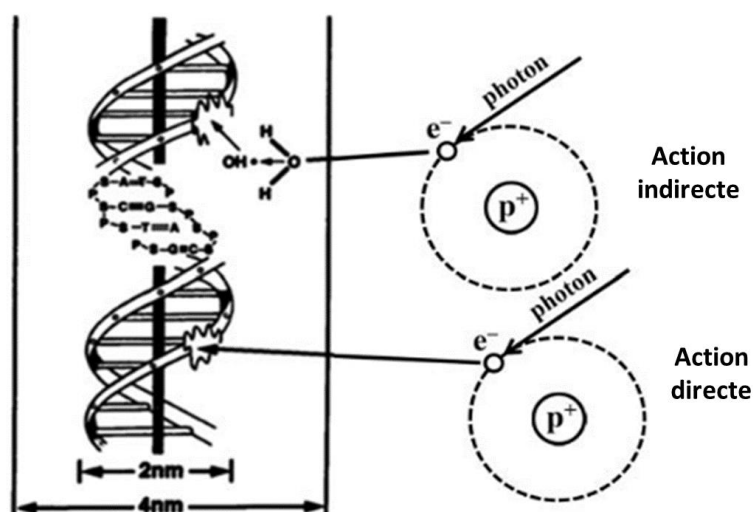
**INTRODUCTION GENERALE DES EFFETS BIOLOGIQUES DES  
RADIATIONS IONISANTES SUR LE SYSTÈME IMMUNITAIRE**

## I. Radiobiologie

La radiobiologie est l'étude (à la fois qualitative et quantitative) des actions des rayonnements ionisants (RI) sur les êtres vivants (Christensen et al., 2014). La connaissance des aspects radiobiologiques des RI est primordiale dans la prise en charge des patients en RT afin d'assurer le contrôle tumoral en minimisant les effets indésirables potentiels sur le corps humain.

### 1. Effets moléculaires : ADN, la cible privilégiée des rayonnements ionisants

Les dommages induits par les RI sur les cibles biologiques sont des actions directes et indirectes par l'intermédiaire de produits de la radiolyse de l'eau cellulaire (Figure 1). L'ADN est la cible principale des RI (Hirayama et al., 2009; Nikjoo et al., 2002). La molécule d'ADN peut être lésée directement par les électrons ou plus souvent indirectement par les radicaux libres issus de la radiolyse de l'eau ; il s'agit de lésions des bases et des sucres, de pontages, ou surtout de ruptures de chaîne, simple ou double (Goodhead, 1994; Tubiana et al., 1986).



**Figure 1** : Dommages directs et indirects aux molécules d'ADN cellulaires, modifiée de (Bailey et al., 2016).

La signalisation des dommages aboutit à l'arrêt transitoire du cycle cellulaire (*checkpoint*) pour permettre la réparation avant les phases critiques du cycle (la réplication et la mitose). Si le nombre des dommages sont trop importants, la cellule sera éliminée par la mort cellulaire programmée. Cela permet d'éviter la propagation de cellules porteuses de dommages.

## 2. Effets cellulaires des rayonnements ionisants : la mort cellulaire

Les effets néfastes des RI dépendent de la dose d'irradiation et de la capacité de réparer les dommages à l'ADN de la cellule irradiée. Après l'exposition aux RI, trois types de lésions de différents degrés sont créés :

- **Lésions létales** : qui sont irréparables, touchent des fonctions vitales pour la cellule et entraînent la mort cellulaire immédiate ou différée.
- **Lésions sub-létales** : qui ne sont pas individuellement létales et sont réparables ; mais leur accumulation dans la cellule peut tout de même conduire à sa mort.
- **Lésions potentiellement létales** : qui peuvent être létales si la division survient rapidement, mais peuvent aussi être réparées si les conditions sont favorables.

Selon le niveau de dommages à l'ADN et le type cellulaire, seront déclenchés l'arrêt du cycle cellulaire, la réparation de l'ADN, la sénescence, l'apoptose ou la nécrose. L'échec d'activer avec précision l'un de ces points de contrôle des dommages à l'ADN dépendant de *p53* peut entraîner la mort cellulaire via une catastrophe mitotique ou la génération de cellules aneuploïdes, contribuant au développement et à la progression du cancer (Figure 2).

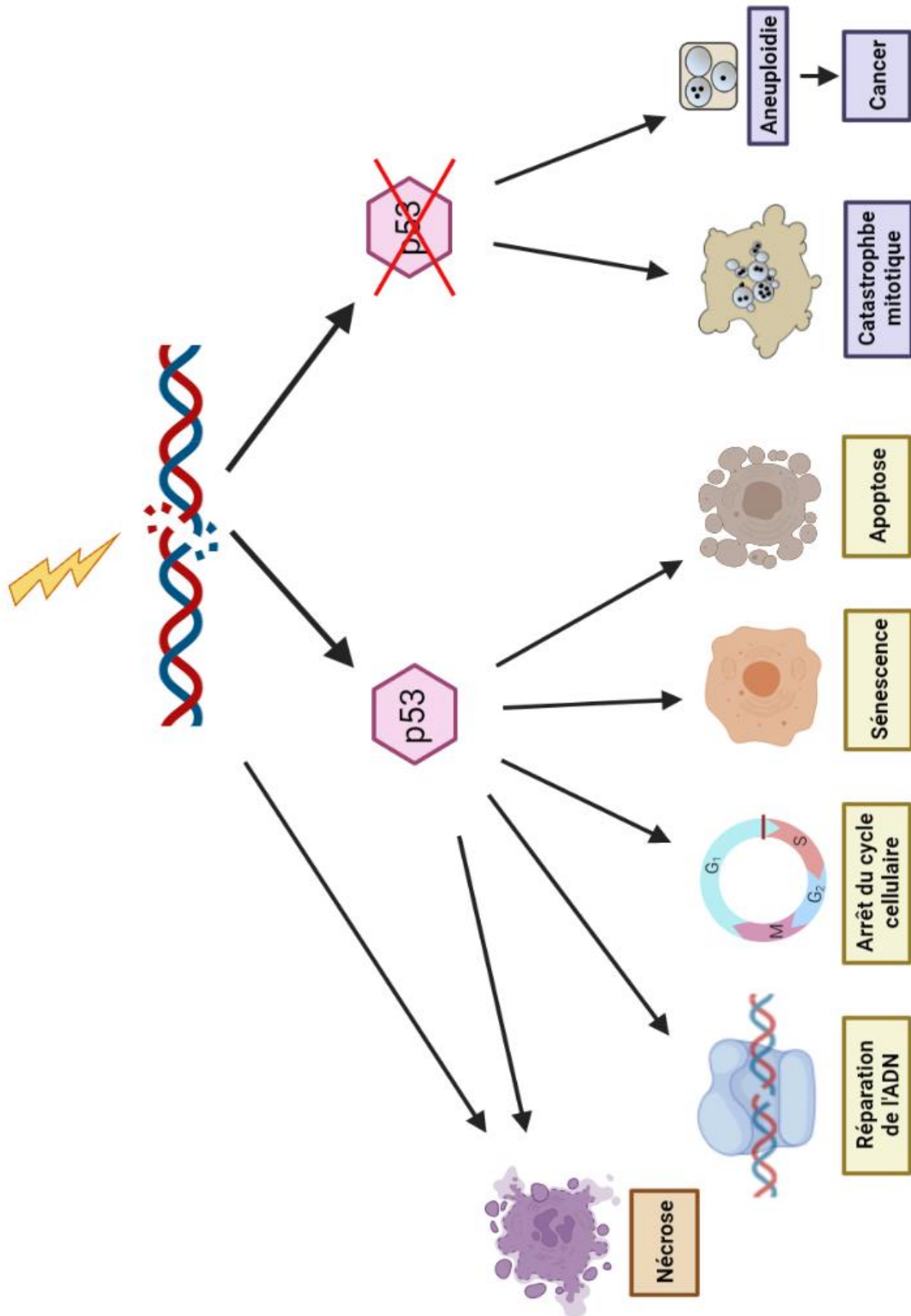


Figure 2 : Réponses cellulaires à l'irradiation.

### **2.1. La mort cellulaire radio-induite et p53**

La nature de la réponse cellulaire à l'irradiation est fortement influencée par l'activation du gène *p53*, souvent désigné comme « le gardien » du génome (Efeyan and Serrano, 2007). L'importance de *p53* en tant qu'un suppresseur de tumeur peut être soulignée par le fait que le gène *p53* est muté dans plus de la moitié des cancers humains (Soussi and Bérout, 2001). P53 est un facteur de transcription avec contrôle des gènes qui influencent l'arrêt du cycle cellulaire, la réparation de l'ADN, l'apoptose, et la sénescence. Après l'irradiation, l'activation de *p53* favorise la survie des cellules par l'arrêt du cycle cellulaire et la réparation des dommages de l'ADN (Yoshida and Miki, 2010). Cependant, en fonction de l'étendue des dommages et du type cellulaire, *p53* peut également activer les voies d'élimination des cellules endommagées (Helton and Chen, 2007; Yoshida and Miki, 2010). Les cellules dont l'activation de *p53* est altérée (mutations, certaines infections virales) peut entraîner des divisions anormales, faciliter l'aneuploïdie, le développement et la progression du cancer. Typiquement, l'exposition de ces cellules aux RI génère une catastrophe mitotique et la mort cellulaire dont quelques formes typiques sont illustrées dans la Figure 2.

### **2.2. La mort cellulaire immunogène**

La radiobiologie traditionnelle stipule que l'effet cytotoxique des RI est principalement dû à la production des CDB de l'ADN engendrant la mort cellulaire. Cette hypothèse a construit les quatre principes de base appelés les **4Rs** de la radiobiologie (Withers, 1975) qui représentent : (i) le **R**éassortiment (des cellules en phases G2/M plus radiosensibles), (ii) la **R**é-oxygénation (des zones hypoxiques), (iii) la **R**éparation (des dommages sublétaux à l'ADN), et (iv) le **R**epeuplement (des cellules survivantes).



Plus tard, la **R**adiosensibilité intrinsèque a été incluse au quatuor (Steel et al., 1989). Ce 5<sup>e</sup> R nous a permis d'expliquer la différence de réponse thérapeutique observée entre différentes maladies au plan mécanistique. Par exemple le séminome ou la plupart des lymphomes sont très radiosensibles, et à contrario le glioblastome ou le mélanome sont très radiorésistants (Deacon et al., 1984). Toutefois, la portée de ce concept est limitée car il ne tient compte ni du microenvironnement tumoral (TME pour *tumor microenvironment*) ni de l'immunité antitumorale de l'hôte ; alors que le recrutement du système immunitaire contribue à la réponse de la RT.

La RT a la capacité de modifier favorablement le TME immunosuppresseur par des mécanismes principalement liés à l'induction de la mort cellulaire immunogène (ICD pour *immunogenic cell death*) (Golden et al., 2012). L'ICD est définie comme une forme de mort cellulaire programmée qui est suffisante pour activer une réponse immunitaire adaptative chez un hôte immunocompétent (Galluzzi et al., 2020).

Trois composants orchestrent la machinerie d'ICD dans les cellules tumorales engagées dans le processus de mortalité et sont nécessaires pour l'amorçage et l'activation immunitaire : (i) la translocation membranaire de la calréticuline (un puissant signal « mange-moi »), et la libération extracellulaire de (ii) HMGB1 (pour *high-mobility group box 1*) et (iii) d'ATP (pour *adenosine triphosphate*) (Galluzzi et al., 2020). Il a été démontré que la RT pouvait induire ces 3 phénomènes, agissant pour favoriser la phagocytose des cellules tumorales par les cellules dendritiques (DC pour *dendritic cell*), la présentation des antigènes dérivés de la tumeur et l'amorçage croisé des CTL CD8+ (Golden et al., 2012).

Cette **R**éactivation de la réponse immunitaire antitumorale pouvant être considérée comme le 6<sup>e</sup> R de la radiobiologie (Boustani et al., 2019), entraîne ainsi une mémoire

immunitaire et des effets systémiques avantageux dont l'effet abscopal (Formenti and Demaria, 2009; Golden and Apetoh, 2015).

### 3. Les effets tissulaires

Les cellules mortellement affectées par l'irradiation peuvent rester fonctionnelles (pendant un certain temps) et mourront lors de la mitose. Les effets cliniques observables des RI sont le résultat du dysfonctionnement cellulaire à une grande échelle

Les effets tissulaires des RI sont généralement divisés en deux catégories :

- Les effets stochastiques sont des effets probabilistes et indépendants de la dose absorbée, ils se produisent par hasard et sont extrêmement rares mais impliquent la carcinogénèse ou des effets génétiques dus à l'irradiation (Scott et al., 2003).
- Les effets déterministes ne se produisent que si une certaine dose seuil est atteinte et sont dose-dépendants. Ce type d'effet est prévisible et reproductible (Dörr, 2015).

En fonction de l'évolution dans le temps des effets, on peut distinguer :

- Les effets précoces : ceux observés dans les 90 premiers jours après le début de la RT. Ils sont plus importants dans les tissus contenant des cellules à prolifération rapide (Stone et al., 2003). Ces derniers disposent également d'une meilleure récupération grâce à une prolifération accélérée des cellules souches basales.

- Les effets tardifs : se développent des mois voire des années après le traitement. Les effets tardifs ont tendance à survenir dans les tissus dont les cellules ont un renouvellement lent (Dörr, 2015; Stone et al., 2003).
- Les effets tardifs consécutifs : ceux qui se développent dans les situations où les réactions aiguës ne guérissent pas complètement et persistent longtemps après le traitement, et deviennent des lésions chroniques (Dörr, 2015).

L'objectif ultime de la RT, dans la grande majorité des cas, est de participer à la guérison d'une maladie mortelle tout en minimisant les risques de toxicités précoces ou tardives (Chauvet et al., 2013).

## II. Système immunitaire et radiothérapie

L'effet tumoricide de la RT a longtemps été expliqué par des effets biologiques directs, qui provoquent des lésions de la molécule d'ADN, notamment des CDB, entraînant la mort cellulaire si elles ne sont pas réparées (Bailey et al., 2016; Hall, 2018). L'avènement de la RT stéréotaxique à des hautes doses par fraction a, par ailleurs, mis en lumière des effets biologiques indirects, dont l'ICD, avec intervention des cellules du TME participant à la mort des cellules tumorales (Garcia-Barros et al., 2003).

### 1. Effets pro-immunogènes antitumoraux de la radiothérapie et effet abscopal

#### 1.1. Effets pro-immunogènes antitumoraux

La RT favorise la présentation des antigènes tumoraux en majorant le pool cellulaire d'antigènes spécifiques et favorisant l'expression du complexe majeur d'histocompatibilité (CMH) de classe I chargé de présenter les antigènes à la surface des cellules tumorales (Reits et al., 2006). Elle génère également une mort cellulaire « immunogène » (ICD) via la libération des motifs moléculaires associés aux dégâts (DAMP pour *damage associated molecular pattern*) (Galluzzi et al., 2020; Golden and Apetoh, 2015). Parmi ces signaux de danger libérés dans le TME, on retrouve l'ATP (adénosine triphosphate) qui induit l'attraction des cellules présentatrices d'antigène (APC pour *antigen-presenting cell*), la protéine HMGB1 qui assure la maturation et l'activation des DC et la translocation membranaire de la calréticuline correspondant à un signal « mange-moi » aboutissant à la phagocytose des fragments cellulaires tumoraux (Golden et al., 2012; Procureur et al., 2021).

Ces phénomènes s'accompagnent d'une réaction pro-inflammatoire induite par l'irradiation, avec libération de cytokines comme les interleukines (IL) IL-1, IL-2, IL-6,

IL-12, les interférons (IFN) IFN- $\alpha$  et IFN- $\beta$  et le facteur de nécrose tumorale  $\alpha$  (TNF pour *tumor necrosis factor*), impliquées dans la réponse immunitaire antitumorale (Nessler et al., 2017). En effet, l'efficacité de « l'ablation tumorale » induite par la RT à haute dose nécessite l'activation d'expression d'IFN de type I dans les DC infiltrant la tumeur via l'activation de cGAS-STING (une voie de signalisation importante du système immunitaire innée qui détecte la présence d'ADN bicaténaire dans le cytosol et déclenche une réponse immunitaire puissantes) (Deng et al., 2014). Les DC activées présentent alors les antigènes tumoraux aux lymphocytes T (LyT) naïfs dans les ganglions lymphatiques permettant la formation de lymphocytes T cytotoxiques (CTL pour *cytotoxic T lymphocyte*) spécifiques des antigènes tumoraux (Formenti and Demaria, 2009). La RT facilite par la suite le recrutement de ces lymphocytes T effecteurs (Teff) en générant la sécrétion des chimiokines et des molécules d'adhésion cellulaire (Gaugler et al., 1997; Matsumura et al., 2008).

L'interaction entre le CTL et la cellule tumorale se fait par contact entre le CMH de classe I présentant l'antigène tumoral à la surface de la cellule tumorale et le récepteur des LyT (TCR pour *T cell receptor*) à la surface des LyT-CD8<sup>+</sup>. L'activité cytotoxique du LyT induisant l'apoptose de la cellule tumorale se fait alors soit par la libération de granules cytotoxiques (perforines, granzymes), soit via l'interaction entre le récepteur de mort Fas de la cellule tumorale et son ligand Fas-L à la surface du LyT. La RT provoque aussi une surexpression du récepteur de mort Fas à la surface des cellules tumorales irradiées, amplifiant l'activité des CTL (Chakraborty et al., 2003). Avec la formation de LyT mémoires circulants pendant des mois voire des années, la RT convertit ainsi la tumeur irradiée en un vaccin antitumoral personnalisé in situ (Golden et al., 2020).

Il a été démontré que les effets des RI peuvent survenir sur les cellules adjacentes aux cellules irradiées, soit à l'intérieur du volume d'irradiation, soit à proximité de ce dernier. Nagasawa et Little ont constaté que, bien que moins de 1% d'une population cellulaire ait été traversée par une particule ionisante, des dommages chromosomiques ont été retrouvés dans 30% des cellules (Nagasawa and Little, 1992). Les cellules du système immunitaire inné peuvent être activées par des RI pour produire des médiateurs pro-inflammatoires de l'instabilité génomique. Dans un modèle murin, les macrophages activés après l'irradiation de 4 Gy ont induit une instabilité chromosomique dans les cellules hématopoïétiques non irradiées via la production de TNF- $\alpha$  et des espèces réactives de l'azote (RNS pour *reactive nitrogen species*) (Lorimore et al., 2001). De tels effets ont été définis comme des « **effets bystander** », incluant l'induction d'une instabilité génomique, de mutations géniques et de la mort cellulaire (Herran, 2014; Nénot and Sugier, 2006).

### 1.2. Effet abscopal

Le terme « abscopal », dérivé du préfixe latin ab- (en dehors, à distance) et du suffixe grec -scopos (la cible), a été introduit par R.H. Mole en 1953 (Mole, 1953). Il s'agit d'un effet non ciblé, correspondant à une régression tumorale après la RT dans des sites situés à distance des volumes irradiés.

L'émergence de l'immunothérapie ouvre de nouvelles opportunités pour faciliter les effets abscopaux. Des travaux précliniques et des observations cliniques ont illustré cette potentielle combinaison synergique entre la RT et les inhibiteurs des points de contrôles immunitaires (ICI pour *immune checkpoint inhibitors*) tels que les inhibiteurs de CTLA-4 (*Cytotoxic T-Lymphocyte Associated protein 4*), PD-1/PD-L1 (*Programmed cell Death protein 1 and its Ligand*) ou les 2 (Reynders et al., 2015).

À l'exception de l'ICD, le mécanisme de l'effet abscopal est un terrain encore largement inexploré. *Camphausen et al.* ont montré que l'irradiation de la patte postérieure (sans tumeur) des souris immunocompétentes syngéniques (adénocarcinome pulmonaire ou fibrosarcome) permettait une inhibition de la croissance tumorale des tumeurs préalablement transplanté dans le dos (Camphausen et al., 2001). Il a été souligné que cet effet était dose-dépendant surtout pour des doses par fraction plus élevées. Il est important de noter que l'effet n'était pas spécifique à la tumeur et nécessitait une fonction intacte de la voie de signalisation *p53* dans le tissu irradié (Camphausen et al., 2001).

Pour que la RT exerce au mieux son rôle en tant que « vaccination *in situ* », il est crucial d'avoir un état inflammatoire tumoral caractérisée par l'infiltration tumorale de LyT CD8+ (van der Burg et al., 2016). La méthode la plus connue est de l'associer avec un boost additionnel, par exemple, vaccines et/ou les ICI (Frey et al., 2019; van der Burg et al., 2016).

Il est aussi important de rappeler que l'effet abscopal après une RT seule reste un phénomène extrêmement rare (Abuodeh et al., 2016). A contrario, il existe des situations où la RT peut provoquer une progression des métastases à distance (Camphausen et al., 2001) associée à une augmentation du TFG- $\beta$  sérique (Biswas et al., 2017) et à une surexpression des molécules témoignant d'une réponse immunosuppressive (Dong et al., 2002; Dovedi et al., 2017).

## 2. Effets immunosuppresseurs de la radiothérapie et immunosénescence

### 2.1. Effets immunosuppresseurs

La RT a été classiquement considérée comme un moyen d'éliminer toutes les cellules immunitaires (Smit et al., 1979; Stjernswärd et al., 1972). Des données plus récentes ont montré une radiosensibilité distincte des cellules immunitaires. Les LyT sont en général plus radiosensibles que les macrophages, les DC ou les cellules killeuses naturelles NK (*natural killer*) (Heylmann et al., 2014). Les cellules de Langerhans dans la peau sont particulièrement radiorésistantes et contribuent à l'immunosuppression par la migration radio-induite des lymphocytes T régulateurs (Treg) vers les ganglions lymphatiques (Price et al., 2015). L'exposition des cellules immunitaires aux RI lors d'une RT locale dépend de leur localisation et de la technique d'irradiation utilisée (Basler et al., 2018). Les cellules immunitaires circulantes dans le système vasculaire tumoral ne reçoivent qu'une faible dose. Toutefois, même s'il existe des preuves que le nombre des cellules immunitaires dans le sang périphérique diminue après la RT, les cellules immunitaires restantes semblent conserver leur fonctionnalité (Belka et al., 1999).

Par conséquent, les effets immunosuppresseurs de la RT ne peuvent pas être expliqués uniquement par la destruction des cellules immunitaires. *Wennerberg et al.* (Wennerberg et al., 2017) rapporte qu'il existe d'autres propriétés immunosuppressives de la RT qui empêchent le système immunitaire de développer une réponse antitumorale :

- Les monocytes sont attirés dans la tumeur par le motif chémokine ligand 2 (CCL2) libérée après RT. Dans le TME, ils se différencient en macrophages associés à la tumeur (TAM pour *tumor-associated macrophages*). Les TAM se



manifestent vers le phénotype M2 pro-tumorigénique et favorisent l'invasion tumorale et les métastases (Qian et al., 2009).

- La RT augmente l'expression du facteur de transcription HIF-1 $\alpha$  (*hypoxia inducible factor 1 subunit alpha*) qui est responsable, entre autres, de la régulation de plusieurs gènes impliqués dans l'immunosuppression, polarisant les TAM vers un phénotype M2 plus immunosuppresseur (Fujimura et al., 2018), favorisant la prolifération des Treg et activant les cellules myéloïdes suppressives.
- La RT active le facteur de croissance transformant bêta (TFG- $\beta$  pour *transforming growth factor beta*), une cytokine immunosuppressive multipotente impliquée dans la régulation de la différenciation cellulaire, la survie et la fonction de différents types de cellules immunitaires (Vanpouille-Box et al., 2015). Il a été démontré que le TFG- $\beta$  inhibe la différenciation des LyT-CD8+ en CTL (Thomas and Massagué, 2005), favorise la différenciation des LyT-CD4+ naïfs en Treg (Yamagiwa et al., 2001) et polarise les macrophages vers un phénotype M2 (Gratchev, 2017).
- Enfin, la RT génère une surexpression de PD-L1 à la surface des cellules tumorales et des cellules myéloïdes présentes dans le TME. Cette augmentation d'expression a pour conséquence de renforcer l'inhibition des LyT et donc de limiter l'élimination du cancer, conduisant ainsi à une récurrence tumorale précoce (Dovedi et al., 2014).

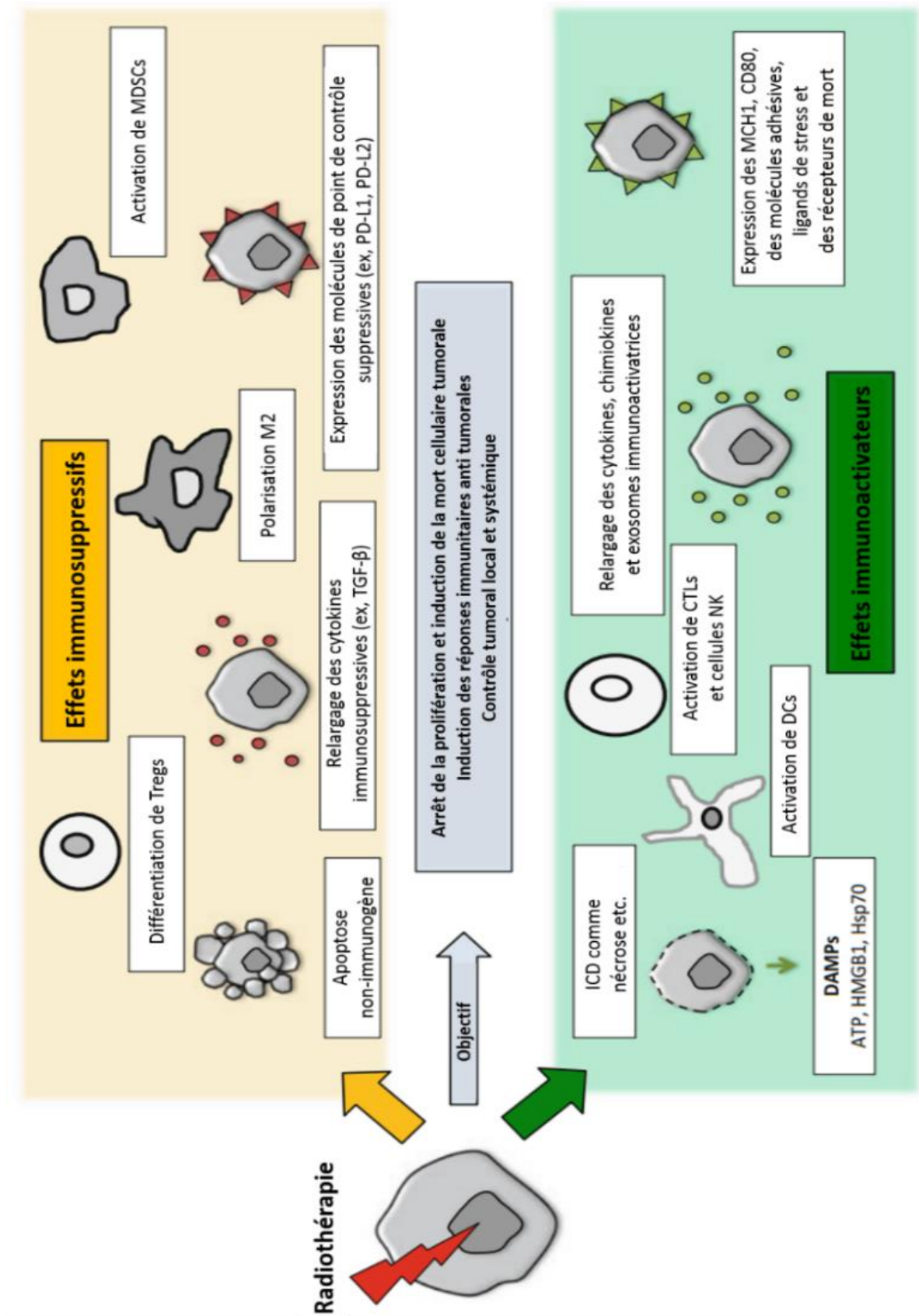
## 2.2. Immunosénescence

Rappelons que le système immunitaire a un rôle ambigu dans le cancer, à la fois essentiel dans la surveillance et la réponse antitumorale, il est aussi étroitement associé à la progression tumorale. De plus, le vieillissement du système immunitaire,

un processus naturel qui survient avec l'âge, entraîne un déclin de la fonction immunitaire augmentant ainsi les risques de cancer (Lian et al., 2020). Ce processus, connu sous le nom d'immunosénescence, a été proposé par Walford en 1964 (Walford, 1964). En plus du vieillissement (Effros, 1998), des stimulations antigéniques répétées et un environnement inflammatoire chronique peuvent également conduire à ce phénomène (Zhang, 2007; Zhang et al., 2021).

L'immunosénescence peut aussi être impliquée dans un contexte oncologique. Un taux élevé de cellules immunitaires sénescentes indique la présence d'un processus tumoral (Göthert et al., 2013; Huang et al., 2020; Trintinaglia et al., 2018), une maladie plus avancée, un pronostic plus sombre et un risque plus élevé de récurrence (Akagi and Baba, 2008; Characiejus et al., 2002; Ferrara et al., 2021; Liu et al., 2019; Moreira et al., 2019; Tang et al., 2020). L'immunosénescence peut également prédire la réponse aux traitements anticancéreux. Un taux bas de LyT sénescents est associé à une meilleure réponse à la radiothérapie stéréotaxique (SBRT) des métastases pulmonaires (Liu et al., 2019), à l'obtention d'une rémission complète (RC) chez les patientes ayant une leucémie aiguë myéloblastique (LAM) (Knaus et al., 2018; Tang et al., 2020), et à une meilleure réponse aux anti-PD1 (Kamphorst et al., 2017). A leur tour, les traitements anticancéreux peuvent provoquer/accélérer la sénescence non répliquative (Bruni et al., 2019), appelée la sénescence thérapie-induite, conférant à une majoration des toxicités et à une mauvaise réponse thérapeutique antitumorale (Arina et al., 2019; Bruni et al., 2019).

La Figure 3 résume les effets activateurs et suppresseurs de la RT sur le système immunitaire.



**Figure 3 :** Effet de la radiothérapie sur l'immunité du TME : immunosuppresseur et immunoactivateur, modifiée de (Frey et al., 2019).

Dans l'ensemble, ces données indiquent que la RT locale peut déclencher des réponses tissulaires complexes qui peuvent avoir des effets systémiques sur la croissance tumorale. La variabilité des modèles, avec différents degrés d'immunocompétence de l'hôte ainsi que les caractéristiques intrinsèques des cellules tumorales, influencent la traduction de cette réponse en un effet systémique anti- ou pro-tumoral. Des effets opposés sont susceptibles de coexister, ainsi il est primordial d'identifier les leviers permettant de favoriser spécifiquement la réponse antitumorale.

Nous allons maintenant nous intéresser à l'impact de l'irradiation dans différents modèles tumoraux impliquant l'immunité : l'effet (i) d'une irradiation corporelle totale (ICT) avant les greffes de moelle osseuse et (ii) d'une irradiation focalisée à la tumeur solide intacte dans le cadre de cancer du sein localement avancé recevant un traitement néoadjuvant préopératoire.



## **PARTIE I : IMPACT DE L'IRRADIATION CORPORELLE TOTALE DANS LES MODELES MURINS D'ALLOGREFFE DE CELLULES SOUCHES HEMATOPOIETIQUES**

Cette partie est dédiée aux effets de l'irradiation corporelle totale (ICT) sur le système immunitaire des modèles murins d'allogreffe. Nous allons nous intéresser à l'alloréactivité des cellules immunes du donneur vis-à-vis du microenvironnement de l'hôte, ainsi que son impact sur la prise de greffe, les effets du greffon contre l'hôte (GVHD pour *graft-versus-host disease*) et du greffon contre la leucémie (GVL pour *graft-versus-leukemia*).

Dans un premier temps, nous allons aborder les notions concernant la transplantation des cellules souches hématopoïétiques, l'alloréactivité ainsi que facteurs influençant le conditionnement pré-greffe.

## I. Allogreffe de cellules souches hématopoïétiques

### 1. Historique

La reconnaissance de la défaillance tardive de la moelle osseuse (MO) après une exposition à la bombe atomique à Hiroshima et Nagasaki a déclenché les pistes de recherche fondamentale et d'application clinique de la transplantation de cellules souches hématopoïétiques (HSCT pour *hematopoietic stem cells transplatation*) (Mathe et al., 1959; van Bekkum and de Vries, 1967). La première série de patients traités par des perfusions intraveineuses de cellules en suspension de MO a été rapportée par l'équipe de Seattle (Thomas et al., 1957). Certains patients ont acquis des signes de chimérisme hématopoïétique du donneur, confirmant ainsi la « preuve du principe » préalablement obtenue dans les modèles précliniques : l'ICT peut remplacer les cellules normales et éradiquer les cellules malignes de la MO chez le receveur. Ainsi, l'infusion de cellules souches hématopoïétiques (CSH) du donneur sain peut restaurer l'hématopoïèse appauvrie du receveur.

Soixante-dix ans de recherche sur la HSCT ont permis de transformer cette thérapie d'une procédure aux complications ingérables à un traitement standard des hémopathies autrement mortelles. La meilleure compréhension du CMH chez l'homme a permis de diversifier et d'élargir les donneurs compatibles ainsi que la source de CSH. Tous ces avancements ont permis une hausse quasi-constante du nombre de HSCT réalisées chaque année depuis 20 ans. On décompte 1857 allogreffes réalisées en France en 2020, contre 799 en 2000 (Agence de la biomédecine, 2020).

## 2. Indications

La HSCT est une thérapie lourde, associée à un fort taux de mortalité et à de nombreuses complications potentielles. Ainsi, elle est réservée aux patients atteints d'hémopathies de moins bon pronostic possédant le plus de risques de rechute. Ce risque est évalué individuellement en fonction du type d'hémopathie, de caractéristiques cytogénétiques et moléculaires au diagnostic et de l'évaluation de la réponse au traitement.

Les hémopathies malignes représentent 90% des indications d'allogreffe (Agence de la biomédecine, 2020). Les quatre principales indications sont : la leucémie aiguë myéloblastique (LAM), la leucémie aiguë lymphoblastique (LAL), la myélodysplasie et les lymphomes non hodgkiniens. D'autres hémopathies voient leur nombre d'indications d'allogreffe diminuer chaque année, suite à l'apparition récente de nouvelles thérapies efficaces permettant de prolonger significativement la survie des patients traités (Nijhof et al., 2018).

Du fait de sa forte toxicité, l'indication de HSCT est aussi conditionnée par des caractéristiques propres au patient, notamment son âge, ses antécédents médicaux et ses comorbidités (Kennedy and Olin, 2021; Sorror et al., 2005). De nouveaux régimes de conditionnements RIC et NMA ont permis l'accès à la greffe à des patients plus âgés ; l'âge moyen des patients allogreffés en France est en augmentation, passant de 37 ans en 2006 à 53 ans actuellement.

## 3. Types de donneurs

Parmi les critères de choix du donneur, le degré de compatibilité du système d'antigènes des leucocytes humains (HLA pour *human leukocyte antigen*), le CMH



chez l'homme, figure au premier rang. Le CMH est un hétérodimère capable de présenter l'antigène aux LyT. Le complexe HLA joue un rôle majeur dans l'auto-reconnaissance, évoquant une réponse immunitaire innée et adaptative à un stimulus antigénique (Klein and Sato, 2000). Ils ont les molécules antigènes les plus immunogènes reconnues au cours du rejet d'une greffe allogénique. En fonction du degré de compatibilité HLA entre le donneur et le patient receveur, on peut distinguer :

- Un donneur compatible dans la fratrie, dit « géno-identique » avec les mêmes gènes du système HLA que le patient ;
- Un donneur volontaire de CSH, dit « phéno-identique » car ayant le même phénotype HLA pour les antigènes testés (HLA A, B, C, DP, DR $\beta$ 1 et DQ $\beta$ 1) ;
- Un donneur alternatif, soit phéno-identique présentant une seule incompatibilité HLA (on parle de « *mismatch* ») et donc 9/10, soit « haplo-identique » ayant la moitié des gènes HLA en commun avec le receveur, soit d'une greffe à partir des unités de sang placentaire.

#### 4. Conditionnement

Avant la HSCT, le conditionnement supprime et élimine fonctionnellement le système immunitaire de l'hôte pour permettre aux CSH du donneur de s'installer dans le microenvironnement de la MO sans risque de rejet. De plus, ce conditionnement éradique la maladie maligne sous-jacente, permettant un contrôle de la maladie à long-terme.

Cette stratégie implique d'utiliser soit plusieurs agents de chimiothérapie (à leur dose maximale tolérable), soit l'ICT à haute dose ou la combinaison des deux. En fonction

de leurs toxicités et de leurs propriétés plutôt myéloablatives/ immunosuppressives, on distingue de façon schématique 3 types de conditionnement :

- Conditionnement myéloablatif MAC (*myeloablative conditioning*) qui visent à détruire le contenu cellulaire de la MO, entraînant une pan-cytopénie généralement irréversible, à moins d'être restauré par une greffe de CSH (Carreras et al., 2019).
- Conditionnement non-myéloablatif NMA (*non-myeloablative conditioning*) incarne l'option d'une cytopénie à visée immunosuppressive plutôt qu'immunoablative.
- Conditionnement à intensité réduite RIC (*reduced-intensity conditioning*) représentant une catégorie intermédiaire entre MAC et NMA, plutôt à visée immunosuppressive que myélotoxique, et pouvant donc être proposée à des sujets plus âgés.

Le choix d'un schéma d'un conditionnement particulier est basé sur la situation clinique des malades, les préférences du centre de greffe et les différents paramètres concernant le receveur comme son âge, ses comorbidités et la source du donneur.

## 5. Surveillance post-greffe

### 5.1. Prise de greffe et chimérisme

Après une allogreffe de CSH, il est essentiel de documenter si l'hématopoïèse nouvellement formée provient du donneur ou du receveur, faisant l'objet d'une analyse de chimérisme. L'origine des LyT est généralement utilisée pour surveiller la prise de greffe : (i) le chimérisme est complet si plus de 95% des cellules CD3+ proviennent du

donneur ; (ii) les cellules CD3+ du donneur de moins de 5% signifiant le rejet de la greffe (Carreras et al., 2019).

Le chimérisme est aussi un paramètre informatif de l'alloréactivité et de la tolérance du greffon. Ainsi, la persistance ou la résurgence des cellules du receveur pourraient correspondre soit (i) à un régime préparatif épargnant des cellules non malignes, (ii) soit à la survie des cellules tumorales, (iii) soit au résultat d'une réapparition de l'hématopoïèse, ou la combinaison des trois.

### **5.2. Contrôles à court et à long terme**

Les patients recevant une allogreffe de CSH ont un risque de complications péri- et post-HSCT. Ils nécessitent des contrôles multidisciplinaires pour prévenir ou traiter ces complications potentielles à court terme et à distance de la greffe. La morbidité résultant du processus de HSCT oblige les patients à adopter un mode de vie compatible, car ils sont généralement immunodéprimés, en particulier ceux atteints de GVHD (Cordonnier et al., 2019).

## **6. Complications**

L'allogreffe est associée à une forte toxicité et à de nombreuses complications, avec une mortalité globale atteignant en moyenne 50% des allogreffés (Gooley et al., 2010).

Au plan carcinologique, entre 25% à 60% des patients ont une récurrence post-greffe dont le pronostic est généralement péjoratif (Mielcarek et al., 2007; Thanarajasingam et al., 2013). La rechute de l'hémopathie initiale constitue ainsi la première cause de décès après l'allogreffe géno-identique (Gratwohl et al., 2005).

Au plan immunologique, la réaction allogénique des LyT recherchée pour éradiquer les cellules leucémiques/tumorales résiduelles (effet GVL/GVT), peut être associée à

la GVHD conduisant à la destruction des tissus sains du receveur, responsable d'une morbi-mortalité majeure après greffe (Ferrara et al., 2009; Gratwohl et al., 2005).

La troisième grande cause de décès après HSCT comporte les complications infectieuses (Gratwohl et al., 2005; Styczyński et al., 2020). Ces dernières peuvent être très diverses (bactériennes, virales, fongiques ou parasitaires) dépendant du degré d'immunosuppression (Carreras et al., 2019).

## II. Maladie du greffon contre l'hôte

### 1. Généralité

La réaction immunitaire allogénique post-greffe est un processus au cours duquel les cellules immunitaires du donneur, principalement les LyT, reconnaissent comme étrangères et attaquent les cellules de l'organisme du receveur. Cette réaction allogénique est à l'origine de deux effets : (i) l'élimination des cellules tumorales par le système immunitaire du donneur (GVL), effet recherché et bénéfique ; (ii) la destruction des tissus sains du receveur, appelé maladie du greffon contre l'hôte (GVHD), effet délétère à éviter.

On reconnaît traditionnellement deux formes de GVHD : la GVHD aiguë (GVHDa) et la GVHD chronique (GVHDc), qui diffèrent sur le plan physiopathologique. La distinction originale entre ces deux formes reposait uniquement sur un critère chronologique : la forme aiguë survenant durant les 100 premiers jours suivant la greffe et la GVHDc après le J100. Cependant, cette définition est devenue insatisfaisante depuis l'application des conditionnements de type RIC et l'infusion des lymphocytes du donneur post-greffe (dans le but d'augmenter l'effet GVL), où la GVHDa retardée (après 100 jours) le syndrome de recouvrement (la survenue concomitante des signes de GVHDa et GVHDc) peuvent être observés. Une nouvelle classification de la GVHD a été élaborée par l'Institute Nationale de la santé Américaine (Filipovich et al., 2005; Jagasia et al., 2015) pour englober ces nouvelles catégories de GVHD.

Dans ce travail de thèse, nous nous intéressons à la GVHDa, qui constitue une complication majeure de HSCT. Globalement, l'incidence de la GVHDa de grade 2 à 4 après allogreffe est d'environ 50% et pour les patients réfractaires au traitement de

première ligne par corticoïdes, la survie à long-terme est mauvaise, de l'ordre de 5 à 30% (Martin et al., 2012). Plusieurs facteurs peuvent influencer la survenue et la sévérité de la GVHDa : le degré de disparité HLA entre le donneur et le receveur, l'intensité du conditionnement, la source du greffon et l'âge du receveur au moment de la greffe (Hahn et al., 2008).

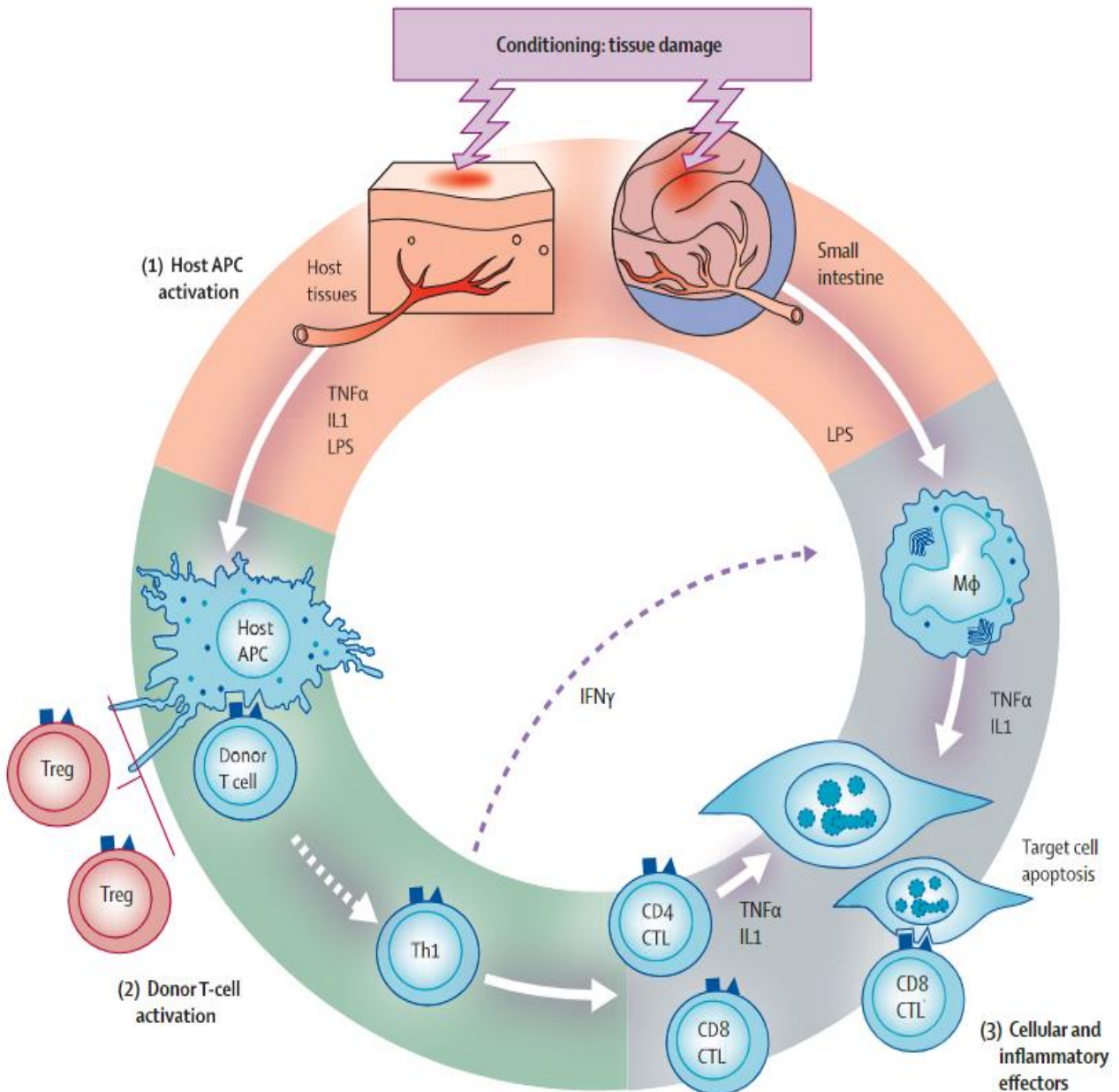
## 2. Physiopathologie

La GVHD a été initialement rapportée comme une « maladie secondaire » (à la guérison des dommages dus à l'irradiation) en l'absence des connaissances du système HLA (Barnes et al., 1962). Elle a été ensuite définie par *Billingham* sous le nom de la « réaction du greffon contre l'hôte » où les cellules immunocompétentes du donneur reconnaissent et attaquent les tissus d'un receveur allogénique immunodéprimé (Billingham, 1966). Les trois conditions nécessaires au développement de la GVHD ont ainsi été établies :

- Le greffon doit contenir des cellules immunocompétentes, notamment les LyT matures, dont le nombre est directement corrélée à la sévérité de la GVHD (Kernan et al., 1986).
- Il existe une incompatibilité entre les tissus du donneur et du receveur (Anasetti et al., 1990; Hobo et al., 2013).
- Le système immunitaire du receveur ne doit pas être capable de monter une réponse effectrice pour éliminer les cellules transplantées.

Les connaissances sur le mécanisme de migration des lymphocytes ont permis d'expliquer la spécificité tissulaire de la GVHD, ajoutant une quatrième condition (Sackstein, 2006) : Les LyT du donneur sont capables de migrer dans les tissus cibles de la GVHD.

La physiopathologie de la GVHDa a été décrite par James Ferrara comme un « orage cytokinique » découpée en trois phases successives représentée dans la Figure 4, communément connu sous le nom de « roue » de Ferrara (Ferrara et al., 2009).



**Figure 4 :** Pathophysiologie de la GVHD aiguë (Ferrara et al., 2009).

### **2.1. Activation des APC du receveur**

La première étape indispensable au déclenchement d'une GVHDa est l'activation des APC du receveur par le conditionnement pré-greffe. Dans les modèles murins de GVHD, ce régime conditionnement est restreint à l'ICT, qui nous a permis de démontrer une corrélation directe entre l'intensité du conditionnement et le degré des dommages tissulaires (Hill et al., 1997).

Les dommages tissulaires affectent en particulier le tube digestif et entraînent par la suite le transfert des endotoxines bactériennes ou lipopolysaccharides (LPS) dans la circulation (Ferrara et al., 2009; Hill et al., 1997), contribuant à l'activation de l' « orage cytokinique », caractérisé par une libération massive des cytokines pro-inflammatoires comme le TNF- $\alpha$ , l'IL-1 et l'IL-6 (Hill et al., 1997). Ces signaux de danger activent les APC dans les tissus permettant à la fois d'amorcer l'activation et de fournir les signaux de costimulation complémentaires aux LyT du donneurs (Hill and Koyama, 2020; Koyama and Hill, 2016).

### **2.2. Activation des lymphocytes T du donneur**

Après avoir été amorcés par les alloantigènes, les LyT du donneur recirculent, prolifèrent et se différencient tout en sécrétant des quantités remarquables de cytokines et/ou en acquérant une fonction cytolytique. La différenciation des LyT du donneur est le poinçon de la seconde étape de la GVHDa (Henden and Hill, 2015).

### **2.3. Phase effectrice**

La phase effectrice proprement dite implique à la fois des acteurs cellulaires et des médiateurs solubles de l'inflammation, comme le TNF- $\alpha$ , l'IFN- $\gamma$ , l'IL-1 et l'oxyde nitrique, qui peuvent exercer leur propre effet cytotoxique direct (Paczesny et al.,



2010). Les principaux effecteurs cellulaires de la GVHDa sont les CTL et les cellules NK (Ferrara et al., 2009; Welniak et al., 2007). Le rôle du TNF- $\alpha$  est particulièrement intéressant, puisqu'il peut être produit à la fois par des cellules du receveur et du donneur et qu'il implique dans les trois phases de la physiopathologie de la GVHD : (i) il active les APC et augmente la présentation par ces dernières d'alloantigènes ; (ii) il recrute les effecteurs cellulaires dans les organes cibles via l'induction de chémokines inflammatoire ; (iii) il induit directement des nécroses cellulaires, comme son nom l'indique (Levine, 2011).

### **3. Caractéristiques cliniques et modèles de la GVHD**

#### **3.1. La GVHD aiguë chez l'homme**

Chez l'homme, la GVHDa endommage 3 organes principaux (Jacobsohn and Vogelsang, 2007) :

- La peau : 80% des patients de GVHDa présentent une éruption maculopapuleuse avec prurit et sensible, pouvant se propager dans tout le corps en épargnant le cuir chevelu.
- Les manifestations gastro-intestinales comprennent une diarrhée abondante parfois sanglante, avec des crampes, des nausées/vomissements, et d'anorexie.
- Les troubles hépatiques représentés par une élévation des enzymes hépatiques et une jaunisse liée à l'hyperbilirubinémie.

#### **3.2. Modèles murins de la GVHD aiguë**

Les premiers travaux expérimentaux dans les modèles murins remontent aux années 50, permettant le développement de la HSCT chez l'homme (Jacobson and Marks,

1949; Lorenz et al., 1951). La première description de ce qui allait être dénommé plus tard GVHD a été faite chez la souris (Barnes et al., 1956; Billingham, 1966). Pour une description précise des manifestations cliniques de la GVHD et évaluer sa sévérité et l'efficacité des traitements étudiés, le modèle murin s'est imposé comme un modèle de choix.

Les organes cibles de la GVHDa murine sont la peau, le foie et le tube digestif (Schroeder and DiPersio, 2011). Malgré de multiples similarités entre les modèles murins et la GVHD humaine, il y a aussi des différences comme l'atteinte hépatique chez la souris généralement plutôt évaluée par l'examen histopathologique que par le dosage de bilirubinémie. De plus, chez l'homme, la GVHDa précède généralement la GVHDc ce qui n'est pas toujours le cas chez la souris.

Plusieurs facteurs sont à prendre en compte en évaluant la GVHDa chez la souris :

- La co-injection de LyT donneurs (prélevés des ganglions et de la rate) est nécessaire pour déclencher la maladie (Cohen et al., 2002; Edinger et al., 2003).
- La dose d'irradiation est directement corrélée aux dommages tissulaires et à l'orage cytokinique qui en résulte (Hill et al., 1997; Schwarte and Hoffmann, 2005). Elle détermine ainsi le degré de myéloablation et la mortalité liée à la GVHDa (Schroeder and DiPersio, 2011).
- Les disparités génétiques
- Les facteurs environnementaux comme la température, le stress causé par le transport, l'irradiation, l'injection et les pathogènes peut varier de manière importante la sévérité de la GVHDa (Leigh et al., 2015).

### 3.3. Classification de la GVHD aiguë chez la souris

Les deux principaux facteurs fréquemment utilisés pour évaluer la GVHDa chez la souris sont la perte de poids et la survie (Schroeder and DiPersio, 2011). Il est rapidement apparu nécessaire de développer un système clinique standardisé évaluant la GVHDa chez la souris. Plusieurs systèmes de score alternatifs ont été proposés, parmi lesquels le premier, et le plus utilisé, a été décrit par l'équipe de Ferrara (Cooke et al., 1996), qui repose sur cinq critères cliniques : la perte de poids, la posture, l'activité, la texture du pelage et l'intégrité de la peau. Chaque critère est évalué et noté de 0 à 2 en fonction de la sévérité de l'atteinte (Tableau 1).

Toutefois, ce système de gradation de Cooke et ceux qui en dérivent sont critiquables sur plusieurs points. Tout d'abord, leur calcul est en grande partie subjectif. Ensuite, il n'évalue pas les atteintes digestives, qui est pourtant l'un des principaux organes cibles de la GVHDa. Enfin, le principal intérêt de l'utilisation d'un tel système repose sur la pertinence clinique des différents grades ainsi non atteint, à savoir : la corrélation entre le système de gradation et les lésions histologiques ; et sa valeur pronostique.

**Tableau 1** : Système de gradation de la GVHDa chez la souris (Cooke et al., 1996).

Critère	Grade 0	Grade 1	Grade 2
Perte de poids	< 10%	10-25%	> 25%
Posture	Normale	Recroquevillement observé seulement au repos	Recroquevillement sévère, limitant la mobilisation
Activité	Normale	Légèrement ou modérément diminuée	Immobile, sauf à la stimulation
Texture du pelage	Normale	Légèrement ou modérément ébouriffée	Sévèrement ébouriffée/mauvais coiffage
Intégrité de la peau	Normale	Desquamation au niveau des pattes ou de la queue	Surfaces de peau dénudée facilement visibles

Afin de répondre à ces questions, mon laboratoire d'accueil a développé un système de gradation de la sévérité de la GVHDa reposant sur une évaluation binaire (oui ou non) de 5 paramètres visuels importants qui reflètent la complexité de la maladie sans nécessité de sacrifier les souris (Naserian et al., 2018). Ces 5 paramètres comprennent : la perte de poids de plus de 10% du poids initial, la posture courbée, les lésions cutanée (desquamation), le poil terne ou ébouriffé, et la diarrhée. Grâce à sa simplicité, l'évaluation peut se faire de façon dynamique, longitudinale au cours de l'expérience afin de surveiller l'évolution de la maladie. Nous avons démontré que ce système de score était cohérent avec la stadification histologique de la GVHDa à travers de plusieurs combinaisons donneuses-receveuses murines, il est également prédictif de la survie de la souris receveuse et est hautement reproductible (Naserian et al., 2018)

### III. Impact du conditionnement par l'irradiation corporelle totale

Le rationnel d'inclure l'ICT dans le régime de conditionnement avant la HSCT dans le cadre des hémopathies malignes est double : (i) éradiquer les cellules leucémiques et (ii) empêcher le rejet de l'allogreffe par ablation du système immunitaire du receveur. La RT cible les cellules leucémiques dans tout le corps, y compris dans les sites sanctuaires où la biodisponibilité de la chimiothérapie est limitée. Après que le rôle myéloablatif de l'ICT ait été établi, plusieurs groupes ont reconnu son potentiel rôle immunosuppresseur. Cette approche peut permettre à un effet immunothérapeutique du greffon du donneur (effet greffon contre tumeur/leucémie ou GVL) de prendre place. Bien que l'ICT ne joue pas un rôle myéloablatif dans ce contexte, elle sert comme moyen d'atténuer la réponse immunitaire de l'hôte qui peut être très importante dans le cadre d'allogreffe lorsqu'une immunosuppression adéquate est essentielle. L'optimisation de l'effet immunosuppresseur des schémas d'ICT tout en limitant la dose aux tissus sains nécessite de prendre en compte la combinaison de la dose totale, du débit de dose, de la dose par fraction et de la durée globale du traitement (Hoeben et al., 2021).

#### 1. La radiosensibilité intrinsèque des cellules hématopoïétiques et leucémiques

La radiosensibilité *in vitro* a été historiquement quantifiée par la valeur  $D_0$ , la dose à laquelle la survie est réduite à 37% de sa valeur initiale (Sparrow et al., 1967). La valeur  $D_0$  des cellules hématopoïétiques normales varie de 0,3 à 1,6 Gy, indiquant une radiosensibilité globalement élevée (Kimler et al., 1985). Dans les modèles animaux, la valeur  $D_0$  *in vivo* est plus élevée que celle *in vitro* avec une sous-population de CSH plus radiorésistante (van Bekkum, 1991). Chez l'homme, la  $D_0$  effective variait de 3,7

à 5,4 Gy pour les LyT dans le sang périphérique, et atteignait 10 Gy pour les granulocytes (Brenda Shank et al., 1983).

Les populations de cellules leucémiques disposent une haute radiosensibilité avec les valeurs  $D_0$  d'environ 0,6-0,7 Gy (Kimler et al., 1985), qui varie entre les types cellulaires. Par exemple, les LAL de la lignée B se sont avérées plus radiorésistantes que les LAL de la lignée T (Uckun et al., 1993), cela étant liée à des changements génétiques impliquant le cycle cellulaire, la réparation d'ADN et la mort cellulaire (Monzen et al., 2016).

Une forte dose par fraction et/ou un débit de dose accru de l'ICT contrera la récupération des cellules leucémiques entre les fractions, mais cela devrait être mis en balance avec la tolérance des tissus sains.

## **2. La dose totale et le fractionnement**

Les premières allogreffes de CSH basées sur un conditionnement reposant uniquement sur une ICT ont été très décevantes en raison d'une absence de prise de greffe, d'une récurrence élevée, et des complications post-greffe dont la GVHD (Bortin, 1970). Les avantages de la combinaison radio-chimiothérapie (ICT 10 Gy en une seule fraction et cyclophosphamide à forte dose) ainsi que les évolutions dans la prise en charge péri-greffe ont permis aux patients atteints de leucémie aiguë de bénéficier de la greffe de CSH (Thomas et al., 1979).

Cependant, comme dans la planification d'autres traitements, la dose doit être mise en balance entre le risque de récurrence et les toxicités radio-induites. Le rapport thérapeutique entre des tissus normaux radiosensibles et des effets immunosuppresseurs et anti-leucémiques d'ICT pouvait être amélioré en diminuant le

débit de dose lors d'une ICT en fraction unique. Toutefois, une ICT à faible débit de dose peut s'étaler sur plusieurs heures, cela pouvant impacter considérablement le planning des traitements dans un service d'oncologie-radiothérapie clinique. L'autre option était de fractionner la dose d'ICT. En effet, de nombreuses études ont montré une supériorité en termes de survie globale et survie sans récurrence d'une ICT fractionnée par rapport à l'ancien standard d'ICT à dose unique de 10 Gy (B. Shank et al., 1983; Thomas et al., 1982), tout en diminuant les taux de pneumopathies interstitielles et de GVHD (B. Shank et al., 1983; Socie et al., 1991).

Le premier schéma fractionné d'ICT appliqué était de 12 Gy en 6 fractions administrées sur 3 ou 6 jours. Afin d'optimiser le rapport thérapeutique, le schéma bifractionné quotidien des doses entre 1,5 à 2 Gy pour une dose totale  $\geq 12$  Gy a été considéré optimal. Un alternatif est de délivrer les 12 Gy d'ICT en fractions quotidiennes de 4 Gy, mais au prix d'augmenter les effets indésirables aigus tels que la mucite et la cystite hémorragique (Belkacemi et al., 2018; Sengeløv et al., 2019). Des approches d'escalade de dose d'ICT sont contrebalancées par ses toxicités (Clift et al., 1998).

### 3. Le débit de dose

Dans les années 1970s, le schéma d'ICT le plus souvent utilisé était 8 à 10 Gy en fraction unique, délivré à des faibles débits de dose sur plusieurs heures, pour équilibrer l'effet thérapeutique et les toxicités (Travis et al., 1985). L'alternatif des faibles débits de dose est de fractionner l'ICT (O'Donoghue, 1986). Dans le but de destruction des cellules leucémiques et de prise de greffe allogénique, l'ICT fractionnée avec un débit de dose plus élevé est préférable à un débit de dose plus faible (O'Donoghue, 1986; Peters, 1980). Des débits de dose  $\leq 4$  cGy/min étaient associés à un risque accru de rechute leucémique chez les patients ayant reçu des

doses d'ICT de 8,4 à 12,5 Gy en 3 jours (Scarpati et al., 1989). Les seuls essais randomisés comparant différents débits instantanés d'ICT (6 vs 15 cGy/min en dose unique et 3 vs 6 cGy/min pour une ICT hexafractionnée) n'ont pas pu démontrer ni de différence en termes de taux de GHVD et de pneumopathie interstitielle ni de différence en termes de contrôle leucémique et de survie globale (Ozsahin et al., 1992).

Des modèles animaux impliquant différents débits de dose en séance unique ou fractionnée ont démontré que l'effet différentiel des tissus sains était principalement influencé par les schémas d'ICT fractionnés. Le bas débit de dose était protecteur seulement en cas d'ICT en séance unique (Joachim Deeg et al., 1988; Tarbell et al., 1987; Travis et al., 1985).

#### **4. L'énergie de la source d'irradiation des modèles expérimentaux**

Un des paramètres importants décrivant une irradiation est lié à l'énergie utilisée, allant de très faibles énergies de quelques dizaines de kilovolts (kV) à des très hautes énergies de quelques centaines de mégavolts (MV).

La recherche fondamentale des effets liés à l'allogreffe de CSH, en particulier dans les modèles murins, implique un conditionnement par l'ICT (Schroeder and DiPersio, 2011). Traditionnellement, l'ICT chez la souris consiste à utiliser les sources radioactives émettant des rayons gamma comme le Cobalt-60 ( $^{60}\text{Co}$ ) ou le Cesium-137 ( $^{137}\text{Cs}$ ). Cependant, pour des raisons de sécurité nucléaire, il y a eu un mouvement mondial vers le remplacement des sources radioactives non scellées par des dispositifs d'irradiation émettant les rayons X (Dodd and Vetter, 2009).



Ce changement de méthode d'irradiation a soulevé une nouvelle question : l'impact de l'énergie de la source d'irradiation sur les résultats des modèles précliniques ? Les sources RI- $\gamma$  émettent les rayons de façon homogène, de 1,25 MeV pour  $^{60}\text{Co}$  et 0,662 MeV pour  $^{137}\text{Cs}$ . En revanche, les dispositifs RI-X disposent de différentes énergies, de 160 à 350 kV pour la plupart des irradiateurs commercialisés pour les petits animaux allant jusqu'à 6 à 25 MV pour les accélérateurs linéaires (AL) utilisés à des fins thérapeutiques. Devant l'indisponibilité des sources d'irradiation les plus fréquemment utilisées (RI-  $\gamma$ ) dans les études publiées et l'accès très restreint aux AL, de nouveaux protocoles d'irradiation utilisant des irradiateurs à rayons X de basses énergies, nécessaire pour assurer la continuité de la recherche expérimentale ont été développés.

## Objectifs

L'impact des paramètres d'irradiation étendue sur les effets aigus et tardifs est bien documenté dans la littérature. Dans le domaine des greffes de CSH, nous avons développé dans les chapitres précédents l'effet de ces paramètres sur de nombreux effets secondaires post-greffe incluant la GVHD et sa sévérité notamment dans les modèles expérimentaux qui souffrent souvent d'une certaine hétérogénéité et de biais liés aux procédures et paramètres d'irradiation utilisés.

Mon arrivée au laboratoire a coïncidé avec l'acquisition d'un irradiateur à rayons X de basses énergies (RxBE), le Faxitron® CP160 aux énergies maximales de 160 kV. Dès mes premières semaines de stage, j'ai commencé à m'intéresser aux effets de ces RxBE chez la souris irradiée en totalité. Étant clinicienne et connaissant la grande différence des distributions des doses générées par les RxBE et les photons de haute énergie (RxHE) des AL, j'ai de suite voulu travailler sur la réponse immunitaire allogénique des modèles murins au laboratoire. J'ai ainsi commencé mes expériences comparant la méthode d'irradiation par les RxBE au CP160® et les RxHE au CLINAC® utilisés pour les traitements des malades atteints de cancers. Il m'a semblé capital d'apporter des données objectives pour les expériences faites dans ce domaine des ICT du petit animal pour permettre une meilleure standardisation des procédures et pouvoir comparer objectivement les résultats publiés dans les recherches sur les allogreffes au laboratoire.

Mon premier travail au laboratoire avait ainsi l'objectif d'évaluer l'impact potentiel de l'énergie d'irradiation sur la réponse immunitaire post-greffe, en comparant les résultats de différents modèles murins de greffe semi-allogénique de CSH, obtenus après une irradiation aux RxHE de 6MV et l'irradiateur à RxBE de 160 kV.

## Résultats

Ce travail, intitulé « *Effect of lethal total body irradiation on bone marrow chimerism, acute graft-versus host disease, and tumor engraftment in mouse models: Impact of different radiation techniques using low and high energy X-rays* » a été soumis au journal « *Strahlentherapie und Onkologie* ».

Nous avons montré pour la première fois que l'énergie d'irradiation impactait l'alloréactivité post-BMT et donc la sévérité de la GVHD de nos modèles de greffe de CSH semi-allogénique. Et en modulant le microenvironnement inflammatoire provoqué par l'irradiation, nous pourrions modifier l'intensité de cette réponse alloréactive.

## **Article 1**

**Effect of lethal total body irradiation on bone marrow chimerism, acute graft-versus host disease, and tumor engraftment in mouse models: Impact of different radiation techniques using low and high energy X-rays.**

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## **ABSTRACT**

**Objectifs :** Pour comparer les effets de différents niveaux d'énergie des rayons X utilisés dans le cadre d'une irradiation corporelle totale (ICT) létale, myéloablative avant la greffe de moelle osseuse (BMT) dans les modèles murins.

**Matériels et méthodes :** Dans différents modèles murins, une fraction unique d'ICT myéloablative à dose létale utilisant deux types d'appareils à rayons X de différentes énergies, soit à faible énergie (cabine d'irradiation de 160 kV), soit à haute énergie (accélérateur linéaire de 6 MV) a été délivrée avant la BMT semi-allogénique pour établir les modèles de chimérisme de la moelle osseuse, de la maladie du greffon contre l'hôte (GVHD), et de la prise de tumeur. Les souris receveuses ont été suivies cliniquement pendant 80 jours après la greffe. La cytométrie en flux a été réalisée pour évaluer le chimérisme du donneur et la prise de tumeur dans la souris receveuse.

**Résultats :** Les deux techniques d'irradiation à rayons X délivrant une fraction unique d'ICT de 10 Gy ont conduit à un effet léthal et ont permis un chimérisme quasi-complet du donneur précocement au jour 13. Cependant, l'irradiation à faible énergie a présenté une alloréactivité des lymphocytes T plus élevée par rapport à l'irradiation à haute énergie, entraînant des conséquences cliniques sur la GVHD et la prise de tumeur. Les différences d'effet alloréactif pourraient être attribuées à la distinction entre les deux méthodes par rapport au statut inflammatoire des receveuses lors de la perfusion de cellules du donneur (à J0). Retarder d'un jour l'injection des cellules du donneur (à J1 de l'ICT létale) a atténué l'alloréactivité des lymphocytes T et amélioré les résultats cliniques dans les modèles murins de GVHD.

**Conclusion :** Les différentes modalités d'irradiation à rayons X conditionnent l'alloréactivité dans la greffe de moelle osseuse médiée par un pic inflammatoire post-ICT quand on utilise un irradiateur à rayons X à faible énergie. Ces informations techniques et biologiques doivent être prises en compte lors de l'interprétation des résultats de GVHD/GVL dans les modèles expérimentaux de BMT chez la souris.

## Strahlentherapie und Onkologie

### Effect of lethal total body irradiation on bone marrow chimerism, acute graft-versus-host disease, and tumor engraftment in mouse models: Impact of different radiation techniques using low and high energy X-rays.

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<b>Abstract:</b>	<p><b>Purpose:</b> Effects of X-ray energy levels used for myeloablative, lethal total body irradiation (TBI) delivery prior to bone marrow transplantation (BMT) in preclinical mouse models.</p> <p><b>Materials and methods:</b> In mouse models, a single fraction myeloablative TBI at a lethal dose was delivered using two different X-ray devices, either low (160 kV cabinet irradiator) or high energy (6 MV linear accelerator) before semi-allogeneic HSCT to ensure bone marrow (BM) chimerism, graft-versus-host disease (GVHD) and tumor engraftment. Recipient mice were clinically followed for 80 days after bone marrow transplantation (BMT). Flow cytometry was performed to assess donor chimerism and tumor engraftment in recipient mice.</p> <p><b>Results:</b> Both X-ray irradiation techniques delivered a 10 Gy single fraction of TBI, presented a lethal effect, and could allow a near-complete early donor chimerism on day 13. However, low-energy irradiation increased T cells alloreactivity compared to high-energy irradiation, leading to clinical consequences on GVHD and tumor</p>

**Article Full Title:** Effect of lethal total body irradiation on bone marrow chimerism, acute graft-versus-host disease, and tumor engraftment in mouse models: Impact of different radiation techniques using low and high energy X-rays.

**Short Running title:** Impact of X-rays energy on BMT mouse model outcomes.

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## ABSTRACT

**Purpose:** Effects of X-ray energy levels used for myeloablative, lethal total body irradiation (TBI) delivery prior to bone marrow transplantation (BMT) in preclinical mouse models.

**Materials and methods:** In mouse models, a single fraction myeloablative TBI at a lethal dose was delivered using two different X-ray devices, either low (160 kV cabinet irradiator) or high energy (6 MV linear accelerator) before semi-allogeneic HSCT to ensure bone marrow (BM) chimerism, graft-versus-host disease (GVHD) and tumor engraftment. Recipient mice were clinically followed for 80 days after bone marrow transplantation (BMT). Flow cytometry was performed to assess donor chimerism and tumor engraftment in recipient mice.

**Results:** Both X-ray irradiation techniques delivered a 10 Gy single fraction of TBI, presented a lethal effect, and could allow a near-complete early donor chimerism on day 13. However, low-energy irradiation increased T cells alloreactivity compared to high-energy irradiation, leading to clinical consequences on GVHD and tumor engraftment outcomes. The alloreactive effect differences might be attributed to the distinction in inflammatory status of irradiated recipients at donor cell infusion (D0). Delaying donor cells administration (D1 after lethal TBI) attenuated T cells alloreactivity and clinical outcomes in GVHD mouse models.

**Conclusion:** Different X-ray irradiation modalities condition T cell alloreactivity in experimental semi-allogeneic BMT mediated by a post-TBI inflammatory burst when irradiating with the low-energy X-ray irradiator. This technical and biological information should be considered in interpreting GVHD/GVL results in mice experimental models of BMT.

**Keywords:** total body irradiation, X-ray devices, hematopoietic stem-cell transplantation, mouse models, bone marrow chimerism, graft-versus-host disease, graft-versus-leukemia



## INTRODUCTION

The success of allogeneic hematopoietic stem-cell transplantation (HSCT) has been limited by post-transplant complications such as severe graft-versus-host disease (GVHD). Although GVHD is initiated by donor T cells recognizing host antigens as non-self-antigens, several other factors, including conditioning regimen toxicities and cytokine dysregulation, may be involved in its pathogenesis [1, 2]. Clinical observations have shown that approaches to reduce GVHD might have a deleterious effect on leukemia relapse control [3], suggesting an important role of alloreactive donor T cells in mediating the graft-versus-leukemia (GVL) effect [4–6].

Preclinical models, especially mice models, have mainly been used to elucidate fundamental understandings of the alloreactive T responses implicated in the two processes and evaluate therapeutic strategies to control GVHD without losing the GVL effect [7]. The first step to trigger donor T cell alloreactivity in immunocompetent mice relies on myeloablative-conditioning regimens generally consisting of total body irradiation (TBI) [8]. This procedure eliminates the recipient's hematopoietic and immune systems to allow reconstitution from the donor's cells while avoiding graft rejection.

Initially, mouse TBI consists in using radioactive sources emitting gamma rays from cobalt-60 ( $^{60}\text{Co}$ ) and cesium-137 ( $^{137}\text{Cs}$ ), namely  $\gamma$ -irradiation ( $\gamma$ -IR) [9]. However, due to security issues, there has been a global move toward replacing these radioactive sources with X-ray irradiation (X-IR) devices [10]. Each type of irradiation device is represented by several parameters, among which beams energy, reflecting penetrating ability, is essential to consider.

$\gamma$ -IR sources have a uniform energy output of 1.25 MeV for  $^{60}\text{Co}$  and 0.662 MeV for  $^{137}\text{Cs}$ . On the other hand, X-IR devices have different energies, ranging from 160-350 kV for most commercially available small animal X-ray irradiators to 6-25 MV for medical linear accelerators (LINACs). Since the most common sources of radiation used in previously published studies, i.e.,  $\gamma$ -IR and LINACs, are either no more available or with very restricted

access, novel radiation protocols using X-ray irradiators are needed to ensure the continuity of experimental research.

Previous studies have investigated the impact of different IR sources ( $\gamma$ -IR vs. X-IR) on HSCT efficacy and toxicity in TBI mouse models [11–14]. However, the impact of different X-IR energies on the fate of transplanted mice remains to be evaluated. Here, we compared the impact of TBI exposure using either a high-energy medical linear accelerator (LINAC<sup>®</sup>, now referred to as LINAC) or low-energy small animal irradiator Faxitron<sup>®</sup> CP160 (Faxitron X-Ray Corp., Wheeling, IL, USA, now referred to as CP160) on GVHD and GVL effects in mice models of BMT. We also address the potential impact of this IR energy difference on alloreactive T responses.

## MATERIEL AND METHODS

### Mice

Female C57BL/6J (B6, H-2<sup>b</sup>), B6xC3HF1 ([B6C3]F1, H-2<sup>kxb</sup>), and (B6xDBA2)F1 ([B6D2]F1, H-2<sup>bxd</sup>) mice were obtained from Charles River Laboratories (France) and used at 10 to 12 weeks of age. Mice were housed under specific pathogen-free conditions. All experiment protocols were approved by the local ethics committee (authorization number N°APAFIS#11511-2017092610086943) and in compliance with European Union Guidelines.

### Irradiation

#### ***Irradiation devices***

*Clinac 2100 CD linear accelerator* (Varian Medical Systems, Palo Alto, CA, USA), used in current radiation oncology, was configured for TBI mouse studies at a dose rate of 100 MU/min (100 cGy/min) using 6 MV flattening filter photon beams. The field size for each beam was set to 40 x 40 cm with an SSD of 100 cm. The gantry was placed at 0° and 180° to ensure

homogenous distribution of IR dose. Mice were positioned in a transparent, plexiglass container box in the IR field's center. During IR, two plexiglass plates of 0.5 cm-thick were placed on top and bottom of the box as a bolus to deal with dose buildup and homogenize the dose distribution. The device is housed in the radiation-oncology department of our institution, with access only available for approved individuals.

Faxitron® model CP160 (Faxitron X-ray Corp., Wheeling, IL, USA) is a commercially available X-ray irradiator designed for animal IR. The unit contains an X-ray tube mounted on the ceiling of a steel, shielded cabinet measuring 85 cm x 85 cm x 110 cm. The X-ray tube has a cathode generator with a power electronics module and a stationary anode made of tungsten, generating negative and positive high voltages to excite the vacuum tube. The tube has a beryllium window of 0.8 mm thick as inherent filtration and a filter holder beyond the exit window to place additional filtration. Beam hardening filters by 0.5 mm thick Copper were used to attenuate low-energy photons preferentially. Mice were placed at the seventh tray at a SSD of 33 cm with IR field size coverage of a zone of 26.7 cm in diameter. The 160-kV X-ray tube provides high output, relatively uniform vertical beam with a maximum output of 1000W, allowing for 0.8 Gy/min delivery at a current of 3 mA. The unit is housed in a secure area with access only available for approved individuals.

### ***Mouse total body irradiation***

Mice were irradiated at a single, lethal dose of 10 Gy either with a medical linear accelerator or a Faxitron® CP160 irradiator. Mice were kept in dedicated boxes previously described [15].

The LINAC device delivered the total IR dose in two sequential anterior and posterior beams. Using the CP160 irradiator, the IR source is fixed on the ceiling of the device, and only a vertically anterior beam was administered. The container was rotated on a turntable during IR exposure to achieve a maximal uniform distribution of IR dose. The same total IR dose was

also delivered equally in two beams to prevent the mice from heatstroke produced inside the cabinet; mice were taken out for 10 minutes between two IR sessions.

### **Bone-marrow transplantation and tumor relapse models**

Recipient [B6C3]F1 (H-2<sup>kxb</sup>) female mice received lethal IR doses followed by retro-orbital infusion of  $10 \times 10^6$  bone-marrow (BM) cells and  $2 \times 10^6$  CD3<sup>+</sup> T cells from C57BL/6 mice. BM and T-cell suspensions were prepared using leg bones and lymph nodes, respectively, as previously described [16]. For tumor relapse models, recipients were [B6D2]F1 mice, BM cells and T cells were reduced by a half, i.e., only  $5 \times 10^6$  BM cells and  $1 \times 10^6$  T cells were needed, and  $1$  or  $2 \times 10^4$  mastocytoma P815-GFP cells (gift from Dr. G. Marodon) were concomitantly injected intravenously into the retro-orbital sinus.

Recipient mice are the offspring F1 (first filial generation) hybrid mice of a cross between C57BL/6J female (B6) and C3H/HeJ males (C3). Donor C57BL/6 mice harbor half of the genome of recipient B6C3HF1. The BMT between these two mice strains is designated as semi-allogeneic BMT.

### **Assessment of GVHD and tumor progression**

After semi-allogeneic BMT, GVHD symptoms and tumor occurrence were evaluated three times per week. Clinical GVHD was evaluated as previously described [15]. Each of the five following parameters was scored 0 if absent or 1 if present: weight loss > 10% of the initial weight, hunching posture, skin lesions, dull fur, and diarrhea attested by liquid stool production at the time of mice evaluation or its presence at the anal area. Dead mice received a score of 5 until the end of the experiment. Mice were sacrificed in case of weight loss > 30% of initial weight or upon reaching the maximal clinical grade (i.e., 5/5). Tumor engraftment was

considered positive in the presence of a visible retro-orbital mass or detection of more than 0.5% of P815-GFP cells among donor splenocytes in peripheral blood.

### **Flow cytometry**

Flow cytometry analyzed the composition of the peripheral blood compartment on day 13 after BMT to assess the engraftment of donor immune cells (in BMT/GVHD models) or leukemic cells (in tumor relapse model). After ACK lysis, peripheral blood cells were stained with the following antibodies: Fluorescein isothiocyanate (FITC)-labeled anti-CD8 (clone REA601) and APC-Cy7-labelled anti CD45 (clone REA737) were purchased from Miltenyi. R-phycoerythrin (PE)-labelled anti-H-2Kb (clone AF6-88,5), PE-labelled anti-H-2Kb (clone SF1-1.1), Brilliant violet (BV)450-labelled anti H-2KK (clone AF3-12.1.3), PE-Cy5-labelled anti-CD3 (clone 553065); PE-Cy7-labelled anti-CD4 (clone RM4-5) were purchased from BD bioscience). Fixable viability dye efluor 506 was purchased from Invitrogen.

Events were acquired on a FACS Canto II flow cytometer using FACS Diva software (BD Biosciences), and data were compensated and analyzed using FlowJo software (Tree Star, Ashland, OR).

For the chimerism assessment: Donor cells were designated as H2Kk- H2Kb+ cells in BMT/GVHD models and identified as H2Kd- H2Kb+ in the tumor relapse model. Also, in the tumor engraftment model, leukemic cells were identified by GFP and H2Kd expression.

### **Cytokine measurement**

Sterile serum was obtained 3 and 24 hours post-TBI from irradiated-only animals without BMT rescue. GM-CSF, IFN- $\gamma$ , IL-6, IL-1b, and TNF- $\alpha$  were quantified using the Premixed Multi-Analyte kit from Bio-Techne with Luminex-based technology as specified by the manufacturer.

## Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). The data of the control and treatment groups were compared using non-parametric tests. For Kaplan-Meier survival curves, groups were compared using the log-rank test. Other data were compared using either a Mann-Whitney test (for two-by-two comparisons) or a Kruskal-Wallis test with Dunn's multiple comparison post-test (for multiple comparisons). Statistical significance is indicated as \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . Population statistics are displayed as the mean  $\pm$  SD/SEM.

## RESULTS

### Lethal total-body irradiation obtained with both devices

We compared each irradiation device to evaluate the lethality of the irradiation dose administered (10Gy). It is generally admitted that an optimal TBI is administered when 100% of the mice die from the second week after irradiation, thus attesting death due to myeloablation but not to radiation injuries. In our hands, all mice irradiated without BMT showed 100% mortality regardless of the irradiation device. The first death from the CP160 occurred on day 7 after irradiation, with most deaths happening on days 8-10. Regarding the LINAC device, the first death was observed on day 10, and most deaths occurred between day 10 and day 12 (Figure 1). The median survival of mice was 10 days and 11 days in the CP160 and LINAC groups, respectively ( $p=0.97$ ). Thus, myeloablation is effective regardless of the type of radiation delivered without inducing excessive radiation damage that could bias the observations.

### Hematopoietic reconstitution after TBI

After semi-allogeneic BMT, flow cytometry analysis was performed on PBMC of grafted mice

on day 13 to evaluate the donor cell engraftment. Leukocyte reconstitution was similar with both irradiation modalities (%CD45<sup>+</sup> cells in PBMC was 94.5% ± 1.9% vs 93.6% ± 1.6% in the CP160 and LINAC group, respectively;  $p=0.21$ ) (Figure 2A). T cell compartment reconstitution was partially achieved on day 13 post-BMT. The proportions of CD8<sup>+</sup> and CD4<sup>+</sup> cells among CD45<sup>+</sup> cells in healthy mice vary between 20-50% [17]. In transplanted mice, T cells compartment was dramatically reduced regardless of the TBI devices (proportions of CD8<sup>+</sup> and CD4<sup>+</sup> cells among CD45<sup>+</sup> cells were 2.35% and 2.19% in the CP160 group, and 2.24% and 2.51% in the LINAC group, respectively,  $p=0.39$  for CD8<sup>+</sup> and  $p=0.59$  for CD4<sup>+</sup>) (Figure 2B, 2C). We did not observe any significant difference between the two radiation devices regarding the presence of recipient and donor cells after semi-allogeneic BMT. C57B6 donor cells harboring the sole H2Kb<sup>+</sup> phenotype constituted the majority of the PMBC (Table1). Most transplanted mice were alive at the end of the study on day 90. Both groups' survival rates were similar, reaching 91.3% and 91.4% in the LINAC and CP160 groups ( $p=0.88$ ), respectively (Figure 3E).

These results confirmed the effect of BM ablation by lethal TBI at 10Gy, and the successful engraftment from donor cells in recipient mice, which were obtained similarly with both radiation modalities.

### **Impact of IR technique and device on incidence and severity of acute GVHD**

In the same model of semi-allogeneic BMT, we next assessed the incidence of acute GVHD (aGVHD) according to the TBI device. To establish aGVHD in mice, we injected donor T cells ( $2 \times 10^6$  cells) with BM cells immediately after a lethal TBI (Figure 3A). We first observed a rapid decline in the weight of grafted mice during the first week following irradiation (Figure 3B), with a more marked effect in mice irradiated with the low-energy CP160 irradiator compared to mice irradiated with the high-energy LINAC device. Compared to the weight of non-irradiated mice, in the BM experimental group, mice's weight on day 6 was reduced by 3.43% and 12.4% in the LINAC and CP160 groups, respectively ( $p=0.0015$ ). In the GVHD

model, the weight loss was measured at 13.8% and 18.3% in the LINAC and CP160 groups, respectively ( $p=0.0032$ ). Interestingly, the weight loss was also more marked in mice receiving CP160-TBI alone without BM rescue (15.8% vs. 4.96% in the LINAC-TBI group,  $p=0.036$ ). Mice in the BM experimental group recovered progressively afterward and regained their initial weight. On day 30, the mice's weight compared to their baseline was not statistically different. In contrast, mice in the GVHD group continued to lose weight, reaching -14.4% and -25.2% on day 30 in the LINAC and CP160 groups, respectively ( $p=0.0047$ ). Besides the weight loss, we also observed acceleration and deterioration of aGVHD in CP160 mice compared to those in the LINAC group (Figure 3C, 3D), objectively evaluated and classified according to the previously published clinical grading system for murine models of aGVHD [15]. We first noted that the onset of aGVHD signs occurred early in the CP160 mice ( $12.34 \pm 3.1$  days,  $n=64$ ) compared to the LINAC group of mice ( $17.95 \pm 5.7$  days,  $n=57$ ) ( $p<0.0001$ ). They also presented a higher clinical grade of aGVHD (AUC of GVHD clinical grade was  $207.6 \pm 29.4$  and  $164.9 \pm 58.7$  in the CP160 and LINAC groups, respectively,  $p<0.0001$ ). Also, aGVHD grade 5 (which means the mouse acquired all clinical aGVHD signs and was sacrificed or found dead on the day of evaluation) was reached earlier in the CP160 mice leading to a worse survival outcome in this group. Indeed, the 30-day survival rates in the CP160 and LINAC groups were 22% and 41%, and the median survivals were 27 and 36 days, respectively (HR=1.89, 95%CI 1.29-2.76,  $p=0.0003$ ) (Figure 3E). Of note, mice irradiated with the CP160 device, regardless of the experimental group, showed typical alopecia in the scalp and dorsal neck region (Supplement Figure 1S) and early skin lesions of dermatitis desquamation type that rapidly healed within the first 2 to 3 weeks following TBI. Since these lesions occur before the onset of visceral GVHD signs, they were not considered GVHD signs. This last observation suggested that besides the BM ablation effect in the BMT model and the donor cells' alloreactivity in the GVHD model, other factors enhancing host inflammatory response could be differently involved depending on the use of the high-energy LINAC or the low-energy CP160 devices.



### Impact of IR technique on tumor growth and GVL effect

Leukemic mouse models that mimic patients that relapse after allogeneic HSCT to evaluate different anti-leukemic therapeutic approaches were previously established [16]. This experimental model was adapted so that alloreactivity induced by donor T cells was insufficient to limit tumor progression or induce GVHD. Tumor cells were concomitantly injected with donor BM cells  $\pm$  donor T cells after a lethal TBI with the LINAC or CP160 device (Figure 4A). When  $2 \times 10^4$  P815 cells were injected with BM cells after TBI using the LINAC device, all studied mice died within 30 days with a retro-orbital mass, attesting tumor evolution. The median survival in this group (tumor engraftment or Tum model) was 17.5 days. When  $1 \times 10^6$  donor T cells (half of the usual number of T cells co-injected in GVHD models) were co-injected with BM cells and tumor cells (anti-leukemia or GVL model), the median survival in this group was 21 days ( $p > 0.99$ ) (Figure 4B), without influencing the incidence of tumor development at the end of the experiment (100%). The median time from BMT to tumor occurrence was 13 and 19 days in the Tum and GVL models, respectively (Figure 4C). Irradiation with the CP160 irradiator does not statistically modify leukemia engraftment depending on whether donor T cells were co-injected or not during semi-allogeneic BMT. Here, the median survivals of mice in the Tum model and the GVL model were 27 and 34.5 days ( $p = 0.099$ ) (Figure 4B). However, when using the CP160 device, tumor incidence varied following BMT with or without T cells co-injection. At the end of the experiments on day 30, tumor incidences in the Tum model were 85.7% (median time to tumor development 16 days) and only 40% in the GVL model (median time to tumor development not reached) ( $p = 0.0325$ ) (Figure 4C).

In order to explain these unexpected GVL effects, and following previous observations of a stronger alloreactivity in the CP160 group, we assumed that mice treated with this irradiation device might develop an increased acute GVHD reaction. Indeed, while only one mouse in the LINAC group presented a moderate acute GVHD clinical grade at death, most mice in the CP160 group died with moderate to severe aGVHD clinical signs (Figure 4D). Also, the AUC

of aGVHD grade was significantly higher in the CP160 group than in the LINAC group (106.4 vs. 27.35, respectively,  $p < 0.0001$ ).

### **Cytokines release post-lethal TBI and their impact on alloreactivity after BMT**

Our observations suggested that mice in the CP160 group presented a stronger alloreactive reaction after semi-allogeneic BMT. Since the pathophysiology of aGVHD is greatly affected by the intensity of conditioning regimens [1], we presumed that TBI with the CP160 device might trigger a more significant cytokine release. Therefore, we evaluated some pro-inflammatory cytokines in irradiated mice (Figure 5). We focused on measuring tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 (IL-1), IL-6, and interferon- $\gamma$  (IFN- $\gamma$ ), based on their well-established roles in GVHD induction [18–21].

Serum samples from non-irradiated B6C3F1 mice contained non-detectable TNF- $\alpha$ , IL-1, IL-6, and IFN- $\gamma$ . In irradiated mice, we observed a significantly higher serum level of TNF- $\alpha$  and IL-1 as early as 3 hours after irradiation in the CP160 group compared to the LINAC group (Figure 5). Interestingly, these cytokines went down rapidly after 24 hours of irradiation in the CP160 group to the same levels as those in the LINAC group, which did not significantly change in the early or later phases after TBI. IL-6 levels did not change in the CP160 mice between 3h and 24h post-TBI. In both groups, serum IFN- $\gamma$  was not detectable at 3 or 24 hours in irradiated mice (data not shown).

So far, our models relied on early injection of donor BM immediately after lethal TBI. We now know this time point corresponds to the peak of inflammation post-irradiation observed in the CP160 group. We hypothesized that we would decrease the post-BMT alloreactivity by delaying the injection of BM cells out of this inflammatory peak. Briefly, we compared BMT and GVHD models when the cells were injected on the day of lethal TBI (D0) or, alternately, the day after (D1) (Figure 6A).

Firstly, we confirmed the difference in GVHD severity between grafted mice irradiated with the LINAC and CP160 device irradiated at D0 (AUC clinical grade of aGVHD = 150.1 vs. 242.7,  $p < 0.0001$ ). By postponing the BMT by one day, we observed a significant change in the AUC clinical grade of aGVHD in the CP160 group (242.7 vs. 203.5 in D0 and D1 grafted mice, respectively;  $p = 0.0126$ ). AUC of aGVHD grades in D1-GVHD-CP160 mice was not statistically significant with those of D0-GVHD-LINAC mice ( $p = 0.079$ ). Mice in the LINAC group did not show a significant difference in aGVHD grade (AUC of clinical-grade of acute GVHD was 150.1 vs. 166.0 with D0 and D1 injection, respectively,  $p = 0.99$ ) (Figure 6B). These observations were in accordance with survival outcomes. The median survival in D0-GVHD-CP160 mice was significantly shorter than in the D0-GVHD-LINAC group (30 days vs. 53.5 days, respectively;  $p = 0.0002$ ). By delaying one day of BMT, the median survival was markedly improved in the CP160 group, passing from 30 days (D0-GVHD) to 47 days (D1-GVHD) (HR=0.33, 95%CI 0.19-0.59,  $p < 0.0001$ ). There was no difference in survival between D1-GVHD-CP160 mice and D0-GVHD-LINAC mice (47 days vs. 53.5 days, respectively,  $p = 0.40$ ) or D0- vs. D1-GVHD-LINAC groups ( $p = 0.99$ ) (Figure 6C).

## DISCUSSION

Since the first allogeneic HSCT pioneered by E.D. Thomas in Seattle [22], research in this field has constantly interchanged between laboratory experiments and clinical trials. Despite many improvements in efficacy and complication management, limiting factors such as aGVHD remain. Animal models demonstrated that the nature (myelo- or immuno-suppressive drugs versus IR) and the intensity of the conditioning regimens could influence aGVHD incidence and severity [9, 23]. In this line, irradiation of small animals is regularly performed for research purposes such as tumor engraftment, adoptive cell transfer, and therapeutic development to control GVHD while maintaining the GVL effect.

Here, we presented the outcomes from different mouse models after lethal irradiation followed by semi-allogeneic BMT. To our knowledge, this is the first study comparing the impact of different energy X-ray devices (the megavoltage high-energy clinical accelerator LINAC machine and the kilovoltage low-energy Faxitron-CP160 irradiator) on clinical outcomes of grafted animals. The major concern between these two irradiation modalities relies on the energy difference of the emitting radiation beams. While the megavoltage LINAC machine is used in the clinical setting to treat human patients of up to a few tens of centimeters thickness, the kilovoltage Faxitron-CP160 is typically reserved for more superficial irradiation of a few centimeter depths.

Firstly, our data showed a near-equivalence of the LINAC and CP160 irradiation modalities in their capacity to deplete the recipient's hematopoietic and immune systems, as demonstrated by the mortality rate of 100% of irradiated mice from the second week in the absence of BMT. This observation is in accordance with previous reports showing that, despite their disadvantageous tissue penetration, low-energy X-ray irradiators have shown interchangeable effects in different BMT models compared to deeper penetration irradiation devices, mainly with <sup>137</sup>Cesium gamma-ray irradiators [11, 12, 14]. However, in our study, typical scalp and dorsal neck alopecia and skin lesions were observed in all mice irradiated with the CP160 device using an IR energy of 160 kV. Other studies evaluating X-ray irradiators using a lower dose of TBI (9Gy) or a higher IR energy (320-350 kV) did not mention such a high rate of alopecia (Table 2). The lower IR energy and the higher the IR dose is, the more energy deposition is increased in superficial tissues, leading to damage to hair follicle stem cells. This likely explains the high rates of alopecia in our study, independently of BMT or donor alloreactive T cell responses.

Many authors agreed on the dose-effect of TBI in recipient BM ablation and donor-type hematopoietic stem cell reconstitution [12, 14]. Notably, T cell lineages and particularly the regulatory T cell subpopulations, are believed to be more radioresistant than other lineages [24, 25]. This could lead to a longer lifespan of these cell types and cause a slower

reconstitution after BMT [17]. In line with these results, our data showed the establishment of BM reconstitution with a very high degree of chimerism (>85% to 95%) of donor hematopoietic source regardless of the radiation modality. The T cell compartment reconstitution remained very low on day 13 (less than 10%), which was coherent with previous observations, where the recovery of this population can take up to two months after BMT to achieve a complete reconstitution [17]. At this stage, we could conclude that the type of irradiation devices did not influence engraftment in our study.

Regardless of BMT models, our study shows an increase in alloreactivity after semi-allogeneic BMT in mice irradiated with the CP160 irradiator compared to those irradiated with the LINAC device, which further translates into either a more severe aGVHD or an increased GVL effect. This observation does not depend on the genetic combination since increased alloreactivity was documented when B6 donor cells were grafted in [B6C3]F1 or [B6D2]F1 recipient mice irradiated with the CP160 irradiator.

It was documented that aGVHD is closely associated with the intensity of conditioning regimens in humans and animals [20, 21, 26]. In SCID immunodeficient mice, *Sakai et al.* observed mild acute GVHD after allogeneic H-2-incompatible BMT without TBI but lethal aGVHD when splenocytes were infused immediately after 2 Gy TBI [27]. Similarly, *Hill et al.* showed an increase in GVHD severity after allogeneic BMT after intensified conditioning of the TBI dose from 9 to 13 Gy, regardless of the compatibilities between donor and recipient mice [20]. Indeed, intensified conditioning regimens generate epithelial and endothelial cell injury leading to a greater amount of lipopolysaccharide (LPS) entering the systemic circulation and inducing the release of inflammatory cytokines [20, 28]. These cytokines enhance the presentation of host alloantigens and the function of donor immune cells. In our study, the mice received the same 10 Gy X-ray dose but delivered with different energy levels. Therefore, we hypothesized that this difference in energy level could also cause differences in alloreactivity intensities. Therefore, we sought to determine whether the induced inflammation could differ according to the device used. Our study showed a significant increase in serum levels of TNF-

$\alpha$  and IL-1 of mice from the CP160 group compared to those from the LINAC group as early as 3 hours after lethal TBI. These cytokine levels then rapidly declined 24 hours after TBI to reach the same levels observed in the LINAC-irradiated mice.

*Xun et al.* showed that 4Gy-TBI alone increased TNF- $\alpha$  and IL-1 $\alpha$  release, followed by IL-6 in the absence of allogeneic transplantation in SCID mice. TNF- $\alpha$ , IL-1 $\alpha$ , and IL-6 levels were significantly augmented and remained high if TBI and H-2-incompatible splenocytes were administered together (TBI +D<sub>0</sub> BMT) compared to TBI and BMT administered separately (TBI + D<sub>4</sub> BMT or TBI + D<sub>7</sub> BMT). Delaying the injection time after the TBI-induced inflammatory cytokine storm from host tissues has markedly reduced cytokine release and acute GVHD mortality [21, 27].

Similarly, our study showed that delaying BMT by one day significantly reduced GVHD severity and mortality in the CP160 mice but not in the LINAC mice, likely due to the cytokine dynamics in these two groups. Thus, we show that in addition to the intensity of X-ray irradiation, the energy level delivered can also influence the level of inflammation in irradiated mice and impact the intensity of GVHD. As this effect is short-lived, a delay in the injection of the donor cells allows their administration in a less inflammatory context, consequently reducing alloreactivity.

## CONCLUSION

Our study was the first one addressing a practical issue on the impact of IR parameters, yet usually neglected in preclinical experiments. Our results suggested that the two studied X-IR modalities (i.e., LINAC vs. CP160) might have the same outcomes regarding lethal effect at 10Gy-TBI and BM engraftment in semi-allogeneic BMT. Despite this similarity, the alloreactive responses vary considerably and are mediated by inflammatory cytokines released after TBI exposure. Delaying BMT injection until the reduction of the initial cytokine burst significantly reduces this alloreactivity. We propose considering this parameter in reporting outcomes of GVHD/GVL in mice models.

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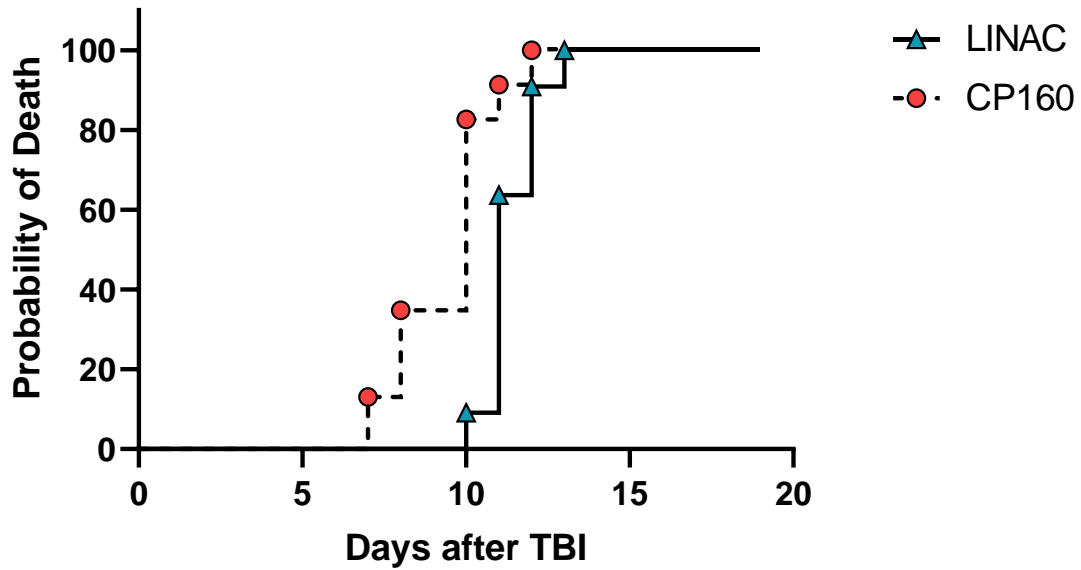
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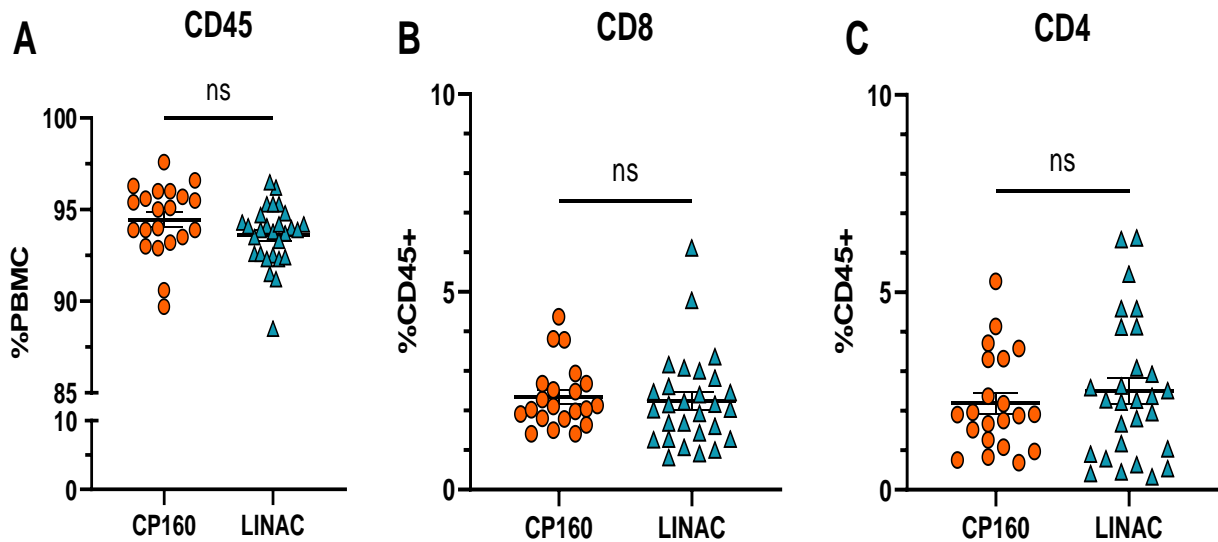
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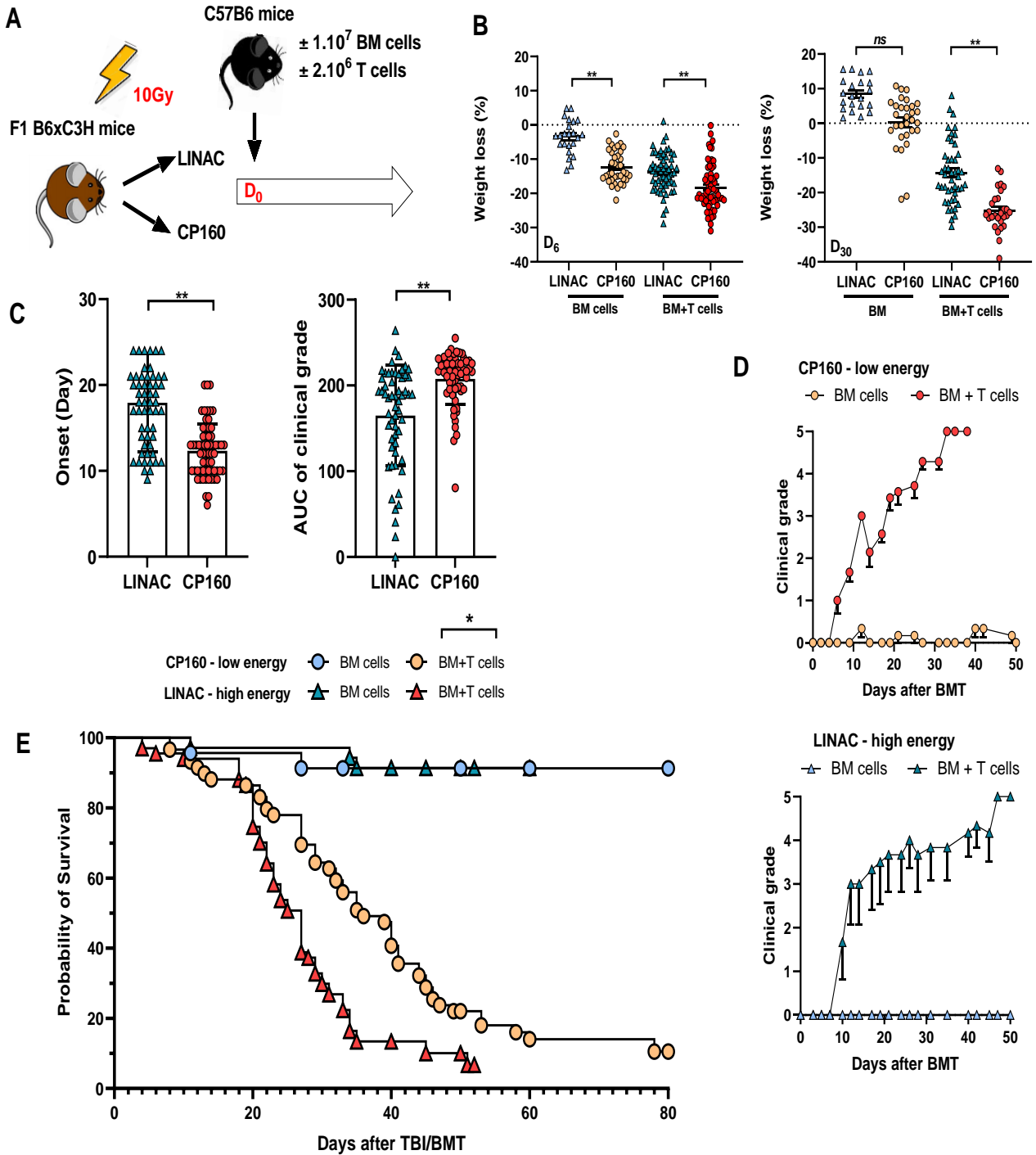
## FIGURES



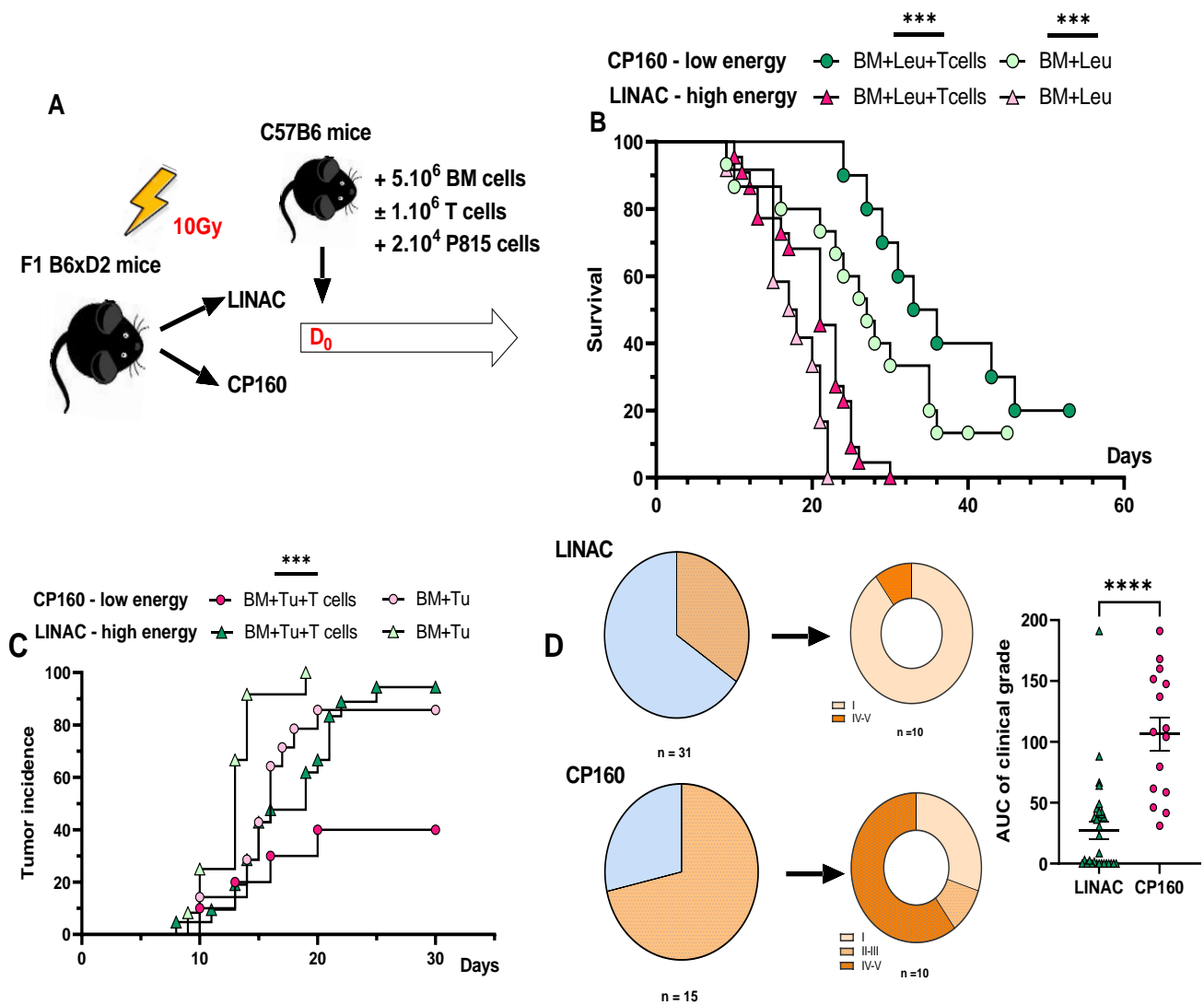
**Figure 1: Lethal-dose curve after 10Gy TBI with the CP160 irradiator and LINAC machine.** Female [B6C3]F1 mice were lethally irradiated with the CP160 irradiator (CP160  $n=11$ ) or with the LINAC machine (LINAC;  $n=23$ ). The Kaplan-Meier survival curve shows the mortality after irradiation. Kaplan Meier curves were compared using the log-rank test. The cumulative data of two independent experiments are shown.



**Figure 2: Early reconstitution in mice transplanted with semi-allogeneic BM cells after a lethal TBI using the CP160 irradiator and the LINAC machine.** After lethally irradiation with the low-energy CP160 irradiator (CP160,  $n=6$ ) or the high-energy LINAC machine (LINAC,  $n=6$ ), [B6C3]F1 mice received  $10 \times 10^6$  BM cells from donor mice were transplanted 13 days after transplantation BMT chimerism, blood was collected for evaluated chimerism by flow cytometry. Donor cells defined as H2Kk- cells were evaluated among the whole leucocyte population defined as CD45+ cells (A) or among CD4 cells (B) or CD8 cells (C). Data are plotted as the mean  $\pm$  SEM. Statistical significance from the controls was determined using Mann-Whitney tests.

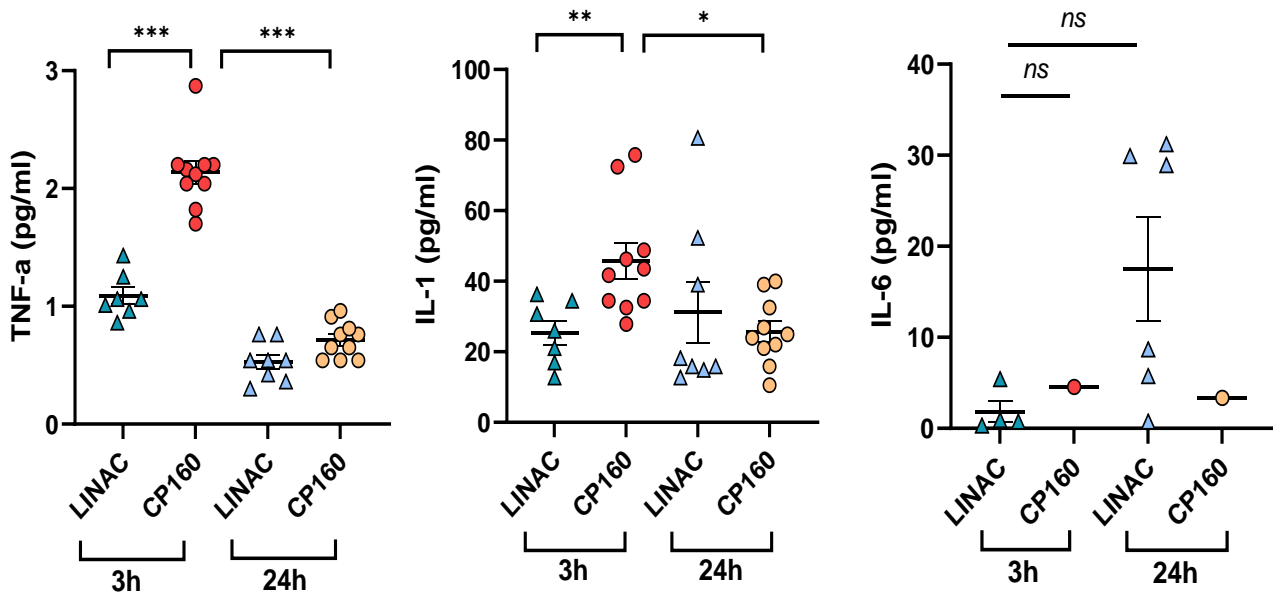


**Figure 3: GVHD mouse models after semi-allogeneic BMT were established with different radiation devices (high-energy LINAC machine and low-energy CP160 irradiator).** A: schematic illustration of the experimental layout. [B6C3]F1 were irradiated at a lethal dose using the high-energy LINAC accelerator (LINAC, n=58) and the low-energy CP160 irradiator (CP160; n=64) and then received semi-allogeneic BMT ( $10 \times 10^6$  BM cells  $\pm 2 \times 10^6$  T cells) on the same day. Weight and GVHD signs were evaluated three times a week. B: weight changes of mice in different models on day 6 (left) and day 30 (right) after TBI/BMT. C, D: occurrence and severity of acute GVHD in GVHD mouse models using LINAC and CP160 devices. E: survival probability of mice in different models using LINAC and CP160 devices. Kaplan-Meier survival curves were compared using the log-rank test. The cumulative data of 6 independent experiments are shown. The AUCs were calculated for the GVHD clinical-grade curve for each mouse and are presented as the mean  $\pm$  SEM. Either Mann-Whitney or Kruskal-Wallis tests were performed, depending on the number of compared groups. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



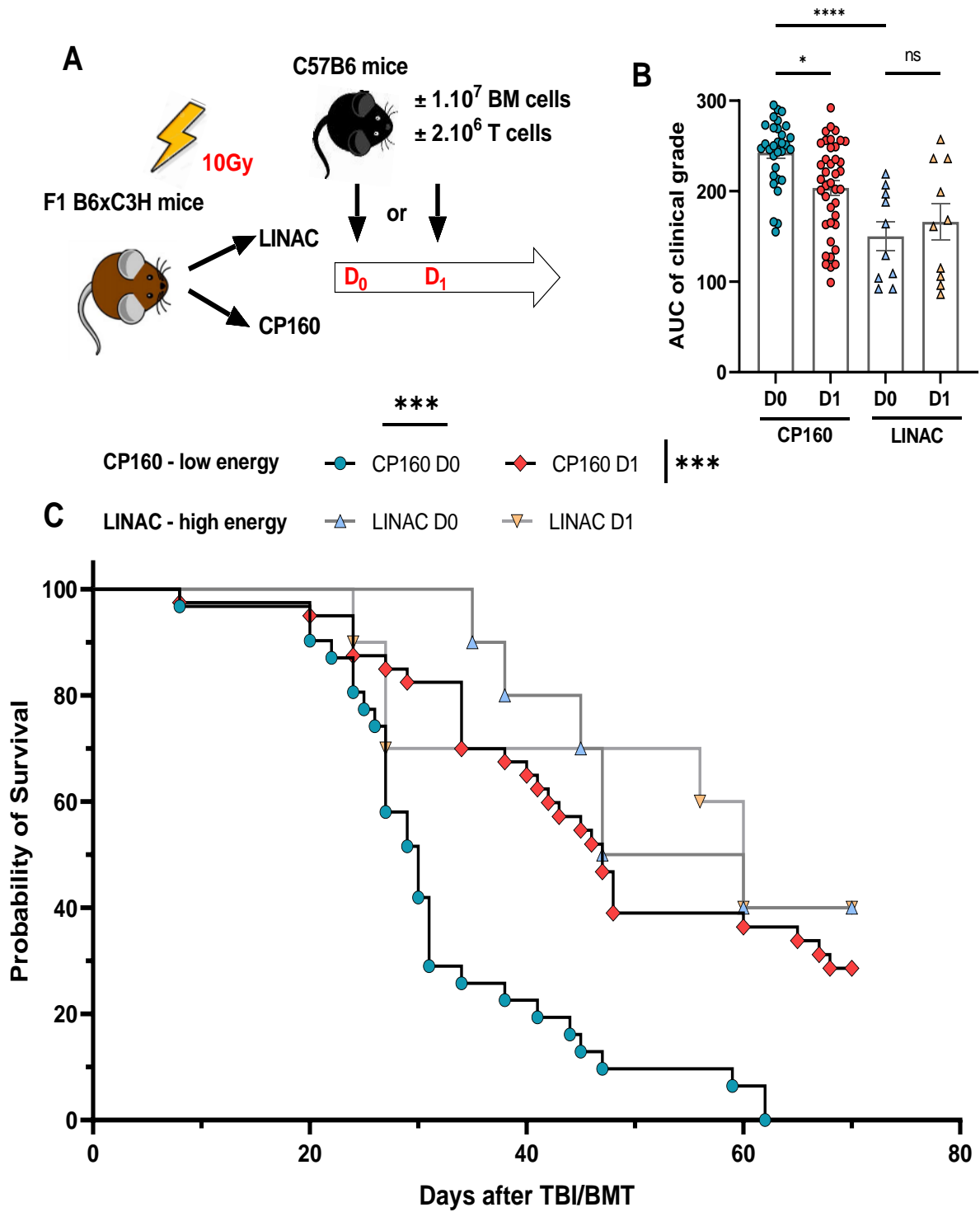
**Figure 4: Leukemia development mouse models after semi-allogeneic BMT was achieved in the LINAC-TBI group but not in the CP160-TBI group.** A: schematic illustration of the experimental layout. [B6D2]F1 were irradiated at a lethal dose using the high-energy LINAC accelerator (LINAC, n=31) and the low-energy CP160 (CP160; n=15) and then received semi-allogeneic BMT ( $5 \times 10^6$  BM cells ±  $1 \times 10^6$  T cells +  $2 \times 10^4$  P815 mastocytoma cells) on the same day. Weight, tumor development, and GVHD signs were evaluated three times a week. B: survival probability of mice in different models using LINAC and CP160 devices. C: tumor development in different models using LINAC and CP160 devices. D: cause of death in mice treated with each device was attributed to either tumor development or GVHD. Deaths

related to acute GVHD were further classified according to clinical GVHD grades and AUC of clinical grades, showing a significant acute GVHD triggered by the low-energy CP160 irradiator. Kaplan-Meier survival curves were compared using the log-rank test. E: Histogram shows the area under the curve (AUC) of GVHD signs. The AUCs were calculated for the GVHD clinical-grade curve for each mouse and are presented as the mean  $\pm$  SEM. Either Mann-Whitney or Kruskal-Wallis tests were performed, depending on the number of compared groups. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



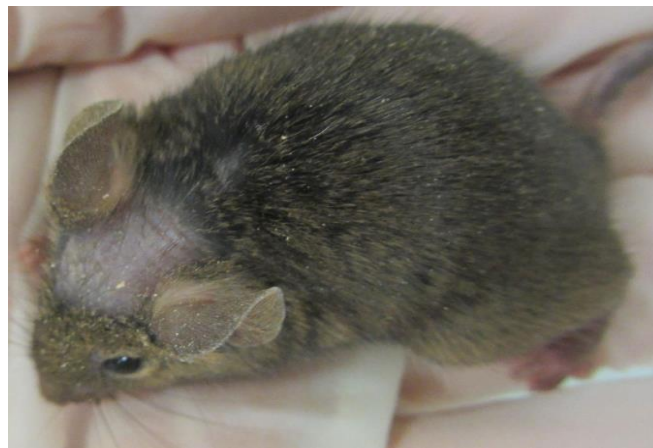
**Figure 5: Pro-inflammatory cytokines released post-TBI in both devices.** Female [B6C3]F1 mice were lethally irradiated with the low-energy CP160 irradiator or with the high-energy LINAC accelerator ( $n = 10$  for each TBI modality); sterile serum was collected 3 and 24 hours later from each mouse. The serum cytokine levels are presented as mean  $\pm$  SEM. Either Mann-Whitney or Kruskal-Wallis tests were performed, depending on the number of compared groups. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .





**Figure 6: Alloreactivity after semi-allogeneic BMT is influenced by injection time after TBI and differentiated according to radiation devices.** A: schematic illustration of the experimental layout. [B6C3]F1 were irradiated at a lethal dose using the high-energy LINAC accelerator (LINAC, n=36) and the low-energy CP160 irradiator (CP160; n=97) and then received semi-allogeneic BMT ( $10 \times 10^6$  BM cells  $\pm$   $2 \times 10^6$  T cells) either on the same day (D0) or the following day (D1). Weight and GVHD signs were evaluated three times a week. B: severity of acute GVHD is influenced by injection time of HSC post-TBI and radiation devices. C: survival probability of mice in different models with different injection times of HSC post-TBI using LINAC and CP160 devices (upper: all studied models, lower: acute GVHD model with different injection time post-TBI using the CP160 irradiator). The cumulative data of 4 independent experiments is shown. Kaplan-Meier survival curves were compared using the log-rank test. The AUCs were calculated for the GVHD clinical-grade curve for each mouse and are presented as the mean  $\pm$  SEM. Either Mann-Whitney or Kruskal-Wallis tests were performed, depending on the number of compared groups. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

**Supplementary Fig 1: Typical alopecia of the scalp and dorsal neck region**



## TABLES

**Table 1: BM cells early reconstitution after semi-allogeneic BMT on day 13**

	Recipient-originated cells (%H2Kk <sup>+</sup> H2Kb <sup>+</sup> )			<i>p</i>	Donor-originated cells (%H2Kk <sup>-</sup> H2Kb <sup>+</sup> )			<i>p</i>
	CP160	LINAC	CP160		LINAC			
<b>CD4<sup>+</sup> cells</b>	4.7 ± 5.8	7.2 ± 11.7	0.108	95.3 ± 5.9	92.8 ± 11.7	0.108		
<b>CD8<sup>+</sup> cells</b>	1.8 ± 0.6	2.2 ± 0.8	0.163	98.2 ± 0.6	97.8 ± 0.9	0.178		
<b>Leukocytes*</b>	0.5 ± 0.2	0.5 ± 0.2	0.374	97.9 ± 1.1	97.8 ± 0.9	0.131		

\*: Leukocytes other than T cells (CD45<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup>)

**Table 2: Effect of different irradiation types on bone marrow transplantation outcomes**

Authors	IR type	Energy (MV)	Dose (Gy)	Dose rate (Gy/min)	BMT type	BMC (cells)	LNC (cells)	Lethal	BM engraftment	GVHD	Skin lesions	GVL
<b>Schwarte, 2007 (29)</b>	Y-IR ( <sup>60</sup> Co)	1.25	9-10	0.85				Yes	-	Yes	No	-
	Y-IR ( <sup>137</sup> Cs) GammaCell	0.662	9	2.5	Allogeneic	5 x 10 <sup>6</sup>	5 x 10 <sup>5</sup>	No <sup>a</sup>	-	Varies	Severe	-
	X-IR (LINAC)	6.0	9.5	1.5				Yes	-	Yes	No	-
<b>Gibson, 2015 (12)</b>	Y-IR ( <sup>137</sup> Cs) GammaCell	0.662	9-11	1.09	Allogeneic	5 x 10 <sup>5</sup>	NA	Yes	Yes	-	No	-
	X-IR (RS 2000)	0.16	9	1.17				Yes	Yes	-	Yes (3%)	-
<b>Gott, 2020 (13)</b>	Y-IR ( <sup>137</sup> Cs) GammaCell	0.662	12	1.0	Syngeneic	10 x 10 <sup>6</sup>	NA	No	Yes	-	NR	-
	X-IR (XRAD 320)	0.32	9.24 <sup>b</sup>	0.9	Allogeneic			No	No	-	NR	-
<b>Eng, 2020 (11)</b>	Y-IR ( <sup>137</sup> Cs) 143-86R	0.662	9	NR	Allogeneic	2-5 x 10 <sup>6</sup>	NA	NA	Yes	-	NR	NR <sup>c</sup>
	X-IR (Multirad 350)	0.35	7	2.0				NA	Yes	-	NR	Yes <sup>c</sup>
<b>Witterborn, 2021 (14)</b>	Y-IR ( <sup>137</sup> Cs) GammaCell	0.662	13	NR	Syngeneic	20 x 10 <sup>6</sup>	NA	Yes	Yes	-	No	-
	X-IR (Multirad 350)	0.35	11	NR				Yes	Yes	-	No	-
<b>Current study</b>	X-IR (LINAC)	6.0	10	1.0	Semi-Allogeneic	10 x 10 <sup>6</sup>	2 x 10 <sup>6</sup> <sup>d</sup>	Yes	Yes	Yes	No	No
	X-IR (CP160)	0.16	10	0.8	Allogeneic		1 x 10 <sup>6</sup> <sup>e</sup>	Yes	Yes	Yes	Yes (100%)	Yes

Legend: BMC: bone marrow cells, BMT: bone marrow transplantation, GVHD: graft-versus-host disease, GVL: graft-versus-leukemia, IR: irradiation, LNC: lymph node cells, NA: not applicable, NR: not reported, a: lethal GVHD, b: dose estimated as equivalent to targeted doses administered by Y-IR, c: delayed tumor growth effect dependent of IR dose, synergistic between IR and the immune checkpoint inhibitor anti-CTLA4 in tumor control of syngeneic tumors, d: BMT protocol using for GVHD models, e: BMT protocol using for BM and leukemic engraftment.

## Discussion

L'utilisation des modèles animaux est essentielle pour évaluer de nouveaux traitements dans le champ de la transplantation de cellules souches hématopoïétiques. Ils nous ont permis de démontrer que la nature des conditionnements (chimiothérapie de haute dose ou ICT myélosuppressive) ainsi que leur intensité pouvaient influencer l'incidence et la sévérité de la GVHDa (Schroeder and DiPersio, 2011; Schwarte and Hoffmann, 2005).

L'irradiation des petits animaux est régulièrement effectuée à des fins de recherche telles que le développement des médicaments protecteurs de la GVHD tout en favorisant l'effet GVL. Ainsi, identifier les paramètres d'irradiation pouvant impacter les modèles de greffe de CSH est essentiel.

### **Impact de l'énergie d'irradiation sur les modèles murins de semi-allogreffe**

Les sources  $\gamma$ -IR ont une énergie en moyenne de 1,25 MeV pour le  $^{60}\text{Co}$  et 0,662 MeV pour le  $^{137}\text{Cs}$ . D'autre part, les appareils X-IR disposent différentes énergies, allant de 160-350 kV pour la plupart des irradiateurs à rayons X pour les petits animaux disponibles à 6-25 MV pour les AL médicaux. Traditionnellement, les  $\gamma$ -RI et la RxHE aux  $^{60}\text{Co}$  ou AL ont été les dispositifs d'irradiation les plus utilisés dans les modèles murins (Schwarte and Hoffmann, 2005), ce qui n'est plus le cas à l'heure actuelle, de nouveaux protocoles d'irradiation avec des dispositifs plus accessibles, les RxBE, sont donc nécessaires pour assurer la continuité de la recherche expérimentale.

Nous avons montré une quasi-équivalente capacité des deux modalités d'irradiation (LINAC<sup>®</sup> et CP160<sup>®</sup>) en déplaçant le système hématopoïétique et immunitaire du receveur, témoigné par le taux de mortalité de 100% des souris irradiées dès la 2<sup>ème</sup>

semaine en l'absence de greffe. Cette observation est en accord avec d'autres études comparant les X-IR à basse énergie aux  $\gamma$ -IR (Eng et al., 2020; Gibson et al., 2015; Wittenborn et al., 2021). L'ensemble de ces résultats confirme l'effet myéloablatif des X-IR à basse énergie par rapport aux irradiations à haute énergie malgré leur désavantage en termes de pénétration tissulaire.

Nous avons également démontré que la prise de greffe dans nos modèles murins de semi-allogreffe n'a pas été influencée par le type de dispositifs d'irradiation. En effet, les taux très élevés de chimérisme à J13 (> 85% à 95%) sont cohérents à ceux obtenus avec la même dose d'irradiation rapporté dans d'autres études (Gibson et al., 2015; Wittenborn et al., 2021).

Cependant, indépendamment du modèle murin de greffe, nos résultats ont montré une augmentation des signes cliniques de GVHDA après la greffe semi-allogénique chez les souris déplétées par l'irradiation au CP160 par rapport à celles irradiées au LINAC. En fonction du modèle étudié, elle conduit soit à une GVHDA plus sévère dans les modèles murins de GVHD soit à un effet GVL accru résultant en une diminution de la croissance tumorale dans les modèles murins de rechute leucémique.

### **Impact de l'énergie d'irradiation sur l'alloréactivité post-greffe**

Il a été documenté que la GVHDA est étroitement liée à l'intensité du conditionnement chez l'homme et chez la souris (Hill et al., 1997; Ringdén et al., 1999; Xun et al., 1994). En effet, les conditionnements plus intensifiés génèrent des dommages au niveau épithélial et endothélial, conduisant à la libération d'une plus grande quantité de LPS entrant dans la circulation systémique et induisant la libération des cytokines inflammatoires (Deeg, 2001; Hill et al., 1997). Ces dernières facilitent la présentation des alloantigènes de l'hôte et la fonction des cellules immunitaires du donneur,

augmentent ainsi l'alloréactivité post-greffe.

Dans nos expériences, les souris ont reçu la même dose d'ICT à 10 Gy délivrée à différentes énergies. Nous avons observé une augmentation significative des taux sériques de TNF- $\alpha$  et IL-1 dans le groupe CP160 dès 3 heures après l'ICT létale. Ces cytokines ont ensuite rapidement diminué pour atteindre les mêmes niveaux observés chez les souris irradiées par le LINAC à 24 heures post-ICT. D'autres études ont également décrit une augmentation des cytokines pro-inflammatoires après l'ICT et BMT, fortement influencées par la séquence entre l'ICT et la BMT (Sakai et al., 1997; Xun et al., 1994).

Dans nos conditions expérimentales, nous avons démontré qu'en décalant la greffe d'un jour, la sévérité et la mortalité de la GVHDA ont été significativement réduites dans le groupe des souris irradiées au CP160 mais pas chez les souris irradiées au LINAC, probablement en rapport avec la dynamique des cytokines inflammatoires dans ces deux groupes. Cet effet étant éphémère, une injection décalée de CSH permet leur administration dans un contexte moins inflammatoire, diminuant ainsi l'alloréactivité post-greffe.

Des approches ciblant les cytokines inflammatoires responsables de la physiopathologie de GVHDA et d'autres maladies auto-immunes et des maladies inflammatoires ont été activement développées. En effet, certains mAb (anticorps monoclonaux) reconnaissant les principales cytokines impliquées dans la GVHD ont été proposés dans le traitement de deuxième ligne de la GVHDA après l'échec de la corticothérapie (Penack et al., 2020). On peut citer parmi eux les mAbs anti-TNF- $\alpha$  (infliximab) ou plus particulièrement le blocage de la voie TNF et son deuxième récepteur TNFR2 qui module efficacement l'alloréactivité en faveur d'un effet GVL ou d'un contrôle de GVHD (Leclerc et al., 2016). Les mAbs antagonistes du récepteur IL-

2 (IL-2R) ont été aussi étudiés. Le daclizumab, un mAb sélectif liant à IL-2R $\alpha$  a été le premier mAb humanisé à être approuvé en pratique clinique, dans la prévention du rejet des greffes rénales (Vincenti et al., 1998). Son effet dans la GVHD est controversé, avec notamment des inquiétudes concernant des effets secondaires infectieux et la perte d'effet GVL (Willenbacher et al., 2001). Une nouvelle approche associant un mAb avec sa cytokine a permis d'améliorer son efficacité ainsi que sa tolérance. Nous allons aborder cette potentielle thérapie dans le prochain paragraphe et l'impact d'énergie d'irradiation sur cette dernière.

### **Impact de l'énergie d'irradiation sur l'efficacité de la thérapie prophylactique et thérapeutique de la GVHDa**

Si l'irradiation à faible énergie conduit à une alloréactivité accrue post-greffe de CSH liée à une augmentation des cytokines inflammatoire post-ICT, est-ce que les thérapies ciblant ces cytokines pourraient atténuer cette réaction ?

Rappelons tout d'abord que les LyT du donneur sont les principaux médiateurs de la GVHD, constituant la première condition nécessaire à son développement. La sévérité de la GVHDa est directement corrélée au nombre des LyT transfusés au receveur (Kernan et al., 1986). Des approches radicales telles que la déplétion des greffons en LyT ont permis d'obtenir des faibles taux d'incidence de GVHD (Horowitz et al., 1990). Cependant, elles s'accompagnent d'une plus forte probabilité de rechute, par perte de l'effet GVL, d'un risque accru de rejet de greffe et d'une fréquence plus élevée des infections post-greffe, notamment virales (Carreras et al., 2019). Pour cette raison, un ciblage plus sélectif des sous populations LyT semble être un alternatif à préconiser.

L'IL-2, une cytokine de type I produite principalement par les LyT conventionnels, est capable soit de stimuler soit d'inhiber la réponse immunitaire (Malek, 2008). D'une

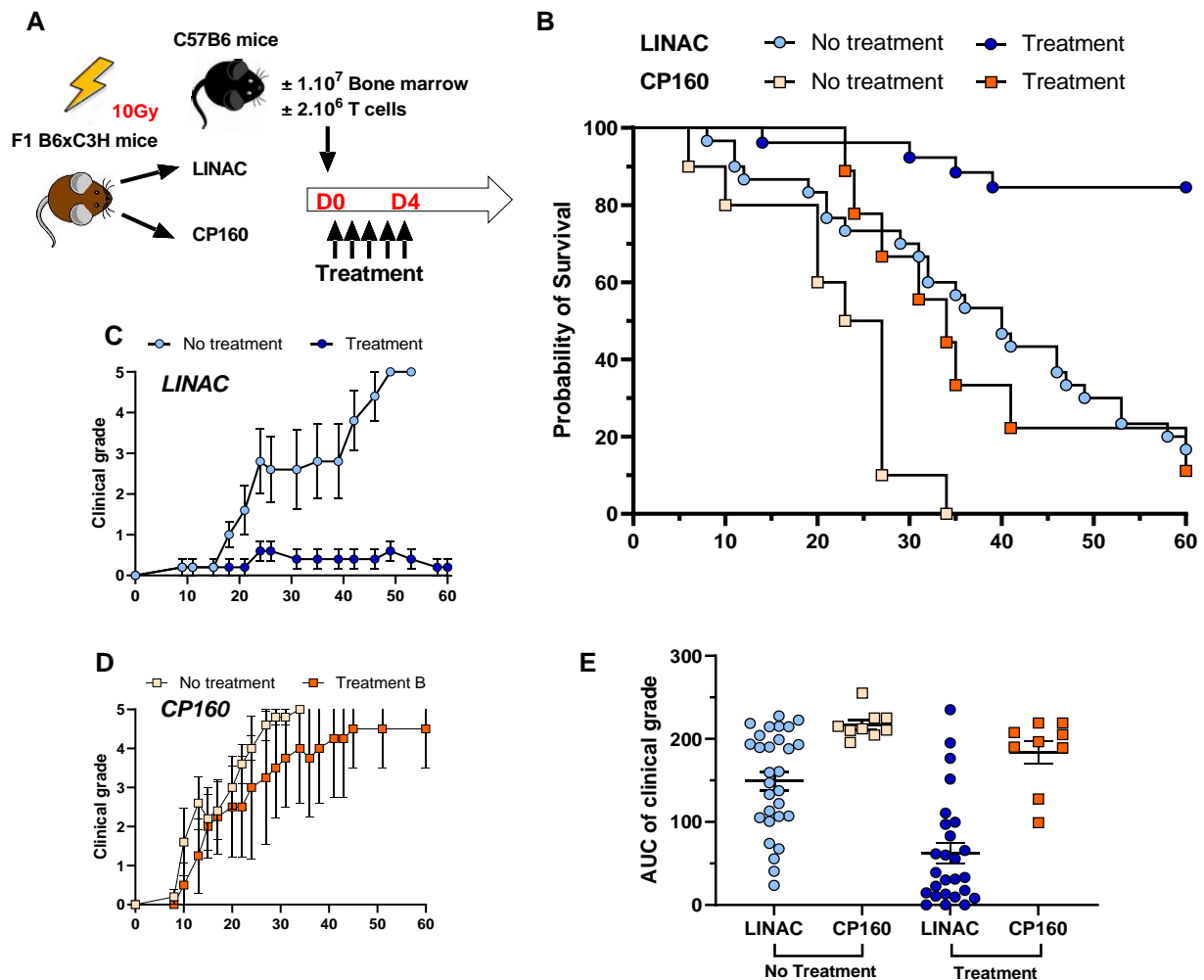


part, l'IL-2 exerce des effets immunostimulateurs en favorisant l'expansion des Teff ; d'autre part, IL-2 peut être immunosuppresseur en induisant la prolifération des Treg, un processus clé dans le maintien de l'homéostasie et de la tolérance.

La thérapie IL-2 a été utilisée en clinique à des doses élevées pour stimuler la réponse immunitaire antitumorale dans les cancer du rein et le mélanome (Rosenberg et al., 1987) et plus récemment, à des faibles doses pour bloquer la réponse immunitaire dans la GVHD (Matsuoka et al., 2013), avec dans les deux cas des effets cliniques limités et des effets secondaires non négligeables. Quand l'IL-2 est associé à un anticorps anti-IL-2, sa pharmacodynamique ainsi que ses toxicités sont améliorées, et en fonction de l'anticorps utilisé, son effet peut être dirigé soit vers des cellules effectrices soit vers des cellules régulatrices de la réponse immunitaire (Boyman et al., 2006).

L'administration du complexe JES6-mAb/IL-2 ciblant la sous-unité alpha du récepteur d'IL-2 (IL-2R $\alpha$  ou CD25) stimule sélectivement l'expansion des Tregs, tandis que le complexe S4B6-mAb/IL-2 ciblant les sous-unités beta et gamma du même récepteur (IL-2R $\beta\gamma$  ou CD122) induit principalement une expansion des LyT CD8+ ayant un phénotype mémoire (Boyman et al., 2006). L'efficacité de cette approche dans les maladies auto-immunes et la GVHD a été démontrée avec le complexe JES6-mAb/IL-2 (Heiler et al., 2018; Robb et al., 2012; Yokoyama et al., 2018). Dans notre laboratoire nous nous sommes appuyés sur le potentiel thérapeutique exceptionnel des complexes IL-2 et anticorps anti-IL-2 (mAb/IL-2) pour moduler finement et séquentiellement la réponse immunitaire dans la greffe de CSH allogénique afin de prévenir à la fois la GVHD et la rechute leucémique. Nous avons confirmé la valeur protectrice du complexe mAb/IL-2 ciblant l'IL-2R $\alpha$  (CX25) dans la prévention de la GVHD (article en préparation).

Toutefois, lorsqu'on a voulu reproduire ce résultat en utilisant l'irradiateur CP160, nous avons perdu l'effet protecteur du complexe CX25 (Figure 5).



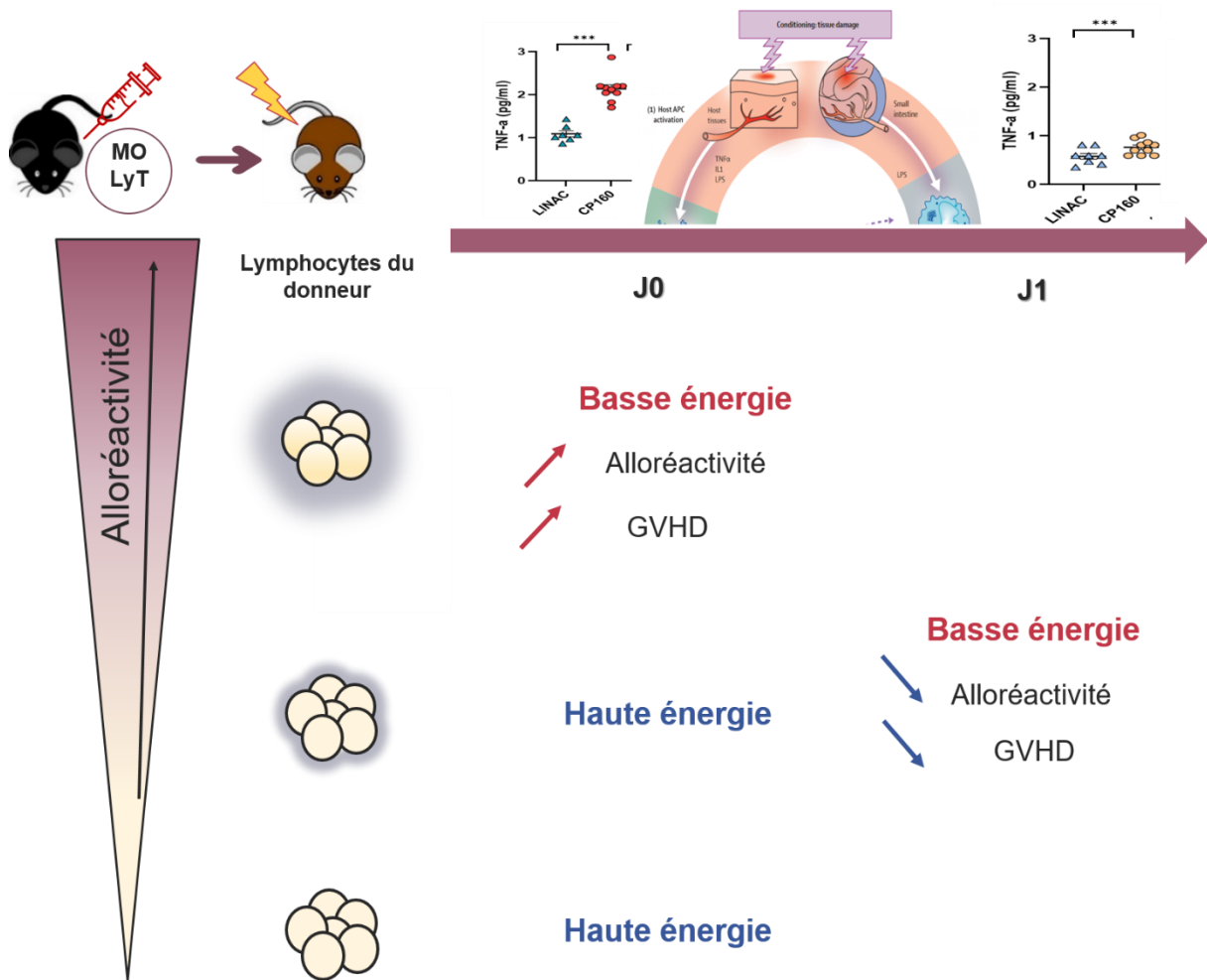
**Figure 5** : Effet du complexe Cx25 chez des souris irradiées avec le LINAC ou CP160. A : Illustration schématique de l'expérience. Les souris [B6C3]F1 ont été irradiées à dose létale à l'accélérateur linéaire LINAC à haute énergie (LINAC, pas de traitement n=30, CX25 n=26) ou à l'irradiateur CP160 à basse énergie (CP160, pas de traitement n=10, CX25 n=9) puis ont reçu une greffe semi-allogénique de MO ( $10 \times 10^6$  cellules MO +  $2 \times 10^6$  LyT). Le traitement prophylactique de la GVHD (CX25) a été injecté quotidiennement pendant 5 jours du J0 au J4. Le poids et les signes de GVHD ont été évalués 3 fois par semaine. B : La survie des souris irradiées au LINAC ou CP160, avec ou sans traitement ; les courbes de survie Kaplan-Meier ont été comparées utilisant le test log-rank. C, D : La survenue et la sévérité de la GVHDa

*chez les souris avec ou sans traitement, irradiées au LINAC ou CP160. E : AUC des scores cliniques de GVHDa en fonction du traitement et de la technique d'ICT.*

La perte d'efficacité protectrice contre la GVHDa des complexes mAb/IL-2 a été observée lorsqu'une alloréactivité est trop importante, dans un modèle « *fully allogeneic* » (McDonald-Hyman et al., 2016), ou quand les premiers signes de GVHD étaient déjà établis (Heiler et al., 2018).

Dans nos expériences, l'irradiation à basse énergie provoque une inflammation plus importante, et en conséquence une alloréactivité plus élevée après une greffe semi-allogénique de CSH. Ces résultats peuvent expliquer la perte d'efficacité des complexes CX25 dans la protection contre la GVHD.

La Figure 6 résume de façon schématique les résultats obtenus dans cette première partie évaluant des effets de l'ICT sur le système immunitaire post-greffe. Dans nos modèles murins de semi-allogreffe, les différentes méthodes d'irradiation employant différents niveaux d'énergie impactent la réponse immunitaire post-greffe. En l'occurrence, l'irradiation à basse énergie entraîne une alloréactivité plus importante après la greffe par rapport à l'irradiation à haute énergie, cette différence en termes d'alloréactivité pouvant être justifiée par le microenvironnement inflammatoire post-ICT chez la souris receveuse et par conséquent, pouvant être modulée par l'ajustement du moment de la greffe.



**Figure 6 :** Schéma récapitulatif des effets de différentes méthodes d'irradiation avec différents niveaux d'énergie sur la réponse immunitaire post-greffe.

## Perspectives

Nous avons vu maintenant qu'il y a une différence transitoire au niveau de l'inflammation post-ICT chez les souris irradiées par deux dispositifs d'irradiation distincts utilisant des niveaux d'énergie différents. Nous avons également montré que le niveau d'inflammation au moment de la greffe influençait les résultats des modèles, suite aux conséquences d'une alloréactivité proportionnelle au degré de la tempête cytokinique. En ajustant l'intervalle entre l'ICT et la greffe, nous pourrions moduler cette réaction immunitaire alloréactive.

La question chez l'homme reste posée. Les quelques études cliniques rétrospectives qui ont posé la question concernant l'intervalle entre le conditionnement incluant ICT et la HSCT (Akahoshi et al., 2015; Holter-Chakrabarty et al., 2015; Kato et al., 2014; Li et al., 2012; Motohashi et al., 2015) n'ont pas suggéré de différence en termes de survie ou de rechute leucémique. La plupart des études n'ont pas démontré un impact de la séquence entre la chimiothérapie et l'ICT dans les conditionnements (ICT avant ou après la chimiothérapie à haute dose) sur les complications post-HSCT (Akahoshi et al., 2015; Holter-Chakrabarty et al., 2015; Motohashi et al., 2015). Elargir l'intervalle entre l'ICT et la HSCT en plaçant l'ICT avant la chimiothérapie a été associé à moins de complications dans une étude (Li et al., 2012) mais a montré une incidence accrue de GVHD de grade  $\geq 2$  dans une autre (Kato et al., 2014). Aucune de ces études n'ont corrélé le taux des cytokines pro-inflammatoires au moment de la HSCT aux complications liées au traitement, laissant une question sans réponse et ouvrant une autre approche de recherche pour la prévention de la GVHDa.





## **PARTIE II : EFFETS DE L'IRRADIATION DANS UN MODELE DE TUMEUR SOLIDE DE CANCER DU SEIN LOCALEMENT AVANCE : BIOMARQUEURS IMMUNOLOGIQUES DE LA REPONSE A LA RADIOTHERAPIE NEOADJUVANTE**

Dans cette partie, nous allons nous intéresser aux effets de l'irradiation dans un modèle de cancer du sein localement avancé agressif de sous-type triple négatif (TN) ou luminal B (LB).

Nous allons présenter l'étude clinique Neo-APBI-01 qui évalue l'impact d'une radiothérapie (RT) focalisée à la tumeur mammaire, délivrée de façon séquentielle à la chimiothérapie néoadjuvante standard dans la prise en charge des cancers du sein TN et LB. La RT néoadjuvante est un vieux concept qui a actuellement un regain d'intérêt dans le cancer du sein grâce au développement technologique et à l'irradiation stéréotaxique à haute dose par fraction. La recherche des biomarqueurs prédisant la réponse est essentielle pour mieux personnaliser la prise en charge des patientes. Cette recherche translationnelle constitue le deuxième travail dans le cadre de ma thèse.

Nous allons d'abord nous rappeler brièvement les généralités des cancers du sein, de l'épidémiologie, du diagnostic à la prise en charge globale, ainsi que le rôle du système immunitaire impliqué dans sa pathologie.



## I. Généralités des cancers du sein

### 1. Épidémiologie

Le cancer du sein a dépassé le cancer du poumon pour devenir le cancer le plus diagnostiqué dans le monde en 2020, touchant 2,3 millions de femmes par an, dont environ 60.000 en France (Sung et al., 2021). Il est également l'une des principales causes de décès chez la femme dans le monde, responsable de 864.996 morts, soit un ratio mortalité/incidence de 0,3 (Łukasiewicz et al., 2021; Sung et al., 2021).

Le processus de transformation néoplasique de la glande mammaire est dirigé par plusieurs facteurs de risques divisés en deux groupes :

- Les facteurs de risques intrinsèques non-modifiables tels que l'âge, le sexe féminin, l'ethnie (femme noire) et la susceptibilité génétique (Hill et al., 2019) ;
- Les facteurs de risques extrinsèques dépendant de l'influence de l'environnement sur l'individu et donc modifiables, comme les traitements hormonaux de substitution (Narod, 2011), l'obésité, la consommation alcoolo-tabagique ou encore une mode de vie sédentaire (Guo et al., 2020; Saxe et al., 1999).

## 2. Diagnostic et Classification

### 2.1. Diagnostic

En France, 90% des cancer du sein sont asymptomatiques et sont découverts grâce au dépistage organisé (HAS, 2014). L'examen clinique et d'imagerie permettent de mieux apprécier les caractéristiques de la tumeur mammaire et son extension locorégionale et à distance. Les biopsies permettent d'obtenir un diagnostic histologique définitif.

Le type histologique permet de caractériser la tumeur en fonction du type de cellules atteintes. L'adénocarcinome est le type histologique le plus fréquent, représentant 95% des cas. Ils se développent à partir des cellules épithéliales de la glande mammaire, soit au niveau des canaux (carcinomes canaux) dans 80% des cas, soit au niveau des lobules (carcinomes lobulaires). Pour chaque type, on peut distinguer les carcinomes in situ lorsque les cellules tumorales sont localisées aux canaux ou aux lobules sans traverser la membrane basale et les carcinomes infiltrants quand la tumeur atteint les tissus conjonctifs adjacents (Tan et al., 2020). D'autres formes de carcinome mammaire ont été aussi identifiées, avec les différentes caractéristiques morphologiques, évolutives et pronostiques, telles que les carcinomes médullaires, les carcinomes mucineux, les carcinomes tubuleux, les carcinomes papillaires et bien d'autres (Tan et al., 2020).

## **2.2. Classification TNM**

En fonction de l'extension de la tumeur mammaire, on peut les distinguer selon la classification TNM ([T] pour *primary tumor*, [N] pour *regional lymph nodes*, [M] pour *distant metastases*). Ce système international de stadification est basé sur les données de pronostic dans la littérature et est révisé périodiquement en fonction de l'évolution des stratégies diagnostiques et thérapeutiques, nous en sommes actuellement à sa 8<sup>e</sup> édition (Brierley et al., 2016; Giuliano et al., 2017) (Annexe 4 : **Classification TNM de cancer du sein, 8e édition**).

Le stade TNM comprend deux catégories :

- cTNM ou le stade TNM clinique basé sur les données cliniques et d'imagerie initiales

- pTNM ou le stade TNM pathologique basé sur les données pathologiques de la pièce opératoire. Après un traitement néoadjuvant, le préfixe « yp » est fondé sur la taille du foyer résiduel le plus volumineux.

Cette classification TNM purement anatomique reste une base solide, demeurant un socle commun obligatoire et universel. L'avancée des connaissances sur la classification des tumeurs, fondée sur des caractéristiques biologiques intrinsèques a été considérée de plus en plus importante.

La valeur ajoutée des biomarqueurs (récepteurs hormonaux et HER2, un récepteur 2 du facteur de croissance épidermique humain), de la prolifération, et, de façon plus récente, des signatures moléculaires pronostiques pour affiner le pronostic et aider à la décision thérapeutique, est désormais établie (niveau de preuve I ou II). Ces biomarqueurs sont maintenant incorporés dans la classification TNM de cette 8<sup>e</sup> édition (Giuliano et al., 2017).

### **2.3. Classification moléculaire**

Au début des années 2000, sur la base d'une analyse de l'expression de complexes d'ARN-messager chez 42 individus présentant différentes pathologies mammaires malignes et bénignes, l'équipe de Stanford a proposé l'identification de quatre sous-groupes moléculaires majeures : récepteur d'estrogène positif (RE+)/*luminal-like*, *basal-like*, Erb-B2+ et *normal breast-like* (Perou et al., 2000). Des études ultérieures ont permis de diviser le groupe *luminal-like* en en deux sous-groupes : Luminal A et B (LA et LB) ; et le sous-groupe normal *breast-like* a ensuite été omis (Sørli et al., 2001). Cette classification moléculaire a été validée dans une plus grande cohorte du projet TCGA (*the Cancer Genome Atlas*) (Cancer Genome Atlas Network, 2012). Un 5<sup>e</sup> sous-type intrinsèque du cancer du sein : *claudin-low*, a été découvert en 2007 dans

une analyse intégrée des tumeurs mammaires humaines et murines (Herschkowitz et al., 2008).

En pratique clinique, la question clé est la discrimination entre les patientes qui bénéficieront ou pas d'une thérapie adjuvante après la chirurgie mammaire. Les consensus de St. Gallen de 2013 ont recommandé d'utiliser la classification des sous-types basée sur les marqueurs d'immunohistochimie (IHC) de substitution pour les décisions cliniques (Goldhirsch et al., 2013). Toutefois, il est important de rappeler que ces marqueurs d'IHC sont seulement ceux de substitution avec des taux de discordance pouvant atteindre les 30% (Prat et al., 2015). Le Tableau 2 résume les caractéristiques des différents sous-types de cancers du sein, avec les marqueurs d'IHC de substitution correspondante.

**Tableau 2** : Caractéristiques des différents sous-types moléculaires des cancers du sein.

Sous-type moléculaire	RE/ RP	HER 2	Ki67/ IM	Caractéristiques des gènes associés	%
Luminal A	+	+	Bas	Gènes activant les facteurs de transcription RO, et faible expression des gènes liés à la prolifération	50-60
Luminal B	+	-/+	Élevé	Expression élevée des gènes liés à la prolifération, faible expression de gènes de l'épithélium luminal (RP,FOXA1)	10
HER2-enrichi	-	+	Élevé	Expresse principalement des gènes et des protéines liés à la prolifération (Erb-B2/HER2, GRB7)	10-15
Basal-like	-	-	Élevé	Kératine 5, kératine 17, integrin b4, laminin, vimentin, EGFR, proliférant	15-20
Claudin-low	-	-	Élevé	Expression basse des gènes liés à l'adhésion cellulaire (claudins 3,4,7, E-cadherine etc.) et expression élevée des gènes de la EMT et de stem cell-like	7-14

% : fréquence parmi les cancers du sein, EMT : epithelial-mesenchymal transition, IM : index mitotique (avec le Ki-67, indiquent la capacité de prolifération), RO : récepteur d'œstrogène, RP : récepteur de progestérone, HER2 : human epidermal growth receptor 2, EGFR : epithelial growth factor receptor.

Outre la biologie intrinsèque des cellules tumorales, des études récentes sur le TME fournissent de nouvelles informations sur la réponse immunitaire dans le cancer du sein. De plus, certaines caractéristiques de l'immunité de l'hôte et de la tumeur semblent être associées au pronostic du cancer du sein (Denkert et al., 2010). Des niveaux élevés des lymphocytes infiltrant dans la tumeur (TILs pour *tumor-infiltrating lymphocytes*) et des signatures immunitaires de LyT auxiliaire de type 1 (Th1 pour *T helper 1*) ont été associés à l'amélioration de la survie des patients ayant un cancer du sein, en particulier chez les sous-types moléculaires agressifs tels que le TN (Savas et al., 2018; Stanton et al., 2016). De plus en plus de données ont également suggéré l'influence de la réponse immunitaire locale sur la réponse thérapeutique des CTNA, évaluées par la pCR, là encore, principalement chez les sous-types agressifs (Denkert et al., 2018). Les tumeurs lumineales sont beaucoup moins immunogènes et donc l'impact de l'immunité sur le pronostic et la réponse thérapeutique est beaucoup moins prononcé (Klopfenstein et al., 2021).

## II. Immunité et cancer du sein

### 1. Le concept d'immunosurveillance et d'« immunoediting » - Théorie des « 3E »

La théorie de l'**immunosurveillance** propose depuis plus d'un demi-siècle que le système immunitaire « pourrait réprimer une fréquence considérable des carcinomes » (Ehrlich, 1909) en agissant comme une sentinelle pour la reconnaissance et l'élimination des cellules néo-transformées (Burnet, 1970).

Cependant, face au constat qu'une tumeur est capable de se développer chez des hôtes immunocompétents, des travaux approfondis révèlent le double rôle du système immunitaire dans le processus de carcinogénèse qui, en éliminant des cellules tumorales sensibles, sélectionne positivement des variants mutés moins immunogéniques. Ce concept est appelé « **cancer immunoediting** » (Schreiber et al., 2011) et consiste en trois phases successives : l'**É**limination, l'**É**quilibre et l'**É**chappement tumoral à la veille immunitaire, d'où le nom la théorie des « **3E** » (Figure 7).

- La **phase d'élimination** englobe le concept original d'immunosurveillance, impliquant une coopération de l'immunité innée et adaptative pour détruire les tumeurs émergentes bien avant qu'elles ne deviennent cliniquement apparentes. Si la destruction des cellules tumorales est complète, l'hôte reste indemne de tumeur, le processus d'immunoediting du cancer prend fin. Toutefois, si un rare variant des cellules cancéreuses n'est pas détruit, il peut alors entrer dans la phase d'équilibre.

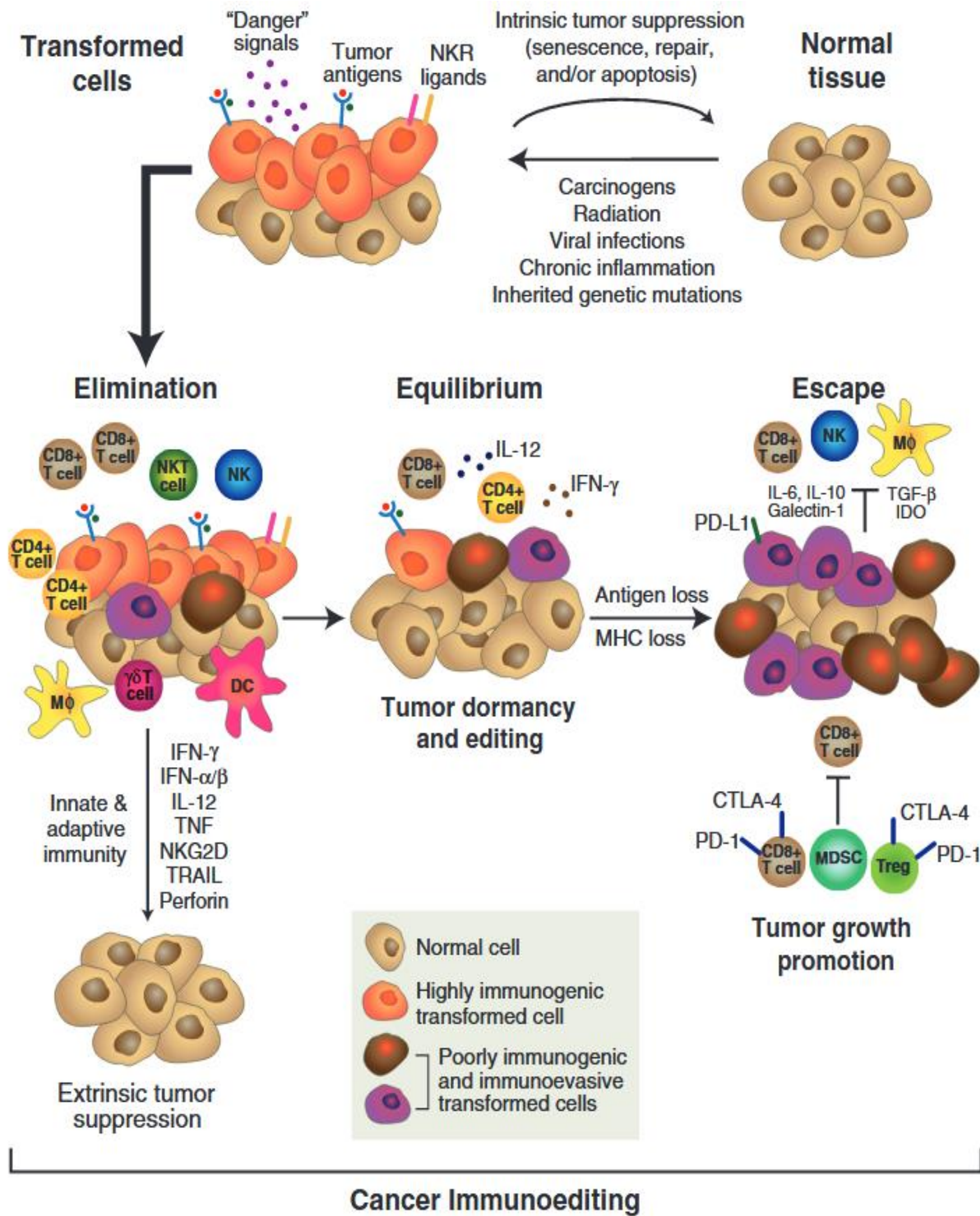


Figure 7 : Concept d'immunoediting du cancer (Schreiber et al., 2011).

- Dans la **phase d'équilibre**, la croissance des cellules tumorales est contrôlée par des mécanismes immunologiques nécessitant la participation des LyT, IL-12 et IFN-γ, avec le maintien de la tumeur en état de dormance fonctionnelle et

la sélection des clones tumoraux de moins en moins immunogènes.

- La ***phase d'échappement*** apparaît quand le système immunitaire ne parvient plus à limiter la croissance des variants tumoraux sélectionnés ayant acquis la capacité de contourner la reconnaissance immunitaire, conduisant à l'apparition d'un cancer cliniquement détectable.

## 2. Le microenvironnement immunitaire du cancer du sein

### 2.1. Le microenvironnement immunitaire de la progression du cancer du sein

Le tissu mammaire normal contient des cellules immunitaires à la fois de la lignée myéloïde (monocytes, macrophages, DC) et lymphoïde (LyT et LyB) (Degnim et al., 2014). Les cellules immunitaires sont principalement localisées dans les lobules, permettant la protection contre les pathogènes ainsi que la surveillance immunitaire et l'élimination des cellules épithéliales en processus de transformation.

Le carcinome canalaire in situ présente une augmentation de l'infiltration des cellules immunitaire, proportionnelle à l'agressivité lésionnelle (Goff and Danforth, 2021).

Le contenu en cellules immunitaires augmente progressivement du tissu mammaire normal au cancer du sein invasif (Azizi et al., 2018; Stanton et al., 2016), avec une hétérogénéité accrue des états cellulaires, probablement dues à la diversité locale des TME dans la tumeur (Azizi et al., 2018).

L'immunogénicité des cancers du sein est très hétérogène, avec différents sous-types moléculaires présentant différents degrés d'infiltration immunitaire. L'immunoprofilage à grande échelle a permis d'élucider davantage la relation entre des sous-populations immunitaires et les sous-types de cancer du sein (Ali et al., 2016; Walens et al., 2021). Les tumeurs TN sont caractérisées comme le sous-type le plus immunogène des



cancers du sein, alors que les tumeurs lumineales sont les moins immune-infiltrées (Denkert et al., 2018).

## **2.2. Les phénotypes immuns du cancer du sein**

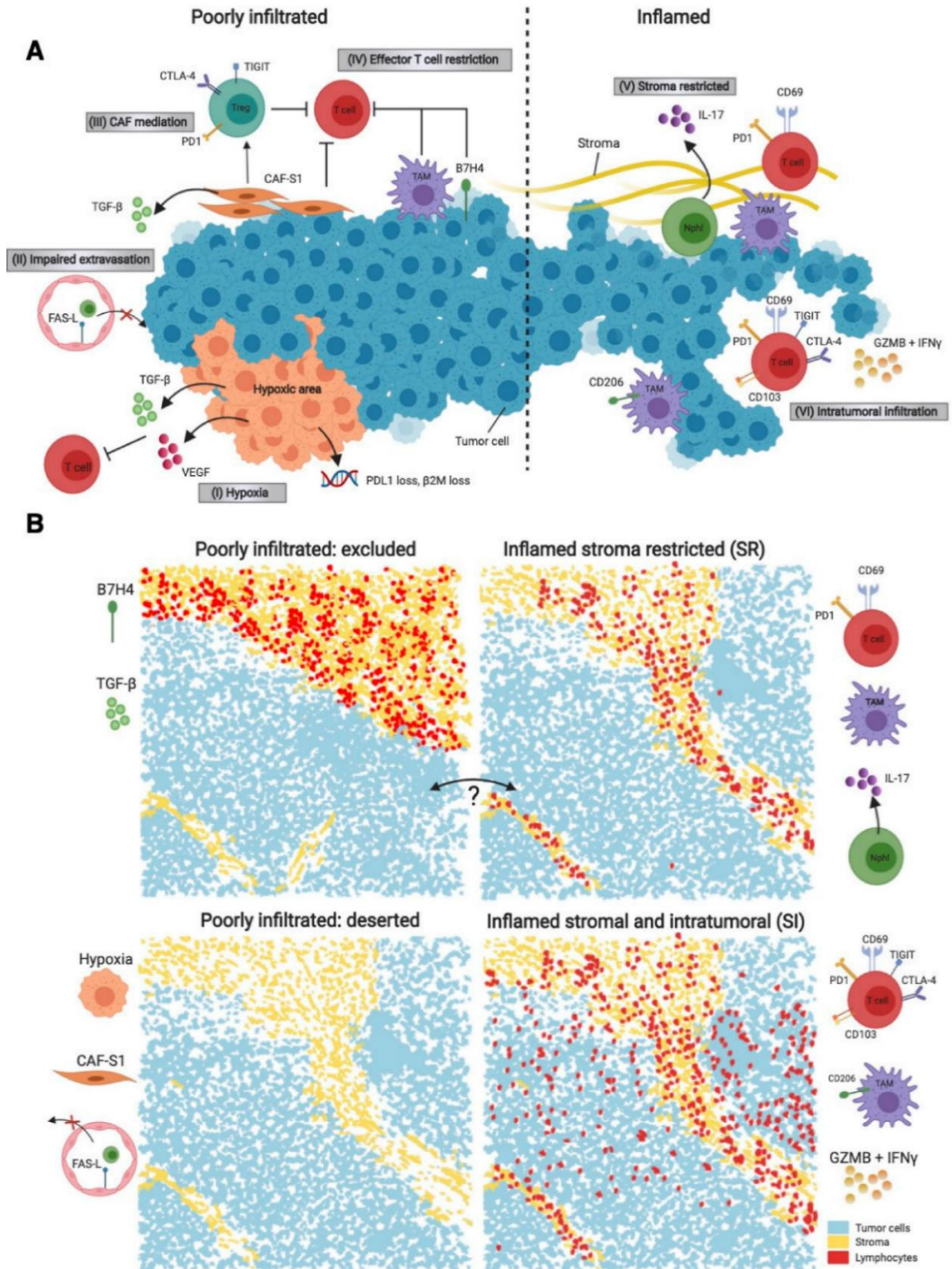
La distribution spatiale des lymphocytes dans le TME est illustrée par trois phénotypes : les tumeurs immuno-enflammées, les tumeurs immuno-exclues et les tumeurs immuno-désertées (Chen and Mellman, 2017). Bien que les cancers triple-négatifs (CSTN) aient un niveau plus élevé de TIL par rapport aux tumeurs RH+ (Denkert et al., 2018), les phénotypes peu infiltrés ne sont pas exceptionnels (Keren et al., 2018).

Les phénotypes immuno-désertés et immuno-exclus sont caractérisés par une infiltration restreinte de lymphocytes dans la tumeur :

- Les tumeurs « **immuno-exclues** » : les lymphocytes sont présents au front invasif mais ne parviennent pas à envahir le lit tumoral.
- Les tumeurs « **immuno-désertées** » : absence totale de lymphocytes au front invasif et dans le lit tumoral, liée à l'extravasation altérée de ces cellules.

Le phénotype enflammé est caractérisé par la présence de lymphocytes dans la tumeur (Chen and Mellman, 2017) et est divisé en deux sous-types : les tumeurs « **immuno-enflammées-SI** » abritent une infiltration des TIL à la fois au niveau stromal et intra-tumoral ; et les tumeurs « **immuno-enflammées-SR** » montrent une infiltration lymphocytaire qui est principalement restreinte au stroma, mais ne parvient pas au sein des tumeurs.

Un résumé des différents phénotypes immuns de cancer du sein avec leurs mécanismes et des marqueurs correspondants sont illustrées dans la Figure 8.



**Figure 8** : Phénotypes spatiaux de l'infiltration immunitaire dans le cancer du sein

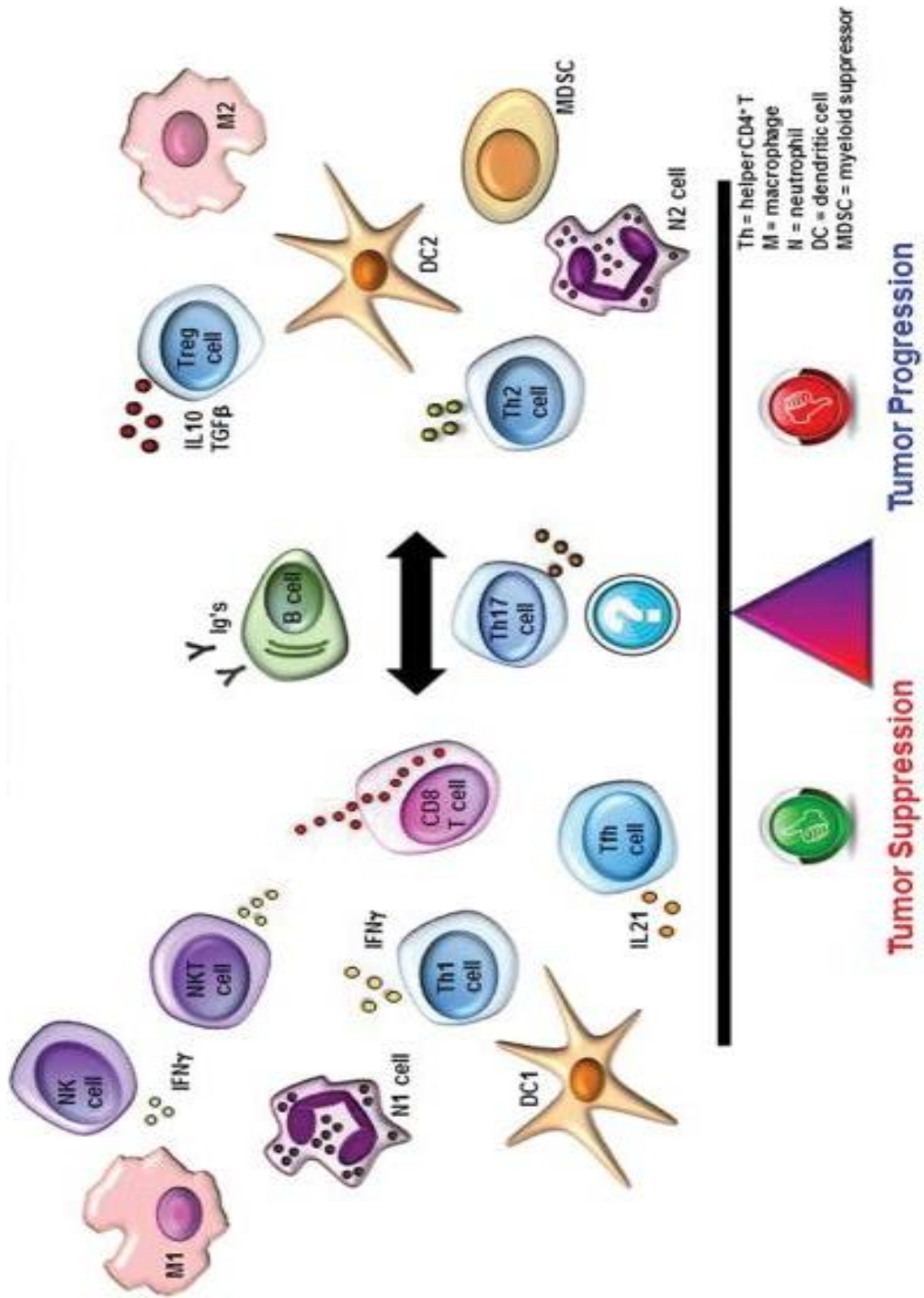
(Nederlof et al., 2021).

### 3. Les acteurs cellulaires principaux dans le microenvironnement du cancer du sein

Les cancers du sein invasifs présentent un niveau plus élevé de TILs, dont les LyT constituent la population dominante, en particulier les CD8+ avec un profil cytotoxique CTL (Azizi et al., 2018; Stanton et al., 2016). La prévalence des LyT auxiliaires évoluent au cours de la carcinogénèse : le phénotype Th1 prédomine pendant la phase d'inflammation aiguë pour limiter la croissance tumorale, puis le phénotype Th2 inhibant la cytotoxicité médiée par les LyT pendant la phase d'inflammation chronique (Kohrt et al., 2005). Les Treg CD4+FOXP3+ ont un rôle immunosuppresseur, contribuant à la réponse immunitaire pro-tumorale (Schreiber et al., 2011).

D'autres types de cellules immunitaires innées et adaptatives sont aussi infiltrés dans le TME favorisant ainsi la tumorigénèse comme les TAM avec à la fois le phénotype M2 pro-tumorigénique ou M1 anti-tumorigénique, les DC, les NK aux propriétés cytotoxiques, les LyB (Goff and Danforth, 2021; Tower et al., 2019). Les acteurs cellulaires principaux dans le TME du cancer du sein sont illustrés dans la Figure 9.

Par ailleurs, l'infiltration des sous-populations immunitaires au sein de la tumeur diffère également entre les sous-types moléculaires du cancer du sein. Les cancers du sein TN, avec une proportion plus élevée des tumeurs à prédominance lymphocytaire, possèdent également le plus haut niveau d'infiltration des LyT CD8+ et les Treg. En revanche, les tumeurs lumineuses avec très peu de tumeurs enflammées, ont un niveau d'infiltration le plus bas de ces LyT (Stanton et al., 2016). Dans une analyse récente de la base de données de TCGA, *Fan et al.* ont montré que les cancers du sein TN ont une infiltration plus élevée des LyB mémoires et des mastocytes en repos, alors que les cancers du sein lumineux sont beaucoup moins infiltrés de cellules dendritiques activées (Fan et al., 2022).



**Figure 9 :** Microenvironnement immunitaire du carcinome mammaire invasif.

Les sous-populations du système immunitaire inné et adaptatif peuvent provoquer à la fois des effets promouvant et supprimant la tumeur (Salgado et al., 2015).



### III. Les traitement néoadjuvants des cancers du sein

#### 1. Généralité et objectifs des traitements néoadjuvants du cancer du sein

Le traitement néoadjuvant (NA) est un traitement systémique et/ou locorégional administré avant le traitement principal (la chirurgie dans la plupart des cas). Dans le cancer du sein, les traitements NA sont en général des traitements systémiques (Cardoso et al., 2019; Korde et al., 2021). Plusieurs arguments justifient la proposition d'une chimiothérapie néoadjuvante (CTNA) ou d'une hormonothérapie néoadjuvante (HTNA) lors de la prise en charge initiale d'un cancer du sein :

- Sa capacité à rendre opérables les tumeurs inopérables ou à augmenter les possibilités de traitement chirurgical conservateur pour les formes localement avancées relevant d'une mastectomie première (Calais et al., 1994).
- L'évaluation *in vivo* de l'activité antitumorale des thérapies administrées, permettant d'accélérer la personnalisation du traitement anticancéreux (Wolff et al., 2008).
- La réponse complète pathologique (pCR pour *pathologic complete response*), ainsi que d'autre biomarqueurs, qui peuvent représenter des paramètres de substitution prédisant un bon pronostic (Cortazar et al., 2014). En outre, la disponibilité de tissus cancéreux avant, pendant et après le traitement permettant à des recherches translationnelles pour élucider les mécanismes de résistance et/ou de sensibilité aux agents utilisés.
- La prise en charge précoce de la maladie micro-métastatique modifie potentiellement l'évolution naturelle de la tumeur en prévenant l'émergence de clones résistants.

## 2. Les traitements systémiques néoadjuvants

### 2.1. La chimiothérapie néoadjuvante

- Les essais des années 1990 ont démontré la non-infériorité de la CTNA par rapport à la chimiothérapie adjuvante en termes d'efficacité antitumorale et de survie, sachant que les protocoles de chimiothérapie n'incluaient pas d'anthracycline (Mauri et al., 2005).
- Dans les années 2000, l'ajout de taxane à la CTNA standard à base de doxorubicine et cyclophosphamide (AC) a amélioré le taux de pCR (26% vs 13%,  $p < 0,001$ ), sans avoir d'impact sur la survie sans maladie (DFS pour *disease-free survival*) et la survie globale (OS pour *overall survival*) (Cuppone et al., 2008; Rastogi et al., 2008).
- L'ajout d'autres agents de chimiothérapie n'a pas montré d'efficacité, que ce soit de la capécitabine (Li et al., 2013) ou de la gemcitabine (Earl et al., 2014).

### 2.2. L'hormonothérapie néoadjuvante

Historiquement proposée chez les femmes âgées ayant un cancer du sein avancé (Horobin et al., 1991), l'hormonothérapie première avait permis avec une certaine efficacité d'obtenir une rémission de la maladie et de longues survies. Les inhibiteurs d'aromatase (AI pour *aromatase inhibitor*) sont les traitements de choix, avec une supériorité démontrée au tamoxifène en termes de taux de réponse objective et de chirurgie mammaire conservatrice (Seo et al., 2009). Ils sont recommandés dans la prise en charge des femmes ménopausées atteintes d'un cancer du sein RH+HER2- (Cardoso et al., 2019; Korde et al., 2021).

### **2.3. Traitement néoadjuvant par blocage de HER2**

L'amplification du gène HER2 et la surexpression de sa protéine sont observées dans environ 20% des cas de cancer du sein et sont associées à une maladie agressive (Slamon et al., 1987). Plusieurs thérapies ciblées ont été développées et ont montré leur efficacité chez les patientes HER2+ par rapport à la chimiothérapie standard chez les patientes métastatiques :

- Simple blocage de HER2 : Trastuzumab, Lapatinib
- Double blocage de HER2 : Trastuzumab-Pertuzumab, Trastuzumab-Lapatinib
- Blocage de HER2 conjugué : Trastuzumab-Emtansine, Trastuzumab-Derexotecan.

En situation néoadjuvante, le simple blocage de HER2 par Trastuzumab ou le double blocage de HER2 par Trastuzumab-Pertuzumab associés à la chimiothérapie à base de taxane est le traitement standard, offrant des taux de pCR allant de 38% à 66% (Gianni et al., 2012, 2010) et une meilleure DFS, notamment celles ayant obtenu la pCR.

### **2.4. Immunothérapie**

Le CSTN, qui se caractérise par une charge mutationnelle tumorale relativement élevée, est associé à une sensibilité accrue aux ICIs (O'Meara and Tolaney, 2021). Les agents anti-PD-1/PD-L1 ont montré leur efficacité en termes de survie et ont été approuvés pour les CSTN au stade avancé (Cortes et al., 2020; Schmid et al., 2018).

En situation néoadjuvante, trois des cinq essais randomisés ont montré une amélioration du taux de pCR (Tableau 3), associée à un bénéfice de survie.

**Tableau 3 :** Principaux essais randomisés de chimio-immunothérapie néoadjuvante dans le cancer du sein triple-négatif de stade précoce, modifié de (Tarantino et al., 2022)

Étude	n	Régime	pCR (%)	Survie
KEYNOTE-522	1174	TCb→AC±pembro (1 an)	64.8% vs 51.2%	3y-EFS 84.5% vs 76.8% 3y-OS 89,7% vs 86,9%
IMpassion031	333	nabT→AC±atezo (1 an)	58% vs 41%	DFS : HR = 0,76 OS : HR = 0,69
NeoTRIPaPDL1	280	nabTCb±atezo + CTA	43,5% vs 40,8% (ns)	En attente
GeparNuevo	174	nabT→AC±durva	53,4% vs 44,2% (ns)	3y-DFS 84,9% vs 76,9% 3y-OS 95,1% vs 83,1%
I-SPY2	107	T→AC ± pembro	60% vs 22%	EFS : HR = 0,6

AC : anthracycline et cyclophosphamide, atezo : atezolizumab, CTA : chimiothérapie adjuvante, Cb : carboplatine, DFS : survie sans maladie, durva : durvalumab, EFS : survie sans événements, HR : hazard ratio, nabT : nab-paclitaxel, OS : survie globale, pembro : pembrolizumab, pCR : réponse pathologique complète, T : taxane (paclitaxel ou docetaxel).

Suite à des résultats positifs en termes de pCR et de la survie sans évènement (EFS pour *event-free survival*) dans l'essai KEYNOTE-522 (Schmid et al., 2022), la haute autorité de santé française, a autorisé le 17/03/2022 l'accès précoce de pembrolizumab, en l'associant à une chimiothérapie dans le traitement NA des patientes atteintes d'un CSTN localement avancé (HAS, 2022), seulement un mois après sa parution dans le journal NEJM.

### 3. La radiothérapie néoadjuvante dans le cancer du sein

La RT des cancers du sein a beaucoup évolué durant les dernières décennies. Alors que son indication en situation adjuvante sur la totalité du sein était incontournable après une chirurgie conservatrice, de nouveaux concepts sont apparus et ont bousculé sa pratique généralisée. Ainsi, l'hypofractionnement de l'irradiation, la focalisation de



l'irradiation au seul lit opératoire et la sélection des malades pour une omission de la RT après une chirurgie conservatrice adéquate, sont devenus des options possibles chez les patientes sélectionnées selon le rapport entre le bénéfice et le risque.

Par ailleurs, l'autre séquence qui a bénéficié d'une évolution ces dernières années est la RT avant la chirurgie d'exérèse, dite radiothérapie néoadjuvante (RTNA), dans le but de booster le système immunitaire antitumoral contre la tumeur mammaire intacte.

Historiquement, la RT première du sein en totalité, seule ou associée à la chimiothérapie, a été surtout proposée chez les patientes non éligibles à la chirurgie mammaire conservatrice d'emblée (Corradini et al., 2019). Les résultats en termes de contrôle et de survie sont modestes au prix d'une augmentation significative des toxicités aiguës et tardives, surtout avec le traitement combiné. Le taux de pCR, qui variait entre 10 et 34% (Adams et al., 2010; Brackstone et al., 2017; Calitchi et al., 2001; Matuschek et al., 2012a), est en faveur des tumeurs RH-.

La RT préopératoire des cancers du sein localisés est devenue un nouveau terrain d'investigation grâce à la maîtrise technique et au concept de l'irradiation mammaire partielle et accélérée (APBI pour *accelerated partial breast irradiation*). L'irradiation préopératoire a une plus grande précision et permet une chirurgie emportant le tissu irradié à haute dose pouvant ainsi limiter le risque de fibrose et la détérioration cosmétique (van der Leij et al., 2015). Au plan carcinologique, l'objectif d'augmenter la pCR dans certains sous types moléculaires, comme les CSTN et HER2+ s'est renforcé au vu des résultats plus significatifs en terme de survie. La pCR est dans ces cas particuliers un vrai « *surrogate marker* » d'une meilleure survie (Broglia et al., 2016; Cortazar et al., 2014).

Au plan biologique, les études translationnelles ont montré une réponse dose-dépendante de l'irradiation, impliquant l'expression de p53, du cycle cellulaire et de la réparation d'ADN à des doses de 4-6 Gy par fraction (Bosma et al., 2020a) voire modulation de l'immunité et de l'inflammation à des doses plus élevées à 18-21 Gy (Horton et al., 2015). Dans l'étude PAPBI, la RTNA a été évaluée chez les patientes RH+, le taux de quasi-pCR était de 7.8% (Bosma et al., 2020a, 2020b).

Une seule étude évaluant le concept d'APBI associée à la chimiothérapie a été publiée (Bondiau et al., 2013). Il s'agit d'une étude d'escalade de dose avec plusieurs paliers allant de 19,5 Gy à 31,5 Gy en trois fractions, délivrée par une technique de haute précision SBRT par CyberKnife®. Le taux de pCR était de 36% dans l'ensemble de la cohorte et 67% pour le palier 3 (25,5 Gy en 3 fractions) (Bondiau et al., 2013). Tous sous-types de cancer du sein ont été inclus et aucune évaluation à long terme n'a été réalisée.

D'autres études se sont intéressées à la combinaison RT-ICI ± CTNA/HNA chez les patientes atteintes d'un cancer de type LB ou CSTN. Actuellement, il y a cinq essais enregistrés dans clinicaltrials.gov. Parmi ces 5 essais, la dose d'APBI la plus utilisée est de 24 Gy en 3 fractions délivrée par la technique SBRT, la RT conformationnelle avec ou sans modulation d'intensité ou encore la proton-thérapie. Le traitement systémique NA inclut des ICI, avec ou sans un traitement systémique standard de type HTNA ou CTNA (Ho et al., 2020b).

#### **4. La réponse pathologique complète en tant que critère de jugement de substitution**

Le traitement NA offre une opportunité unique d'évaluation rapide de l'efficacité des médicaments anticancéreux d'une tumeur en place, avec certains critères d'évaluation à court terme étant en corrélation aux résultats cliniques à long terme. Le résidu

tumoral après CTNA par exemple, nous informe sur l'agressivité de la maladie, sa chimio-sensibilité et donc, logiquement, son pronostic (Luen et al., 2019). Au contraire, la pCR se présente comme un potentiel marqueur prédictif de la survie à long terme. Rappelons que dans la plupart des premières études étudiant l'intérêt de la CTNA, le bénéfice en termes de survie n'a pas été démontré. Cependant, les patientes ayant obtenu une pCR avaient aussi une meilleure DFS et OS (Rastogi et al., 2008).

Afin de confirmer ces résultats prometteurs et d'encourager l'approbation accélérée de nouveaux agents anticancéreux pour les patientes atteintes de cancer du sein à haut risque (FDA, 2012), des méta-analyses à grande échelle des essais randomisés étudiant la CTNA ont été menées (Broglia et al., 2016; Cortazar et al., 2014). La pCR définie comme ypT0/Tis ypN0 était fortement associée à des taux supérieurs de l'EFS et OS.

L'efficacité des traitements NA varie entre les sous-groupes de patientes, influencée par le statut des RH et HER2. Des taux élevés de pCR en réponse à la CTNA sont plus souvent observés chez les patientes RH- (Broglia et al., 2016; Cortazar et al., 2014). De plus, la corrélation entre la pCR et une meilleure survie est plus prononcée dans les sous-types plus agressifs de cancer du sein comparée au sous-groupe LA (Cortazar et al., 2014).

Dans l'ensemble, ces données suggèrent que la pCR après une CTNA est associée à une amélioration de la DFS, notamment dans les cancers du sein plus agressifs. Il est donc essentiel d'identifier des biomarqueurs efficaces pour prédire la pCR et concevoir des essais cliniques pour cibler ces nouveaux biomarqueurs afin d'améliorer davantage la pCR et la survie à long terme.

## **IV. Les facteurs pronostiques et prédictifs des cancers du sein**

Le domaine de biomarqueurs en oncologie évolue rapidement, porté par une meilleure compréhension de la biologie tumorale et la connaissance du génome humain. Cependant, seule une petite proportion de marqueurs s'est finalement avérée cliniquement utile. Par définition, un facteur pronostique fournit des informations sur les résultats cliniques au moment du diagnostic, indépendamment de la thérapie. Un facteur prédictif renseigne sur la probabilité de réponse à une modalité thérapeutique donnée. Bien qu'ils puissent être classés séparément, plusieurs facteurs d'au cancer du sein sont à la fois pronostiques et prédictifs (Febbo et al., 2011).

### **1. Les facteurs prédictifs**

Les facteurs qui peuvent aider à la personnalisation des traitements adjuvants sont les plus importants car ils permettraient idéalement de sélectionner les patientes pour une thérapie tout en épargnant aux autres des traitements potentiellement inefficaces mais toxiques. L'identification de ces marqueurs prédictifs cliniquement utiles est malheureusement moins probante que celle des marqueurs pronostiques. Les marqueurs prédictifs les mieux établis dans le cancer du sein non-métastatique à ce jour sont le récepteur d'œstrogène (RO) (pour l'hormonothérapie) et le HER2 (pour la thérapie anti-HER2). Les signatures génomiques peuvent aussi participer dans le choix de la chimiothérapie adjuvante (CTA).

## 2. Les facteurs pronostiques utilisés en routine clinique

### 2.1. Les facteurs liés à la patiente

- L'âge jeune de moins de 35-40 ans est souvent associé à un pronostic plus sombre, notamment chez les sous-types luminaux (Adami et al., 1986; Fredholm et al., 2009; Partridge et al., 2016). À l'inverse, les femmes âgées de plus de 65-75 ans sont aussi exposées à plus de risque de décès global et lié au cancer (van de Water et al., 2012).
- Les patientes ménopausées semblent d'avoir un meilleur résultat (Swain et al., 2010).
- Les disparités raciales peuvent être dues en partie à des raisons socio-économiques (Silber et al., 2013), mais également liées à une biologie tumorale plus agressive chez les Afro-Américaines (Carey et al., 2006; Keenan et al., 2015).
- La consommation tabagique est associée à un risque accru de décès lié au cancer (Passarelli et al., 2016).

### 2.2. Les facteurs liés à la tumeur

- Le stade tumoral ou le stade TNM anatomique fournit un outil standardisé pour la prise de décision thérapeutique, mais également des informations pronostiques. Dans le cancer du sein, le taux de OS à 5 ans est de plus de 90% pour les stades I et seulement 50% pour le stade IIIC (Brierley et al., 2016; Zhang et al., 2019).
- Type histologique : les CCI sont les plus fréquents (70%) ayant plus de risque de récurrence que les CLI dans les 6-10 premières années après le traitement du cancer (Pestalozzi et al., 2008).

- Grade histo-pronostique SBR-EE : plus le grade est élevé, moins bon est le pronostic (Elston and Ellis, 1991; Rakha et al., 2008).
- Envahissement lympho-vasculaire (LVI) péri-tumoral : il semble être un indicateur de mauvais pronostic, en particulier dans les tumeurs de haut grade (Pinder et al., 1994).
- Des niveaux élevés de Ki-67 : des niveaux élevés sont associés à un risque plus élevé de récurrence et une moins bonne survie (de Azambuja et al., 2007; Stuart-Harris et al., 2008).

### **2.3. Les récepteurs hormonaux et HER2**

L'expression des récepteurs hormonaux est généralement associée à de meilleurs résultats carcinologiques, du moins au cours des 5 premières années suivant les traitements du cancer. Néanmoins, après 5 ans, ces patientes présentent un risque de récurrence annuelle plus élevé et qui continue constamment même au-delà de 20 ans (Colleoni et al., 2016; Colzani et al., 2011). Les tumeurs RO+ se propagent principalement dans le os, alors que les tumeurs RO- développe des métastases viscérales, associées ainsi à une survie plus courte (Insa et al., 1999).

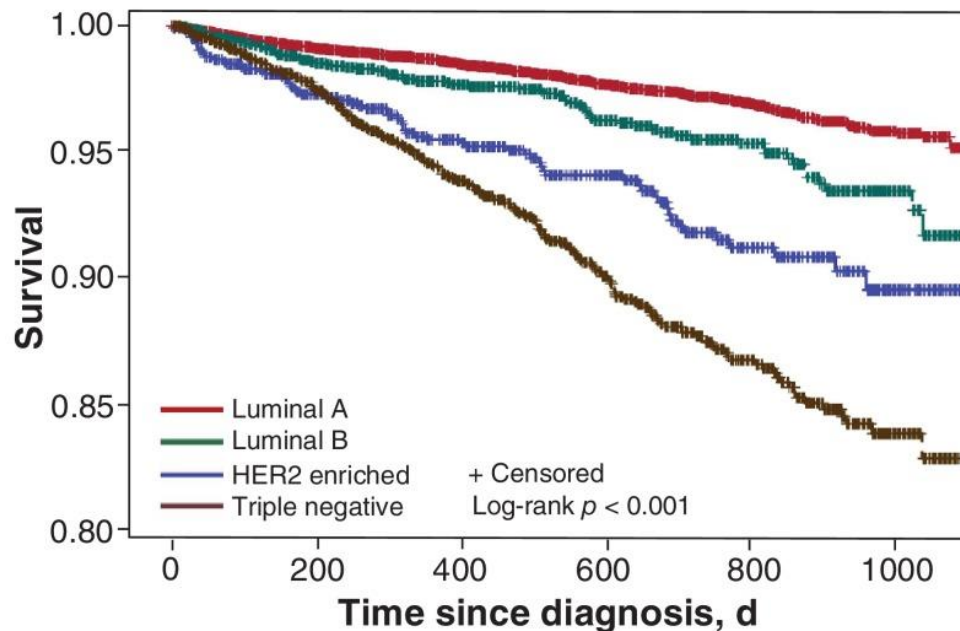
La surexpression de HER2 représente un pronostic défavorable, en particulier si les patientes ne sont pas traitées par chimiothérapie et des thérapies anti-HER2 (Gusterson et al., 1992), même à des stades très précoces des tumeurs de moins de 1 cm sans atteinte ganglionnaire (Jaraj et al., 2020).

### 3. Autres facteurs pronostiques

#### 3.1. Sous-types moléculaires

L'hétérogénéité cellulaire et moléculaire du cancer du sein souligne l'importance des études génomiques à haut débit. En dépit de l'hétérogénéité de la maladie, l'analyse sur presque 30.000 femmes ayant eu un cancer du sein a permis de déceler que le LA était le sous-type le plus fréquemment diagnostiqué (59%) avec la plus grande survie, tandis que le sous-type *basal-like* (CSTN) avait la survie la plus faible (Fallahpour et al., 2017) (Figure 10).

Le risque métastatique dépend aussi des sous-types moléculaires : les LB ont plus de risque de métastases osseuses ; alors que les HER2-enrichi et les CSTN font plus de métastases viscérales mais moins de métastases osseuses que les tumeurs RH+ (Xiao et al., 2018).



**Figure 10** : Survie globale par sous-type moléculaire (Fallahpour et al., 2017).

### 3.2. Signatures multigéniques

Depuis la découverte des sous-types moléculaires du sein par *Perou et al.*, plusieurs puces à ADN (*microarrays*) ont été développées et commercialisées comme outils fiables pronostiques et prédictifs en pratique clinique. Malgré la différence dans la quantité et la spécificité des gènes testés dans chaque plate-forme, il y a étonnamment une forte corrélation parmi les tests génomiques (Oncotype DX, PAM50, MammaPrint et EndoPredict) évaluant très peu de gènes en commun (Fan et al., 2011, 2006). Il est important de souligner que ces signatures multigéniques classent à haut risque presque toutes les patientes atteintes d'un cancer RH-. En revanche, chez les patientes avec un cancer RO+ qui constituent un groupe plus difficile à catégoriser en se basant uniquement sur les critères cliniques et pathologiques conventionnels, ces tests sont plus intéressants et utiles (Wesolowski and Ramaswamy, 2018). La valeur pronostique et décisionnelle thérapeutique ajoutée de ces critères « biologiques intrinsèques » a permis leur intégration dans la dernière classification de TNM dans le cancer du sein (Giuliano et al., 2017).

## 4. Les facteurs immunologiques

Nous avons vu dans la section « immunité et cancer du sein » l'implication du système immunitaire dans le développement de cancer du sein. Un regain d'intérêt de l'immunité a encouragé des recherches à grande échelle s'intéressant aux valeurs pronostiques des facteurs immunologiques dans cette maladie.

### 4.1. Les lymphocytes infiltrants dans la tumeur (TILs)

Les TILs sont principalement composés de LyT, mais peuvent également inclure des LyB, NK, DC et TAM (Keren et al., 2018; Savas et al., 2018). Pour l'utilisation clinique,



seuls les cellules mononucléaires (lymphocytes et plasmocytes) sont évaluées (Salgado et al., 2015).

Dans une analyse groupée de 2 essais randomisés allemands de CTNA, il a été démontré que les tumeurs inflammatoires à prédominance lymphocytaires LPBC (*lymphocyte-predominant breast cancer*) (définie par un taux de TILs > 60%), présentait le meilleur taux de pCR à 42% (par rapport à 3% pour les tumeurs sans infiltrat lymphocytaire), et une augmentation de TILs post-traitement NA a été corrélée à un pronostic plus favorable (Denkert et al., 2010). D'autres observations ont aussi confirmé la valeur pronostique de TILs en situation adjuvante (Loi et al., 2013) et métastatique (Luen et al., 2017).

Le phénotype LPBC a été identifié plus fréquemment dans les tumeurs CSTN (20%) et HER2-enrichies (16%) que dans les tumeurs luminales (6%) (Stanton et al., 2016). Bien que la valeur prédictive de la pCR et la valeur pronostique des TIL aient été confirmées dans les tumeurs TN et HER2+ ; le phénotype LPBC dans les tumeurs luminales RH+ avait un meilleur taux de pCR, mais était associé à une survie plus courte, suggérant une biologie différente de l'infiltrat immunitaire (Denkert et al., 2018).

La distribution spatiale des lymphocytes a aussi un impact sur le pronostic des patientes. Chez les patientes CSTN ayant reçu une CTNA, les TILs dans le stroma (sTILs) en nombre élevé étaient associés à un meilleur pronostic, chaque 10% d'augmentation de sTILs réduisait 14% le risque de récurrence ou de décès (Adams et al., 2014). D'autres études ont montré la valeur pronostique négative des Treg FOXP3+ intra-tumoraux (iTILs) (Liu et al., 2011) et la valeur pronostique positive des iTILs CTL CD8+ (Ali et al., 2014; Egelston et al., 2019).

#### 4.2. Les sous-populations des TILs

**Les lymphocytes T** constitue la sous-population dominante des TILs (Azizi et al., 2018), notamment les LyT CD8+ présentant les caractéristiques de cellules mémoires résidentes (CD63+CD103+) avec un profil cytotoxique (Savas et al., 2018). Les données robustes sur la valeur pronostique et prédictive des TIL-LyT proviennent notamment des études s'intéressant au sous-type TN. Un nombre total élevé des TILs CD8+ et CD4+ a été associé à une meilleure survie chez les patientes CSTN ayant été traitées par la CTA (Liu et al., 2012; Schmidt et al., 2018). En situation NA, des niveaux élevés de TILs CD3+, CD8+, CD4+, ainsi qu'un ratio élevé de CD8+/FOXP3+ ont été positivement associés à la pCR et au pronostic des patientes (Seo et al., 2013; West et al., 2011). Dans les cancers luminaux HER2-, les résultats sont moins consistants. Alors qu'en situation adjuvante, les CTL CD8+ étaient négativement corrélés à la survie (Criscitiello et al., 2020), des niveaux élevés de TIL-CD8+ et du ratio CD8+/FOPX3+ ont été associés à la pCR (Goto et al., 2018). En revanche, la présence des TILs CD4+FOPX3+ a été constamment associée à un mauvais pronostic, surtout en l'absence d'infiltration de TILs CD8+ (Liu et al., 2014).

**Les lymphocytes B** ne se retrouvent qu'à des niveaux mineurs dans la tumeur (Chung et al., 2017). Des données cliniques solides des LyB sont encore manquantes, mais quelques évidences préliminaires ont suggéré une corrélation entre la présence des TILs LyB et une meilleure survie dans les tumeurs TN/*basal-like* (Iglesia et al., 2014) ou un taux de pCR plus élevé après un traitement NA par anti-HER2 sans chimiothérapie (De Angelis et al., 2020).

**Les macrophages** sont parmi les premières cellules immunitaires à confronter aux cellules tumorales transformées (Ruffell et al., 2012). Ils représentent une population hautement hétérogène de cellules immunitaires mais peuvent être dichotomisés en 2

phénotypes polarisés, M1 anti-tumorigénique et M2 pro-tumorigénique (Azizi et al., 2018). L'infiltration des TAM dans le TME du cancer du sein est généralement associée à des caractéristiques clinico-pathologiques défavorables (Tiainen et al., 2015), mais leur valeur pronostique et prédictive reste inconsistante et non concluante (Dieci et al., 2021).

**Les cellules NK** sont des membres du système immunitaire inné, représentant un médiateur crucial de l'étape d'élimination dans le processus *d'immunoediting* du cancer et de l'immunosurveillance (Schreiber et al., 2011). Quelques données préliminaires des petites cohortes de patientes ayant un cancer du sein ont suggéré leur impact positif sur la survie sans récurrence (Ascierto et al., 2013) et la pCR (Verma et al., 2015).

#### 4.3. PD-1 et PD-L1

Le PD-1 et l'un de ses ligands (PD-L1) font partie du processus du point de contrôle limitant la réponse immunitaire des LyT (Dong et al., 2002). L'expression de ces molécules a été corrélée à un taux de pCR plus élevé et à un meilleur pronostic dans le cancer du sein (Pelekanou et al., 2018; Zerdas et al., 2020).

La valeur prédictive à la réponse à l'immunothérapie de PD-L1 dans le cancer du sein est moins robuste par rapport aux autres histologies comme le cancer du poumon ou le mélanome, surtout en cas d'une monothérapie par pembrolizumab (Adams et al., 2019; Chung et al., 2020; Schmid et al., 2020).

#### 4.4. Statut immunitaire systémique

Les réponses inflammatoires et immunitaires de l'hôte dans la tumeur et son TME sont des composants critiques dans le développement et la progression du cancer. La

réponse inflammatoire systémique induite par la tumeur entraîne des altérations des globules blancs du sang périphérique (Batalha et al., 2021). L'utilisation de biomarqueurs immunitaires du sang périphériques pourrait servir de méthode de *monitoring* plus accessible, plus rapide et moins coûteuse. Des études récentes ont rapporté la valeur prédictive et/ou pronostique des indices dérivés des cellules immunitaires périphériques dans différents cancers dont le cancer du sein, tels que :

- Le nombre absolu des lymphocytes du sang périphérique (ALC pour *absolute lymphocyte count*) est le moyen le plus précoce et le plus simple d'évaluer la capacité immunitaire d'un patient. Plusieurs études ont montré avec constance qu'une valeur initiale élevée d'ALC est un indicateur d'un meilleur pronostic (Batalha et al., 2021; Lee et al., 2018; Papatestas and Kark, 1974) et d'un taux accru de pCR (Qian et al., 2018).
- Le rapport entre neutrophiles et lymphocytes (NLR pour *neutrophil-to-lymphocyte ratio*) : des taux de NLR plus élevés correspondaient à une probabilité de 4 à 6 fois plus élevée de décès à 5 ans (Azab et al., 2013, 2012), alors qu'un NLR bas a été associé à une pCR plus élevée (Zhou et al., 2021). Un taux élevé de NLR est souvent associé à d'autres indicateurs de mauvais pronostic comme le processus métastatique, RH-, et HER2+ (Koh et al., 2015).
- Le rapport entre lymphocytes et monocytes (LMR pour *lymphocyte-to-monocyte ratio*) : le LMR est proportionnellement corrélé à la survie, notamment chez les CSTN (Goto et al., 2018; Hu et al., 2018; Marín Hernández et al., 2018). Des valeurs élevées de LMR ont été trouvées chez les patientes ayant une maladie moins avancée (He et al., 2016), RH+ (Ni et al., 2014), et une infiltration TILs CD8+ plus élevée (Lee et al., 2018).

- Le rapport entre plaquettes et lymphocytes (PLR pour *platelet-to-lymphocyte ratio*): Le développement du cancer s'accompagne souvent d'une thrombocytose, et les plaquettes elles-mêmes peuvent également affecter la tumorigénèse, l'angiogénèse et la croissance tumorale (Jiang et al., 2017). Un PLR élevé indique en général un moins bon pronostic (Zhang et al., 2017).
- L'indice d'inflammation immunitaire systémique (SII pour *systemic immune-inflammation index*), établie par le nombre absolu (ou le pourcentage) des neutrophiles x plaquettes / lymphocytes. Le SII est corrélé négativement à la survie chez les patientes avec un cancer du sein HER2+ (Sun et al., 2019) et les patientes ayant reçu une CTNA (Jiang et al., 2020), avec une utilité pronostique supérieure à celle du NLR et PLR.

La nécessité de développer des tests simples et non invasifs est un réel besoin dans la prise en charge initiale, le « *monitoring* » et l'adaptation du traitement du cancer du sein. Ainsi, des biomarqueurs pronostiques et prédictifs dérivés de l'immunité obtenus à partir du sang périphérique permettraient une prise de décision plus rapide, moins chère et plus facilement individualisée. Malgré un nombre croissant de publications des dernières années, des incertitudes sur la relation entre le système immunitaire périphérique et le cancer du sein persistent, en raison des biais de sélection, de l'absence de standardisation et l'inclusion de populations de malades hétérogènes dans les essais. Une évaluation de ces biomarqueurs en fonction de sous-types moléculaires du cancer du sein est aussi nécessaire, compte tenu des profils immunogéniques de chacun de ces sous-types. L'identification de biomarqueurs immunitaires périphériques cliniquement pertinents stimulerait le développement de modèles d'interaction du cycle cancer-immunité, ouvrant ainsi des nouvelles approches thérapeutiques potentielles.

## V. Étude Néo-APBI-01

Nous avons vu, dans les précédentes sections, les potentiels concernant l'effet synergique entre la RT et la CTNA, les avancements techniques de la RT et le concept de la RT partielle de la glande mammaire permettant une meilleure administration de cette dernière en limitant les toxicités surajoutées du traitement combiné. Avec tous ces arguments, les équipes des Centres Hospitaliers Universitaires de Grenoble et d'Henri Mondor ont mené un essai clinique prospectif évaluant l'intérêt d'une telle association en situation néoadjuvante dans certains sous-types de cancer du sein agressif : il s'agit de l'étude Néo-APBI-01.

### Objectifs

L'étude Néo-APBI-01 (NCT02806258) est un essai de phase 2, multicentrique, randomisé, comparant un traitement NA séquentiel incluant une CTNA et une APBI versus une CTNA seule, chez les patientes ayant un cancer du sein localement avancé de sous-type TN et LB proliférant.

Le but de l'étude est d'évaluer la RTNA dans une population de patientes sélectionnées sur les critères biologiques. L'objectif principal est d'obtenir une augmentation des taux de pCR par l'adjonction de la RT en cours de la CTNA. Il est attendu une différence de 15% de pCR en faveur du bras expérimental comportant l'APBI, avec possiblement un impact sur la survie.

Le choix des sous-types TN et LB a été fait pour cibler les sous-types moléculaires agressifs avec un taux de Ki67 élevé dont l'obtention de la pCR après CTNA est plus

favorable et associée à une amélioration de la survie par rapport au sous-types plus indolents (Adams et al., 2010; Cortazar et al., 2014).

Il s'agit également d'évaluer les paramètres prédictifs de réponse à l'association RT-CT d'une tumeur mammaire en place avec tout son TME. Les résultats préliminaires de cette recherche translationnelle constitue la deuxième partie de ma thèse.

## Résultats

Les résultats de cette deuxième partie de ma thèse peuvent être résumés dans les deux articles présentés ci-dessous :

Dans le premier article (**Article 2**) nous allons présenter deux patientes qui ont eu des réponses atypiques à la combinaison séquentielle de la CTNA et de l'APBI.

Dans le deuxième article (**Article 3**), les résultats préliminaires de l'étude sur les 42 premières patientes seront présentés. C'est la première fois qu'une étude translationnelle des biomarqueurs dérivés de l'immunité prédisant la réponse à la RT-CT préopératoire est rapportée.

## Article 2

### **Atypical responses to neoadjuvant chemotherapy combined with accelerated partial breast tumor-directed radiotherapy: two cases and considerations for future clinical trials.**

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**Contexte :** Dans la première étape de l'étude, évaluant la dose maximale tolérable de la RT combinant à la CTNA, nous avons constaté deux patientes ayant des réponses inhabituelles par rapport à la réponse à une CTNA standard. Nous avons réalisé des analyses des biomarqueurs tissulaires et collecté des analyses du sang périphérique afin de mieux connaître les biomarqueurs potentiels pouvant nous aider à expliquer cette différence en répondant à l'association RT-CT néoadjuvante.



## **ABSTRACT**

La radiothérapie néoadjuvante (RTNA) non-palliative est en cours d'évaluation comme un *boost* de la réponse immunitaire dans le cancer du sein. Nous rapportons deux cas de réponse atypique au traitement dans un essai clinique évaluant l'irradiation mammaire partielle et accélérée (APBI) focalisée à la tumeur en association séquentielle à la chimiothérapie néoadjuvante (CTNA) standard dans les cancers du sein triple-négatif (TN) et luminal B HER2-. Nous discutons de la biologie sous-jacente, et fournissons des propositions pour la prise de décision par l'équipe multidisciplinaire dans chaque situation. La réponse exceptionnellement bonne (REx) comportait une destruction massive du tissu tumoral dans les adénopathies axillaires, bien qu'elles n'aient pas été irradiées. La réponse exceptionnellement mauvaise (NREx) s'est présentée comme une progression de la maladie sous traitement, avec une grosse tumeur résiduelle et le développement rapide de métastases. Les différences les plus importantes entre ces deux cas étaient : a) le statut tumoral p53/pRb +/- dans REx et +/- dans NREx, et b) une forte augmentation pré-APBI du rapport NLR chez NREx et une augmentation similaire du rapport PLR chez REx mais post-APBTI. La tumeur NREx résiduelle était très hétérogène, TN, HER2<sub>low</sub>, en transition épithélio-mésenchymateuse. Ces observations soulignent le besoin d'effectuer un prélèvement de tissu tumoral pendant le traitement néoadjuvant en cas de réponse atypique et que les valeurs prédictives du statut p53/pRb tumoral et les marqueurs dérivés du bilan sanguin périphérique, requièrent une évaluation plus approfondie dans les essais de CRTNA dans le cancer du sein.

## REPORTS OF PRACTICAL ONCOLOGY AND RADIOTHERAPY

### **Atypical responses to neoadjuvant chemotherapy combined with accelerated partial breast tumor-directed radiotherapy: two cases and considerations for future clinical trials**

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Keywords:	triple-negative breast cancer, neoadjuvant chemoradiation therapy, exceptional response

**Full title:** Atypical responses to neoadjuvant chemotherapy combined with accelerated partial breast tumor-directed radiotherapy: two cases and considerations for future clinical trials

**Running title:** Atypical responses to a neoadjuvant chemo-radiotherapy for breast cancer

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## Abstract

Neoadjuvant non-salvage radiotherapy is under clinical testing as a booster of immune response to breast cancer. We report two atypical responses to treatment in a neoadjuvant trial of accelerated partial breast tumor irradiation (APBTI) sequentially combined with standard chemotherapy in triple-negative and luminal/HER2- BC, discuss the biology behind, and provide suggestions for decision-making by multidisciplinary teams in such situations. The exceptionally good response (ExR) featured massive tumor tissue destruction in the axillary lymph nodes, although they were not irradiated. The exceptionally poor response (ExNR) presented as progression under treatment, a large residual tumor and rapid development of metastatic disease. The most important differences between these cases were: a) the p53/pRb tumor status, +/+ in ExR and -/- in ExNR, and b) a strong pre-APBTI increase in neutrophil-lymphocyte ratio in ExNR, and a similar increase in the platelet-lymphocyte ratio, but post-APBTI, in ExR. The residual ExNR tumor was highly heterogeneous, triple negative, HER2low, in epithelial-mesenchymal transition. These observations confirm the need for on-treatment tumor tissue sampling in case of atypical response to any neoadjuvant anticancer treatment and highlight the p53/pRb tumor status and blood cell counts as predictive biomarkers worth further evaluation in trials of preoperative chemoradiation in breast cancer.

**Keywords:** *triple-negative breast cancer, neoadjuvant, radiotherapy, exceptional response, p53/pRb.*

## Introduction

Neoadjuvant radiotherapy (RT) has been increasingly tested in clinical trials due to its well-documented capacity to induce and/or boost the anticancer immune response [1]. The introduction of irradiation in breast cancer (BC) neoadjuvant treatment has gotten particularly facilitated by recent technical advances in RT, which allow more precise radiation delivery and fewer postoperative complications [2].

Neoadjuvant association of accelerated partial breast tumor-directed irradiation (APBTI) and chemotherapy in BC is expected to fully exploit the synergy of radiation and cytotoxic drugs, with acceptable side effects, especially on long-term cosmetic outcomes. This approach is currently being evaluated in a French multicentric randomized phase 2 trial, NeoAPBI-01 ([NCT02806258](#)). The trial compares patients with triple-negative (TN) or luminal B/HER2- locally advanced BC receiving a standard anthracycline-taxane-based regimen and patients receiving the same regimen sequentially combined with a short-course APBTI (5 consecutive days, 2.5 Gy bi-daily).

Here we present two patients from the NeoAPBI-01 trial, one with an exceptionally good and another with an exceptionally poor response to the regimen with APBTI. We elaborate on tumor tissue characteristics, and blood cell counts, which could have predicted such unusual responses to therapy, and provide suggestions for improvement of patient management in future trials of neoadjuvant APBTI in BC.

### Case 1: Exceptional responder (ExR)

*Clinical features (Table 1):* a 56-year-old African black woman without a family history of cancer or comorbidities was diagnosed with TNBC stage T3 N2 M0. She first received four cycles of 5-fluorouracil-epirubicin-cyclophosphamide (FEC) and then APBTI, followed by two

cycles of docetaxel. The treatment was stopped due to several toxicities, and four weeks later, breast-conserving surgery with complete axillary LN dissection (ALND) was performed. Six weeks after breast surgery, adjuvant RT at a total dose of 50 Gy in 25 fractions of 2 Gy was delivered to the whole breast and the internal mammary and medial supraclavicular (IM-MS) LN regions without boosting the lumpectomy bed. The patient is alive and disease-free five years after enrollment into NeoAPBI-01.

*Blood counts:* at baseline, slight anemia (Hb 10.6 g/dL) and leukopenia ( $3.5 \times 10^9/L$ ), normal platelet count ( $293 \times 10^9/L$ ). The neutrophil-to-lymphocyte ratio (NLR) and the platelet-to-lymphocyte ratio (PLR) were 1.1 and 205, respectively. The NLR remained relatively low throughout the NAT (Fig. 1A). Compared to the pre-APBTI value, the PLR doubled after the irradiation but returned to the pre-APBTI level before surgery (Fig. 1B).

*Histopathology* (detailed in Table 2): at diagnosis, an invasive BC of nonspecific type (IBC-NS), intermediate grade, without lymphovascular invasion (LVI). Immunohistochemistry (IHC): a TN, basal-like BC (50% tumor cells expressing cytokeratin 5/6), diffusely and strongly positive for p53 (corresponding to the missense type *TP53* mutation [10], Fig. 2A), with the retinoblastoma protein (pRb) present in all cells (Fig. 2B). After NAT, the breast contained less than 100 viable tumor cells, single or in small groups. Most of the tumor bed was replaced by scar tissue, with a high number of elastic fibers and foamy macrophages. Three out of 13 excised LNs were replaced by acellular tissue, corresponding to the hyaline change (Fig. 2C). In six other LNs, 30-80% of the lymphoid tissue was destroyed and replaced by fibrin deposits or hemorrhage.

## **Case 2: Exceptional non-responder (ExNR)**

*Clinical features* (Table 1): a 31-year-old Caucasian woman without a family history of cancer or comorbidities was diagnosed with TNBC stage T2 N0 M0. After the first four cycles

of FEC, a clinically suspected progression was confirmed by magnetic resonance imaging showing a bigger primary tumor and three centimetric satellite nodules. The NAT was continued with interceding APBTI between two of the four cycles of docetaxel, to be finalized by mastectomy and complete ALND. The surgical specimen contained a large residual tumor without involved LNs. Eight weeks post-surgery, adjuvant treatment consisted of RT only, at a total dose of 50 Gy normofractionally delivered to the chest wall and IM-MS regions. No further adjuvant systemic treatment was indicated according to national and institutional breast cancer management guidelines at that time. The patient developed a solitary metastasis in the right lower lung lobe three months after adjuvant RT. The lesion was not accessible for a biopsy to exclude lung cancer, so the treatment was continued with three cycles of a carboplatin-paclitaxel-bevacizumab regimen, to be completed with a lobectomy and mediastinal lymphadenectomy. Histopathological analysis confirmed BC metastasis without therapeutic effect. Three months after, new metastases in the lungs, the pleura, and the mediastinal LNs were observed by computerized tomography. The patient died 15 months after the lung surgery and three years after the BC diagnosis.

*Blood counts:* at baseline, Hb, leucocyte, and platelet counts were within the normal range; NLR and PLR were 1.8 and 200, respectively. The NLR and PLR dynamics were strikingly opposite to the one of the ExR patient: a very high NLR (6.1) right before APBTI dropped to 1.6 post-irradiation (Fig. 1A) while the PLR remained relatively stable throughout the therapy (Fig. 1B).

*Histopathology* (detailed in Table 2): at diagnosis, an IBC-NS of high grade, without LVI. IHC: a TNBC without basal-like characteristics (< 1% tumor cells CK5/6+). Both p53 and pRb were absent (Figs. 2D, 2E), indicating the presence of *TP53* mutation of the “null” type [10] and *RBI* loss or a “null” mutation. The post-NAT residual tumor was highly histologically heterogeneous, with >30% represented by loose epithelial cells and sarcomatous tissue. IHC:

absence of hormone receptor expression, with many zones of HER2 score 1 or 2 (Fig. 2F) but without HER2 gene amplification (details in Table 2). The tumor cells expressed CK5/6, CK8/18, KIT(CD117), CD56, SOX10, and ZEB1 in multiple large foci, SOX2 in small foci, while being negative for EGFR, BCL-2, androgen receptor, chromogranin, synaptophysin and PD-L1 (Table 2). This IHC profile corresponded to a basal-like TNBC in epithelial-mesenchymal transition.

## Discussion

According to our best knowledge, this is the first report of an exceptionally good or poor BC response to a NAT containing RT, which is not a salvage treatment.

No evaluated baseline clinico-pathological feature could indicate that the ExR patient will respond by the total elimination of tumor cells in the non-irradiated LNs. The hyaline change observed in some LNs after NAT indicates tissue destruction that happened well before the surgery and suggests an early response to the treatment, which could have been induced by chemotherapy, the abscopal effect of the APBTI, or both. The increased tumor cell sensitivity to DNA damaging agents, well documented in the basal-like TNBC subtype [3], could have resulted in sufficient activating of the cancer-immunity cycle [4] and almost total elimination of malignant cells over time. This underlines the need for an extensive assessment of DNA damage repair (DDR) proficiency before any DNA damage-inducing therapy, as the DDR pathway deficiencies likely are among the strongest predictors of good response to this type of anticancer treatment.

The only pre-treatment feature indicative of potential high resistance to treatment of the ExNR patient's tumor was the p53-/pRb- status, unique among the first 25 patients enrolled in the trial (data not shown). The simultaneous inactivation of the p53 and the pRb pathway has



been shown to predict breast cancer resistance to DNA damage in vivo [5]. In addition, *RBI* deficiency is implicated in promoting stemness and metastatic progression [6] and is associated with poor clinical outcomes in several cancer types [7]. Interestingly, prostate cancers with p53/pRb loss, resistant to many therapeutics [8], were radiosensitized by PARP1 inhibitors (PARP1i) [9], making a combination of PARP1i and APBTI worth clinical testing in p53-/pRb- breast cancers.

Our patients markedly differed in blood parameters right before APBTI. The ExNR patient had more than 3-fold higher NLR than the ExR patient, mainly due to a much higher neutrophil count. Neutrophilia, alone or combined with lymphopenia, is well demonstrated to be strongly unfavorable for response to treatment and prognosis in breast and other cancers [10]. While it remains to be validated, we believe that a high neutrophil count in a patient under an experimental therapy and with suspected progression should be discussed by the multidisciplinary team (MDT) as a potential stop signal to prevent harmful effects of the upcoming treatment.

Without available on-treatment tumor biopsies, we cannot conclude whether APBTI stimulated the metastatic progression of the ExNR patient's tumor. Resistance to chemotherapy was suspected well before the APBTI started, so re-biopsying the breast tumor was already indicated at that time. That could have revealed the HER2<sub>low</sub> tumor status, observed only after NAT, and initiated a discussion about the exclusion of the patient from the trial and her eventual enrollment into a trial of HER2 antibody-drug conjugates, shown to be efficacious in HER2<sub>low</sub> BCs [11].

Molecular tumor profiling before treatment (for example, PAM50 gene panel for determination of molecular subclass and the BRCAness assays) would have been helpful in better elucidating why these unusual responses occurred. In addition, if the patient agrees, analyses of the germline mutational status should be undertaken in all situations of

unexpected/unusual/exceptional response to a novel treatment. Certain germline anomalies, like mutations in *BRCA1/2* or/and other genes involved in DDR, can be responsible for particularly good responses to chemo- or/and radiotherapy [12].

In conclusion, the p53-/pRb- tumor status and blood cell counts are biomarkers worth testing in future trials of neoadjuvant chemoradiation for BC. By this report, we encourage MDTs to demand additional tumor tissue and/or blood samples in any situation of atypical response to neoadjuvant anticancer treatment and to connect with the consortia dedicated to a deep exploration of such cases.

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**Table 1.** Patient clinical features and treatment

	<b>ExR patient</b>	<b>ExNR patient</b>
<b>Clinical features</b>		
Age at diagnosis	56	31
Breast tumor size (mm)	50	35
TNM stage	T3 N2 M0	T2 N0 M0
<b>Neoadjuvant treatment</b>		
Chemotherapy	4 x FEC + 2 x docetaxel	4 x FEC + 4 x docetaxel
APBI	20 Gy (2 Gy BID x 5 days)	25 Gy (2.5 Gy BID x 5 days)
Breast surgery type	lumpectomy	mastectomy
ALND	yes	yes
Pathological response	near-pCR	ypT2, ypN0
Toxicities	grade 2 hematologic, GI, vascular, neurologic	no
<b>Adjuvant treatment</b>		
Chemotherapy	no	no
RT	WBI + ILN	chest wall + ILN
<b>Recurrence</b>		
Local	no	no
Metastatic	no	yes
Site(s) of metastases	NA	lung, pleura, mediastinum
Treatment of metastases	NA	7 lines of systemic CT
<b>Disease-free survival</b>	60 months	12 months
<b>Overall survival</b>	60 months	36 months

*Legend: ALND, axillary lymph node dissection; APBI, accelerated partial breast irradiation; BID (bis in die), twice a day (two fractions of radiotherapy with an interval of 6 hours per day); CT, chemotherapy; ExR, exceptionally good response; ExNR, exceptionally poor response; FEC, 5-fluorouracil-epirubicin-cyclophosphamide regimen; GI, gastrointestinal; ILN, internal lymph nodes (including supraclavicular and internal mammary lymph nodes); pCR, pathologic complete response; RT, radiation therapy; WBI, whole breast irradiation.*

**Table 2.** Histological and immunohistochemical characteristics of pre-NAT and post-NAT

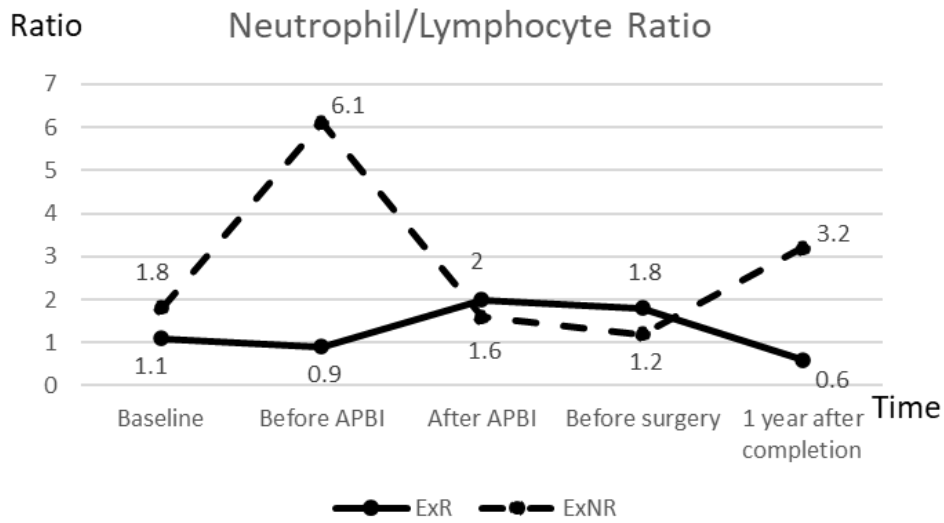
breast tumors and axillary lymph nodes

	<b>ER patient</b>	<b>ENR patient</b>
<b>Pre-NAT features</b>		
histological type	IBC-NS	IBC-NS
histological grade (Elston-Ellis SBR)	intermediate	high
presence of LVI	no	no
axillary LNs	2 biopsied, both involved	not biopsied
ER	0 %	0 %
PR	0 %	0 %
HER2 score	1	0
<i>ERBB2</i> amplification status	non-amplified	non-amplified
Ki67	80 %	95 %
molecular subtype	triple negative	triple negative
E-CADH	100 %, strong	100%, moderate
CK5/6	50 %, multifocal	< 1%
CK14	0 %	0 %
CK8/18	0 %	30 %, multifocal
KIT (CD117)	30 %, multifocal	80 %
EGFR	< 1%	0 %
p53	100%, strong	absent
pRb	100 %	0 %
<b>Post-NAT features</b>		
Breast		
tumor bed size	60 mm	45 mm
residual tumor size	9 mm	45 mm
residual tumor histotype	NA	IBC-NS with a metaplastic/sarcomatous component
residual tumor grade	intermediate	high
LVI	no	no
TILs	NA	< 1 %
Axillary LNs		
excised (n)	13	5
involved (n)	0	0
altered (n)	9	0
IHC profile of residual tumor		

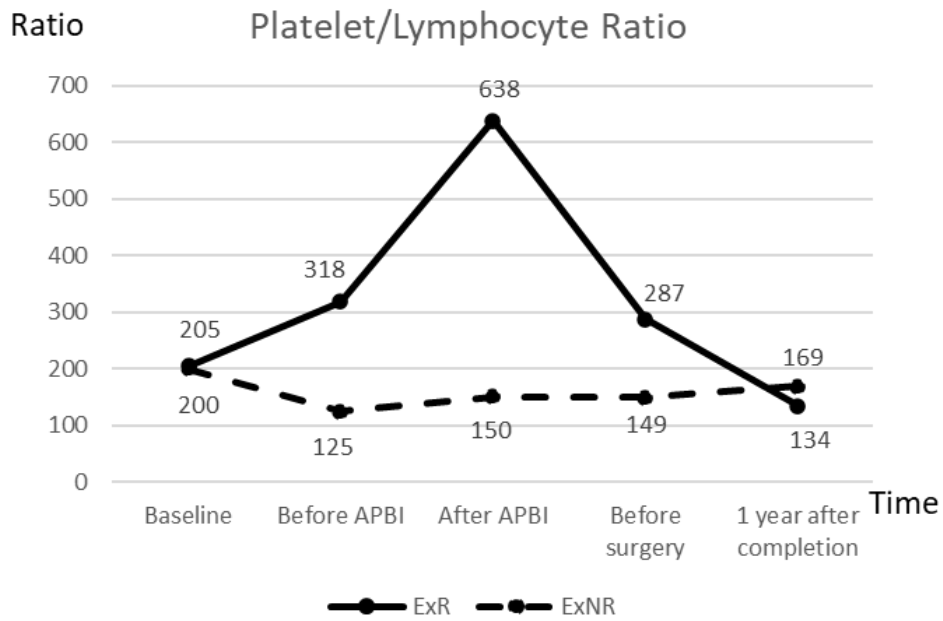
ER	0 %	0 %
PR	0 %	0 %
HER2 score	0	heterogeneous, areas with score 0, 1 and 2
<i>ERBB2</i> amplification status	nd	non-amplified
Ki67	10 %	90 %
molecular subtype	triple negative	triple negative
E-CADH	100 %	100 % in the ribbon and solid pattern areas, 0% in the loose cell and sarcomatous areas
CK5/6	100 %	heterogeneous, 1% - 90%
CK14	nd	0 %
CK8/18	nd	50%, multifocal
KIT (CD117)	nd	70 %, multifocal
EGFR	nd	0 %
p53	100 %, strong	0 %
pRb	100 %	0 %
SOX2	nd	10 %, small foci
SOX10	nd	80 %
ZEB1	nd	50% in average (100% in the loose cell and sarcomatous areas)
chromogranin	nd	0 %
synaptophysin	nd	0 %
CD56	nd	30 %, multifocal
BCL-2	nd	0 %
AR	nd	0 %
PD-L1	nd	< 1 %

*Legend: AR, androgen receptor; CK, cytokeratin; E-CADH, E-cadherin; Elston-Ellis SBR, the Elston & Ellis modified Scarff-Bloom-Richardson grading system; ER, estrogen receptor; IBC-NS, invasive breast carcinoma of nonspecific type; LNs, lymph nodes; LVI, lymphovascular invasion; NA, not applicable; NAT, neoadjuvant treatment; PR, progesterone receptor; pRb, retinoblastoma protein.*

**A**

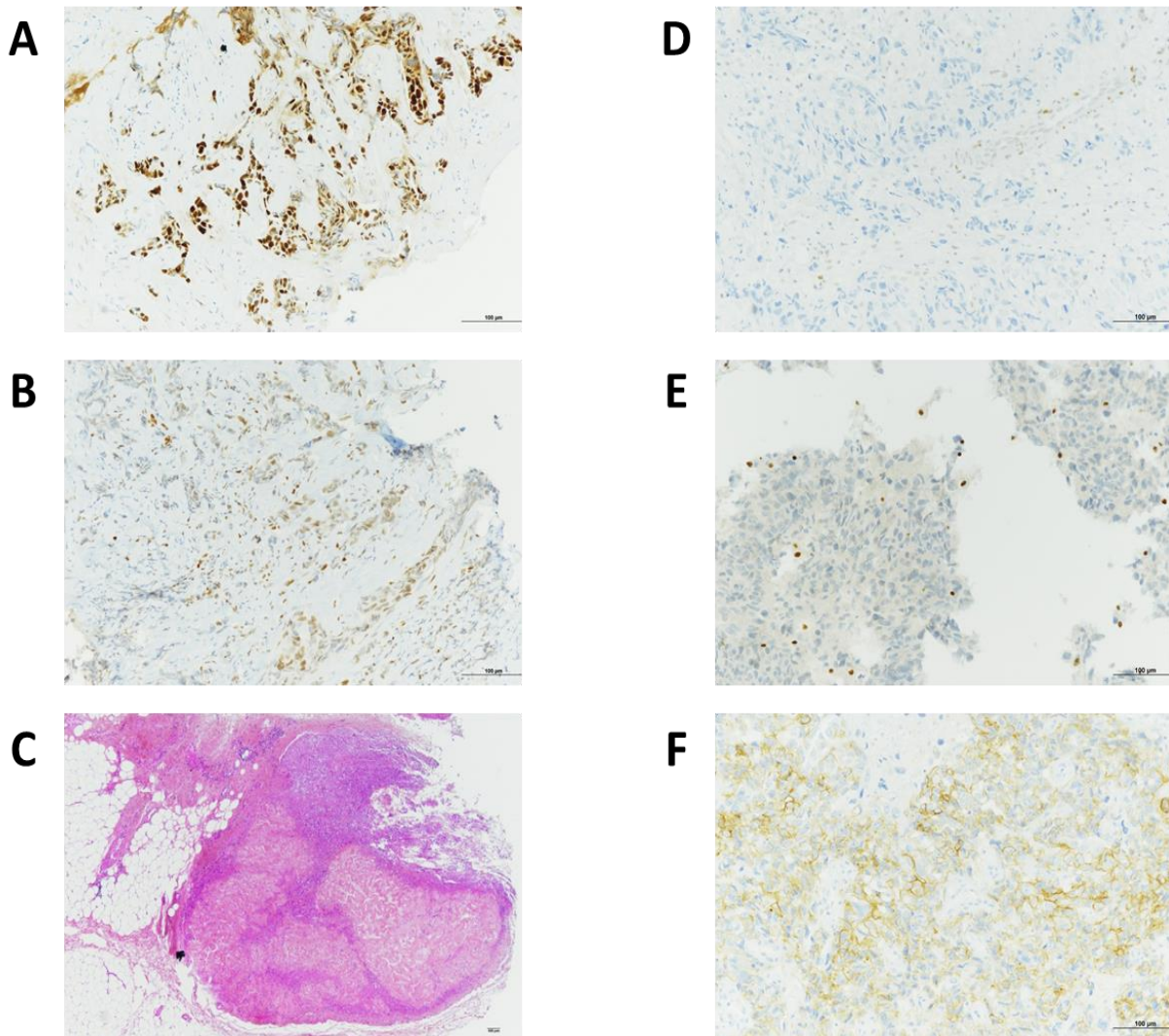


**B**



**Figure 1:** Dynamics of the neutrophil/lymphocyte ratio and the platelet-lymphocyte ratio and throughout the clinical follow-up. Legend: APBI, accelerated partial breast irradiation; ExR, the exceptional responder patient; ExNR, the exceptional non-responder patient.





**Figure 2:** Histological and immunohistochemical characteristics of patients' pre-treatment biopsies and post-treatment surgical specimens. A, ExR patient, pre-treatment, immunohistochemistry (IHC) for p53, x200; B, ExR patient, pre-treatment, IHC for pRb, x200; C, ExR patient, post-treatment, axillary lymph node, hyaline change, H&E, x40; D, ExNR patient, pre-treatment, IHC for p53, x200; E, ExNR patient, pre-treatment, IHC for pRb, x200; F, ExNR patient, post-treatment, an area of HER2 score 2, IHC for HER2, x200.

### **Article 3**

**Pathologic response to neoadjuvant sequential chemoradiation  
therapy in locally advanced breast cancer:  
preliminary, translational results from the French Neo-APBI-01 trial.**

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## **ABSTRACT**

**Contexte :** La radiothérapie (RT), comme étant une nouvelle approche de booster la réponse immunitaire anticancéreuse, a été étudiée progressivement dans le cadre d'un traitement néoadjuvant dans le cancer du sein.

**Objectif :** Nous avons cherché à évaluer les indicateurs dérivés de l'immunité de la réponse à la chimio-radiothérapie néoadjuvante (CRTNA) dans le cancer du sein pour une meilleure personnalisation du traitement.

**Patients et Méthodes :** Nous avons analysé les données des 42 premières patientes incluses dans l'essai randomisé de phase 2 Néo-APBI-01 comparant la chimiothérapie néoadjuvante (CTNA) standard et le schéma CRTNA dans les cancers du sein localement avancés de sous-type triple-négatif (TN) et luminal B (LB). Les paramètres clinico-pathologiques, le nombre des globules et leur index dérivés, l'infiltration totale des lymphocytes dans la tumeur (TILs) et l'infiltration de leur sous-population, ainsi que le statut mutationnel de TP53 ont été évalués comme prédicteurs de réponse.

**Résultats :** Vingt-et-une patientes ont été assignées de façon égale dans chaque groupe. La réponse pathologique complète (pCR) était de 33% et 38% dans les groupes CTNA et CRTNA, respectivement, avec un effet dose-réponse. Une seule tumeur LB a atteint la pCR après la CRTNA. Plusieurs paramètres associés à la réponse ont été identifiés, qui différaient selon le traitement reçu. Dans le groupe CRTNA, un taux initial d'hémoglobine  $\geq 13$  g/dL et un indice de masse corporelle (IMC)  $< 26$  étaient fortement associés à la pCR. Un rapport initial de neutrophiles-lymphocytes plus élevé, un nombre plus élevé des TILs et des T-effecteurs étaient favorable pour la pCR.

**Conclusion :** Cette analyse préliminaire a identifié les tumeurs LB et à faible TILs comme de mauvais répondeurs à la CRTNA, qui délivrait la RT après plusieurs cycles de chimiothérapie. Ces résultats permettront, dans l'amendement prévu, de modifier la sélection des patientes à inclure dans l'essai et contribueront à une meilleure conception des futurs essais de CRTNA dans le cancer du sein.

## Breast Cancer Research and Treatment

### Pathologic response to neoadjuvant sequential chemoradiation therapy in locally advanced breast cancer: preliminary, translational results from the French Neo-APBI-01 trial.

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Abstract:	<p>Background: Radiation therapy (RT), as a novel approach to boost the anticancer immune response, has been progressively evaluated in the neoadjuvant setting in breast cancer (BC).</p> <p>Purpose: We aimed to evaluate immunity-related indicators of response to neoadjuvant chemo-radiation therapy (NACRT) in BC for better treatment personalization.</p> <p>Patients and Methods: We analyzed data of the first 42 patients included in the randomized phase 2 Neo-APBI-01 trial comparing standard neoadjuvant chemotherapy (NACT) and NACRT regimen in locally advanced triple negative (TN) and luminal B (LB) subtype BC. Clinicopathological parameters, blood counts and the derived parameters, total tumor-infiltrating lymphocytes (TILs) and their subpopulation, as well as TP53 mutation status, were assessed as predictors of response.</p> <p>Results: Twenty-one patients were equally assigned to each group. The pathologic</p>

**Full title:** Pathologic response to neoadjuvant sequential chemoradiation therapy in locally advanced breast cancer: preliminary, translational results from the French Neo-APBI-01 trial.

**Running title:** Factors associated with response to neoadjuvant chemoradiation in breast cancer.

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**Abstract:**

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**Purpose:** We aimed to evaluate immunity-related indicators of response to neoadjuvant chemo-radiation therapy (NACRT) in BC for better treatment personalization.

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**Results:** Twenty-one patients were equally assigned to each group. The pathologic complete response (pCR) was 33% and 38% in the NACT and NACRT groups, respectively, with a dose-response effect. Only one LB tumor reached pCR after NACRT. Numerous parameters associated with response were identified, which differed according to the assigned treatment. In the NACRT group, baseline hemoglobin of  $\geq 13\text{g/dL}$  and body mass index of  $< 26$  were strongly associated with pCR. Higher baseline neutrophils-to-lymphocytes ratio, total TIL, and T-effector cell counts were favorable for pCR.

**Conclusion:** This preliminary analysis identified LB and low-TIL tumors as poor responders to the NACRT protocol, which delivered RT after several cycles of chemotherapy. These findings will allow amending the selection of patients for the trial and help prepare a better design for future trials of NACRT in BC.

**Keywords:** neoadjuvant, radiotherapy, triple negative breast cancer, luminal B, neutrophils-to-lymphocytes ratio, tumor-infiltrating lymphocytes.

## Introduction

Breast cancer (BC) management is a multimodal approach integrating surgery, radiation therapy (RT), and systemic treatment. Recent decades have witnessed the emergence of new treatment paradigms, introducing neoadjuvant treatment (NAT) with more effective anticancer agents. Advances in knowledge of cancer immunoediting [1] and the cancer-immunity cycle [2] have enabled the development of modern cancer immunotherapy, with immune checkpoint inhibitors (ICIs), to reach an important inflection point in the history of cancer treatment. Recent studies evaluating the addition of an ICI (e.g., pembrolizumab or atezolizumab) have significantly increased the pathologic complete response (pCR) rate compared with neoadjuvant chemotherapy (NACT) alone in HER2-negative BC [3, 4]. Novel combinations boosting antitumoral T cell response are actively under investigation to overcome resistance to ICIs, such as platinum agents [5, 6], cyclin-dependent kinase inhibitors [7], or RT [8].

Accelerated partial breast irradiation (APBI) has been increasingly incorporated into the management of early-stage BC in the adjuvant setting. Recent technical advances in RT have facilitated treatment delivery, especially when RT is directed to the tumor in the preoperative setting [9]. In addition, knowledge of distinct effects on the microvasculature and the immune system of higher doses of ionizing radiation (IR) has allowed the design of novel approaches to improve clinical outcomes in BC patients [8]. The neoadjuvant association of tumor-directed APBI and chemotherapy is expected to fully exploit the synergistic effect of radiation and cytotoxic agents with acceptable side effects.

Only a few prospective studies have been published on RT responses of BC patients with intact tumors [10–12], as RT is traditionally given after breast surgery. An ongoing prospective study assessing neoadjuvant chemo-radiation therapy (NACRT), the Neo-APBI-01 trial (NCT02806258), was specifically designed for aggressive HER2-negative locally advanced BC, i.e., triple negative (TN) and luminal B (LB) subtypes. Neo-APBI-01 aimed to exploit the ability of RT to induce immunogenic cell death, enhance the cancer-immunity cycle, and, thus, improve the pCR rate of TN and LB tumors as a surrogate marker for better long-term outcomes [13, 14]. Furthermore, the trial also provided a unique opportunity to assess *in vivo* the response of breast tumors and to evaluate potentially predictive

biomarkers, helping to adjust the treatment individually. There is growing clinical evidence of the prognostic and predictive values of immunity/inflammation-related biomarkers, such as tumor-infiltrating lymphocytes (TILs) [15–17]. In addition, the patient's systemic immune status, easily accessible by blood tests, has drawn attention as a promising and non-expensive biomarker of response to several anticancer therapies [18].

In this report, we present the results of the translational analysis of the Neo-APBI-01 trial, associated with an insight into potential predictive biomarkers. The conclusions we have drawn could be of consideration for the design of future NACRT trials in BC.

## **Patients and methods**

### **1.1. Patients**

Between October 2016 and September 2021, 42 patients were treated according to the Neo-APBI-01 protocol. The eligibility criteria were: age  $\geq 18$  years, histologically proven invasive non-specific type or lobular breast carcinoma, and locally advanced disease of TN or LB/HER2- subtypes.

The patients were randomly assigned into two groups. The standard group received anthracycline/taxane NACT, and the experimental group had the same NACT regimen incorporating a sequential, short-course tumor-directed APBI. Neoadjuvant RT consisted of 10 fractions of 2.5 Gray (Gy), two fractions per day (BID) over five days, or eight fractions of 3.125 Gy, one fraction per day (QID) over one week and a half. Breast surgery, lumpectomy or total mastectomy, and axillary lymph node dissection were performed 4-6 weeks after NAT completion. Postoperative RT was delivered according to the discretion of the local physician. No additional RT was added to the experimental NACRT group. Adjuvant hormonotherapy was prescribed for hormonal receptor-positive (HR+) patients.

Twenty-one patients were assigned to NACT only, and 21 received NACRT. Patients had consented to use their tumor tissue samples and blood test data for translational research analyses. The institutional review board approved the project (N°IDRCB: 2015-A01062-47).



### **1.2. Histopathological assessments**

Pretreatment histological type, grade, lymphovascular invasion, and molecular subtype by immunohistochemistry (IHC) were determined by local pathologists and reviewed by the central pathologist (NRR) for the study of biomarkers. The positivity for estrogen and progesterone receptors (ER and PR, respectively) was defined using the 10% cutoff. HER2 status was determined according to the ASCO/CAP criteria [19]. The molecular subtype was determined according to the Saint Gallen consensus 2013 [20].

pCR was defined as the absence of residual invasive cancer in the breast and the axillary lymph nodes (ypT0/Tis ypN0) without evidence of metastatic deposits [21].

IHC for p53, pRb, and PD-L1 was interpreted by the central pathologist as presented in Supplementary Table 1.

### **1.3. Assessment of tumor-infiltrating lymphocytes (TILs)**

The number of stromal TILs was assessed in H&E-stained tumor biopsies according to the International Immuno-Oncology Biomarker Working Group on Breast Cancer [22]. The tumors were classified into inflamed (IM), immune-excluded (IE), and immune-deserted (ID), according to [23].

TIL subpopulations expressing CD8, CD4, FoxP3, CD20, or PD-L1 were visualized by IHC performed as presented in Supplementary Table 1. QuPath software [24] was used to count stromal and intratumoral cells of each TIL subpopulation in a region of interest delineated to correspond to 10 consecutive high-power (x400) microscopic fields (field diameter 500  $\mu\text{m}$ ) from the tumor invasive front to the tumor center. The number of cells per  $\text{mm}^2$  was calculated for each subpopulation.

PD-L1 expression was expressed as the percentage of PD-L1+ tumor cells or PD-L1+ immune cells.

### **1.4. Blood cell counts and the derived parameters**

Blood tests were routinely performed at different time points (baseline, before and after APBI, and before surgery). Neutrophils-to-lymphocytes ratio (NLR) was calculated by dividing the absolute neutrophil count (ANC) by the absolute lymphocyte count (ALC). Platelets-to-lymphocytes ratio (PLR) was obtained by dividing absolute platelet count (APC) by ALC. Lymphocytes-to-monocytes ratio

(LMR) was obtained by dividing the ALC by the absolute monocyte count. Systemic immune-inflammation index (SII) was defined as  $(ANC \times APC)/ALC$ . The blood cell count-derived parameters dynamic changes (i.e., delta-NLR, delta-PLR, delta-LMR, delta-SII) were defined as the post-NAT value minus the pre-NAT value of a given ratio.

### 1.5. Statistical analysis

Optimal biomarker cutoffs for pCR prediction were calculated considering the maximum (sensitivity and specificity) point of the Receiver Operating Characteristic (ROC) curve [25]. Associations between clinicopathological, blood cell-derived or TIL-related parameters and response to NAT were evaluated by the Chi-squared test or Fisher's exact test. Stromal TILs were assessed as continuous variables or predefined cutoffs of 10%, 30%, or 50%. Tumor or immune cell PD-L1 expression was evaluated as a binary variable, with cutoffs at 0%, 1%, and 10%. Univariate analyses were performed using the logistic regression model. Multivariate analyses were not performed due to less than 10 events in the analyzed cohorts [26]. The odds ratio (OR) was reported with the corresponding 95% confidence intervals (95% CI). The p values of  $< 0.05$  were considered statistically significant, and those of  $< 0.1$  and  $\geq 0.05$  as tendencies. Statistical analysis was performed using the R software, version 4.2.0 (R-Project, GNU GPL, <https://cran.r-project.org>).

## Results

### 1.6. Patient and tumor characteristics

Patients and tumor baseline characteristics were comparable in both groups (Table 1). The median age at diagnosis was 48 years in the NACT group and 45 years in the NACRT group. The median tumor size was 30 mm (interquartile range, IQR: 25-38 mm) in the NACT group and 32 mm (IQR: 25-35 mm) in the NACRT group. The NACT group contained more patients with involved lymph nodes (n=12, 57%) than the NACRT group (n=8, 38%).

All patients presented with invasive carcinoma of non-specific type; most had histologic grade 3 and a very high Ki67 index. About one-third had a LB tumor, and two-thirds had a TNBC in both groups.

Immunity-related biomarkers in the tumor microenvironment (TME) and blood in both groups were relatively similar. Supplementary Tables 2 and 3 present IHC and systemic inflammation markers, respectively. The immune deserted (ID) phenotype was most common, accounting for nearly half of the patients, corresponding to low TIL levels (< 10%) in a majority of the tumors. About three-fourths of tumors had a *TP53* mutation (equally the null type and the missense type) and present pRb. Absolute blood cell counts and the derived ratios were also comparable in both groups, except for a slightly higher number of platelets in the NACRT group (median 309 G/L compared to 271 G/L in the NACT group,  $p=0.05$ ). Consequently, platelet-related ratios, i.e., PLR and SII, tended to be slightly higher in the NACRT group.

### 1.7. Response to treatment

There was no statistically significant difference in the pCR rate between the NACRT and the NACT group (38% vs. 33%, respectively,  $p = 0.7$ ). All but three pCR patients had a TNBC, and only one LB tumor responded to NACRT by pCR. Details of the response patterns are given in Table 2.

Among patients who received the total dose of 25 Gy, 43% of those who received 10 x 2.5 Gy BID ( $n=6/14$ ) and 67% of those who received 8 x 3.125 Gy QID ( $n=2/3$ ) reached a pCR, whereas no pCR was observed in patients receiving preoperative RT of less than 25 Gy ( $n=4$ ).

### 1.8. Associations of putative biomarkers and response to treatment

To evaluate the predictive capacity of clinical, blood, and tumor tissue parameters, we performed a logistic regression analysis. Globally, we observed that different parameters were associated with pCR in the NACRT cohort than in the NACT cohort.

Table 3 presents the associations between clinical parameters, peripheral blood counts, blood count-derived ratios, and pCR. In the cohort treated by NACRT, Hb level and body mass index (BMI) were the most significant parameters associated with pCR. None of the patients with Hb < 13 g/dL and BMI > 26 reached pCR. Furthermore, baseline NLR > 2.2 and delta-PLR > 3.3 G/L were associated with a higher probability of pCR. Finally, delta-NLR, baseline PNN, pre-APBI LMR, and the absence of involved LNs tended to correlate with pCR (Table 3). In the cohort treated by NACT, younger age ( $\leq 48$  years) and a lower baseline SII ( $\leq 252$ ) were favorable for pCR.

Table 4 presents the associations between tissular parameters and pCR. In the NACRT group, a very high Ki67 index ( $\geq 90\%$ ,  $p=0.03$ ) and higher tumor infiltration by T cells (the sum of CD8+ and CD4+ cells) ( $p=0.05$ ) were markedly associated with pCR. The TN subtype ( $p=0.08$ ), TILs  $\geq 10\%$  ( $p=0.1$ ), an immune phenotype other than the ID subtype ( $p=0.1$ ), and mutated *TP53* ( $n=6/6$ ) tended to correlate with pCR. On the other hand, a higher number of each assessed TIL subpopulation was associated with pCR in NACT-treated patients, as well as a higher ratio of CD8+ to FOXP3+ or CD8+ to CD4+ TILs. Patients with a higher expression of PD-L1 in immune cells ( $\geq 10\%$ ) tended to have a pCR to NACT.

## Discussion

To the best of our knowledge, this is the first study of a fractionated regimen of preoperative APBI associated with NACT to treat TN and LB tumors. Preoperative RT provides a unique opportunity to study radiation response and to gain more insight into the radiation sensitivity of BC.

Only a few studies have reported the outcomes of preoperative tumor-directed APBI in BC. Historically, APBI has been considered an option in selected patients of  $>50$  years with HR+ early BC with a favorable prognosis [27]. In the neoadjuvant setting, one dose-escalating phase 1 study of tumor-directed APBI associated with taxane-based NACT reported an overall pCR rate of 36%, with a dose-dependent effect [10]. Table 5 summarizes data on prospective, tumor-directed APBI in the literature.

In our study pCR, rate was 38% in the NACRT arm, similar to pCR rates in previously published trials of chemo-RT in BC. A much higher pCR rate was observed in patients with a TN (54%) than with a LB tumor (13%). The TN subtype, harboring a higher proportion of lymphocyte-predominant tumors, has been well-known to respond more favorably to NACT [15, 16]. Our study showed the same tendency of tumor response by subtypes; 38% and 25% of TN and LB patients, respectively, reached a pCR after NACT.

By evaluating clinicopathological and systemic immune parameters, we identified several factors associated with response, which differed according to the assigned treatment. In the NACRT group, the absence of anemia, a normal BMI, and systemic inflammation-related index are markedly observed in

pCR patients. In contrast, well-established favorable factors for a better response to NACT, such as young age, a higher TIL, and TIL subpopulations, were correlated to pCR.

The parameters strongly associated with pCR in the NACRT cohort were Hb  $\geq$  13 g/dL and BMI  $<$  26. Tissue oxygenation is a key component modulating responsiveness to ionizing radiation. Accumulating evidence has strongly suggested that poor intratumoral oxygenation and the presence of anemia can adversely influence the survival of numerous cancer patients [28] and outcomes following curative-intent RT [29]. The negative impact of pretreatment anemia (using a Hb level threshold of 9-14.5 g/dL) on locoregional control and survival has been consistently documented in patients with various solid tumors (e.g., head and neck, uterine cervix, lung, anus, prostate) [29]. All patients in our study who obtained a pCR after NACRT had a pretreatment Hb level of  $\geq$  13 g/dL. Adequate oxygenation has recently been demonstrated to suppress immunosuppressive metabolites and cytokines in the TME and confer a favorable immunity-mediated response to treatment [30].

Other immunity-related factors could also alter the response to anticancer therapy. Excessive body fatness is linked to systemic and intratumoral chronic and TME inflammation, which diminishes the anticancer immune response. Inflammation is considered a hallmark of cancer establishment and progression [31]. It has been reported that overweight and obese patients (defined as BMI  $\geq$  25 and 30, respectively) experienced reduced efficacy and increased anticancer treatment-related toxicities, as well as inferior outcomes [32]. These patients also had a lower pCR rate when treated by NACT in comparison to the patients with under-/normal weight [33, 34]. In line with these findings, all the patients in our study who reached a pCR after NACRT had normal weight. Undoubtedly, maintaining a healthy weight is an important topic in patients' lifestyle education, helping the improvement of the quality of life and treatment outcomes.

In contrast with most published studies on NACT in BC, high pretreatment NLR ( $>$  2.2) was favorable for pCR in our NACRT-treated cohort. Neutrophils are the most abundant leukocytes in the blood and are emerging as important regulators of cancer [35]. Elevated counts of neutrophils and a neutrophil-derived parameter NLR were found in advanced malignant disease and considered an indicator of poor prognosis in several cancers [36]. Recent research has revealed the multifaceted roles of neutrophils; in

some contexts, these cells may exert strong antitumoral activities [35]. These findings might explain the controversies about NLR as a prognostic and predictive indicator in different cancers, including BC [36–38]. RT to the intact breast tumors produces inflammatory cytokines, such as TNF- $\alpha$  and IFN- $\gamma$  which can promote neutrophil differentiation towards the N1 antitumor phenotype, which also might explain why more pretreatment neutrophils can be favorable for tumor elimination by a NACRT [35].

Although the level of statistical significance was not reached, likely due to the small number of patients, the presence of at least 10% TIL, as well as higher numbers of T effector cells (CD8+ and CD4+), was favorable for pCR to NACRT in our study. These associations were more significant in the NACT group and concordance with the literature [39]. An immune-effective TME was necessary for the survival benefit of RT in BC [40]. Placing preoperative APBI in the middle of NACT when the immune TME was impoverished might have been why the immunogenic antitumoral effect of RT was not observed in the study. The lack of the immunity-boosting effect of RT could have been even greater in the LB than in the TN tumors, as the former typically contained less TIL than the latter [15]. Interestingly, the only LB tumor that responded by pCR in our NACRT cohort was much richer in TILs than the other tumors of this subtype (data not shown). A fraction of TIL-rich BCs, greater in the TN than in the LB subtype, have defects in the DNA repair pathway and are more chemo/radiosensitive. This intrinsic tumor cell characteristic is likely a major biomarker of response to any DNA-damaging therapy, including RT, so it should be assessed by dedicated tests in future trials of NACRT in BC.

Related to tumor DNA repair, an interesting observation in this study was that no tumor without mutated *TP53* reached pCR to NACRT. The only LB tumor which had a pCR was also *TP53*-mutated\*. Most of these tumors had a missense *TP53* mutation\*. None of the *TP53*-mutated tumors had the ID phenotype, whereas among the ID tumors (all non-pCR), only one was *TP53*-mutated\* (\*data not shown). The association between the missense *TP53* mutation and higher TIL levels has been recently described [42]. In the LB subtype, the presence of *TP53* mutation is associated with more aggressive disease [43] and a higher frequency of other mutated genes involved in DNA repair [44]. Therefore, screening for *TP53* mutations by IHC might provide insight into cancer chemo/radiosensitivity and help better select BC patients for NACRT approaches.

The pRb status, assessed by IHC, was not associated with response in this small cohort of patients. However, the only patient with a null-type *TP53* mutation and absent pRb (corresponding to the p53 loss/pRb loss status) among the 21 treated by NACRT was primo-resistant to the treatment and experienced a rapid fatal metastatic relapse (To et al., Reports of Practical Oncology and Radiotherapy, In Press). As in the case of *TP53* mutations, an IHC-based assessment of the pRb status seems worth further evaluation in the NACRT trials.

Several limitations are worth noting in our present study. Notably, the low number of included patients and, consequently, the low number of complete responders make it insufficient to detect significant and clinically relevant differences in potential predictive factors. Blood tests were performed according to patients' convenience in different laboratories, and inter-laboratory and inter-individual differences might exist. Despite these limitations, to the best of our knowledge, this is the first cohort of sequential NACRT-receiving patients in which immunity-related potential predictive biomarkers were evaluated.

## **Conclusion**

This preliminary analysis of the association of NACRT with a short course of tumor-directed APBI revealed the poor response of LB and low-TIL tumors to a regimen having a low-dose RT placed after several cycles of chemotherapy. These findings will allow us to adapt the selection criteria by excluding patients with LB tumors in the planned amendment of the Neo-APBI-01 trial. Also, this will constitute reflection support for the design of future trials. Finally, each NACRT will likely have its specific predictive biomarkers; therefore, translational studies associated with NACRT trials should be deeply explorative.

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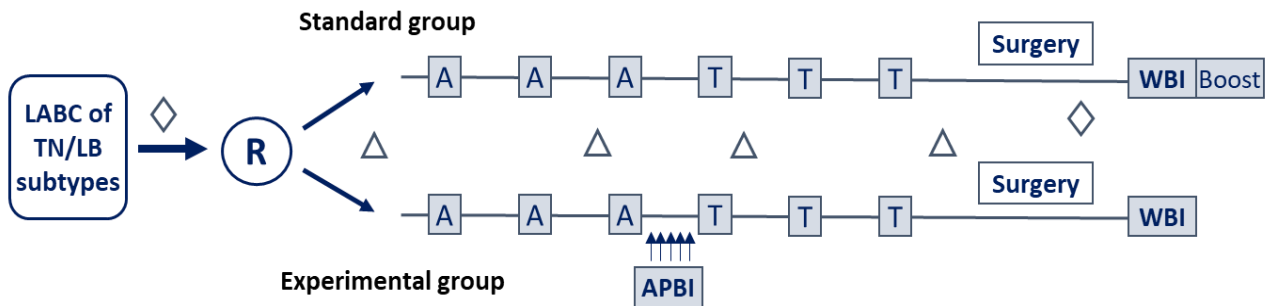
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Figure:



**Figure 1:** Schematic overview of the study. Patients were randomized to receive either standard treatment consisting of anthracycline/taxane-based NACT or experimental treatment with the same NACT and the addition of a short course of tumor-directed APBI between two chemotherapy cycles. *Abbreviation:* A: anthracycline-based chemotherapy (FEC or EC: fluorouracil, epirubicin, cyclophosphamide), APBI: accelerated partial breast irradiation, LABC: locally advanced breast cancer, LB: luminal B, R: randomization, RT: radiation therapy, T: taxane, TN: triple-negative, WBI: whole breast irradiation (or parietal irradiation),  $\Delta$ : blood test,  $\diamond$ : histological analysis (biopsy before randomization and operative piece after breast surgery).

**Table 1:** Standard clinical and histological characteristics of the analyzed cohorts

Variable	N	NACT, N = 21	NACRT, N = 21	<i>p</i> -value <sup>1</sup>
<b>Age (y) Median (IQR)</b>	42	48 (41, 52)	45 (40, 50)	0.3
<b>Menopausal status</b>	42			>0.9
Premenopause		16 (76%)	17 (81%)	
Menopause		5 (24%)	4 (19%)	
<b>Body Mass Index</b>	42			0.5
Normal		10 (48%)	12 (57%)	
High		11 (52%)	9 (43%)	
<b>Tumor size</b>	42			0.2
cT 1		3 (14%)	0 (0%)	
cT 2		15 (71%)	19 (90%)	
cT 3		3 (14%)	2 (9.5%)	
<b>Nodal status</b>	42			0.5
cN 0		9 (43%)	13 (62%)	
cN +		12 (57%)	8 (38%)	
<b>Histological grade</b>	42			0.5
Grade 2		7 (33%)	5 (24%)	
Grade 3		14 (67%)	16 (76%)	
<b>Ki67 Median, (IQR)</b>	42	80 (60, 95)	85 (60, 95)	>0.9
<b>Molecular subtype</b>	42			>0.9
Triple-negative		13 (62%)	13 (62%)	
Luminal B		8 (38%)	8 (38%)	

<sup>1</sup> Wilcoxon rank sum exact test; Fisher's exact test; Pearson's Chi-squared test; Wilcoxon rank sum test

*Legend:* cN: clinical nodal status (-: not involved, +: involved), cT: clinical tumor status, IQR: interquartile range, NACT: neoadjuvant chemotherapy, NACRT: neoadjuvant chemo-radiotherapy



**Table 2:** Response to neoadjuvant therapy

	<b>NACT, N = 21</b>	<b>NACRT, N = 21</b>
<b>pCR, n(%)</b>	<b>N = 7 (33%)</b>	<b>N = 8 (38%)</b>
<b>Triple-negative</b>	5 (71%)	7 (88%)
<b>Luminal B</b>	2 (29%)	1 (12%)
<b>Response to neoadjuvant therapy</b>		
<b>Any primary tumor downstaging</b>	20 (95.3%)	20 (95.3%)
<b>Mean tumor size reduction (mm)</b>	26.6	23.0
<b>cN- to ypN-</b>	8 (38.1%)	12 (57.1%)
<b>cN+ to ypN-</b>	9 (42.9%)	2 (16.7%)
<b>cN- to ypN+</b>	1 (4.8%)	1 (4.8%)
<b>cN+ to ypN+</b>	3 (14.3%)	6 (28.6%)

*Legend: cN: clinical node classification, NACT: neoadjuvant chemotherapy, NACRT: neoadjuvant chemo-radiation therapy, pCR: pathologic complete response, ypN: pathologic nodal stage after neoadjuvant treatment*

**Table 3:** Associations between clinical and peripheral blood parameters and response to neoadjuvant treatment

Parameters	NACT				NACRT			
	pCR+	pCR-	OR (95% CI)	p-value	pCR+	pCR-	OR (95% CI)	p-value
<b>Age (years)</b>	<b>0.004</b>				<b>0.52</b>			
≤ 48	7	4	—		6	8	—	
> 48	0	10	0		2	5	0.53 (0.1-3.5)	
<b>Body mass index</b>	<b>&gt;0.99</b>				<b>0.018</b>			
≤ 26	4	8	—		8	6	—	
> 26	3	6	1 (0.2-6.3)		0	7	0	
<b>LN involvement</b>	<b>&gt;0.99</b>				<b>0.08</b>			
Negative	3	6	—		7	6	—	
Positive	4	8	1 (0.1-6.8)		1	7	0.1 (0.1-1.0)	
<b>Baseline PNN</b>	<b>0.34</b>				<b>0.08</b>			
< 3.3 G/L	5	7	—		1	7	—	
≥ 3.3 G/L	2	7	0.4 (0.1-2.6)		7	6	8.2 (1-177)	
<b>Baseline Hb</b>	<b>0.34</b>				<b>0.006</b>			
< 13 g/dL	5	7	—		0	8	—	
≥ 13 g/dL	2	7	0.4 (0.1-2.6)		8	5	8.2 (1-177)	
<b>Baseline NLR</b>	<b>&gt;0.99</b>				<b>0.03</b>			
≤ 2.2	5	9	—		3	10	—	
> 2.2	2	5	1 (0.1-7.2)		5	3	10 (1.5-101)	
<b>Baseline SII</b>	<b>0.05</b>				<b>0.15</b>			
≤ 252	4	7	—		0	2	—	
> 252	3	7	0.1 (0.01-1)		8	11	3x10 <sup>7</sup> (0-NA)	
<b>Pre-APBI LMR</b>	<b>&gt;0.99</b>				<b>0.08</b>			
< 1.9	3	7	—		2	9	—	
≥ 1.9	4	7	1.3 (0.2-9)		6	4	8.2 (1-177)	

<b>Delta-NLR</b>							<b>0.06</b>
	>0.99						
< 0.8	4	7	—	2	7	—	
≥ 0.8	3	7	1 (0.2-6.4)	6	6	6.8 (1-63)	
<b>Delta-PLR</b>							<b>0.05</b>
	0.74						
< 120	2	5	—	2	8	—	
≥ 120	5	9	1.4 (0.2-12)	6	5	11 (1.4-245)	

*Legend: CI: Confidence Interval, Hb: hemoglobin, LMR: lymphocytes-to-monocytes ratio, LN: lymph node, NACT: neoadjuvant chemotherapy, NACRT: neoadjuvant chemo-radiotherapy, NLR: neutrophils-to-lymphocytes ratio, OR: Odds Ratio, PLR: platelets-to-lymphocytes ratio, pCR: pathologic complete response (+: yes, -: no), PNN: polymorphonuclear neutrophils, SII: systemic immune inflammation index.*

**Table 4:** Associations between tissular parameters and response to treatment

Characteristics	NACT				NACRT			
	pCR+	pCR-	OR (95%CI) <sup>†</sup>	p-value	pCR+	pCR-	OR (95% CI) <sup>†</sup>	p-value
<b>Subtype</b>				0.52				0.08
Triple-negative	5	8	—		7	6	—	
Luminal B	2	6	0.5 (0.1-3.5)		1	7	0.1 (0.1-1.0)	
<b>Ki-67 index</b>				0.54				0.03
< 90%	3	8	—		2	10	—	
≥ 90%	4	6	1.8 (0.3-12)		6	3	10 (1.5-101)	
<b>TILs (%)</b>				0.68				0.1
< 10	4	8	—		3	8	—	
≥ 10	3	4	1.5 (0.2-11)		5	5	4.8 (0.8-43)	
<b>ID phenotype</b>				0.87				0.1
No	5	3	—		8	8	—	
Yes	2	9	1.2 (0.1-9.9)		0	4	0	
<b>TIL-CD8<sup>+</sup></b>				0.01				0.25
< 930/mm <sup>2</sup>	2	11	—		4	9	—	
≥ 930/mm <sup>2</sup>	5	1	28 (2.8-725)		4	3	3 (0.5-23)	
<b>TIL-CD4<sup>+</sup></b>				0.08				0.36
< 1360/mm <sup>2</sup>	2	9	—		3	8	—	
≥ 1360/mm <sup>2</sup>	5	3	6 (0.8-64)		5	4	2.3 (0.4-16)	
<b>TIL-FOXP3<sup>+</sup></b>				0.04				0.55
< 480/mm <sup>2</sup>	3	11	—		5	9	—	
≥ 480/mm <sup>2</sup>	4	1	15 (1.5-356)		3	3	1.8 (0.3-13)	
<b>TIL-CD20<sup>+</sup></b>				0.01				0.36
< 428/mm <sup>2</sup>	1	11	—		3	7	—	
≥ 428/mm <sup>2</sup>	6	1	66 (5-2,648)		5	5	2.3 (0.4-16)	
<b>TIL-T cells</b>				1				0.05

< 3076/mm <sup>2</sup>	3	12	—	3	10	—
≥ 3076/mm <sup>2</sup>	4	0	4x10 <sup>8</sup> (0-NA)	5	2	8.3 (1.2-86)
<b>TIL-CD8/FOXP3</b>				<b>0.02</b>		0.46
< 1.9	2	9	—	4	4	—
≥ 1.9	5	3	18 (2-426)	4	8	0.5 (0.1-3.1)
<b>TIL-CD8/CD4</b>				<b>0.03</b>		0.46
< 0.4	2	9	—	4	4	—
≥ 0.4	4	3	15 (1.6-360)	4	8	0.5 (0.1-3.1)
<b>PD-L1 ic</b>				<b>0.07</b>		0.51
< 10%	3	11	—	5	5	—
≥ 10%	3	1	11 (1-276)	2	4	0.5 (0.1-3.9)
<b>TP53 mutation</b>				0.68		0.2
No	1	3	—	<b>0</b>	4	—
Yes	6	9	1.5 (0.2-11)	<b>6</b>	7	9x10 <sup>7</sup> (0-NA)

*Legend: CI: Confidence Interval, ID: immune-deserted, NACT: neoadjuvant chemotherapy, NACRT: neoadjuvant chemo-radiotherapy, OR: Odds Ratio, pCR: pathologic complete response (+: yes, -: no), PD-L1 ic: programmed death-ligand 1 presented on immune cells, TIL: tumor-infiltrating lymphocyte.*

**Table 5:** Prospective studies assessing preoperative, tumor-directed APBI

Author	N	Tumor characteristics	RT Dose	NACT	Time to surgery	pCR rate
Bondiau et al., 2013 [10]	25	Unifocal, HER2-, BCS unsuitable	3x6.5-10.5Gy	Yes	4-8 weeks	36%
Van der Leij et al., 2015 [49]	70	> 60 yo, unifocal $\leq$ 3 cm, SLN-	10x4Gy or 5x6Gy	No	6 weeks	10% (near-pCR)
Nichols et al., 2017 [12]	27	Unifocal, < 3 cm, cN0	10x3.85Gy bid	No	21 days	15%
Horton et al., 2018 [45]	32	$\geq$ 55 yo, T1, cN0, ER/PR+ HER-	1x15-21Gy	No	10 days	NR
Yaremko et al., 2018 [50]	39	Unifocal, < 3 cm, cN0, ER+	1x21Gy	No	1 week	NR
Current study	21	Locally advanced TNBC, LB	10x2.5Gy bid or 8x3.125Gy qid	Yes	4-6 weeks	38%

*Legend: BCS: breast-conserving surgery, bid: twice a day, ER: estrogen receptor, HER2: human epidermal growth factor receptor 2, ICI: immune checkpoint inhibitor (pembrolizumab), LB: luminal B, mTNBC: metastatic triple-negative breast cancer, N: number of patients, NA: not applied, NACT: neoadjuvant chemotherapy, NR: not reported, ORR: overall response rate, pCR: pathologic complete response, PR: progesterone receptor, qid: once a day, SLN: sentinel lymph node, yo: years old.*

**Supplementary Table 1:** Immunohistochemical staining

Antigen	Clone	Distributor	Dilution	Incubation	Detection	Interpretation
<b>p53</b>	DO-7	Agilent	1/200	40 min	ultraView DAB	According to [51]. Strong diffuse nuclear staining in tumor cells: TP53 missense mutation(s) No nuclear staining in tumor cells with heterogeneous moderate/weak staining in benign cells: TP53 'null' mutation Heterogeneous moderate/weak nuclear staining in tumor cells: no TP53 mutation
<b>pRb</b>	358	Novocastra	1/50	60 min	OptiView DAB	pRb present: tumor cell nuclear staining*
<b>CD8</b>	SP16	ThermoFisher Scientific	1/200	60 min	OptiView DAB	CD8+ TILs: with membranous staining*
<b>CD4</b>	SP35	CellMarque	1/50	60 min	ultraView DAB	CD4+ TILs: with membranous staining*
<b>CD20</b>	SP32	CellMarque	1/200	60 min	ultraView DAB	CD20+ TILs: with membranous staining*
<b>FoxP3</b>	SP97	ThermoFisher Scientific	1/100	60 min	ultraView DAB	FoxP3+ TILs: with nuclear staining*
<b>PD-L1</b>	28-8	Abcam	1/50	60 min	OptiView DAB	% of tumor cells with membranous staining* % of immune cells (TILs and macrophages) with membranous staining*

*Legend: ultraView DAB, ultraView Universal DAB Detection Kit (Ventana/Roche, Meylan, France); OptiView DAB, OptiView DAB IHC Detection Kit*

*(Ventana/Roche, Meylan, France); \*any intensity; TILs, tumor-infiltrating lymphocytes. All stainings were performed in Benchmark Ultra automates*

*(Ventana/Roche, Meylan, France).*

**Supplementary Table 2:** Immune-related histological markers

Variable	N	NACT, N = 21 <sup>1</sup>	NACRT, N = 21 <sup>1</sup>	p-value <sup>2</sup>
<b>Immuno-phenotype</b>	39			0.8
Inflamed		5 (26%)	7 (35%)	
Excluded		9 (47%)	9 (45%)	
Deserted		5 (26%)	4 (20%)	
<b>Tumor-infiltrating lymphocytes</b>	40			0.5
< 10%		12 (63%)	10 (48%)	
≥10% - <30%		4 (21%)	7 (33%)	
≥30% - <50%		0 (0%)	2 (9.5%)	
≥50%		3 (16%)	2 (9.5%)	
<b>TIL subpopulations (per mm<sup>2</sup>)</b>				
TIL CD8+	39	399 (235, 1,097)	583 (269, 1,069)	0.6
TIL CD4+	38	1,227 (595, 1,577)	1,255 (855, 2,099)	0.4
TIL FOXP3+	39	234 (95, 445)	331 (140, 562)	0.5
TIL CD20+	39	226 (53, 604)	446 (216, 1,238)	0.2
TIL T lymphocytes	39	1,502 (770, 2,420)	2,011 (1,020, 3,365)	0.4
TIL total lymphocytes	39	1,902 (1,002, 2,819)	2,502 (1,249, 4,603)	0.5
<b>TP53 mutation status</b>	36			>0.9
No mutation		4 (21%)	4 (24%)	
Mutated type null		8 (42%)	6 (35%)	
Mutated type missense		7 (37%)	7 (41%)	
<b>pRb status</b>	36			>0.9
Absent		5 (26%)	4 (24%)	
Present		14 (74%)	13 (76%)	

<sup>1</sup> n (%); Median (IQR)<sup>2</sup> Fisher's exact test; Wilcoxon rank sum exact test

*Legend: NACT: neoadjuvant chemotherapy, NACRT: neoadjuvant chemo-radiotherapy, TIL: tumor-infiltrating lymphocyte.*



**Supplementary Table 3:** Immune-related systemic markers

Variable	N	NACT, N = 21 <sup>1</sup>	NACRT, N = 21 <sup>1</sup>	p-value <sup>2</sup>
Leukocyte (G/L)	42	5.80 (4.60, 6.70)	6.60 (5.70, 7.84)	0.2
Neutrophil (G/L)	42	3.06 (2.10, 4.22)	3.94 (2.92, 5.24)	0.11
lymphocyte (G/L)	42	1.70 (1.57, 2.48)	1.83 (1.41, 2.35)	>0.9
Monocyte (G/L)	42	0.48 (0.34, 0.50)	0.50 (0.40, 0.62)	0.2
Platelet (G/L)	42	271 (222, 301)	309 (265, 361)	<b>0.05</b>
Hemoglobin (g/dL)	42	12.90 (12.70, 13.60)	13.40 (12.50, 13.90)	0.5
NLR	42	1.77 (1.02, 2.24)	1.77 (1.22, 3.29)	0.3
PLR	42	149 (109, 186)	201 (125, 211)	<b>0.10</b>
LMR	42	4.00 (3.31, 4.71)	3.55 (3.13, 4.61)	0.4
SII	42	513 (253, 825)	650 (378, 1,018)	<b>0.087</b>

<sup>1</sup> Median (IQR)<sup>2</sup> Wilcoxon rank sum test; Wilcoxon rank sum exact test

*Legend: LMR: lymphocyte-monocyte, NACT: neoadjuvant chemotherapy, NACRT: neoadjuvant chemo-radiotherapy, NLR: neutrophil-lymphocyte ratio, PLR: platelet-lymphocyte ratio, SII: systemic immune-inflammation index.*

## Discussion

Dans cette section, notre objectif est d'apporter des éléments plus approfondis sur les résultats obtenus lors de mes recherches dans les deux articles consacrés aux cancers du sein, et de discuter des perspectives dans le domaine des biomarqueurs prédictifs de réponse.

Les progrès de techniques d'irradiation ont permis de proposer de nouvelles approches thérapeutiques et d'améliorer le résultat oncologique des patientes atteintes de cancer du sein (Corradini et al., 2019; Ho et al., 2020b). La diffusion des schémas hypofractionnés à hautes doses apporte des perspectives de développement très ambitieux de traitements RI combinés à de nouvelles molécules, telle l'immunothérapie, sous-tendu d'un rationnel biologique robuste.

L'irradiation tumorale à haute dose, seule ou associée à d'autres traitements systémiques, peut provoquer une réponse immunitaire favorable et augmenter ainsi la réponse objective (Bondiau et al., 2013; Ho et al., 2020a). La RTNA, effectuée avant la chirurgie, fournit également une occasion unique d'évaluer de façon *in vivo* la réponse des tumeurs mammaires aux RI et permet d'étudier les marqueurs de réponse potentiels contribuant à la perspective de personnalisation des traitements.

### **La réponse à la CRTNA dans l'étude Néo-APBI-01**

Dans l'analyse préliminaire des 42 premières patientes de Néo-APBI-01, les taux de pCR dans le groupe CRTNA et CTNA était de 38% et 33%, respectivement ( $p=0.7$ ). Ces taux de réponse sont corrélés à ceux rapportés dans la littérature. Des études plus anciennes évaluant la RT du sein en totalité en préopératoire ont été réalisées chez les patientes avec une maladie localement avancée non éligible à une chirurgie.

La CRTNA a permis un taux de pCR entre 27 et 34% des patientes et a rendu une grande majorité opérable (Adams et al., 2010; Bollet et al., 2012; Brackstone et al., 2017; Matuschek et al., 2012b). Cependant, les complications postopératoires et tardives fréquentes ont largement limité son utilisation (Brackstone et al., 2017).

Parmi les quelques études rapportées sur la RT préopératoire limitée à la tumeur dans les tumeurs mammaires, une seule évaluant l'association de la APBI aux taxanes a montré un taux de pCR de 36% (Bondiau et al., 2013). De plus, cette étude a montré un effet dose-réponse, observé avec 3 x 8.5 Gy pour un taux de pCR atteignant 67% (n=4/6) (Bondiau et al., 2013). Cet effet dose-réponse est également observé chez nos malades. La pCR n'a été obtenue chez aucune des patientes ayant reçu une dose RT de moins de 25 Gy (n=4) alors que 43% (n=6/14) et 67% (n=2/3) des patientes ont eu une pCR après des fractions de 2.5 Gy, deux fois par jour en 5 jours consécutifs ou des fractions quotidiens de 3.125 Gy, cinq jours par semaine, pour une dose totale de 25 Gy.

Certaines remarques doivent être faites sur les résultats préliminaires de notre étude translationnelle de Néo-APBI 01, notamment une grande disparité en termes de réponse en fonction du sous-type moléculaire.

Les cancers du sous-type LB semblent être plus sensibles à la CTNA seule (pCR à 25%) qu'à la CRTNA (pCR à 12%). Pour les CSTN, l'effet semble être inversé, avec 54% de pCR pour la CRTNA vs. 38% de pCR pour la CTNA. Selon les résultats obtenus après la CTNA dans la littérature, une moins bonne réponse à la CRTNA dans le sous-type LB est attendue. Il en est de même pour les anciennes études de CRT dans les cancers du sein souvent localement avancés (Lightowers et al., 2017). En revanche, une perte d'efficacité dans le contexte d'un traitement combinant de façon séquentielle une APBI à la CTNA chez les patientes LB était totalement inattendue.

L'immunité locale peut contribuer considérablement à la réponse à la RT au-delà de la radiosensibilité intrinsèque. En effet, un TME immune-effectif peut être nécessaire pour obtenir un bénéfice avec la RT dans le cancer du sein (Cui et al., 2018). La délivrance de l'APBI préopératoire, au milieu de la CTNA, où l'immunité locale est appauvrie, pourrait être une des raisons de l'inexploitation l'effet immunomodulateur antitumoral. Cet effet peut être plus profond dans le sous-type LB dont les niveaux de TILs sont plus bas que les tumeurs TN (Denkert et al., 2018; Stanton et al., 2016) expliquant la différence en termes de réponse des sous-types de cancers du sein de notre étude.

Malgré la meilleure réponse aux traitements, il est important de souligner que la plupart des patientes récidivantes ont aussi le sous-type TN (86%, n=6/7). Cette observation a été rapportée dans une autre étude associant la RT et l'immunothérapie, des réponses encourageantes à la combinaison avaient été notées, néanmoins, toutes sauf une patiente ont progressé ultérieurement (Ho et al., 2020a).

Une des premières conclusions de ce travail préliminaire est probablement d'exclure les LB de la suite des inclusions dans l'étude Néo-APBI-01 et de continuer à développer les marqueurs prédictifs de réponse à l'association CRT afin de mieux sélectionner les malades qui en bénéficieront.

### **Les potentiels paramètres associés à la réponse à la CRTNA**

L'une des premières questions que nous nous sommes posés dès le début des travaux a concerné les réponses, ou a contrario, les échecs inattendus après la CRTNA. Nous avons ainsi étudié les profils biologiques tumoraux chez deux patientes ayant un cancer du sein du sous-type TN et qui ont présenté des réponses inhabituelles à l'association CRT. Aucune caractéristique clinico-pathologique de base pouvait

indiquer que la patiente avec une atteinte ganglionnaire initiale allait présenter une réponse complète exceptionnelle (REx) au niveau tumoral mais surtout ganglionnaire axillaire non irradié. En revanche, la patiente non répondeuse exceptionnelle (NREx) s'est présentée avec une progression de la maladie sous traitement, une grosse tumeur résiduelle et le développement rapide de métastases.

Dans le profil biopathologique tumoral de cette patiente NREx, le statut p53-/pRb- a été le seul paramètre retrouvé comme indicatif de la forte résistance aux traitements parmi les patientes incluses dans l'étude. Dans la littérature, l'activation des voies de signalisation p53 et pRb ont été corrélée *in vivo* à la résistance aux dommages à l'ADN (Knappskog et al., 2015). Par ailleurs, la déficience de RB1 est aussi impliquée dans la promotion de cellules souches cancéreuses et la progression métastatique (Zacksenhaus et al., 2017). A noter que chez la patiente REx, la tumeur présente un statut double positif p53/pRb, pouvant expliquer la meilleure réponse obtenue après la CRT (Darb-Esfahani et al., 2016; Gupta et al., 2012). En plus de la différence du statut p53/pRb entre ces deux patientes, les paramètres sanguins et leur cinétique se distinguaient aussi considérablement. Nous avons donc décidé, après ces résultats, d'inclure ces biomarqueurs dans une analyse exhaustive chez la totalité des patientes de l'étude Néo-APBI-01.

En évaluant les paramètres clinico-pathologiques et ceux de l'immunité, nous avons identifié plusieurs facteurs associés à la réponse, qui se différencient par rapport aux traitements assignés. Chez les patientes ayant reçu la CRTNA, nous avons observé une corrélation entre la réponse et certains paramètres rapportés dans la littérature pour d'autres cancers (ORL, col utérin) comme le taux d'hémoglobine (Hb) ou encore l'indice de masse corporelle (IMC) et les index dérivés de l'inflammation systémique. Par ailleurs, nous avons retrouvé les paramètres bien établis corrélés à la pCR après

CTNA comme le jeune âge, la proportion élevée des TILs et les sous-populations de TILs.

Dans le groupe des patientes ayant été traitées par la CRTNA, un taux d'Hb  $\geq 13$  g/dL et un IMC  $< 26$  sont fortement associés à la pCR. L'impact négatif de l'anémie (en utilisant un seuil d'Hb de 9 à 14,5 g/dL) est significativement corrélé à des mauvais résultats cliniques et à la résistance à la RT dans différentes tumeurs solides (Harrison et al., 2002). Toutes les patientes avec un taux de Hb  $\geq 13$  g/dL ont répondu complètement à la CRTNA dans notre étude. Il a été démontré récemment qu'une oxygénation adéquate pouvait supprimer les métabolites et les cytokines immunosuppresseurs dans le TME et entraîner une réponse immunité-médiée favorable à la RT (Riera-Domingo et al., 2020).

D'autres facteurs liés à l'immunité pourraient également modifier la réponse aux traitements anticancéreux. Des études ont montré qu'une graisse corporelle excessive est liée à une inflammation chronique, qui diminue la réponse immunitaire antitumorale (Hanahan and Weinberg, 2011). Il a été rapporté que les patients en surpoids et obèses (définis comme avoir un IMC  $\geq 25$  et 30, respectivement) bénéficiaient de moins d'efficacité et présentaient plus de toxicités lors des traitements anticancéreux traditionnels (Di Cosimo et al., 2020; Wang et al., 2021).

Les cellules immunitaires, acteurs principaux de l'immunité, à la fois dans le sang périphérique et dans le TME, ont contribué à la réponse à la CRTNA dans notre cohorte. Contrairement aux publications sur la CTNA dans le cancer du sein, des taux élevés avant traitement de PNN ( $\geq 3,3$ ) et de NLR ( $> 2,2$ ) sont favorable pour la pCR dans le groupe CRTNA. Les neutrophiles (PNN pour *polynuclear neutrophil*) sont les leucocytes les plus abondants dans le sang et émergent comme d'importants régulateurs du cancer (Hedrick and Malanchi, 2022). Des études récentes ont révélé

les fonctions diverses des PNN ; qui, dans certains contextes, peuvent exercer des fortes activités antitumorales (Hedrick and Malanchi, 2022). De plus, la RT à la tumeur mammaire en place produit les cytokines inflammatoires telles que TNF- $\alpha$ , IFN- $\gamma$  promouvant la différenciation des PNN vers le phénotype N1 antitumoral. Ces résultats expliquent les controverses sur la valeur pronostique et prédictive du rapport NLR dans différents cancers (Corbeau et al., 2020; Suppan et al., 2015). Dans le cancer du sein, des taux bas de NLR ont été souvent rapportés comme étant un facteur pronostique et prédictive favorable (Corbeau et al., 2020). En revanche, dans notre cohorte, des taux élevés de PNN et de NLR étaient associés à une moins bonne réponse à la CRTNA.

Au niveau tumoral, une proportion plus élevée de TILs, ainsi qu'un nombre plus élevé des LyT (CD8+ et CD4+) sont favorables pour la pCR à la CRTNA dans notre étude. Comme évoqué dans la section précédente, le fait d'intégrer la RT au milieu de la CTNA peut être la raison pour laquelle l'effet immunogène antitumoral de la RT n'ait pas été observé. Ce manque d'effet est encore plus probant dans le sous-groupe LB dont le niveau d'infiltration de TILs est typiquement faible (Stanton et al., 2016). La seule tumeur LB ayant une pCR après la CRTNA avaient un TIL beaucoup plus riche par rapport aux autres tumeurs LB de la cohorte.

De plus, une fraction des cancers du sein riche en TIL présente des défauts dans la voie de réparation de l'ADN et serait plus résistante aux thérapies induisant des dommages à l'ADN, dont la RT. Une autre observation intéressante est qu'aucune patiente sans mutation de *TP53* a une pCR après la CRTNA. La plupart des mutations sont de type *missense* et aucune tumeur mutée *TP53* n'a le phénotype immunitaire ID (dont aucune n'a eu une pCR à la CRTNA). Le dépistage des mutations *TP53* en IHC

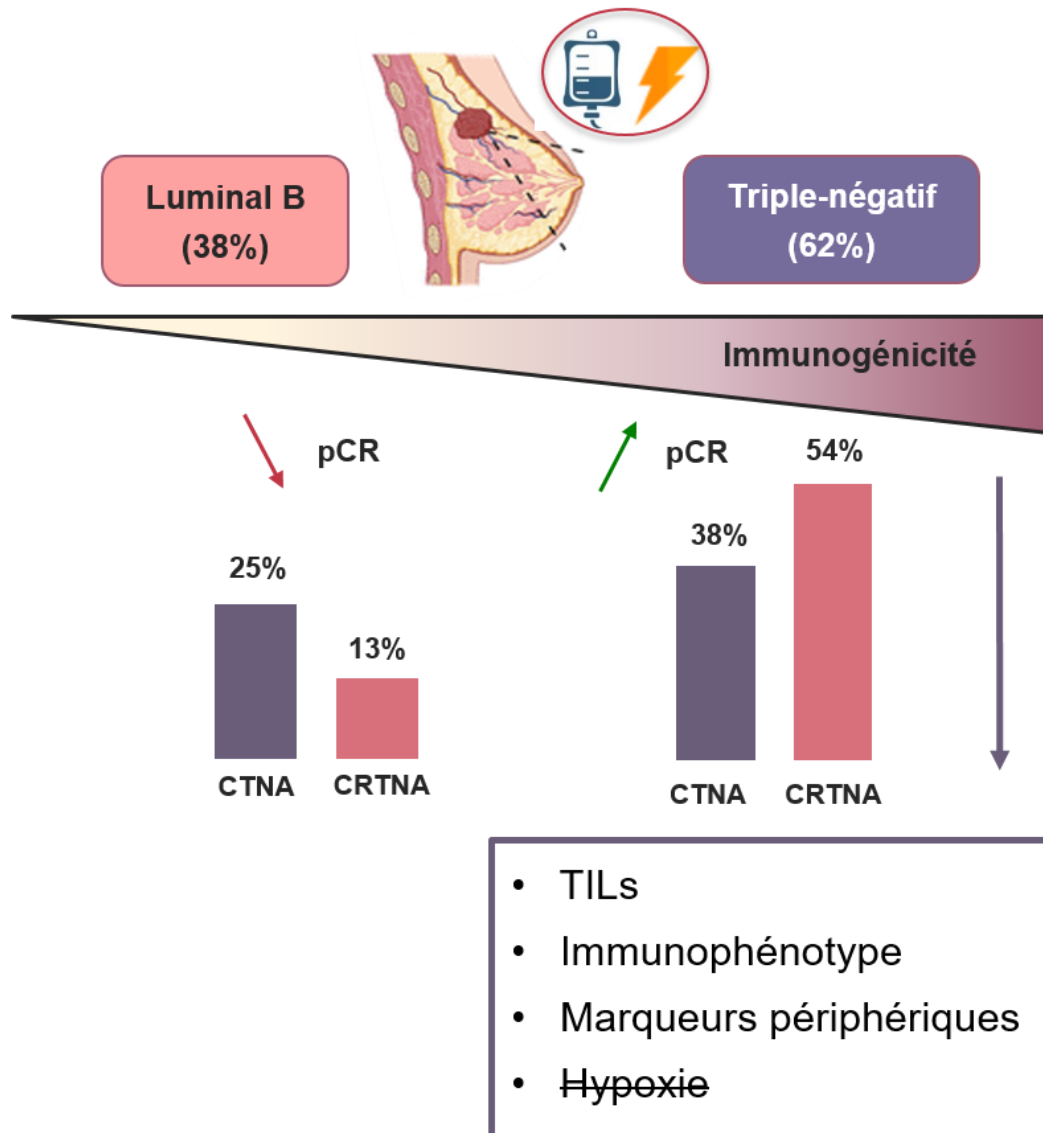
pourrait donner un aperçu de la sensibilité à la CRT et ainsi nous aider à mieux sélectionner les patientes avec une telle approche.

Toutefois, la recherche des biomarqueurs prédictifs à la réponse à la RT reste un encore un vaste domaine à étudier, compte tenu de la relation complexe entre les RI, la tumeur et l'hôte. Il est donc nécessaire de poursuivre les recherches des marqueurs théranostiques et leurs potentielles thérapies afin de rendre le traitement par RT dans le cancer du sein plus adapté et plus efficace.

La Figure 11 résume de façon schématique des résultats de cette deuxième partie évaluant les effets potentiels sur le système immunitaire d'une radiothérapie préopératoire sur la tumeur mammaire en place chez des patientes ayant un cancer du sein agressif. En effet, la réponse à la CRTNA est fortement conditionnée par l'immunité tumorale des cancers du sein. En l'occurrence, les tumeurs luminales B ainsi que les tumeurs peu immunogènes ont répondu défavorablement à l'ajout de la RT à la CTNA. En revanche, les CSTN et les tumeurs mieux infiltrées ont présenté un meilleur taux de pCR avec cette association par rapport à la CTNA seule.

Cette immunogénicité peut être témoignée par l'infiltration lymphocytaire tumorale et l'immuno-phénotype, mais aussi par des biomarqueurs périphériques dérivés de l'immunité, et de façon indirecte, par l'absence d'anémie et d'hypoxie.





**Figure 11** : Schéma récapitulatif des effets de la radiothérapie sur le système immunitaire impliqués dans le modèle clinique de cancer du sein agressif.

## Perspectives

Plusieurs décennies de recherche et de développement des traitements anticancéreux dans le cancer du sein ont permis d'identifier des marqueurs robustes de la réponse à une thérapie spécifique, tels que le statut de RO pour l'hormonothérapie et HER2 pour les anti-HER2 (Harris et al., 2007). L'expression génique a été largement étudiée, permettant ainsi de distinguer des sous-types moléculaires distincts (Perou et al., 2000) et a fourni une vraie valeur pronostique et prédictive dans le cancer du sein. Des signatures génomiques comme Oncotype DX et MammaPrint ont été approuvées pour sélectionner les patientes pour une CTA (Kalinsky et al., 2021; Sparano et al., 2018).

Des efforts pour mieux comprendre l'effet de la RT dans le cancer du sein sont en cours. Malgré le bénéfice incontestable de la RT adjuvante dans les cancers du sein de stade précoce, 21% à 66% des patientes ont eu une récurrence, confirmant ainsi l'hétérogénéité de la maladie (EBCTCG (Early Breast Cancer Trialists' Collaborative Group), 2014). Il y a eu un grand intérêt d'intégrer des informations sur la biologie tumorale en plus des paramètres cliniques et pathologiques pour améliorer la prise de décision et la personnalisation de la RT mammaire (Cui et al., 2018; Servant et al., 2012; Sjöström et al., 2019; Tramm et al., 2014). Des signatures génomiques à haut débit, incluant les gènes impliqués dans le cycle cellulaire, la prolifération et les activités kinase, ont été évaluées, mais la plupart des études n'ont pas pu être validées dans des bases de données indépendantes (Servant et al., 2012), ni prédire le bénéfice de la RT (Tramm et al., 2014). La signature génomique combinée de la radiosensibilité intrinsèque et d'immunité intratumorale développée par Cui et al. a été prédictif du bénéfice de la RT en termes de survie spécifique à la maladie dans une cohorte rétrospective composée principalement de patientes mastectomisées (Cui et al., 2018). L'ARTIC (*Adjuvant Radiotherapy Intensification Classifier*) a été validé

comme étant un score prédictif de la RT adjuvante dans l'essai phase 3 SweBCG91-RT, mais seulement une minorité des patientes avait reçu un traitement systémique adjuvant, ce qui ne reflète pas la pratique actuelle (Sjöström et al., 2019).

Des études prospectives, randomisées incluant les signatures génomiques sont en cours. Le but est d'identifier les patientes à faible risque qui ne nécessiteraient pas de RT adjuvante après une chirurgie mammaire conservatrice adéquate (IDEA, NCT02400190 ; PRECISION, NCT02653755 ; LUMINA, NCT01791829) ou n'auraient besoin que d'une RT mammaire sans nécessité d'une irradiation ganglionnaire (TAILOR RT, NCT03488693).

En parallèle, des biomarqueurs théranostiques avec une potentielle thérapeutique ont suscité de grands intérêts. Parmi eux, les micro-ARNs (miRs) sont activement investigués.

Les micro-ARNs (miRs) sont une classe majeure de molécules d'ARN de simple brin non codantes endogènes de très petites tailles (19-25 nucléotides), qui jouent un rôle régulateur dans l'expression des gènes au niveau post-transcriptionnel dans de multiples voies de signalisation (Ambros, 2004) impliqués dans différents processus du cancer (Peng and Croce, 2016). De plus, leur spécificité et leur stabilité font des miRs des biomarqueurs théranostiques potentiels du cancer (Bertoli et al., 2015).

La dérégulation des miRs peut se traduire par une réponse favorable ou défavorable aux RI dans les cellules tumorales. En général, la « uprégulation » des miRs oncogènes comme miR-21, miR-27a, miR-221 ou la « downrégulation » des miRs suppresseurs de tumeurs comme la famille de let-7 ou miR-34 confère une radorésistance. En corrigeant ces dérégulations des miRs, plusieurs équipes ont pu

montrer une nouvelle approche thérapeutique sensibilisant les cellules tumorales aux RI, surmontant ainsi leur radiorésistance intrinsèque.

Le rôle émergent des miRs en tant que biomarqueurs théranostiques et régulateurs modifiant la radiosensibilité dans le cancer du sein TN a été abordé dans une revue systématique de la littérature que j'ai réalisée au cours de ma thèse (Annexe 5).



## CONCLUSION

En conclusion des travaux réalisés au cours de cette thèse, nous avons pu observer un impact significatif de la radiothérapie sur le système immunitaire de différents modèles d'expérimentation.

Dans les modèles murins d'allogreffe, nous avons abordé, pour la première fois, une question pratique sur l'impact des paramètres des RI souvent négligés dans les expériences précliniques. Nos résultats ont suggéré que les deux modalités d'irradiation à rayons X (RxHE par LINAC et RxBE par CP160) pourraient avoir les mêmes conséquences en termes d'effet létal et de la prise de greffe post-transplantation. Cependant, les réponses alloréactives varient considérablement entre les deux méthodes d'ICT, et sont médiées par une sécrétion différentielle des cytokines inflammatoires post-RT. Nous avons ensuite démontré qu'en retardant l'injection des CSH, cette réaction immunitaire pouvait être modulée. Ainsi, nos travaux ont montré la nécessité de bien définir les paramètres de l'ICT du petit animal pour permettre une meilleure interprétation homogène des modèles murins de GVHD/GVL.

Dans le modèle clinique de cancer du sein, nous avons présenté l'analyse translationnelle préliminaire de l'essai Néo-APBI-01 évaluant l'association CRTNA chez les patientes ayant un cancer du sein localement avancé de sous-type moléculaire TN et LB. Nous avons observé pour la première fois, l'effet délétère en ajoutant une RTNA focalisée à la tumeur au cours d'une CTNA standard dans les tumeurs de sous-type LB et des tumeurs avec des taux faibles de TILs. Nous avons aussi identifié différents paramètres associés à la pCR après le traitement néoadjuvant. Nos travaux permettent d'ouvrir des perspectives pour la recherche de nouveaux facteurs prédictifs de la réponse et personnaliser les futures stratégies

thérapeutiques dans les cancers TN. De plus, ce travail préliminaire va permettre un ajustement majeur du protocole thérapeutique en cours en excluant les cancers LB initialement prévus dans l'essai et en ajoutant la possibilité de faire bénéficier les patientes de la synergie entre la RTNA et l'immunothérapie devenue un standard pour les cancers TN.

Au total, nos travaux confirment que les effets de la RT sont en partie médiés par le système immunitaire et inversement. Une meilleure connaissance de cette relation complexe entre le système immunitaire et la RT est un enjeu thérapeutique majeur pour une prise en charge plus efficace et plus adaptée à l'ère de la personnalisation des traitements.







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## ANNEXES

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Annexe 1 : Certificat de formation RERF





## Annexe 2 : Revue de la sénescence radio-induite

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## Ionizing radiation-induced cellular senescence promotes tissue fibrosis after radiotherapy. A review



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## ABSTRACT

Ionizing radiation-exposure induces a variety of cellular reactions, such as *senescence* and *apoptosis*. Senescence is a permanent arrest state of the cell division, which can be beneficial or detrimental for normal tissue via an inflammatory response and senescence-associated secretion phenotype. Damage to healthy cells and their microenvironment is considered as an important source of early and late complications with an increased risk of morbidity in patients after radiotherapy (RT). In addition, the benefit/risk ratio may depend on the radiation technique/dose used for cancer eradication and the irradiated volume of healthy tissues. For radiation-induced fibrosis risk, the knowledge of mechanisms and potential prevention has become a crucial point to determining radiation parameters and patients' intrinsic radiosensitivity. This review summarizes our understanding of ionizing radiation-induced senescent cell in fibrogenesis. This mechanism may provide new insights for therapeutic modalities for better risk/benefit ratios after RT in the new era of personalized treatments.

## 1. Introduction

Ionizing radiation (IR) exposure can induce a variety of cellular reactions, such as *apoptosis* and *senescence*. Cellular senescence plays an important role in embryonic development and in shaping organogenesis, wound healing and tumor suppression (He and Sharpless, 2017). A senescent cell is a potent anti-cancer mechanism that can occur in virtually any cell, including fibroblasts, epithelial cells, melanocytes, endothelial cells, and astrocytes (He and Sharpless, 2017; Chinta et al., 2015). It remains viable and metabolically active even though it does not undergo cell division and permanently arrests cell proliferation (He and Sharpless, 2017; Campisi and d'Adda di Fagagna, 2007).

At the tissue level, cellular senescence increases with age in many renewable tissues which are discovered at sites of age-related diseases, including degenerative disorders, malignant and benign diseases. In addition, researches on senescence biomarkers have to take into account specific contexts and different cell types. In the lung tissue, senescent fibroblasts could trigger fibrogenesis (Schafer et al., 2017),

while senescent activated hepatic stellate cells could diminish liver cirrhosis (Krizhanovsky et al., 2008). The impact of cellular senescence in tissue may be beneficial or harmful, which depend on the triggered factors, tissue, and cell type (Schafer et al., 2018). At the cellular level, the long-drawn presence of senescent cells in normal tissues may stimulate potency damage, predominantly due to the persistence of related inflammatory responses (Lasry and Ben-Neriah, 2015). Conversely, cellular senescence can have a beneficial response and prevent the growth of premalignant cells and activate cancer immune surveillance.

IR-induced cellular senescence as a promoter of fibrotic sequelae has been shown in several models recently. In lung, data from Beach et al. have suggested radiation-induced pulmonary fibrosis as a step in progressive fibrosis (Beach et al., 2017). Recently, Schafer et al. showed that IR-induced senescent fibroblasts contribute to lung fibrosis via their profibrotic secretomes (Schafer et al., 2017). Korpela and Liu reported that radiation-induced senescence is involved in endothelial cells (Korpela and Liu, 2014). Hong et al. reported articular

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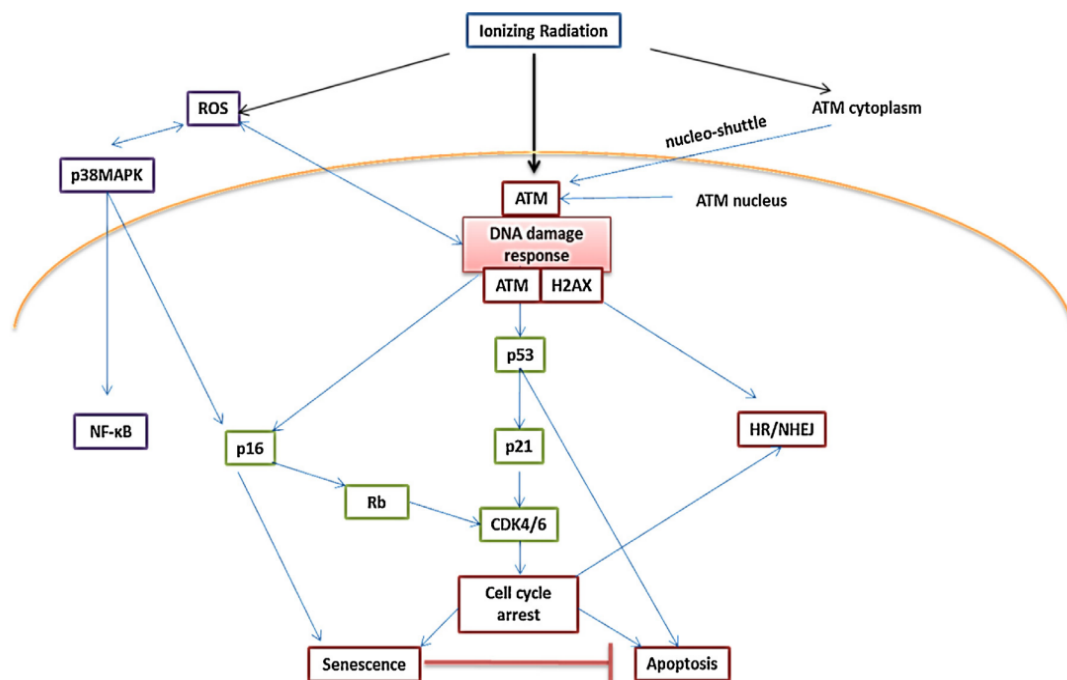


Fig. 1. DNA damage response induced senescence by ionizing radiation.

IR induced nucleo-shuttling of ATM. The ATM kinase activated, permit accurate phosphorylation of H2 AX and other DNA repair protein by two main repairs NHEJ, HR. The DNA damage response (DDR) after IR leads to stimulate p38 mitogen-activated protein kinase (MAPK) through ROS, ROS also produced after radiolysis of water, in turns activate transcription factor nuclear factor kappa-B, in turn production of p16INK4A, p16 INK4A activates the pRb tumor suppressor, which block certain proliferative genes, ultimately induces durable cell-cycle arrest. DDR activates not only p16/Rb but also p53 tumor suppressor, then turns on the p21 WAF1 gene transcription, ultimately causes senescence permanent growth arrest. Both the p53/p21 and p16INK4a/pRb pathways are clearly of major importance in cellular senescence by inhibiting the cyclin-dependent kinase (CDK) to exit from cell division cycle, thus involve in cellular arrest and oncogene-induced senescence, which is characterized by overexpression of p16, p53 or p21 proteins and resistant to apoptosis. (ATM: Ataxia Telangiectasia Mutated protein; CDK: cyclin-dependent kinase; DNA: Deoxyribonucleic acid; H2 AX: histone H2A family; IR: Ionizing radiation; NF-κB: nuclear factor kappa-B; NHEJ: non-homologous end joining; p38MAPK: p38 mitogen-activated protein kinase; pRb: retinoblastoma protein ROS: Reactive Oxygen Species).

chondrocytes damage after radiation via the senescence pathway (Hong et al., 2010). In the heart, Liu et al. recently reported that radiation can induce myocardial fibrosis after RT for thoracic tumors (Liu et al., 2017). However, the authors have not shown that IR-induced senescence promotes a fibrotic myocardium.

In this review, we aimed to focus on IR-induced senescence accelerated tissue fibrosis. We hypothesize that this mechanism may provide new insights into novel therapeutic modalities in RT to prevent or cure radiation-induced fibrosis in patients who have a long-term outcome after irradiation.

## 2. Methods

### 2.1. Data sources

We searched for relevant studies by using the electronic resources of the “Pubmed” database from 1977 to 2018. The search language was limited to publications written in English and French.

### 2.2. Search strategy

A literature search strategy involving the keywords “radiation-induced toxicity”, “radiotoxicity” “radiation-induced senescence”, “radiation-induced fibrosis”, “radiotherapy-induced toxicity”, “radio-sensitivity AND radioresistance”, “radiation AND senescence”, “senescence AND fibrosis”, “radiation AND fibrosis”, “senescence AND senotherapeutics”, “senescence AND senolytics”, “senescence AND senomorphics”, and “senescence AND SASP inhibitors” was used. We screened keywords, titles and abstracts of all retrieved articles and selected significant articles for full text. Electronic links to related articles

and references of selected articles were hand-searched. Eligible articles included meta-analyses, prospective studies, multicenter studies, clinical trials, reviews and systematic reviews.

## 3. Results

### 3.1. Radiation-induced senescent cells mechanisms

Exposure to IR can lead to many cellular responses, including a modification of gene expression as well as deoxyribonucleic acid (DNA) damage which occurs either as a direct effect of radiation on DNA molecules or by an indirect action by the free radicals genesis and reactive oxygen species (ROS). DNA double-strand breaks (DSBs) are one of the most genotoxic lesions in the genome, with 5% unable to be repaired. Unrepaired DSBs after irradiation can lead to cell death, such as apoptosis, senescence, mutation or genomic instability. IR induces cell apoptosis and senescence via multiple pathways (Islam, 2017; Liauw et al., 2013a; Santivasi and Xia, 2014).

Several authors have shown that radiation can lead to the oxidation of ataxia telangiectasia mutated (ATM) protein dimers converted into ATM monomers. The resulting ATM monomers are nucleo-shuttled, to allow DSB recognition via the phosphorylation of H2 AX ( $\gamma$ -H2 AX) and execute DSBs repair by two main repair pathways non-homologous end joining (NHEJ), prevalent throughout all phases of the cell cycle and homologous recombination (HR), prevalent during late S-phase and G2-phase (Islam, 2017; Santivasi and Xia, 2014; Belkacemi et al., 2016; Bodgi et al., 2013; Bodgi and Foray, 2016; COPERNIC project investigators et al., 2016). The DNA damage response (DDR) after IR leads to stimulating p38 mitogen-activated protein kinase (p38 MAPK) and protein kinase C (PKC), activating transcription factor nuclear



factor kappa-B (NF- $\kappa$ B) which produces cytokines and chemokines as well as contributing to increased intracellular ROS, in turn activating the Cyclin-dependent kinase inhibitor 2 A (CDKN2A) locus, producing p16 INK4A (p16) and p19 INK4D (ARF). p16 INK4A activates the retinoblastoma protein (pRb) tumor suppressor, which blocks certain proliferative genes by heterochromatinization, ultimately inducing durable cell-cycle arrest. DDR activates not only p16/Rb but also the p53 tumor suppressor, then turns on the p21 WAF1 (p21) gene transcription, ultimately causing senescence and permanent growth arrest (Fig. 1). Both the p53/p21 and p16/pRb pathways are clearly of major importance in cellular senescence, by inhibiting the cyclin-dependent kinase (CDK) from exiting from the cell division cycle, involved in cellular arrest and oncogene-induced senescence which is characterized by overexpression of p16, p53 or p21 proteins (Bernardes de Jesus and Blasco, 2012) (Fig. 1).

Additionally, Kim et al. examined the characterization of DNA damage that can induce endothelial cell senescence after IR by performing a DNA microarray analysis in human umbilical vein endothelial cells (HUVECs) irradiated in comparison with the multi-passage HUVECs-induced senescence. These authors demonstrated that IR-induced gene alterations are associated with senescence (Kim et al., 2014a). Recently, Baselet et al. determined that IR induced senescence in human telomerase-immortalized coronary artery endothelial (TICAE) cells with a single X-ray dose (0.05, 0.1, 0.5 and 2 Gy) and measured at days 1, 7 and 14 after exposure to irradiation. The authors elucidated IR-induced premature senescence by functional gene alterations that resulted in cell cycle changes and inflammation in endothelial cells (Baselet et al., 2017).

### 3.2. Senescent cells and microenvironment after X-ray exposure

In order to update the hallmarks of cancer, Fouad and Aanei showed that senescent cells can be induced by different oxidative stresses which activate tumor-suppressors and unbalance mitogenic signaling (Fouad and Aanei, 2017). Additionally, the characterization of the senescence-associated secretory phenotype (SASP) or senescence-messaging secretome by cellular senescence is capable of remodeling the tissue microenvironment, which can be detrimental to tissues by promoting inflammation, stimulating angiogenesis, and contributing to metastasis (Campisi, 2013; Coppé et al., 2011, 2008; Demaria et al., 2017; Kulman and Peepers, 2009; Rodier et al., 2009) (Fig. 2).

#### 3.2.1. Senescence-associated secretory phenotype

The senescence-associated secretory phenotype (SASP) is one of the hallmarks of cellular senescence, which can facilitate wound healing, tissue repair and development, and serve several biological functions, either beneficial or deleterious. SASP constituents include a various number of cytokines, chemokines, growth factors and proteases (Campisi, 2013; Demaria et al., 2017; Baker et al., 2017). Additionally, SASP can extend the stress response and impact in the tissue microenvironment by cell-cell communication or cell-matrix interaction. The SASP provides a strong link between senescence and inflammation (Lasry and Ben-Neriah, 2015). On the other hand, inflammatory genes can be observed in various types of senescence cells that are activated by different triggers (RT, chemotherapy, UV, smoking), secrete proteins and affect their neighboring environment. The stimulation of SASP is activated by the transcription factors nuclear NF- $\kappa$ B by way of producing a diversity of soluble inflammatory chemokines, pro-inflammatory cytokines (interleukin (IL), IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8), growth factors (hepatocyte growth factor (HGF), transforming growth factor beta (TGF- $\beta$ )), granulocyte-macrophage colony-stimulating factor (GM-CSF) and matrix components, protease released (matrix metalloproteinases (MMP)-1,-3,-13) and small molecular weight metabolites, which create a pro-inflammatory environment and multiply reactions with the immune cell, and ultimately cause tissue injury (Lasry and Ben-Neriah, 2015). Senescence-associated IL-6 and IL-8 cytokines can be induced by

paracrines, autocrines, or endocrines, which reinforce the senescent milieu as well as cause inflammatory microenvironment (Ortiz-Montero et al., 2017).

#### 3.2.2. Radiation-induced senescence in fibroblasts

Fibroblasts play a crucial role in the fibrosis process and tissue scarring, as they are responsible for the deposition and remodeling of extracellular matrix (ECM) components. Fibrogenesis occurs through the transition from fibroblasts to myofibroblasts via environmental inflammation mediated inflammatory cytokines (Kendall and Feghali-Bostwick, 2014). Papadopoulou and Kletsas investigated human lung fibroblasts premature senescence after exposure to IR. In their study, the researchers showed that generated ROS and activated TGF- $\beta$ , as well as activated p38 MAPK by IR, can accelerate premature senescence (Papadopoulou and Kletsas, 2011). Schafer et al. also demonstrated IR-induced senescence in primary human fibroblasts, which triggered proinflammatory and profibrotic SASP factors, promoted lung fibrosis (Schafer et al., 2017). In primary human mammary fibroblasts (HMFs), Tsai et al. showed that IR-induced senescent stromal fibroblasts rendered nearby radioresistant cell lines in breast cancer (BC) (Tsai et al., 2009). Similarly, Liakou and colleagues demonstrated that IR induced premature senescence in human breast stromal fibroblasts *in vivo* and *in vitro*, which can lead to overexpression of proteoglycan syndecan 1 (SDC1) at the cell surface through an autocrine TGF- $\beta$  loop formation (Liakou et al., 2016).

#### 3.2.3. Radiation-induced epithelial cell senescence and pathways

IR-induced epithelial cells senescence has been investigated in many studies of different organs. In this paragraph, we will discuss radiation-induced senescence according to the irradiated tissue types of epithelium, such as pulmonary, breast, skin and crystalline lens.

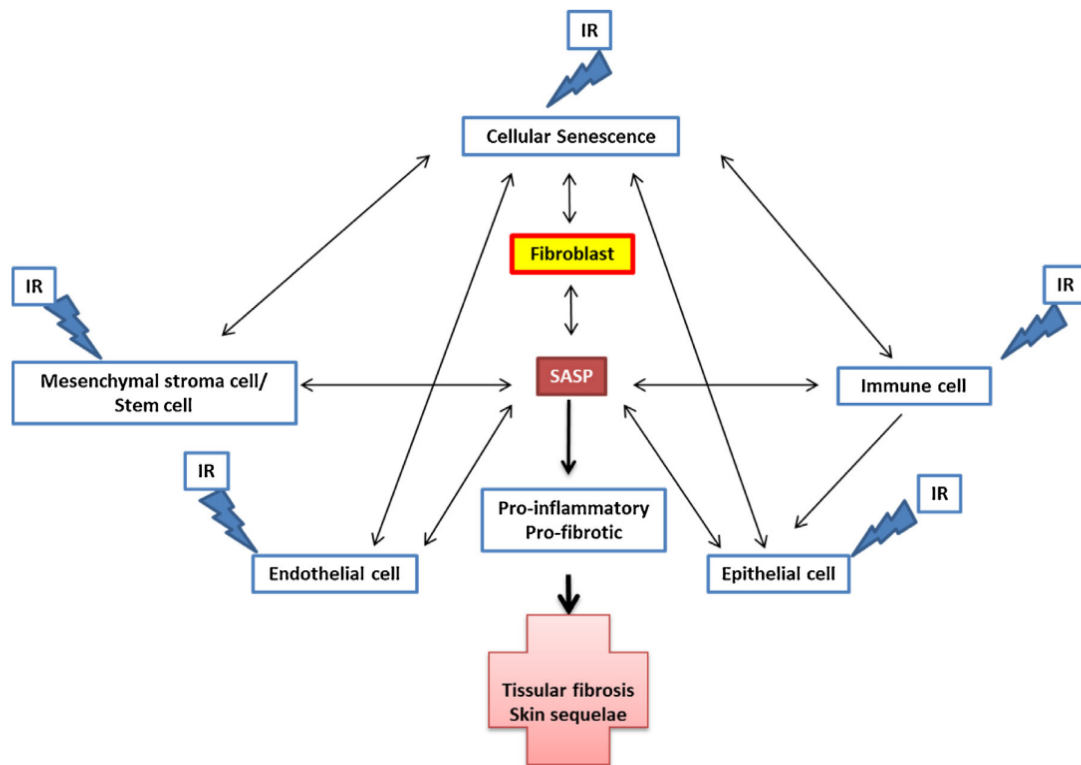
**3.2.3.1. Pulmonary.** Radiation-induced pulmonary fibrosis is one of the main complications. Oxidative stress can induce it in the oxygenated medium, thus can be at the origin of these lethal consequences in different types of cellular mortality and organ dysfunction. Chung et al. found that radiation-induced senescence promoted pulmonary fibrosis in alveolar epithelium (Chung et al., 2016a). Another research group investigated bronchio-alveolar epithelial cells from the lungs of mice, which had induced senescence after total body irradiation (Klein et al., 2015). Several studies elucidated that epithelial senescence in culture has been shown in the up-regulation of a range of pro-inflammatory gene transcripts that include IL-6 and chemokines (CXCL1, CCL2), implicated in the emergence of radiation-induced pulmonary fibrosis (Beach et al., 2017; Coppé et al., 2011; Coppé et al., 2008; Rodier et al., 2009; Johnston et al., 2010).

**3.2.3.2. Breast and skin.** After RT for BC, breast and skin fibrosis can significantly alter aesthetic score and quality of life. The mechanism of radiation-induced fibrosis has been extensively studied in the literature. It depends on the technical modalities of the irradiation and the intrinsic radiosensitivity of the patients (Azria et al., 2012). Senescence may be at the origin of radiation-induced fibrosis.

In the human mammary epithelium, Mukhopadhyay et al. demonstrated that IR induced accelerated cellular senescence *in vitro* (Mukhopadhyay et al., 2010). Similarly, Gao et al. showed that IR-induced the senescent cell line MCF-10A (non-tumorigenic mammary epithelial) at day 10 following an acute dose of 10 Gy (Gao et al., 2016).

Apart from the mammary tissue, cutaneous tissue elements may also be affected by senescence. McCart and colleagues recently demonstrated that accelerated senescence occurs in the human keratinocyte following radiation exposure at 18 Gy in a murine model. Interestingly, the investigators found that the fibrosis occurred 80 days following irradiation (McCart et al., 2017).

**3.2.3.3. Crystalline lens.** The crystalline lens is a radiosensitive



**Fig. 2.** Interrelationships between senescence-associated secretory phenotype (SASP) and the cellular microenvironment.

Ionizing radiation (IR) can induce senescence cells in epithelium, endothelium, immune cell, and in mesenchymal stromal cells/stem cells. SASPs interact to neighboring cells through endocrine or a paracrine stimulatory loop, which excrete pro-inflammatory cytokines such as IL-1, TNF- $\alpha$  as well as the pro-fibrotic cytokine TGF- $\beta$ . TGF- $\beta$  signaling results in activation of SMAD 2/3 pathway, can stimulate fibroblast proliferation and collagen production, involve in ECM remodeling and ultimately contribute to the progression of fibrosis. (ECM: extracellular matrix; IL: Interleukin; IR: Ionizing radiation; SASP: Senescence-associated secretory phenotype; TGF: transforming growth factor beta; TNF: tumor necrosis factor).

avascular organ and opacification can be observed at a low dose. Apoptosis of crystalline epithelial cells is involved in cataractogenesis (Belkacémi et al., 2001; Belkacémi et al., 2000). Recently, Hamada found that IR induced senescence in primary normal human lens epithelial cells, dependent on the ATM and DNA-PK pathways (Hamada, 2017). These results suggest that IR-induced senescence in human lens epithelial cells might result in cataracts.

### 3.2.4. Radiation-induced endothelial cell senescence and pathways

In endothelial tissues, many cellular types can be affected by senescence via different mechanisms and signaling pathways. For each cell type, it is possible to have the involvement of one or more signaling pathways to promote radio-induced senescence. In this section, we will describe the processes in HUVECs as well as in human pulmonary arterial endothelial cells (HPAECs), human lung microvascular endothelial cells (HMVECs), cerebrovascular endothelial cells (CMVECs) and TICAE cells. Accelerated endothelial senescence after IR has been elucidated by various signal transductions from the transmembrane tyrosine kinase receptor (IGF-1R/PI3K/Akt/mTOR), leading to the activity of cytoplasmic signaling pathways (p38 MAPK pathways, mitochondrial dysfunctions) and ultimately nucleus alterations (NF- $\kappa$ B pathway).

**3.2.4.1. Transmembrane tyrosine kinase receptor and cytoplasmic signaling pathways.** Senescence is a process that may involve all transmembrane and cytoplasmic signaling pathways. In membrane, the insulin-like growth factor receptor (IGF-1R) was studied by Day et al. IR-induced endothelial cell senescence has been investigated through the effect of the IGF-1R/phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt/mechanistic target of rapamycin/mammalian target of rapamycin

(mTOR) pathway signaling. Several *in vitro* studies showed that IR-induced senescence has been associated with reduced pro-survival PI3K in HUVECs as well as in HPAECs by triggering IGF-1R-induced PI3K/Akt/mTOR activity upstream of both p53 and p21 (Day et al., 2014; Panganiban and Day, 2013; Wang et al., 2016; Yentrapalli et al., 2013).

In cytoplasm, p38 MAPK is widely implicated in senescence. p38 MAPK is a signaling pathway which responds differently, depending on the dose of exposure to radiation. Exposure of endothelial cells to a high dose (> 10 Gy) of irradiation can induce apoptosis via the relationship of plasma membrane (acid sphingomyelinase (ASMase)/ceramide) and p38 MAPK pathways by the relocation of ASMase at the cell membrane, allowing the formation of the sphingolipid ceramide from sphingomyelin, leading to the formation of enlarged lipid platforms and, finally, yielding the signal to p38 MAPK activation and apoptotic cell death (Niaudet et al., 2017). Interestingly, IR exposure of endothelial cells to low or moderate doses (0.5–10 Gy) can induce cellular senescence via p38 MAPK pathways by up-regulating p16 expression (Wang et al., 2016).

Mitochondrial dysfunction after irradiation exposure was recently studied by Lafargue's group. The authors showed that IR can induce endothelial cell senescence through mitochondrial respiratory complex II dysfunction and superoxide generation in primary human lung microvascular endothelial cells (Lafargue et al., 2017). Moreover, the NF- $\kappa$ B pathway has been considered as an important element in the promotion of inflammatory genes under the governance of p38 MAPK. Dong et al. reported that radiation-induced DSBs allowed NF- $\kappa$ B pathway activation and, ultimately, induced HUVECs to enter a senescence-like phenotype. In this study, the authors revealed that IR-induced senescence can reduce, by inhibiting of NF- $\kappa$ B activation and ultimately decreasing of IL-6, one of the major pro-inflammatory



cytokines (Dong et al., 2015).

NF- $\kappa$ B signaling is the crucial signaling pathway which prompts the emergence of SASP. Ungvari et al. found that IR-induced senescence promotes SASP formation activation in CMVECs. The authors showed that DNA alterations occurred with the role of increased DNA damage and decreased DNA repair capacity after IR induced senescence and contributed to the impairment of angiogenic capacity in CMVECs (Ungvari et al., 2013).

### 3.2.5. Radiation-induced mesenchymal stromal/stem cell senescence

Stem cells are able to differentiate in bone, cartilage, and fat, and contribute to tissue regeneration and body homeostasis. Radiation-induced mesenchymal stem cell senescence can promote cytotoxic effects via accelerated senescence. In this paragraph, we introduce the effect of irradiation in cell-derived mesoderm and ectoderm to senescence.

#### 3.2.5.1. Cell-derived mesoderm

**3.2.5.1.1. Preadipocyte.** By analyzing the expression of p16 and p21 in the preadipocyte cells, Xu et al. revealed that IR induced preadipocyte senescence. The authors then analyzed in depth the SASP components, which increased messenger ribonucleic acid (mRNA) levels IL-1 $\alpha$ , IL-6, IL-8, and MMP-3 in preadipocyte senescence-mediated radiation compared with control non-senescence (Xu et al., 2015). Zhu et al. have also demonstrated radiation-induced senescence in primary human preadipocytes after a single-dose of 10 Gy (Zhu et al., 2015).

**3.2.5.1.2. Bone marrow cells.** The medullary cells are highly radiosensitive. Radiation can damage bone marrow stem cells via the senescence process. Alessio's group demonstrated that radiation-induced senescence in human mesenchymal stromal cells (MSC) of bone marrow derived from the mesoderm was sensitive to very low dose irradiation (40 mGy) and triggered senescence due to impaired autophagy and DNA repair capacity. Interestingly, the authors showed that an increase in senescence was progressive from 40 mGy to 2 Gy; notably, exposure to 2 Gy of dose radiation induced senescence rather than apoptosis (Alessio et al., 2014). Similarly, Carbonneau's group found IR-mediated senescence in murine bone marrow-derived stromal cell populations (Carbonneau et al., 2012).

Hong's team described radiation-induced senescence of mesenchymal stromal cells in articular chondrocytes by negative post-translational regulation of SIRT1 (a mammalian Sir 2 ortholog) via ROS-dependent p38 kinase activation pathway; an increase of the expression of SIRT1 significantly decreased the IR-induced cellular senescence (Hong et al., 2010). Additionally, Havelek's laboratory found that radiation-induced senescence and premature differentiation of human dental stem cells shifted to odonto-/osteoblast lineages *in vitro* (Havelak et al., 2013).

**3.2.5.2. Cell-derived ectoderm.** IR can induce senescence either in mesodermal stem cells or in ectodermal stem cells. We next present radiation-induced senescence in neural stem cells and skin stem cells, which are original ectoderm.

**3.2.5.2.1. Neural stem cells.** Contrary to medullary cells, neural stem cells are radioresistant. Schneider et al. investigated the DNA damage in mammalian neural stem cells (NSC) by inducing X-ray. This study found that IR induced senescent NSC by leading cell cycle arrest and down-regulation of DDR signaling by acute exposure to 10 Gy X-ray irradiation (Schneider et al., 2013).

**3.2.5.2.2. Skin stem cells.** Radiation can induce dermatitis and ulceration, which affect the quality of life of cancer patients after RT. The loss of the adult stem cell population can induce tissue damage via the senescence process. Recently, McCart's team demonstrated radiation-induced senescence in adult skin stem cells in mice within 14 days after 18 Gy of irradiation. The authors observed that the p21/waf1 expression was increased through 30 days post-irradiation, which correlated with multifocal ulceration and chronic-active

pygranulomatous dermatitis (McCart et al., 2017).

### 3.2.6. Radiation-induced immune cellular senescence

**3.2.6.1. Radiosensitivity of immune cells.** The immune cells are particularly sensitive to IR. B-cells, as well as natural killer (NK) cells, are radiosensitive, whereas memory T cells, effector T cells (Teffs) and regulatory T cells (Treg) from healthy donors are radioresistant (Wilkins et al., 2002). IR not only affects the Treg/Teffs immune balance but also reshapes the ratios of T helper 1 (Th1)/T helper 2 (Th2) (Schaue, 2017). Bauer and colleagues revealed that monocytes are more radiosensitive than macrophages (Bauer et al., 2011). The immune reactivity after IR is a potential major benefit but can increase the risk of normal tissue sequelae and chronic inflammation of cancer patients in the long term (Schaue, 2017).

**3.2.6.2. Immunosenescence and T cell function.** The induction of senescence by oxidative stress that results from IR exposure can alter leukocyte and T cell function; therefore, senescent T cells might have implications for enhancing human immunity (Akbar et al., 2016). In addition, senescent T cells have the capacity to secrete large quantities of pro-inflammatory cytokines and cytotoxic mediators, called SASP (Yu et al., 2016).

The CD8 + T cell senescence revealed its mitochondrial dysfunction, which augmented ROS production and had a negative effect on mitochondrial biogenesis (Henson et al., 2014). Ye et al. showed that Treg cells can induce senescence in naïve T cells and Teffs (Ye et al., 2012). Interestingly, Treg cells have been found to contribute fibrotic diseases in the lung by promoting a pro-fibrotic microenvironment after IR (Wirsdörfer and Jendrossek, 2016). In the study of Xiong's group, the authors demonstrated that Treg cells promoted epithelial-to-mesenchymal transition (EMT) in radiation-induced lungs fibrosis through the  $\beta$ -catenin signaling pathway (Xiong et al., 2015).

While Th1 cells have shown that anti-fibrotic activity by their production of IFN- $\gamma$  can antagonize the pro-fibrotic activity of TGF- $\beta$ 1, Th2 cells act as a potent driver of progressive fibrosis, enhance inflammation and promote tissue damage. Th2 cells are known by the production of IL-4, IL-5 and IL-13, linked to the development of fibrosis (Duffield et al., 2013; Wynn and Ramalingam, 2012). Likely, Th2 and Th17 cells are also pro-inflammatory and pro-fibrosis cells. Th17 cells can produce the pro-inflammatory cytokine IL-17 A, which plays a central role in driving the fibrosis in the pathogenesis of pulmonary fibrosis, hepatitis-induced hepatic fibrosis, especially radiation-induced lung fibrosis, myocardial fibrosis, colonic mucosal damage and fibrosis, as has been highlighted in many studies (Duffield et al., 2013; Wynn and Ramalingam, 2012; Bessout et al., 2015; Wang et al., 2014a, b).

**3.2.6.3. Pre-clinical and clinical results.** According to a study by Ye et al. *in vitro*, IR-induced senescence in naïve CD4 + T cells were observed at day 5 after 5 Gy (Ye et al., 2012). One recent work described the immune alteration in peripheral T lymphocytes after exposure to low doses of IR in mice. The authors provided evidence that low-dose radiation accelerated aging of the T-cell receptor repertoire with more T lymphocytes surviving (Candéias et al., 2017). Additionally, in the study of Klein and colleagues, the authors demonstrated that IR induced senescence in the leukocyte infiltration in lung tissue from mice exposed to whole-thorax irradiation at 15 Gy (Klein et al., 2015). Interestingly, radiation-induced senescence of CD4+ and CD8 + T cells has been reported in one patient undergoing extracorporeal photopheresis in graft-versus-host disease (Lee et al., 2004).

### 3.2.7. Radiation-induced senescent cells promotes fibrosis

Radiation-induced DNA damage leads to a chronic inflammatory state and senescence. A landmark in the study of Beach's team was identifying the merging of a molecular target involved in DNA damage response, inflammatory response and senescent cells in radiation-induced pulmonary fibrosis by using RNA sequencing technology. The



data strengthen the fact that radiation-induced cellular senescence related to changes in ECM and promoted tissue fibrosis (Beach et al., 2017). Additionally, cellular senescence may exist for longer periods in tissues and can be dependent on adjacent cells by producing a myriad of molecules, such as pro-inflammatory mediators. In this paragraph, we will describe how these factors can promote fibrosis in IR-induced senescence.

**3.2.7.1. Senescent fibroblasts.** As described above, fibroblasts contribute to fibrogenesis by activating fibroblast-to-myofibroblast transition, which promote excessive ECM production (Kendall and Feghali-Bostwick, 2014). IR-induced senescent fibroblasts bring out the SASP with a range of pro-inflammatory cytokines, chemokines, and growth factors which exacerbated fibrotic process in the naïve fibroblasts (Schafer et al., 2017). A vicious circle in senescent fibroblasts induced senescence in the others vicinal cells or fibroblasts, multiplied signal SASP with pro-inflammatory factors, driven fibrosis.

**3.2.7.2. Pro-inflammatory factors.** IR can induce senescent cells in epithelium and endothelium, in fibroblast and mesenchymal stromal cells/stem cells, as well as in immune cells. The SASP interacts with neighboring cells through paracrine, autocrine and/or endocrine stimulatory loops, which excrete pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6, IL-8, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin (also known as CD62 antigen-like family member E (CD62E) or endothelial-leukocyte adhesion molecule 1), and platelet endothelial cell adhesion molecule (PECAM-1) expressions via activation of NF- $\kappa$ B, inducing attraction of the immune cells. Polarization of neutrophil infiltration, macrophage subpopulations and monocytes activate in the damage position and release the TGF- $\beta$ 1, which stimulates ECM synthesis by local fibroblasts. TGF- $\beta$ 1, together with ras homolog (Rho)/ rho-associated protein kinase (ROCK) signaling pathway, plays an important role in radiation-induced fibrosis (Liu et al., 2017; Monceau et al., 2010; Taunk et al., 2015).

**3.2.7.3. ROS formation.** Cellular senescence can generate increased ROS levels by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which damage neighboring cells. Therefore, Citrin et al. clarified the mechanism of radiation-induced senescence-promoted lung fibrosis via NADPH oxidases (NOX) generation, in turn leading to senescent pneumocytes-stimulated fibroblast proliferation and collagen deposition, and ultimately to lung injury and fibrosis (Wang et al., 2016; Citrin et al., 2013; Citrin et al., 2017).

**3.2.7.4. TGF- $\beta$ .** TGF- $\beta$  activated at injury sites after IR exposure plays an important role in pathological processes. Senescence-mediated IR is associated with the SASPs, which secrete TGF- $\beta$ . TGF- $\beta$  signaling results in activation of the Smad 2/3 signaling pathway, reacts to ROS formations, and expands various pro-fibrotic signals to promote myofibroblast accumulation and production of ECM and, ultimately, fibrosis (Ahamed and Laurence, 2017). Interestingly, the myofibroblasts store in the space of an organic structure plays an important role in fibrotic formation through producing ECM protein pro-fibrosis. Moreover, myofibroblasts are the primary effector cells in tissue reconstruction and fibrosis through the activation of immune cells by secreting a number of cytokines, including TGF- $\beta$ 1, TNF- $\alpha$ , IL-1, IL-6, IL-17 A, IL-13, and platelet-derived growth factor (PDGF) (Duffield et al., 2013; Wynn and Ramalingam, 2012; Citrin et al., 2017; Wynn, 2008). In addition, the renin-angiotensin system plays a pivotal role in hemodynamic regulation in multiple organ systems. TGF- $\beta$  is activated by up-regulation of angiotensin II, thus contributing to fibrosis.

TGF- $\beta$  and ROS have a reciprocal relationship in the pathophysiology of radiation-induced fibrosis, a concept known as redox-fibrosis. Immediate induction of ROS by radiation induces damage of DNA,

protein, and lipids, thus releasing TGF- $\beta$  (Ahamed and Laurence, 2017). Moreover, TGF- $\beta$  is associated with pro-inflammatory effects involving the development and subset commitment of IL-17 producing Th17 cells, which promote inflammation (Raphael et al., 2015). Thus, the triplets of TGF- $\beta$ , ROS and pro-inflammatory factors might play an important role in promoting fibrosis via accelerated senescence in fibroblast after IR. Conversely, in animal models exposed to carbon tetrachloride (CCl<sub>4</sub>), the senescent activated stellate cells decreased hepatic fibrosis via the enhancing immune surveillance by natural killer cells (Krizhanovsky et al., 2008). The role of the senescent cell is a paradox in the fibrotic tissue, advantageous or disadvantageous fibrogenesis is dependent on oxidative stress parameters and cellular types (Schafer et al., 2018). The pathway of IR-induced senescence boosts fibrosis which might be a relevant hypothesis for selectively killing the senescent cell in choosing treatment. Further studies will be investigated to help a better understanding of the fibrotic mechanisms involved in multi-element and multi-step events in radiation-induced multi-tissue senescence (Fig. 2).

### 3.3. Prediction of intrinsic radiosensitivity by cellular senescent tests

In fundamental research, cellular senescence might be identified in fibroblasts by cellular phenotype (specific morphology, senescence-associated  $\beta$ -galactosidase activity), cell proliferation arrest (decreased Ki-67), inhibiting the cyclin dependent kinase (p16, p21, p53, p38 MAPK expression), apoptosis exclusion (cleaved caspase-3), DDR and repair (ATM,  $\gamma$ -H2AX) and secretory proteins (IL-6, IL-8, CXCR2) (Schafer et al., 2017; Akbar et al., 2016; Ewald et al., 2010). By analyzing characteristic features of senescence after IR-induced senescence, we might predict IR-induced fibrotic tissue. Thus, we hypothesize that the more IR-induced senescence, the more radiosensitivity and, ultimately, the more tissue injury with fibrosis. Use of the senescence biomarker assay could reflect indirectly radiosensitivity in translational research and predict the complication of prospective RT patients in clinical practice. Herein, we describe different tests to predict radiosensitivity: senescence-associated  $\beta$ -galactosidase, protein expression assays, SASP assay, senescence-associated secretory phenotype, DDR and apoptosis assays (Table 1).

#### 3.3.1. Senescence-associated $\beta$ -galactosidase assay (SA- $\beta$ -gal assay)

**3.3.1.1. Method.** SA- $\beta$ -gal activity can be measured at pH 6.0 by immunohistochemistry or by staining with X-gal (5-Bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside), a chromogenic substrate of  $\beta$ -gal. X-gal is cut by  $\beta$ -gal in the lysosome, resulting in an insoluble blue dye in cytoplasm. SA- $\beta$ -gal reflected an increase in lysosomal mass in the senescent cells. Actually, SA- $\beta$ -gal provides strong evidence for identifying cellular senescence with changes in morphology and increased side scatter in flow cytometry (Bernardes de Jesus and Blasco, 2012; Kurz et al., 2000).

**3.3.1.2. Experiment.** By using SA- $\beta$ -gal assay, many cellular types of radiation-induced senescence have been identified, such as naïve T cells at day 5 after exposure to 5Gy, human breast stromal after 5 days followed by irradiation in a dose of 4 Gy, and mammalian neural stem cells observed at 1, 3, 5 and 7 days after 10 Gy (Liakou et al., 2016; Schneider et al., 2013; Ye et al., 2012).

#### 3.3.2. Gene and protein expression assay

As noted earlier, prematurely senescent cells have an over-expression of cell cycle inhibitors p21 and p16, and an absence of the hyper-phosphorylated form of the pRb. The expression of p16 strongly correlates with the overall burden of senescence cells. *in vitro* and *in vivo* experiments have shown the increase of senescent biomarkers of p21 or p16 expression after hours and up to 30 weeks following radiation treatment by using the Western blot analysis, immunofluorescence staining or reverse transcription polymerase chain reaction (RT-PCR)

**Table 1**  
Prediction of intrinsic radiosensitivity by cellular senescent/apoptotic tests.

Tests	Methods	Models/Dose	Results in senescence/apoptosis	References
SA- $\beta$ Gal assay	staining with X-Gal /Immunohistochemistry	human breast stromal (4 Gy) naïve T cell (5 Gy) mammalian neural stem cell (10 Gy)	blue dye in cytoplasm/senescence	(Liakou et al., 2016), (Schneider et al., 2013), (Ye et al., 2012)
Genes/Proteins expression	p21 (p21 WAF1), p16 (p16 INK4a)/Western Blot, RT-PCR	murine stromal bone marrow (6 Gy)	protein overexpression/senescence	(Carbonneau et al., 2012)
SASP assay	Pro-inflammatory cytokine dosage/ ELISA kits or Luminex xMAP	human preadipocyte (10 Gy)	increased level expression of cytokine/senescence	(Xu et al., 2015)
DNA damage response assay (DDR assay)	$\gamma$ -H2 AX foci/Immunofluorescence	human lymphocyte (2 Gy) human skin biopsy (2 Gy)	radiation toxicity related to a decline of $\gamma$ -H2 AX foci decay	(Belkacemi et al., 2016), (van Oorschot et al., 2017)
Apoptotic assay	cleaved caspase-2/3/9 Radiation-induced T-lymphocyte apoptosis (RILA)	peripheral blood (1 Gy, 2 Gy, 4 Gy) lymphocyte (2, 8 Gy)	protein overexpression/apoptosis high value RILA/apoptosis	(Santos et al., 2017), (Ozsahin et al., 2005), (Azria et al., 2015)

Abbreviations (Table 1).

ELISA: enzyme-linked immunosorbent assay (ELISA); Luminex xMAP: Luminex (multi-analyse profiling); RILA: Radiation-induced T-lymphocyte apoptosis; RT-PCR: reverse transcription polymerase chain reaction SA- $\beta$  Gal: senescence-associated  $\beta$ -Galactosidase; SASP: senescence-associated secretory phenotype; X-Gal : 5-Bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside.

analysis. The study of McCart's team showed that p21 expression was increased in areas of adult skin stem cells following radiation-induced accelerated senescence (McCart et al., 2017). In the *in vivo* study of Carbonneau, p16 expression levels were found to be increased in murine stromal bone marrow after 6–8 weeks of 6 Gy total body irradiation (Carbonneau et al., 2012). Measurements of p16 levels can be performed in the tissue or in peripheral blood lymphocytes (Beach et al., 2017; Kim et al., 2014a; Panganiban and Day, 2013; Citrin et al., 2013; Salama et al., 2014). The p16 pre-treatment status in patients might be predictive of the degree of radiotoxicity: the higher the threshold, the higher the toxicity. Therefore, p16 expression might be promising as a biomarker to measure intrinsic radiosensitivity.

### 3.3.3. Senescence-associated secretory phenotype assay (SASP assay)

**3.3.3.1. Method.** As mentioned earlier, senescent cells down-regulated the production of proliferative proteins but up-regulated the production of pro-inflammatory and pro-fibrogenesis cytokines, a behavior termed the SASP. The level of these cytokine expressions can be measured by using immunoassays based on either enzyme-linked immunosorbent assay (ELISA) kits or Luminex xMAP (multi-analyse profiling). High-level expressions of IL-6, IL-8 and TGF- $\beta$ 1 cytokine-mediated the SASP significantly linked to radiation-induced senescence and radiotoxicity.

**3.3.3.2. Experiment.** Xu et al. demonstrated that the most highly produced cytokines of radiation-induced senescence in preadipocytes were IL-6 (19-fold) and IL-8 (46-fold) in comparison to un-irradiated cells *in vitro* (Xu et al., 2015). Recent studies have shown that increased levels of IL-8 and TGF- $\beta$ 1 in the plasma of patients undergoing RT functioned as a biomarker in combination with mean lung dose to predict the risk of radiation-induced lung toxicity in non-small cell lung cancer (Wang et al., 2017). Altogether, quantification of cytokine expression could show promise as a bio-indicator to predict radiation-induced toxicity via the mechanism of the SASP factor induced by senescent cells at the systemic level.

### 3.3.4. DNA damage response assay (DDR assay)

Several studies have shown the use of  $\gamma$ -H2 AX as a predictive monitor of DDR and senescence on *in vitro*, *in vivo* and translational research (Lafargue et al., 2017), (Oorschot et al., 2013; van Oorschot et al., 2017; Pouliliou and Koukourakis, 2014; van Oorschot et al., 2014; Vandersickel et al., 2010). Biosampling for  $\gamma$ -H2 AX studies can be applied to most types of cells or tissues, such as tumor cells,

lymphocytes, plucked cells, oral cells and skin biopsy, exposed to radiation *ex vivo* and quantification of  $\gamma$ -H2 AX foci *in vitro* by microscopy or by flow cytometry (Belkacemi et al., 2016; COPERNIC project investigators et al., 2016; Oorschot et al., 2013; van Oorschot et al., 2017; Pouliliou and Koukourakis, 2014, 2014; Andrievski and Wilkins, 2009; Borràs et al., 2015; Golfier et al., 2009; Kunogi et al., 2014; Olive and Banáth, 2004). Oorschot's team found that late radiation toxicity was related to a decline of  $\gamma$ -H2 AX foci decay and decreased DDR repair genes in peripheral lymphocytes of prostate cancer patients undergoing RT (van Oorschot et al., 2017). Hence, the  $\gamma$ -H2 AX assay might show promise as a standardized method in clinical practice to monitor DSB damage and tissue complication by IR-induced senescence.

### 3.3.5. Apoptotic assay

Resistance to radiation-induced apoptosis has been described in cellular senescence. This mechanism has been demonstrated *in vitro* and *in vivo* by determining the ratio of pro-apoptotic proteins and anti-apoptotic B-cell lymphoma 2 (Bcl-2) family members, which determine whether and when apoptosis was triggered. Cellular senescence has both a stable expression of anti-apoptotic Bcl-2, Bcl-W and Bcl-XL proteins and lower levels of pro-apoptotic protein expression (p53 up-regulated modulator of apoptosis (PUMA), BCL2-antagonist/killer (Bak), Bcl-2 related ovarian killer (Bok), Bcl-2-interacting killer (Bik)) (Campisi and d'Adda di Fagagna, 2007; Kim et al., 2014a; Demaria et al., 2017; Monceau et al., 2010). Seluanov et al. demonstrated that the existence of apoptosis resistance in senescent cells *in vitro* following IR low-dose cisplatin (Seluanov et al., 2001).

**3.3.5.1. Methods.** Apoptotic analysis can be studied by different methods (flow cytometry or immunofluorescence staining) with different markers, such as cleaved PARP (poly (ADP-ribose) polymerase), cleaved caspase-2/3/9 or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

**3.3.5.2. Cleaved caspase-3.** Active caspase-3 expression levels were established as a bio-indicator of individual radiosensitivity in peripheral blood samples of 10 healthy individual donors by using flow cytometry, *ex vivo* at 1, 2, 4 Gy and *in vitro* incubation after 24, 48 and 72 h. The data suggested that incubation during 24 h was considered the better condition to distinguish dose of IR; the proposed assay could be used as a test for detecting radiosensitivity of patients before undergoing RT (Santos et al., 2017).



**3.3.5.3. Radiation-induced T-lymphocyte apoptosis.** Radiation-induced T-lymphocyte apoptosis (RILA) has been investigated since 1997. This assay has provided a rapid means of determining radiosensitivity with a high negative predictive value, able to discriminate differences in radiation-induced cytotoxicity between individuals (Wilkins et al., 2002; Ozsahin et al., 2005; Ozsahin et al., 1997). In a French prospective multicentric study, Azria et al. demonstrated that patients with a low RILA (< 12%) present grade  $\geq 2$  breast fibrosis, whereas patients with a high RILA (> 16%) are not affected by late toxicity after RT. Radio-induced apoptosis in CD8 + T lymphocyte was significantly associated with late effects in various cancers (prostate, head and neck, cervix) in prospective trials by using flow cytometry with staining of the markers of propidium iodide/annexin V (Ozsahin et al., 2005, 1997; Azria et al., 2017, 2015; Azria et al., 2010; Brengues et al., 2017).

#### 3.4. Therapeutic perspectives

Despite recent advances in RT techniques, approximately 5–15% of patients suffer from radiation-toxicity that can impede effective treatment and also quality of life. Radiation-induced fibrosis is one of the toxicities that influences morbidity and function in patients after RT. In order to extend the therapeutic window between tumor eradication and the induction of adverse events in normal tissue, the development of advance therapeutics aimed at eliminating the detrimental effects of cellular senescence might mitigate radiation-induced toxicity of normal tissue. Table 2 and Fig. 3 summarize some molecular targets investigated with intracellular signaling pathways.

##### 3.4.1. Drug products

**3.4.1.1. Anti-oxidants and free radical scavengers.** The work of Yuhas and that of Liauw showed that radioprotective compounds can act as free-radical scavenger agents, which can absorb radicals generated by IR (Liauw et al., 2013b; Yuhas et al., 1977). The thiol-containing drug amifostine was approved by the FDA for clinical use as a protector of radiation-induced cell death. Amifostine can reduce the severity of mucositis, xerostomia and dysphagia in head and neck cancer patients treated with RT (Hall et al., 2016). These antioxidants and free radical scavenger products can also reduce ROS production after IR, thus possibly decreasing the senescent process via preventing ROS formation.

**3.4.1.2. Anti-inflammatory agents.** Much evidence indicates that anti-inflammatory agents, such as dexamethasone, benzydamine, paravastatin, and pentoxifylline, can prevent and treat radiation-induced toxicity. These drugs might effectively reduce IR-induced senescence-mediated toxicity by reducing the pro-inflammatory cytokines. Wang et al. found that dexamethasone might provide a protective role in radiation-induced lung injury in mice via reduction of the IL-17, which is a cytokine that has powerful pro-inflammatory effect (Wang et al., 2014b).

Benzydamine, a topical non-steroidal anti-inflammatory drug, can decrease both the incidence and severity of oral mucositis while undergoing RT (Hall et al., 2016; Sheibani et al., 2015).

An inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase repressed late radiation-induced submucosal fibrosis in the intestine (Hall et al., 2016). Doi et al. found that pravastatin can protect normal intestines and lung tissues after irradiation. The effect of pravastatin was associated with a decrease in the level of radiation-induced DNA damage (Doi et al., 2017). Similar works showed that pravastatin reduced pro-inflammatory and pro-fibrotic cytokines, including IL-6 and IL-8, in lung endothelial cells exposed to radiation (Hall et al., 2016; Gaugler et al., 2005).

Pentoxifylline, a methyl xanthine derived, has been shown to be favorable in decreasing the risk of radiation-induced lung toxicity in patients with breast and lung cancer by the mechanisms of anti-inflammation and anti-oxidant. Additionally, sulfasalazine, a 5-

aminosalicylate compound, with the same mechanisms as pentoxifylline, has been shown to be beneficial in reducing the gastrointestinal manifestations associated with radiation toxicity (Hall et al., 2016).

The mechanism used by angiotensin-converting enzyme (ACE) inhibitors to alleviate tissue injury in IR-induced senescence is not completely understood. Nevertheless, ACE inhibitors reduced the rate of radiation pneumonitis in patients undergoing thoracic radiation for lung cancer in the retrospective study of (Kharofa et al. (2012)). Similarly, Harder's publication reported that treatment with the pharmacological inhibition of ACE was associated with less radiation pneumonitis and fibrosis (Harder et al., 2015). Captopril can reduce adverse events and severity of radiation-induced proctitis (Alashkham et al., 2016a; Alashkham et al., 2016b). A new clinical trial (NCT01754909) of enalapril is ongoing, to evaluate those drugs that can alleviate radiation-induced lung injury (Citrin et al., 2017). We hypothesize that ACE inhibitors down-regulated TGF- $\beta$  and, thus, can reduce senescence-mediated IR by decreasing TGF- $\beta$  via the SASP.

##### 3.4.2. Targeted therapies of senescence

**3.4.2.1. IGF-1R.** Pharmacological inhibition of IGF-1R has great therapeutic potential to prevent radiation-induced senescence. *in vitro*, the primary endothelial cell IGF-1R inhibitor AG1024 significantly inhibited cellular senescence from a radiation dose of 10 Gy. It offers promise in pre-clinical research and a clinical trial in the future (Day et al., 2014; Panganiban and Day, 2013).

**3.4.2.2. PI3K/ Akt/ mTOR.** PI3K/Akt/mTOR pathway is involved in cellular proliferation, Akt activation causes pre-mature senescence via increased production of ROS. Blocking PI3K activation by the PI3K inhibitor LY294002 significantly reduced radiation-induced endothelial cellular senescence in the *in vitro* study of Panganiban's laboratory. Moreover, the mTOR inhibitor with rapamycin has been shown as a molecular target to prevent accelerated senescence in cells exposed to DNA-damaging agents *in vitro* (Panganiban and Day, 2013; Iglesias-Bartolome et al., 2012). Recent *in vivo* studies found that rapamycin might alleviate radiation-induced lung injury and mucositis in a murine model, by preventing the senescent process through decreasing of ROS formation and prohibiting the SASP (Iglesias-Bartolome et al., 2012; Chung et al., 2016b; Suzuki and Yamashita, 2014, 2014).

**3.4.2.3. Senolytics and senomorphics.** Interestingly, another promising target to reduce toxicity due to radiation-induced senescence is the use of senotherapeutics, namely, senolytics and senomorphics. The others senotherapeutics are targeting SASP factors and immune system-mediated senescent cell clearance, were described in the reviews of Schafer et al. and Childs et al. (Schafer et al., 2018; Childs et al., 2017). By using senolytic drugs, radiation-induced injury in tissue can be mitigated and treated via anti-inflammatories and by selectively killing senescent cells. In addition, senolytic drugs not only target individual harmful inflammatory molecules (cytokine, chemokine) and suppress SASP, but also improve tissue repair and regeneration, improving the microenvironment tissue (Wang et al., 2016). The first investigated senolytics was carried out by the laboratory at Robert and Arlene Kogod center on aging in America. The authors discovered the selective destruction of cellular senescence by the combination of dasatinib and quercetin *in vivo* after IR (Zhu et al., 2015). Two years later, Scharfer et al. as well as Lehmann et al. demonstrated dasatinib plus quercetin could attenuate fibrotic pulmonary disease by selectively clearance of senescence cell (Schafer et al., 2018; Lehmann et al., 2017; Mailleux and Crestani, 2017). Further clinical studies should be investigated to evaluate the benefit/risk ratio in patients (Mailleux and Crestani, 2017). Apart from senolytics, targeting senescent cells by senomorphics showed clearly an efficient in *in vitro* and *in vivo*. In the study of Yosef et al. found the ABT-737 was efficiently eradicated senescent cells after ultraviolet radiation through inhibiting BCL-W and BCL-xl (Yosef et al., 2016). In an *in vivo* study in Pan's laboratory, ABT-

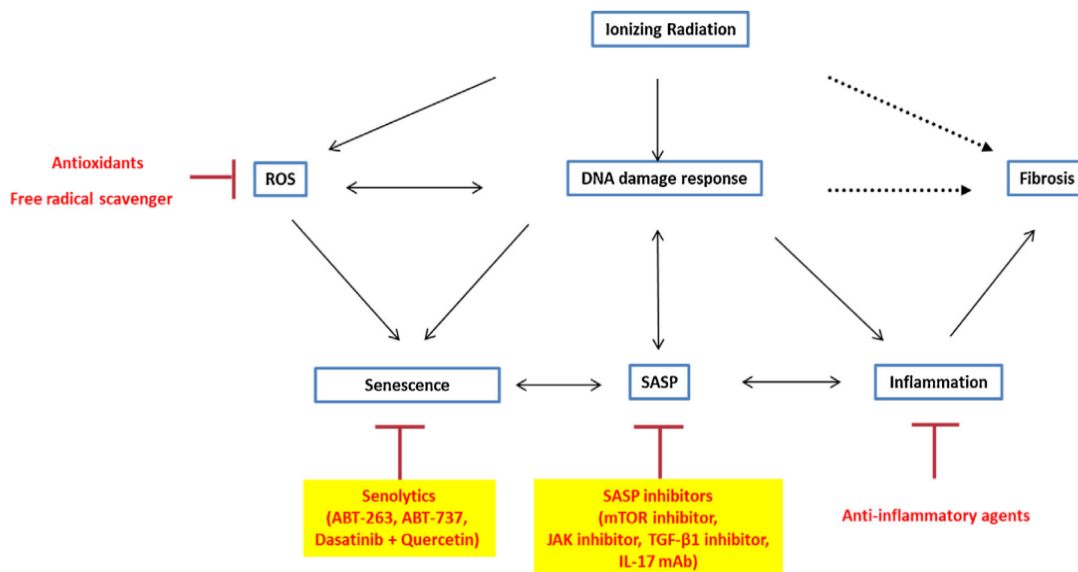


**Table 2**  
Current status of molecular interventions for irradiation-induced toxicity.

Drug products	Target	Substance	IR-induced toxicity	Mechanism	Impact on senescence	Status of Project Trial	Indication	References
	Antioxidants - free radical scavenger	Amifostine	Radiotoxicity in head and neck cancer patient	Reduction of ROS	Unknown	FDA approved 2007	200 mg/m <sup>2</sup> (IV) Once daily, starting 15-30 minutes prior to standard fraction radiation therapy	<a href="https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/020221s024lbl.pdf">https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/020221s024lbl.pdf</a>
	Anti-inflammatory	BIO300 (genistein)	Chemoradiation in non-small cell lung cancer	Reduction of ROS, modulation of the inflammatory cytokine response	Unknown	NCT02567799	Oral	<a href="https://clinicaltrials.gov/ct2/show/NCT02567799">https://clinicaltrials.gov/ct2/show/NCT02567799</a>
		HMG-CoA Dexamethasone	IR-induced intestinal injury	Reduced the level of Anti-inflammatory by reducing IL-17	Unknown	<i>In vivo</i>		(Doi et al., 2017)
		ACE inhibitors (Enalapril)	IR-induced lung injury	Anti-inflammatory	Unknown	<i>In vivo</i>		(Wang et al., 2014b)
		Benzylamine	IR-induced pneumonitis and fibrosis	Anti-inflammatory	Unknown	NCT01754909		<a href="https://clinicaltrials.gov/ct2/show/NCT01754909">https://clinicaltrials.gov/ct2/show/NCT01754909</a>
		Pentoxifylline AG1024	IR-induced endothelial senescence	Anti-inflammatory IGF-1R blockade	Unknown			(Hall et al., 2016), (Sheibani et al., 2015)
Targeted therapies of senescence	IGF-1R	LY294002 Rapamycin	Radiation-induced lung fibrosis	PI3K blockade mTOR inhibitors	Reduction SASP inhibition	<i>In vitro</i>		(Hall et al., 2016)
		TGF-β receptor 1 inhibitor	Radiation-induced tissue fibrosis	Reduction of ROS Anti-inflammatory	Reduction of ROS	<i>In vitro</i>		(Panganiban and Day, 2013)
		JAK/STAT	Radiation-induced senescence in preadipocytes and in HUVECs	JAK inhibitors	Reduction of ROS Anti-inflammatory SASP inhibition	<i>In vivo</i>		(Panganiban and Day, 2013) (Chung et al., 2016b)
		NF-κB	Radiation-induced senescence in HUVECs	Inhibition of the DSB/NEMO/NF-κB signal pathway	SASP inhibition	<i>In vitro</i>		(Schafer et al., 2018), (Ahamed and Laurence, 2017), (Avraham et al., 2010)
		IL-17	Radiation-induced lung injury	Immunoregulation, restoration of the immunological balance	SASP inhibition	<i>In vitro</i>		(Xu et al., 2015)
		Senolytic	Radiation-induced lung injury	Bcl-xL inhibitor	Killing senescent cell	<i>In vivo</i>		(Dong et al., 2015)
		Dasatinib + Quercetin	Radiation-induced hepatic fibrosis		Killing senescent cell	<i>In vivo</i>		(Wang et al., 2014a)
		ABT-737	Radiation-induced senescence	BCL-W and BCL-XL inhibitors	Killing senescent cell	<i>In vivo</i>		(Wang et al., 2016), (Pan et al., 2017)
		Stem cell mobiliser	Skin/intestines/lung/Bones/Salivary glands	Regeneration of radiation-induced tissue damage	Unknown	<i>In vivo</i>		(Monesek et al., 2018)

**Abbreviations (Table 2).**

ACE: Angiotensin-converting enzyme; BCL-XL: B-cell lymphoma-extra large; DDR: DNA damage response; DSBs: DNA double-strand breaks; FDA: The Food and Drug Administration; HGF: hepatocyte growth factor; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A reductase; HUVECs: human umbilical vein endothelial cells; IGF-1R: insulin-like growth factor receptor; IL: Interleukin; IL-17mAb: Interleukin 17 monoclonal antibody; IR: ionizing radiation; JAK: Janus kinase; MSC: mesenchymal stem cells; mTOR: mechanistic target of rapamycin/mammalian target of rapamycin; NEMO: nuclear factor kappa B essential modulator; NF-κB: nuclear factor kappa-B; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; RNAi: RNA interference; ROS: Reactive Oxygen Species; SASP: Senescence-associated secretory phenotype; STAT: signal transducer and activator of transcription; TGF-β: transforming growth factor beta; UV: Ultraviolet.



**Fig. 3.** Interrelationships between DNA damage, inflammatory response and cellular senescence induced by IR and therapeutic perspectives.

Radiation-induced senescent cell by triggering the DNA damage responses, activating the senescence-associated inflammatory signaling and increasing the SASP, which is composed different inflammatory proteins, chemokines and pro-fibrogenesis of cytokines, which are well-known players in fibrotic sequelae. Several strategies described above are promising target for either radiotoxicity approaches. These include antioxidants, free radical scavenger, mTOR inhibitor, senolytic drugs, and anti-inflammatory agents. (DNA: Deoxyribonucleic acid; IGF-1R: insulin-like growth factor receptor; IL-17mAb: Interleukin 17 monoclonal antibody; JAK: Janus kinase; mTOR: mechanistic target of rapamycin/mammalian target of rapamycin; SASP: Senescence-associated secretory phenotype; ROS: Reactive Oxygen Species; TGF- $\beta$ : transforming growth factor beta).

263, also a specific Bcl-2/xl inhibitor and a newly identified senolytic drug, was used to treat IR-induced tissue injury and fibrosis in the lungs. Researchers have demonstrated that this senolytic can reverse pulmonary fibrosis at week 16 after exposure in C57BL/6 mice to a single dose of 17 Gy (Pan et al., 2017). To date, no clinical studies are available investigating ABT-263 in the context of radiation-induced fibrosis. In the same mechanism of Pan's study, Moncsek et al. recently found a novel senolytics targeting killed senescent cholangiocytes improve hepatic fibrosis in *in vivo* (Moncsek et al., 2018). These findings emerged a new era in optimal treatment IR-induced fibrosis in radiosensitivity patients via the suppressed senescent cells.

**3.4.2.4. Targeting SASP factors.** Senotherapeutics by targeting SASP factors might decrease radiation-induced fibrosis through inhibiting proinflammatory cytokines and profibrogenic factors. We next describe the SASP inhibitors such as TGF- $\beta$ , NF- $\kappa$ B/NEMO and JAK/STAT pathways, and IL-17 A antibody (Fig. 3).

**3.4.2.5. TGF- $\beta$ .** TGF- $\beta$  is strongly linked to radiation-induced fibrosis. The TGF- $\beta$  signaling pathway can activate and amplify these profibrotic signals via ROS. Therefore, pharmacological inhibition of the TGF- $\beta$  receptor 1 (LY-364947) has been shown to significantly decrease radiation-induced tissue fibrosis on lymphatic endothelial cells (Ahamed and Laurence, 2017; Avraham et al., 2010; Kim et al., 2014b) Targeted TGF- $\beta$  might reduce radiotoxicity-related senescence by reducing ROS formation and proinflammatory, thus inhibiting SASP factors. In the recent review of Schafer showed that TGF- $\beta$ , as well as IL-6, is considered as a promise senotherapeutics but difficult approach due to the plurality of proinflammatory, profibrotic and proremodeling factors with the senescence-messaging secretome by cellular senescence (Schafer et al., 2018).

**3.4.2.6. NF- $\kappa$ B/NEMO and JAK/STAT pathways on the SASP.** One *in vitro* study demonstrated that a specific small-molecule inhibitor of NF- $\kappa$ B essential modulator (NEMO) PS1145 may inhibit radiation-induced senescence via inhibition of NF- $\kappa$ B activation and reduce the SASP, such

as IL-6 formations (Dong et al., 2015). By the same mechanism in decreasing of the SASP in radiation-induced senescent preadipocytes, Xu et al. found that pharmacological inhibition of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway could alleviate frailty after exposure to irradiation via SASP inhibition. Targeting the JAK pathway has the potential to be developed as a new treatment for radiotoxicity via senescent cells (Xu et al., 2015). However, to date, no clinical data are available and further studies will need to be confirmed in animal experiments before being transferable to humans.

**3.4.2.7. IL-17A antibody.** In a study performed in rats, Bessout et al. found that IL-17 played an important role in colonic smooth muscle cells to induce expression of pro-inflammatory genes, which can be associated with the development of radiation-induced fibrosis (Bessout et al., 2015). As mentioned earlier, the effect of dexamethasone can reduce IL-17 in mice following IR-induced toxicity (Wang et al., 2014b) Not long after these experiments were performed by Wang et al., the IL-17 A antibody was determined to attenuate the radiation-induced fibrotic lung in mice through blocking of IL-17A, reducing inflammation, and inactivation of the SASP. This finding may offer a new treatment option for radiation-induced toxicity (Wang et al., 2014a). These findings strongly suggest that IL-17 is an important target for reducing late side effects induced after irradiation.

Targeting senescent cells by using the molecules selected into the signaling pathway senescence, such as IGF-1R/PI3K/Akt/mTOR, NF- $\kappa$ B/NEMO and JAK/STAT pathways in the SASP, IL-17 A antibody, and the others senotherapeutics such as senolytics and senomorphics might show a promise perspective in the prevention and treatment of IR-induced toxicity via the senescence process.

#### 3.4.3. Stem cell therapy

Hematopoietic stem cells, as well as mesenchymal stem cells (MSC), are known to be radioresistant to apoptosis by activating the senescence pathway. In the review of Nicolay's team, the authors revealed that MSC-based treatments might afford an exciting new approach to the



therapy and could prevent radiation-induced toxicity (Nicolay et al., 2015; Nicolay et al., 2015). Similarly, the study by Haubner et al. showed that human adipose-derived stem cells (ASC) reduced the overexpression of IL-6, basic fibroblast growth factor (FGF) and adhesion molecules (ICAM-1, VCAM-1) in human dermal microvascular endothelial cells (HDMEC) following irradiation with sequential doses of 2–12 Gy (Haubner et al., 2013). In addition, *in vivo* experiments were performed by Bessout's group, and the investigators determined that adipose-derived mesenchymal stromal cells can reduce radiation-induced epithelial injury with decreased collagen production (Bessout et al., 2015). We hypothesize that therapy-based stem cells can replace IR-induced cellular senescence and can reduce the radiotoxicity-mediated senescence process.

Taken together, these results clearly support the clinical practice that stem cells may have an effect when injected into the irradiated tissue injury, providing therapeutic opportunities aimed at reducing severe lesions induced after RT via decreasing IR-induced senescence.

## 4. Discussion

### 4.1. Mechanisms

Radiation induced accelerated senescence by triggering the DNA damage response machinery, arresting the cellular cycle and ultimately senescent states or called apoptosis-resistant states. Cellular senescence significantly contributes to the onset and progression of fibrotic tissues by secreting pro-inflammatory cytokines and pro-fibrogenic molecules via the SASP. IR promotes senescence from molecular alterations in DNA to protein expressions in different cellular types, such as epithelium, endothelium, immune and stem/stroma cells, cell-derived ectoderm, and cell-derived mesoderm through multicytokine-mediated cell-cell and cell-microenvironment interactions, thus also injured tissues, dysfunctional organs and ultimately morbidities, such as fibrosis, that decrease quality of life. Fibroblasts play a central role in fibrogenesis via the mechanism of the fibroblast-to-myofibroblast transition in response to oxidative stress (RT, UV, chemotherapy, smoking) (Kendall and Feghali-Bostwick, 2014). IR senesces fibroblasts, stimulate pro-fibrotic process in naïve fibroblasts through their SASP factors (Schafer et al., 2017). Better understanding of radiation-induced senescence, accelerated fibrotic tissue, and skin sequelae will be able to help the radiation oncologist in the choice of the best personalized therapy, thereby optimizing outcomes in patient cancer assigning RT.

### 4.2. Prognostic tests and clinical impacts

Senescence markers and their characteristics could also be used to assess the diagnosis and anticipate the prognosis of radiosensitivity in normal tissues by different tests, such as SA- $\beta$ -gal, p16 expression, SASP assay, apoptosis assay, and DDR assay. In clinical studies, *ex-vivo* radiation induced apoptotic lymphocytes from blood samples or radiation-induced ATM nucleoshuttling from fibroblast of skin biopsies have been developed to evaluate intrinsic radiosensitivity. By using the RILA assay based CD8 + T lymphocytes in a prospective multicenter study for late breast fibrosis in BC patients undergoing RT, Azria and colleagues have shown an increase of RILA values at 8 Gy, which was observed for a decreasing percentage of grade  $\geq 2$  in breast fibrosis. The authors proposed the use of RILA as a rapid screening test for predicting the risk of breast fibrosis pre-treatment RT (Ozsahin et al., 2005, 1997; Azria et al., 2017, 2015; Azria et al., 2010; Brengues et al., 2017).

Another test can reflect the senescence process through a DDR assay via ATM and  $\gamma$ -H2 AX residual foci in fibroblasts. This assay was shown in the COPERNIC's retrospective study, determined into three groups of human radiosensitivity based on the speed of ATM nucleoshuttling (COPERNIC project investigators et al., 2016). The cytoplasm to nuclear fast translocation of the ATM after radiation in radioresistance can induce a lower senescent cell risk due to a decrease of ATM in the

cytoplasm which might impede ATM interaction with the lysosome and reduce the mitochondrial dysfunction, thus alleviating senescence (Kang et al., 2017). Further prospective studies are necessary to confirm the predictive value before being used in routine practice.

### 4.3. Therapeutic potentials

As discussed in this review, there is a growing list of novel therapeutic potentials to mitigate radiation-induced fibrosis via targeting senescent cells with a selective molecule. The trend of therapeutic perspectives in radiation-induced accelerated senescence has been focused on the tyrosine kinase cascade. Pharmacological inhibition of IGF-1 G/PI3K/Akt/mTOR signal transduction pathway might become a promising approach to reduce tissue injury due to radiation-induced senescence. Similarly, NF- $\kappa$ B/NEMO and JAK/STAT pathways play an important role in SASP production after radiation; therefore, their inhibitors have promise for treating radiotoxicity via senescent cell formations. These pathways are plausible, although not yet proven in clinical practice of radiation-induced toxicity. Further investigations need to be performed in humans to evaluate their safety and efficacy.

In addition, stem cell therapy, senolytics and senomorphics are the new options to treat radiation-induced senescence dysfunction. While MSC-based treatments have shown beneficial effects in different organ-specific toxicities *in vitro* and *in vivo*, senolytics and senomorphics have shown favorable effects to reduce IR-induced senescence and pulmonary fibrosis *in vitro*, and amelioration of *in vivo* fibrotic status. Senotherapeutics for treatment of radiation-induced fibrosis are continuing to be a focus of research with increasing enthusiasm in the future. These will be nominated in other translational research and in clinical trials to evaluate and guarantee patient safety and reproducible treatment efficacy. Further studies in different selective molecules will be investigated to clarify these unknowns as well as characterize in more detail the molecular mechanisms of radiation-induced cellular senescence promoting sequelae.

In the scenario of IR-induced cellular senescence promoting fibrotic sequelae, the biomarkers for prediction of outcome in radiation-induced senescence are not validated in patients. Senescent assay by analyzing skin tissue biopsy via fibroblast or liquid biopsy via lymphocyte tests could be conceptualized as predictive IR-induced fibrosis in patients after RT. Moreover, there is no existential recommendation of the prophylactics and treatment in clinical practice. Looking ahead to a more promising future for cancer patients with radiotoxicity, further preclinical studies and clinical trials will be indispensable to contribute the intrinsic individual marker as well as the therapeutics in radiation-induced senescence-promoted fibrosis in cancer patients.

## 5. Conclusion

Ionizing radiation-induced cellular senescence is a double-edged sword in the defense against cancer. It is not only a benefit in tumor-suppression, but also acts in a deteriorative process to promote fibrogenesis. It is vitally important to identify predictive biomarkers for intrinsic radiosensitivity of patients in clinical practice. Use of senescence biomarkers might assess the radiosensitivity of prospective RT patients. A better understanding of radiation-induced cellular senescence in fibrotic tissue and skin sequelae will be able to help the radiation oncologist in the choice of the best personalized therapeutic course of action, thereby optimizing outcomes in assigning RT.

### Conflicts of interest

The authors have no conflict of interest.

### Author contributions

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Literature review and data collection: Yazid Belkacemi, Hoang Quy Nguyen

Revision and approval of the manuscript: All authors.

Manuscript writing: Yazid Belkacemi, Hoang Quy Nguyen

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## Annexe 3 : Article sur l'effet antitumoral du blocage TNFR2

Open access

Original research



# TNFR2 blockade of regulatory T cells unleashes an antitumor immune response after hematopoietic stem-cell transplantation

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## ABSTRACT

**Background** Targeting immune checkpoints that inhibit antitumor immune responses has emerged as a powerful new approach to treat cancer. We recently showed that blocking the tumor necrosis factor receptor-type 2 (TNFR2) pathway induces the complete loss of the protective function of regulatory T cells (Tregs) in a model of graft-versus-host disease (GVHD) prevention that relies on Treg-based cell therapy. Here, we tested the possibility of amplifying the antitumor response by targeting TNFR2 in a model of tumor relapse following hematopoietic stem-cell transplantation, a clinical situation for which the need for efficient therapeutic options is still unmet.

**Method** We developed appropriate experimental conditions that mimic patients that relapsed from their initial hematological malignancy after hematopoietic stem-cell transplantation. This consisted of defining in allogeneic bone marrow transplantation models developed in mice, the maximum number of required tumor cells and T cells to infuse into recipient mice to develop a model of tumor relapse without inducing GVHD. We next evaluated whether anti-TNFR2 treatment could trigger alloreactivity and consequently antitumor immune response. In parallel, we also studied the differential expression of TNFR2 on T cells including Treg from patients in post-transplant leukemia relapse and in patients developing GVHD.

**Results** Using experimental conditions in which neither donor T cells nor TNFR2-blocking antibody per se have any effect on tumor relapse, we observed that the coadministration of a suboptimal number of T cells and an anti-TNFR2 treatment can trigger alloreactivity and subsequently induce a significant antitumor effect. This was associated with a reduced percentage of activated CD4<sup>+</sup> and CD8<sup>+</sup> Tregs. Importantly, human Tregs over-expressed TNFR2 relative to conventional T cells in healthy donors and in patients experiencing leukemia relapse or cortico-resistant GVHD after hematopoietic stem cell transplantation.

**Conclusions** These results highlight TNFR2 as a new target molecule for the development of immunotherapies to treat blood malignancy relapse, used either directly in grafted patients or to enhance donor lymphocyte infusion strategies. More widely, they open the door for new perspectives to amplify antitumor responses against solid cancers by directly targeting Tregs through their TNFR2 expression.

## INTRODUCTION

Regulatory T cells (Tregs) play a key role in the fine tuning of immune responses in allogeneic hematopoietic stem-cell transplantation (alloHSCT).<sup>1–5</sup> Cell therapy using Treg infusions to prevent graft-versus-host disease (GVHD) has shown very promising results in the clinic.<sup>6–11</sup> Conversely, we performed ex vivo Treg depletion from donor lymphocyte infusions (DLIs) in 2010 to enhance the graft-versus-leukemia/graft-versus-tumor (GVL/GVT) effect in patients who relapsed after alloSCT without previously developing GVHD.<sup>12</sup> This initial clinical trial suggested that Treg depletion can trigger alloreactivity and its associated GVL/GVT effect. A confirmatory, randomized, double-blind trial following the same approach is currently ongoing (DLI-Boost, ClinicalTrials.gov, NCT03236129).

A major advance in our understanding of the mechanisms of action of Tregs occurred with the identification of their preferential surface expression of tumor necrosis factor (TNF) receptor-type 2 (TNFR2). Indeed, it was first demonstrated that TNFR2 is critical for the stability and suppressive functions of Tregs.<sup>13–16</sup> In cancer, most studies have shown that Tregs are TNFR2<sup>hi</sup> and that TNFR2 antagonism can lead to solid tumor regression, mainly in TNFR2-expressing cancers, such as ovarian cancer or Sézary syndrome.<sup>17–21</sup> In experimental alloHSCT, Chopra *et al* initially demonstrated that TNFR2 activation using a homemade agonist molecule can control life-threatening GVHD in mice without abrogating the GVL effect.<sup>22</sup> Conversely, we previously showed the complete dependency of Tregs on TNF- $\alpha$  to maintain their suppressive capacity in vivo. We demonstrated that the protective effect of therapeutic Tregs against GVHD was completely abolished when mice were treated



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with a TNFR2-blocking mAb at the time of alloHSCT in a Treg-based cell therapy approach. In addition, we conclusively demonstrated in the aforementioned setting that therapeutic Tregs rely on their expression of TNFR2 and require TNF- $\alpha$  production by donor T cells to control the disease.<sup>23</sup>

Here, we hypothesized that blocking the TNF- $\alpha$ /TNFR2 pathway would provide space for a powerful and amplified GVL/GVT effect to emerge, which could be useful in the treatment of blood malignancy relapses after alloHSCT. Indeed, originally proposed to treat TNFR2-expressing solid tumors or cutaneous T-cell lymphoma,<sup>18,19</sup> TNFR2 blockade has been underappreciated and never tested to trigger an allogeneic immune response and hence an antitumor effect in the specific setting of alloHSCT. The aim is to induce a strong GVL/GVT effect by blocking the effects of TNFR2-expressing Tregs in a targeted population preferentially consisting of patients who relapse without previously developing GVHD, as in our aforementioned clinical trial.<sup>12,24</sup>

To test this hypothesis, we developed a dedicated experimental model in which the number of donor T cells infused in recipient mice did not allow GVHD development and was also insufficient to mediate a complete GVL/GVT effect. After tumor cell infusion, this very sensitive model allowed us to evaluate the *in vivo* effect of treatments that target immune cells by simply detecting either clinical signs of alloreactivity or the produced GVL/GVT effects through macroscopic and/or blood tumor detection. Our data show that the *in vivo* administration of anti-TNFR2 treatment triggers strong alloreactivity associated with a potent GVL/GVT effect. In addition, we have also generated promising data using samples collected from post-transplant patients with relapsing leukemia or GVHD showing that Tregs preferentially overexpress TNFR2 relative to conventional T cells. These observations provide further support for the efficiency and versatility of TNFR2-modulation strategies to block Tregs and trigger an antitumor or allogeneic immune response, as observed here, or to induce Tregs with TNFR2 agonists to dampen the immune response, as recently suggested.<sup>22</sup>

## METHODS

### Mice

Female C57BL/6J (B6, H-2<sup>b</sup>), (B6xDBA2) F1 ((B6D2) F1, H-2<sup>bx/d</sup>), (BalbCxB6) F1 ((CB6)F1, H-2<sup>bx/d</sup>), and NSG (NOD.Cg-Prkdc<sup>scid</sup>IL2rg<sup>tm1Wjl</sup>/SzJ) mice were obtained from Charles River Laboratories (France) and used at 10–12 weeks of age. Mice were housed under specific pathogen-free conditions.

### Bone-marrow transplantation and tumor relapse models

Recipient (B6D2)F1 female mice received 10Gy irradiation (X-ray) followed by retro-orbital infusion of 5 $\times$ 10<sup>6</sup> bone-marrow (BM) cells and 1 $\times$ 10<sup>6</sup> CD3<sup>+</sup> T cells from C57BL/6 mice. BM and T-cell suspensions were prepared using leg bones and lymph nodes, respectively, as previously described.<sup>12</sup> P815-GFP mastocytoma cells (gift from Dr G. Marodon) were also injected intravenously (one or 2 $\times$ 10<sup>4</sup> per

mouse) into the retro orbital sinus of (B6D2)F1 female mice at the time of transplantation. A20 cells were purchased from the American Type Culture Collection. A20 cells were transfected with a lentiviral vector encoding GFP gene (Vectalys) according to the manufacturer's instructions. After Transfection, A20-GFP cells were sorted using a MoFlo Legacy instrument (Beckman Coulter, Villepinte, France) with a purity of 99%. Female (CB6)F1 recipient mice received 9Gy of irradiation (X-ray) followed by retro-orbital infusion of 5 $\times$ 10<sup>6</sup> BM cells and 1 $\times$ 10<sup>6</sup> CD3<sup>+</sup> T cells from B6 mice. In addition, 1 $\times$ 10<sup>6</sup> A20-GFP cells were injected into the retro orbital sinus of mice at the time of bone-marrow transplantation (BMT). After semi-allogeneic BMT, GVHD symptoms and the apparition of tumors were evaluated three times per week. Clinical GVHD was evaluated as previously described.<sup>23</sup> Each of the five following parameters was scored 0 if absent or one if present: weight loss >10% of the initial weight, hunched posture, skin lesion(s), dull fur, and diarrhea. Mice were considered to be positive for tumors if a tumor mass was visible near the eye and/or if more than 0.5% of P815-GFP, or A20-GFP cells were detected in their peripheral blood.

### Histopathological examination

Liver, skin, small intestine, and colon samples were preserved in Bouin's fixative and embedded in paraffin. For these organs, 5  $\mu$ m thick sections were stained with H&E for histological examination as previously described.<sup>23</sup> Slides were analyzed by a pathologist in a blinded fashion to assess the intensity of GVHD. GVHD lesions in each sample were scored according to a semi-quantitative scoring system previously described.<sup>25</sup>

### Antibody treatment

Anti-TNFR2 (TR75-54.7), anti-CTLA-4 (9H10), and anti-PD-1 (RMP1-14) mAbs were purchased from BioXCell (West Lebanon, NH). TNFR-2 blockade was achieved by performing three intraperitoneal mAb injections of 500  $\mu$ g each on day 0, day 2, and day 4 after BM transplant or a single intraperitoneal 500  $\mu$ g injection on d10 after BMT. CTLA-4 or PD1 blockades were achieved by performing three intraperitoneal mAb injections of 200  $\mu$ g each on day 0, day 3, and day 6 after BMT.

### Patients

Relapsing patients and those experiencing GVHD were transplanted in adult hematology unit from Saint Louis hospital, Paris, France and Henri Mondor hospital, Créteil, France. All patients and donors signed an informed consent for (i) registration in the Promise database and (ii) cryopreservation of biological material for research purposes. This study was conducted in accordance with the Declaration of Helsinki.

### Human peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (Laboratoires Eurobio, Les Ulis, France) and resuspended in phosphate-buffered saline (PBS; Life Technologies; Thermo Fisher Scientific, Waltham, Massachusetts, USA)





containing 3% fetal bovine serum (FBS; Gibco, Life Technologies; Thermo Fisher Scientific).

#### Short-term in vivo xenogeneic human lymphocyte activation

NSG mice were used to test the effect of a blocking anti-human TNFR2 mAb (clone UTR1) on healthy donor immune cells in vivo. PBMCs isolated from two healthy donor blood samples were pretreated with either UTR1 or a control immunoglobulin of the same isotype (mIgG1) for 2 hour at 37°C (10 µg Ab/mL for 10<sup>7</sup> PBMCs). NSG mice were then injected intravenously with pretreated cells (n=3 mice/donor). After 6 days, their spleens were collected, and their content analyzed by flow cytometry.

#### Phenotypic analysis of immune cell populations

Spleens were harvested on day 12 and splenocytes stained with the antibodies listed in online supplemental table 1. Non-specific binding was blocked using anti-CD16/CD32 (Miltenyi Biotec). After isolation, PBMCs from healthy donors or patients were stained with the antibodies listed in online supplemental table 2). Non-specific binding was blocked using FC block (BD bioscience). For intracellular staining, cells were fixed and permeabilized with fixation/permeabilization solution (ThermoFischer), according to the manufacturer's instructions. Data acquired with a BD LSR-Fortessa flow cytometer were compensated and exported into FlowJo (version 10.0.8, TreeStar Inc.). An unbiased analysis was performed on splenocytes using the t-distributed stochastic neighbor embedding (t-SNE) algorithm tool (Flowjo software) to reduce the flow cytometry data to two dimensions. Live cells from mice in the same group were merged. Results are shown as a t-SNE map displaying the repartition of the expression of a given marker among randomly sampled cells from the indicated parent population.

#### Statistical analysis

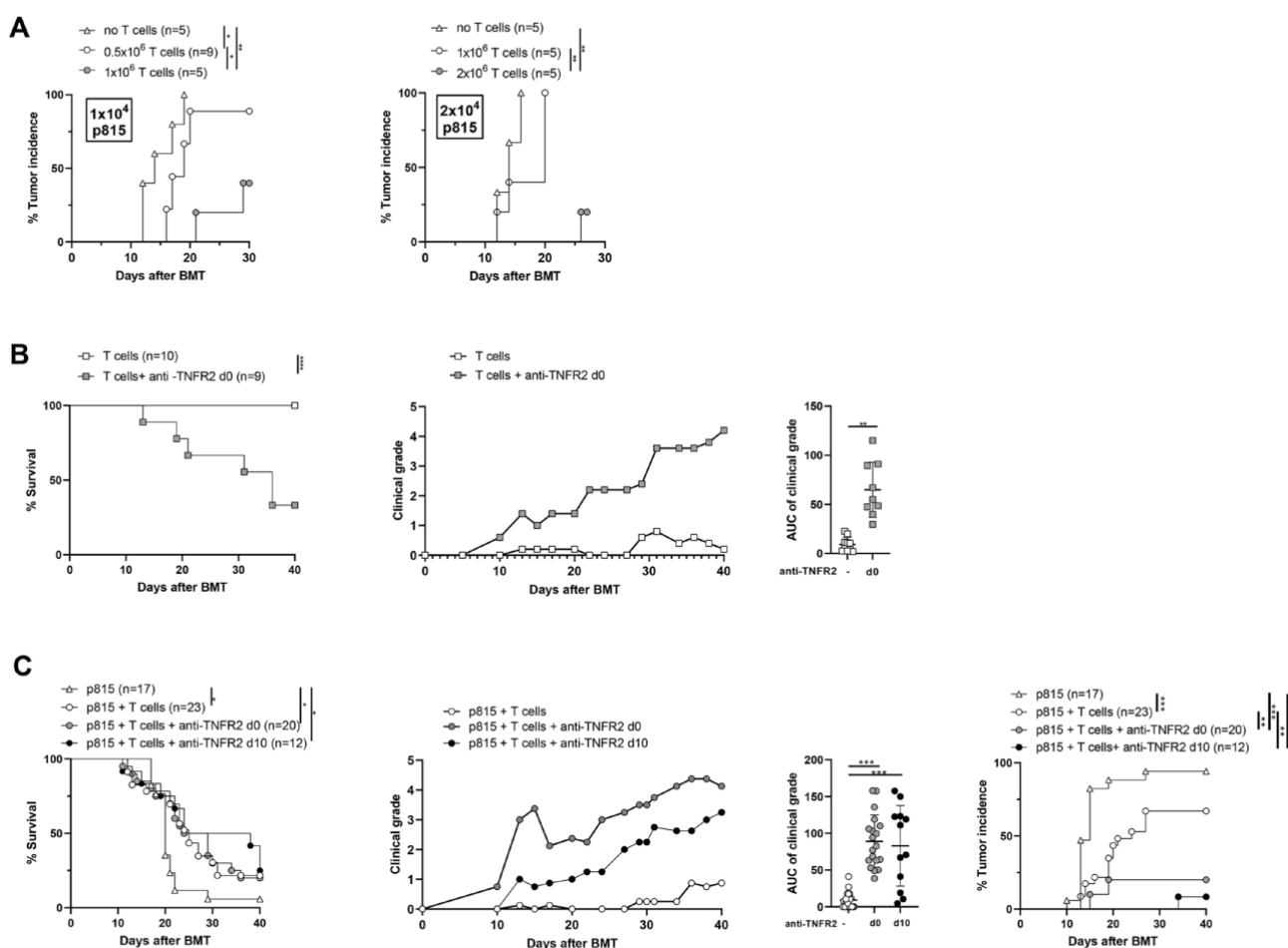
Statistical analysis was performed using GraphPad Prism nine software (GraphPad Software, San Diego, California, USA). The data of control and treatment groups were compared using non-parametric tests. For Kaplan-Meier survival curves, groups were compared using the log rank test. Other data were compared using either a Mann-Whitney test (for two-by-two comparisons) or a Kruskal-Wallis test with Dunn's multiple comparison post-test (for multiple comparisons). Statistical significance is indicated as \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, and \*\*\*\*p<0.0001. Population statistics are displayed as the mean±SD.

## RESULTS

### Anti-TNFR2 treatment triggers a GVL/GVT effect after hematopoietic stem-cell transplantation

We first performed a series of experiments to define the maximum number of required tumor cells and T cells to infuse into recipient mice to develop a model of tumor relapse without inducing GVHD. The appropriate experimental conditions that mimic those of patients relapsing after alloHSCT from their initial hematological

malignancy were achieved using 2×10<sup>4</sup>P815 tumor cells (mastocytoma) and 1×10<sup>6</sup>T cells. Indeed, we never observed any clinical signs of GVHD under these experimental conditions and the tumor incidence was 100% on day 20 (figure 1A). In the absence of P815 cells, we next evaluated whether anti-TNFR2 treatment could trigger alloreactivity with a deliberately restricted number of donor cells. We triggered an alloreactive immune response in mice receiving 1×10<sup>6</sup>T cells and treated with an anti-TNFR2 mAb on day 0, day 2, and day 4. This was shown by elevated clinical grades of GVHD throughout the experiment, quantified by a significantly higher area under the curve (AUC) of the GVHD score and a lower survival rate at day 40 than for untreated mice, for which no signs of alloreactivity were detected (figure 1B). Thus, under experimental conditions in which a very low number of T cells is injected, brief treatment with anti-TNFR2 makes it possible to initiate a significant allogeneic immune response. We then reproduced these experiments to validate our hypothesis, adding P815 cells at the time of grafting to mimic the occurrence of a tumor relapse after alloHSCT (figure 1C). Tumor cells were detected in 16 of 17 mice that had no T-cell infusion, of which 94% were dead by the experimental endpoint (MST=20 days). Tumors were detected in 14 of 23 (60%) mice infused with 10<sup>6</sup>T cells, suggesting that the mortality observed for all mice (MST=25 days) was related to GVHD for only a small percentage. Indeed, no or very low clinical grade GVHD was consistently observed in this group of mice. In contrast, the administration of the anti-TNFR2 mAb on day 0, day 2, and day 4 resulted in a dramatic decrease in tumor incidence, with only 4 of 20 (20%) mice harboring P815 cells. The mortality observed in this group (MST=26.5 days) could be mainly attributed to an allogeneic immune response, as attested by elevated clinical grades of GVHD. We tested the versatility of this approach by delaying anti-TNFR2 treatment. We, thus, repeated the same experiments and compared the effect of anti-TNFR2 treatment initiated on day 0 or day 10. Remarkably, a single injection of anti-TNFR2 on d10 was sufficient to trigger a potent GVL/GVT effect. A body of clinical data suggests that delaying and reducing the dose of anti-TNFR2 treatment may be safer. Indeed, the time of death of the mice was slightly delayed (MST=31 days) and their mean GVHD clinical scores reduced throughout the follow-up, whereas the antitumor effect was preserved similarly to that of mice treated on day 0, day 2, and day 4; P815 cells were detected in only 1 of 12 mice (figure 1C). Dedicated experiments were repeated to evaluate potential histopathological manifestations after anti-TNFR2 treatment. We assessed the consequences of triggered alloreactivity on target tissues of GVHD in treated mice on d12. This allowed us to evaluate the short-term (for mice treated on d10) and more long-term (for mice treated on day 0, day 2, and day 4) histological consequences of anti-TNFR2 treatment after its administration. Two days after treatment, we observed several histological manifestations of GVHD, with a trend toward an increase in

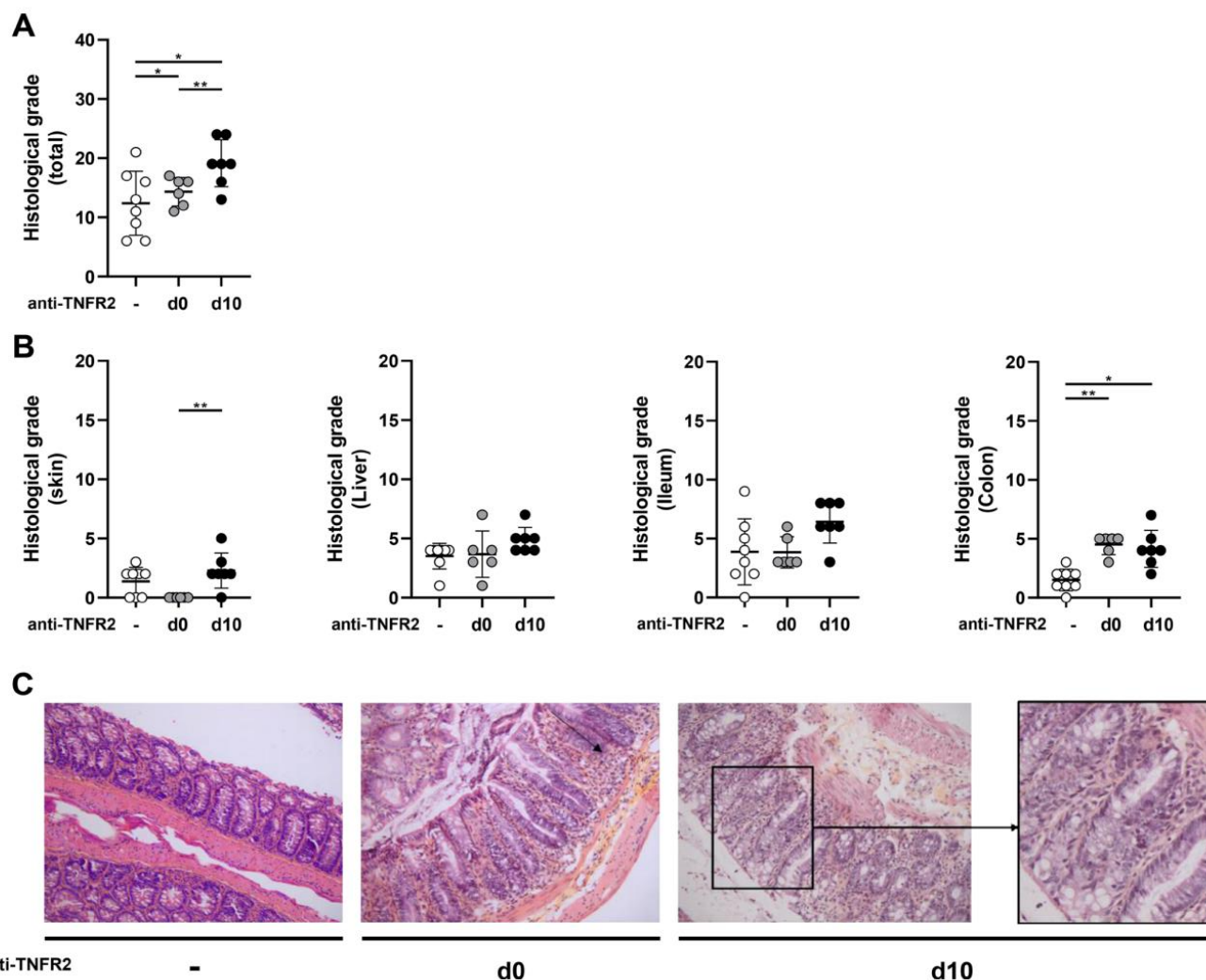


**Figure 1** TNFR2 blockade using an anti-TNFR2 mAb triggers alloreactivity and the associated graft-versus-leukemia/graft-versus-tumor (GVL/GVT) effect after alloHSCT. (A) Lethally irradiated (B6D2)F1 recipient mice received  $5 \times 10^6$  BM cells,  $1$  or  $2 \times 10^6$  T cells, and  $1 \times 10^4$  (left) or  $2 \times 10^4$  (right) P815 cells. The tumor incidence observed for each dose of P815 cells is depicted. (B) Female (B6D2)F1 mice underwent (total body irradiation) TBI, followed by BMT with  $1 \times 10^6$  T cells (T cell group, n=10) and treated with anti-TNFR2 mAb administered on day 0, day 2, and day 4 (T cell + anti-TNFR2 group, n=9). Survival curves, clinical grade evolution (right), and the area under the curve (AUC) of graft-versus-host disease (GVHD) manifestations are presented. The cumulative data of two independent experiments are shown. (C) Mice underwent TBI followed by BMT with  $1 \times 10^6$  T cells and  $2 \times 10^4$  P815 cells with or without anti-TNFR2 (500  $\mu$ g) treatment administered on day 0, day 2, and day 4 (labeled as d0), or with a single injection on day 10 (d10). Survival curves, clinical grades, and the AUCs, as well as tumor incidence are depicted. The cumulative data of four independent experiments are shown. Kaplan-Meier survival curves were compared using the log-rank test. The AUCs were calculated for the GVHD clinical grade curve for each mouse and are presented as the mean  $\pm$  SD. Either Mann-Whitney or Kruskal-Wallis tests were performed, depending on the number of groups that were compared. \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

the histological grade for the skin, liver, and small intestine, and a statistically significant increase for that of the colon of d10-treated mice, leading to a significant difference on summing all of the clinical grades of the target tissues relative to those of the control group (figure 2), in accordance with clinical observations. Six days after the end of treatment, histopathological manifestations were observed only in the colons of mice treated on day 0, day 2, and day 4. Thus, anti-TNFR2 treatment rapidly led to histological signs of GVHD in target organs, demonstrating a surprisingly prompt physiopathological translation of this treatment.

We ruled out the possibility that the observed effects were limited to the P815 tumor model used in a specific genetic combination of alloHSCT by repeating the same type of experiment using A20 cells (B-cell leukemia) in the B6 background in the B6xBalb/C genetic combination. First, we reproduced the result that TNFR2 treatment given at the time of the graft leads to increased alloreactivity in this second model of alloHSCT (online supplemental figure S1A). We also observed spontaneous alloreactivity of donor T cells, leading to higher clinical grades of GVHD than in the previously used model. This was illustrated by partial induction of a GVL effect observed





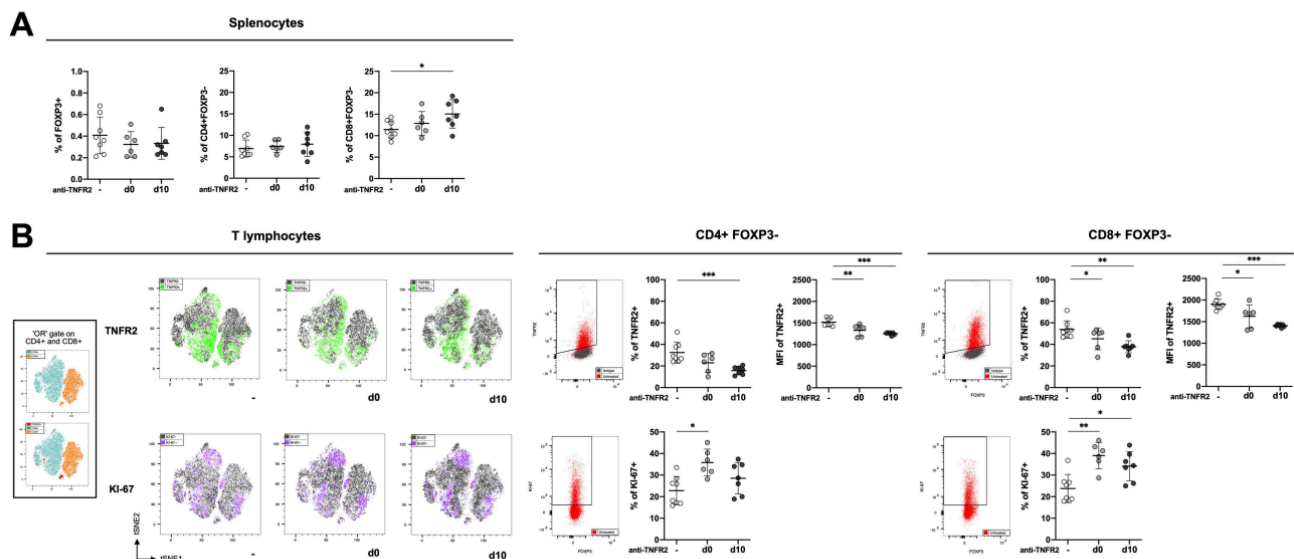
**Figure 2** Effect of TNFR2 blockade on target organs of GVHD. (B6D2)F1 recipient mice underwent TBI followed by bone-marrow transplantation (BMT) with  $1 \times 10^6$  T cells and  $2 \times 10^4$  P815 cells with or without anti-TNFR2 (500  $\mu$ g) treatment administered on day 0, day 2, and day 4 or with a single injection on day 10. On day 12, mice were sacrificed and GVHD target organs collected and analyzed. (A) the histological grades of each target organ of GVHD were summed to give a global histological score. (B) Histological grade of skin, liver, ileum, and colon. (C) Severe aGVHD lesions were observed in the colon of mice treated with anti-TNFR2, either at day 0 or day 10, as attested by the global loss of crypt morphology and crypt hyperplasia, marked crypt-cells apoptosis, and the presence of a substantial lymphocytic infiltrate (example enlarged on the right). Results are presented as the mean  $\pm$  SD and each plot represents one mouse. P values were determined by one-way analysis of variance. \* $P < 0.05$ , \*\* $p < 0.01$ .

when A20 cells were added, although it was not statistically significant. Leukemic cells were detected in 71% of mice receiving T cells vs 100% of mice not receiving T cells. As in the P815 setting, anti-TNFR2 treatment increased the clinical manifestations of alloreactivity relative to those of untreated mice. Indeed, only 28% of mice developed leukemia when anti-TNFR2 treatment was administered on day 0, day 2, and day 4 (online supplemental figure S1B). Importantly, anti-TNFR2 treatment alone did not show any direct effect in the absence of T lymphocytes, neither on P815 nor A20 tumor development, leading to an unchanged tumor incidence (online supplemental figure S2). Thus, we demonstrated that anti-TNFR2 treatment starting on day 0 or day 10 strongly induced a

potent GVL/GVT effect in a setting in which injected T cells alone were inefficient in two relapse models using two different genetic combinations of alloHSCT.

#### Anti-TNFR2 treatment alters the phenotype of both CD4<sup>+</sup> and CD8<sup>+</sup> Treg populations after hematopoietic stem-cell transplantation

We evaluated the biological effects of both anti-TNFR2 modalities of treatment on T cells. First, we performed a competition experiment *ex vivo* to verify whether we could still detect the surface expression of TNFR2 despite treating the mice with an anti-TNFR2 mAb. The addition of various doses of therapeutic mAb to C57BL/6 splenocytes did not impair the fixation and further detection



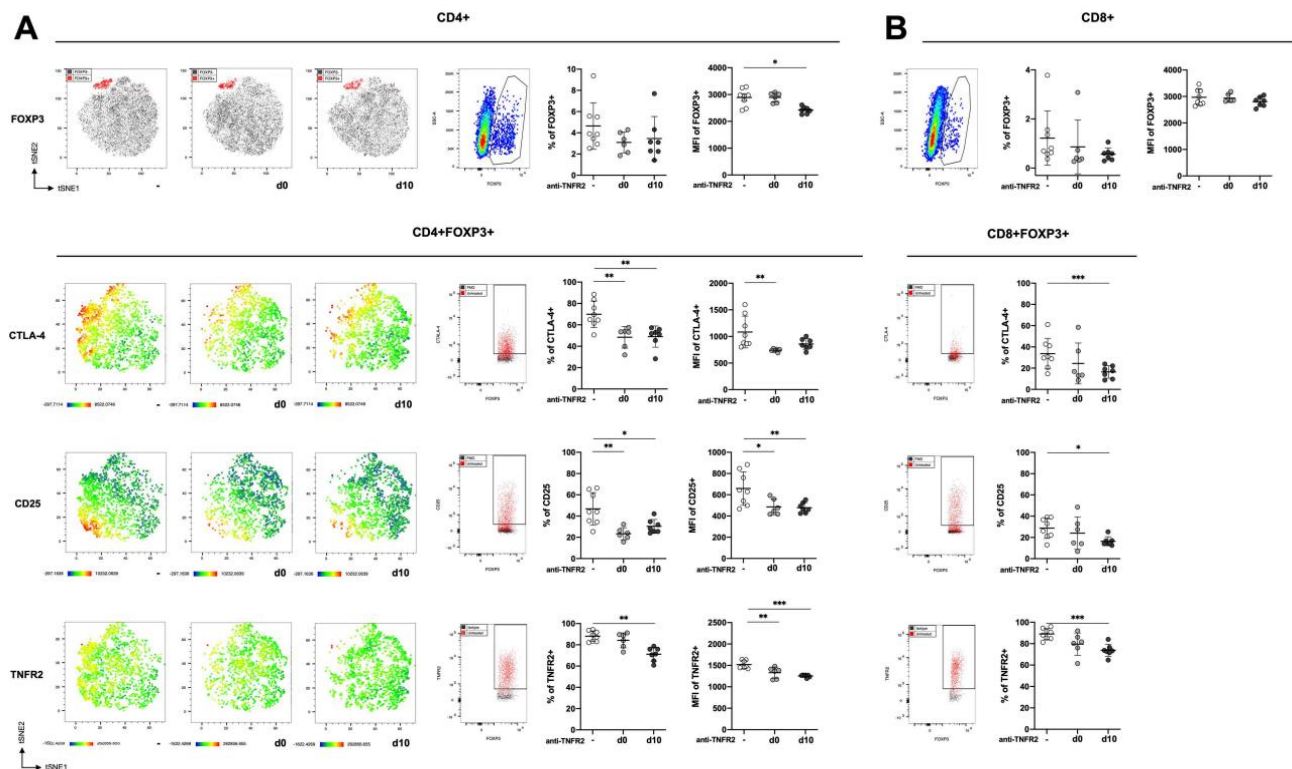
**Figure 3** Mechanism of action of anti-TNFR2 treatment on conventional T cells. Splenocytes of the mice described in figure 2 were analyzed by flow cytometry. (A) percentage of FOXP3<sup>+</sup>, CD4<sup>+</sup>FOXP3<sup>-</sup>, and CD8<sup>+</sup>FOXP3<sup>-</sup> lymphocytes among live splenocytes. (B) cell clustering using a t-distributed stochastic neighbor embedding (t-SNE) algorithm performed on an « or » gate, including both live CD4<sup>+</sup> and CD8<sup>+</sup> cells. The localization of FOXP3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cell clusters is indicated in the subfigure on the left. The localization of TNFR2<sup>+</sup> (in green) and KI-67<sup>+</sup> (in purple) cells among the aforementioned lymphocyte populations were mapped following anti-TNFR2 mAb administration on day 0, day 2, and day 4 (n=6) or day 10 (n=7) or without (n=8). The gating strategy and percentage of TNFR2<sup>+</sup> and KI-67<sup>+</sup> cells among the CD4<sup>+</sup>FOXP3<sup>-</sup> and CD8<sup>+</sup>FOXP3<sup>-</sup> cells are shown for each group, as well as their mean fluorescence intensity. Data are plotted as the mean±SD statistical significance from the controls was determined using Mann-Whitney tests. \*P<0.05, \*\*p<0.01, \*\*\*p<0.001.

of TNFR2 at the lymphocyte membrane by flow cytometry, excluding the possibility of antigen masking by the treatment (online supplemental figure S3). Then, grafted mice were treated on day 0, day 2, and day 4, or day 10 and their spleens collected on day 12, that is, 6 or 2 days after the end of treatment and around the time of the apparition of the first tumors in control mice (figure 1C). This endpoint provided the best window to observe the influence of anti-TNFR2 administration on the immune responses of mice with yet no symptoms of tumors or GVHD, but on the verge of developing one or the other. At this specific timepoint, the frequency of CD8<sup>+</sup>FOXP3<sup>-</sup> cells was higher in day 10-treated mice than the controls (15.07%±3.33% and 11.45%±2.00%, respectively), whereas that of CD4<sup>+</sup>FOXP3<sup>-</sup> and FOXP3<sup>+</sup> cells remained unchanged (figure 3A). Early anti-TNFR2 administration (day 0) did not induce any modification in the frequency of these lymphocyte populations. We then quantified CD107a, Grz B, and IFN $\gamma$  on CD8 cells, with or without anti-TNFR2 treatment. We did not observe any alterations in Grz B expression or INF $\gamma$  production (not shown). However, CD107a expression increased in CD8<sup>+</sup>FOXP3<sup>-</sup> cells, with a marked and statistically significant effect when mice were treated at day 10 (18.47%±2.98 vs 14.91±0.90 for untreated mice, p<0.05), suggesting improved cytolytic activity for CD8<sup>+</sup> T cells.<sup>26</sup> We then addressed the changes in expression of several lymphocyte function-related markers after anti-TNFR2 treatment using an unbiased approach. T-SNE maps were created to display the repartition of TNFR2 and KI-67

expression among randomly sampled CD4<sup>+</sup> and CD8<sup>+</sup> cells from summed mouse cells in each group (figure 3B, left). Importantly, proliferating cells (KI-67<sup>+</sup>) in our model were not restricted to the TNFR2<sup>+</sup> or TNFR2<sup>-</sup> population, despite the well-described involvement of TNFR2 in NF- $\kappa$ B pathway-related proliferation in conventional T cells,<sup>27–29</sup> as apparent when comparing the TNFR2 and KI-67 maps of the same group. The percentage and surface density of expression of TNFR2 were dramatically lower in both CD4<sup>+</sup>FOXP3<sup>-</sup> and CD8<sup>+</sup>FOXP3<sup>-</sup> conventional T cells after anti-TNFR2 treatment for the day 0, day 2, and day 4 group and day 10 group, compared with untreated control mice. In contrast, the frequency of KI-67<sup>+</sup> cells was higher than that of conventional T cells of control mice for the CD8<sup>+</sup>FOXP3<sup>-</sup> population after the day 10 treatment and for both CD4<sup>+</sup>FOXP3<sup>-</sup> and CD8<sup>+</sup>FOXP3<sup>-</sup> lymphocytes after the day 0, day 2, and day 4 treatment. Thus, conventional T-cell proliferation can be triggered by anti-TNFR2, consistent with the previously described alloreactivity manifestations and despite a reduction of TNFR2 at their surface in these settings.

We then focused our analysis on CD4<sup>+</sup>FOXP3<sup>+</sup> Tregs, the main targeted cell population of our anti-TNFR2 therapeutic strategy. First, t-SNE mapping revealed a slight diminution in the FOXP3-expressing cell cluster (red) associated with a statistically significant decrease in FOXP3 MFI when anti-TNFR2 was administered at d10 (figure 4A). Anti-TNFR2 treatment did not directly affect the number of Tregs, consistent with our previous observations.<sup>23</sup> Nevertheless, we observed a decrease in FOXP3





**Figure 4** Mechanism of action of anti-TNFR2 treatment on CD4<sup>+</sup> and CD8<sup>+</sup> regulatory T cells. Splenocytes of the mice described in figure 2 were analyzed by flow cytometry. (A) Cell clustering using t-distributed stochastic neighbor embedding (t-SNE) algorithm performed on the CD4<sup>+</sup> (top) or the CD4<sup>+</sup>FOXP3<sup>+</sup> (bottom) cell gates. The localization of Foxp3<sup>+</sup> (in red) or CTLA-4<sup>+</sup>, CD25<sup>+</sup>, and TNFR2<sup>+</sup> (heatmap color gradient) cells among the aforementioned lymphocyte populations was mapped following anti-TNFR2 mAb administration on day 0, day 2, and day 4 or day 10 or without. The gating strategy and percentage of FOXP3<sup>+</sup> cells among CD4<sup>+</sup> cells and CTLA-4<sup>+</sup>, CD25<sup>+</sup>, and TNFR2<sup>+</sup> cells among CD4<sup>+</sup>FOXP3<sup>+</sup> cells, as well as their mean fluorescence intensity, are plotted for each group of mice. (B) Gating strategy, percentage, and mean fluorescence intensity of FOXP3<sup>+</sup> cells among CD8<sup>+</sup> cells (top) and gating strategy and percentage of CTLA-4<sup>+</sup>, CD25<sup>+</sup>, and TNFR2<sup>+</sup> cells among CD8<sup>+</sup>FOXP3<sup>+</sup> cells (bottom) for each group of mice. Data are plotted as the mean±SD statistical significance from the controls was determined using Mann-Whitney tests. \*P<0.05, \*\*p<0.01, \*\*\*p<0.001.

density on Tregs after treatment, echoing the results of a recent publication showing that TNFR2 signaling prevents DNA methylation at the FOXP3 promoter.<sup>30</sup> In addition, the t-SNE representation revealed the almost complete disappearance of various clusters of Tregs highly expressing CTLA-4, CD25, and TNFR2 after both treatment modalities, suggesting the loss of the highly suppressive cluster of Tregs in mice treated on day 0, day 2, and day 4 and those treated on day 10. In mice treated on day 10, the percentage of Tregs expressing TNFR2, CD25, and CTLA-4, as well as the levels of expression of TNFR2 and CD25 among Tregs, was lower than that of control mouse Tregs. Mice treated on day 0 showed comparable but less marked effects (figure 4A). This is consistent with a decrease in the percentage of Tregs expressing ICOS, CD39, KLRG1, NRP1, and CD103, as all are membrane markers associated with the suppressive functions and activation of Tregs<sup>31</sup> (online supplemental figure S4).

A population of CD8<sup>+</sup>FOXP3<sup>+</sup> T cells with suppressive functions that emerge in the early stages of GVHD has been identified in mice<sup>32</sup> and ex vivo generated in

humans.<sup>33,34</sup> In our model, without any anti-TNFR2 treatment, we also identified a population of CD8<sup>+</sup>FOXP3<sup>+</sup> T cells that exhibited elevated expression of TNFR2 (89.01%±5.10%), in addition to CD25 (28.76%±3.41%) and CTLA-4 (33.68%±5.10%) markers (figure 4B). As for CD4<sup>+</sup>FOXP3<sup>+</sup> T cells, anti-TNFR2 treatment on d10 only tended to decrease their percentage, but markedly reduced their expression of TNFR2, CD25, and CTLA-4. We did not observe any major modifications 6 days after the end of anti-TNFR2 treatment initiated on day 0, either because the CD8<sup>+</sup>FOXP3<sup>+</sup> population had not yet emerged at the time of treatment or because it had the time to reconstitute before the endpoint. Heinrichs *et al* reported that alloreactive CD8<sup>+</sup>FOXP3<sup>+</sup> T cells moderately attenuate GVHD, while sparing the GVL effect.<sup>35</sup> Here, we demonstrated that partially decreasing the frequency of these cells via anti-TNFR2 treatment and, more importantly, decreasing their state of activation, as illustrated by the diminution of CTLA-4<sup>+</sup>, CD25<sup>+</sup>, and TNFR2<sup>+</sup> CD8<sup>+</sup> Tregs, did not impair the triggering of alloreactivity or the associated GVL effect.

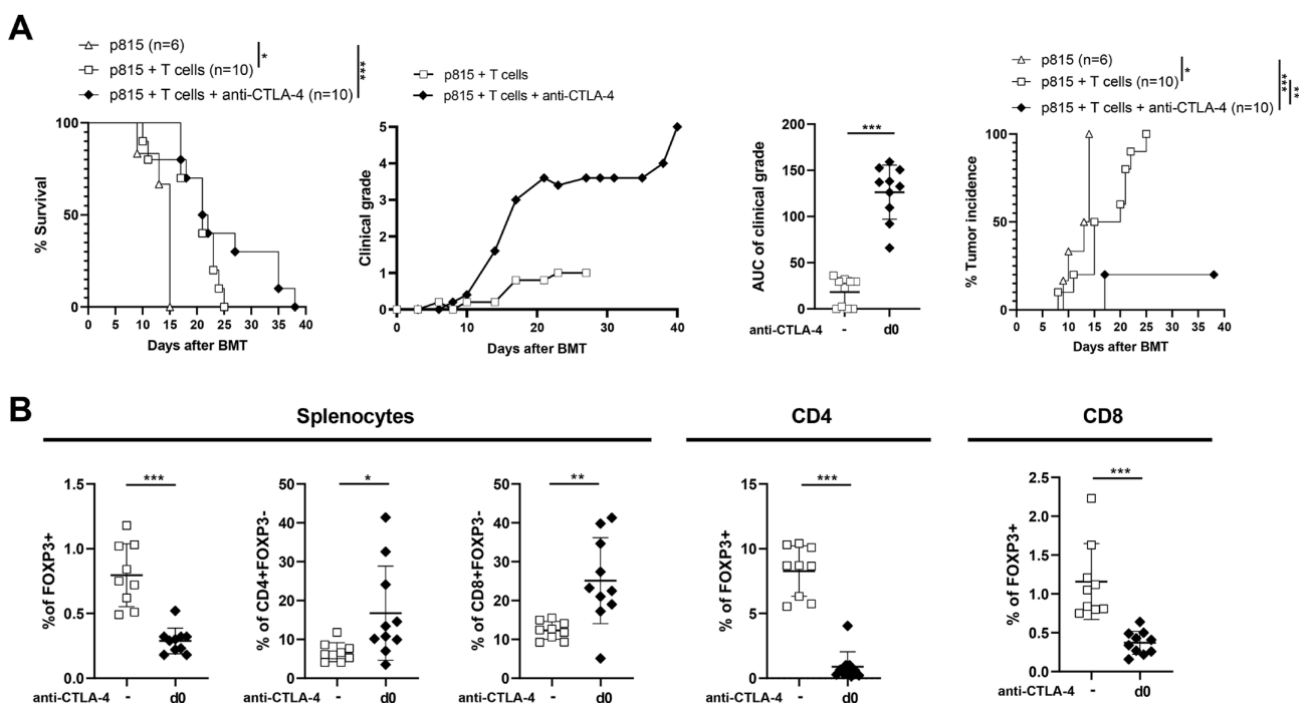


As some cells may coexpress TNFR1 and TNFR2, we also evaluated the effect of anti-TNFR2 treatment on TNFR1 expression. First, TNFR1 was expressed by a small portion of CD4 T cells, regardless of their FOXP3 status, and anti-TNFR2 treatment did not alter TNFR1 expression in these cell populations. The same was true for CD8<sup>+</sup>FOXP3<sup>+</sup> cells. Whether TNFR1 expression can be observed in a small percentage of CD8<sup>+</sup>FOXP3<sup>+</sup> cells (this cell population representing less than 2% of splenocytes), treatment at day 0, but not day 10, induced a slight increase in the percentage of TNFR1-expressing CD8<sup>+</sup> Tregs. By contrast, 60% of non-CD3 splenocytes expressed TNFR1 and anti-TNFR2 treatment had no effect on these cells. In no case was the MFI of TNFR1 modified by anti-TNFR2 treatment (online supplemental figure S5). In summary, anti-TNFR2 treatment had little or no impact on TNFR1 expression by splenocytes of grafted mice.

### Preclinical considerations of the anti-TNFR2 strategy in light of existing therapies

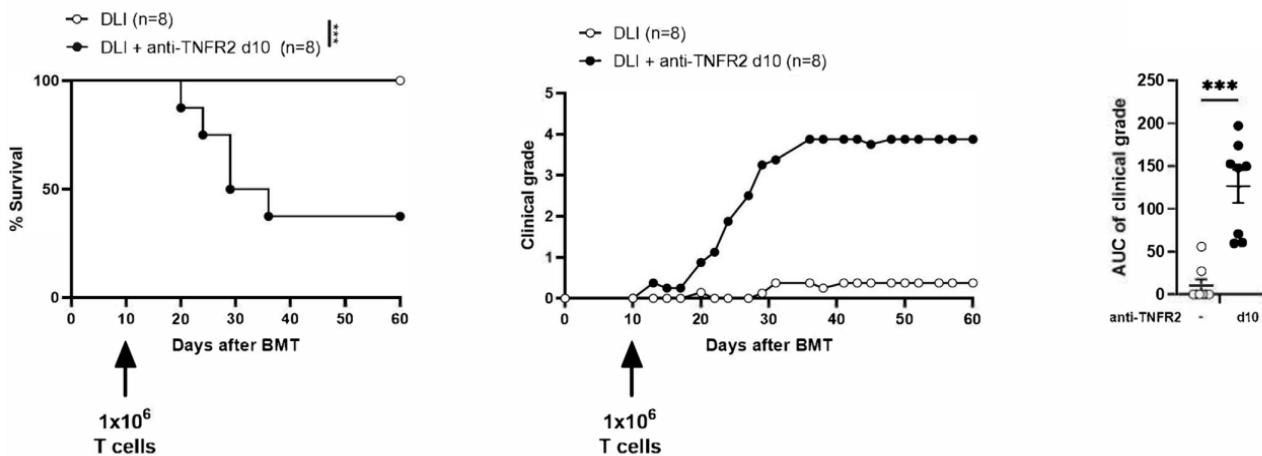
The side effects and benefits of anti-PD-1 and anti-CTLA-4 mAb administration in post-transplant settings to treat relapsing patients have been previously described.<sup>36–39</sup> Notably, severe GVHD and autoimmune complications have been observed. We, thus, evaluated anti-PD1 and

anti-CTLA-4 treatment using the exact same conditions established to test anti-TNFR2 treatment. Compared with anti-CTLA4, anti-PD1 treatment only produced moderate alloreactivity and a partial antitumor effect (online supplemental figure S6). We then focused our efforts on anti-CTLA4 treatment, for which we observed important clinical effects since anti-CTLA-4 treatment increased alloreactivity, whereas tumor incidence was markedly reduced (figure 5A). Merging all the data from the experiments performed in this study (online supplemental figure S7) showed that the clinical intensity of GVHD was stronger after anti-CTLA-4 administration than after anti-TNFR2 treatment on day 0, day 2, and day 4 and that on day 10 (AUC=89 and 82 for anti-TNFR2 on day 0 and day 10, respectively, vs an AUC=126 for anti-CTLA-4,  $p<0.05$ ). At the time of spleen collection (day 12), the Treg frequency among CD4<sup>+</sup> T cells of anti-CTLA-4 treated individuals was markedly lower than that of control mice (from  $8.27\% \pm 1.94\%$  to  $0.89\% \pm 1.15\%$ ) (figure 5B). In our hands, anti-TNFR2 treatment mainly modified the activation state of Tregs, without considerably modifying their frequency (figure 4), whereas anti-CTLA-4 administration led to the quasi-disappearance of the Treg population. The subpopulation of CD8<sup>+</sup>FOXP3<sup>+</sup> Tregs among CD8<sup>+</sup> T



**Figure 5** Anti-CTLA-4 treatment triggers alloreactivity and its associated antitumor effect after alloHSC. (A) Female (B6D2) F1 mice underwent TBI followed by bone-marrow transplantation (BMT) with  $1 \times 10^6$  T cells ( $n=10$ ) and were treated with anti-CTLA-4 mAb administered on day 0, day 3, and day 6 ( $n=10$ ). Survival curves, clinical grades, the AUC of clinical grade, and tumor incidence from two independent experiments are depicted. Kaplan-Meier survival curves were compared using the log-rank test. The AUCs for the GVHD clinical grade curve were calculated for each mouse and are presented as the mean  $\pm$  SD. A Mann-Whitney test was performed. (B) On day 12, splenocytes were collected and the T cells analyzed. Each plot represents one mouse and the data are presented as the mean  $\pm$  SD. The cumulative data of two independent experiments is shown. The Mann-Whitney test was performed to compare the effect of anti-CTLA-4 mAb treatment with the control. \* $P<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , . AUC, area under the curve; GVHD, graft-versus-host disease.





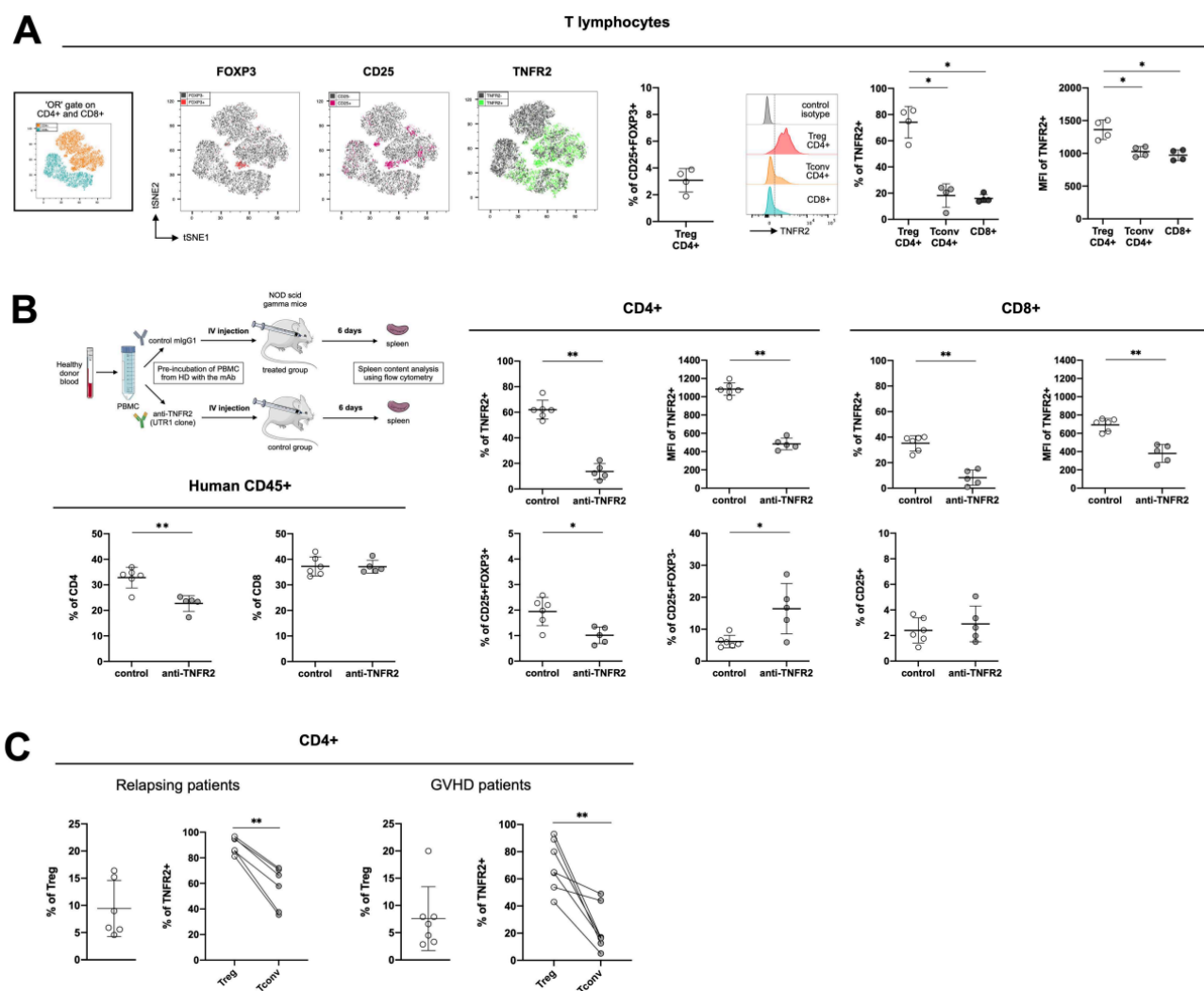
**Figure 6** TNFR-2 blockade in donor lymphocyte infusion (DLI). Lethally irradiated (B6D2)F1 recipient mice received  $5 \times 10^6$  BM cells and were infused 10 days later with  $1 \times 10^6$  T cells (labeled as DLI,  $n=8$ ) and treated with anti-TNFR2 mAb (labeled as DLI + anti-TNFR2,  $n=8$ ). Survival curves, clinical grades, and the AUC of clinical grade are presented. Kaplan-Meier survival curves were compared using log-rank tests. The AUCs of the GVHD clinical grade curve was calculated for each mouse and are presented as the mean  $\pm$  SD. A Mann-Whitney test was performed. results are presented as the mean  $\pm$  SD. \*\*\* $P < 0.001$ . AUC, area under the curve; BMT, bone-marrow transplantation; GVHD, graft-versus-host disease.

cells was also reduced (from  $1.16 \pm 0.49\%$  to  $0.37 \pm 0.15\%$ ). Conversely, we observed an increase in both  $CD4^+FOXP3^+$  ( $16.75\% \pm 12.13\%$ ) and  $CD8^+FOXP3^+$  ( $25.12\% \pm 11.06\%$ ) T cell populations when mice were treated with anti-CTLA-4 (figure 5B), compared with untreated mice ( $CD4^+$ :  $6.70\% \pm 2.43\%$ ;  $CD8^+$ :  $12.30\% \pm 2.30\%$ ). In summary, we were unable to demonstrate any difference either in the  $CD4^+FOXP3^+$  or  $CD8^+FOXP3^+$  T-cell populations relative to those of untreated mice with an anti-TNFR2 treatment beginning on day 0. Only late treatment with anti-TNFR2 increased the frequency of  $CD8^+$  T cells (figure 3). Anti-CTLA-4 administration on day 0 led to a rapid increase in both  $CD4^+FOXP3^+$  and  $CD8^+FOXP3^+$  populations but at the expense of more severe GVHD and the quasi-complete disappearance of both the  $CD4^+FOXP3^+$  and  $CD8^+FOXP3^+$ Treg populations.

DLIs are mostly administered after alloHSCT to treat relapses of hematological malignancy. Such delayed administration of T cells is often unsuccessful in controlling malignancy, thus leaving space for anti-TNFR2 treatment to further boost the alloreactivity of DLIs. Thus, we attempted to strengthen our observations by testing the anti-TNFR2 strategy in a model of delayed DLI. Recipient mice, therefore, received bone marrow cells on day 0 and T cells on day 10, with or without anti-TNFR2 treatment on day 10 (figure 6). In this experimental setting, we did not observe any mortality nor clinical signs of GVHD in untreated control mice. On the contrary, anti-TNFR2 treatment at the time of T-cell infusion immediately led to increased alloreactivity, shown by higher clinical grades of GVHD. This suggests that anti-TNFR2 treatment is a perfectly relevant therapeutic approach to trigger a GVL effect in the setting of delayed DLI that would have an insufficient antileukemic effect on its own.

### TNFR2 is a relevant therapeutic target on human T cells in the steady state and after alloHSCT

We first evaluated TNFR2 expression in healthy volunteers (figure 7A), generating a t-SNE map of  $CD4^+$  and  $CD8^+$  T cells. Displaying FOXP3, CD25, and TNFR2 expression on the map allowed us to discriminate between conventional  $CD4^+$  and  $CD8^+$  T cells and  $CD4^+$  Tregs via their coexpression of CD25 and FOXP3. Among the four donors considered for this analysis,  $CD4^+$  Tregs represented a mean of  $3.07\% \pm 0.88\%$  of the lymphocyte population. TNFR2 expression was distributed equally between the  $CD4^+$  and  $CD8^+$  clusters, with a seemingly higher density in the  $CD4^+$  Treg cluster. The frequency and density of TNFR2 expression were indeed significantly higher than for conventional  $CD4^+$  and  $CD8^+$  T cells. We evaluated whether such preferential expression of TNFR2 by Tregs remained in vivo in settings in which conventional T cells are highly activated using cells from two of the healthy donors described above to generate a xenogeneic model. Half of the PBMCs were incubated with UTR-1, a mouse anti-human TNFR2 antagonistic mAb and the rest with a control mIgG1 for 2 hours and then used to infuse immunodeficient NSG mice. After 6 days, we collected the spleens of the grafted mice and evaluated any modifications of the  $CD4^+$  and  $CD8^+$  cell populations resulting from UTR-1 treatment (figure 7B). Among the human  $CD45^+$  cells, we noted a reduction in the percentage of  $CD4^+$  but not  $CD8^+$  T cells. Relative to the initial frequency on the  $CD4^+$  and  $CD8^+$  T cells before injection into the mice (figure 7A), the frequency of TNFR2 6 days after mouse antibody stimulation was higher on  $CD8^+$  ( $15.98 \pm 3.12\%$  to  $35.15\% \pm 5.97\%$ ) and markedly higher on  $CD4^+$  ( $18.14 \pm 8.98\%$  to  $61.98\% \pm 7.37\%$ ), consistent with the strong lymphocyte stimulation induced in



**Figure 7** Tumor necrosis factor receptor-type 2 (TNFR2) expression on human cells from healthy donors and from hematopoietic stem-cell transplantation (HSCT) patients. (A) cell clustering using a t-distributed stochastic neighbor embedding (tSNE) algorithm performed on an « or » gate including both CD4<sup>+</sup> and CD8<sup>+</sup> cells collected from healthy donors (n=4). The localization of CD4<sup>+</sup> and CD8<sup>+</sup> cell clusters is indicated in the subfigure on the left. The localization of Foxp3<sup>+</sup>, (in red), CD25<sup>+</sup> (in pink), and TNFR2<sup>+</sup> (in green) cells among the aforementioned lymphocyte populations are mapped. The percentage of CD25<sup>+</sup>FOXP3<sup>+</sup> (Treg) cells among CD4<sup>+</sup> cells and the gating strategy and percentage of TNFR2<sup>+</sup> cells, as well as their mean fluorescence intensity among CD4<sup>+</sup> Tregs (CD25<sup>+</sup>FOXP3<sup>+</sup>) and Tconv (FOXP3<sup>-</sup>) are indicated. (B) Peripheral blood mononuclear cells (PBMCs) from two healthy volunteers were pre-treated 2 hours at 37°C with an anti-hTNFR2 mAb (UTR-1 clone) or a control mIgG1 and injected into NSG mice, as depicted. The spleens were analyzed on day six for the percentage of CD8<sup>+</sup>, CD4<sup>+</sup>, TNFR2<sup>+</sup>, CD25<sup>+</sup>, and Foxp3<sup>+</sup> cells and the mean fluorescence intensity of TNFR2<sup>+</sup> cells. (C) TNFR2 expression on CD4<sup>+</sup> Tregs (CD25<sup>+</sup>FOXP3<sup>+</sup>) and Tconv (FOXP3<sup>-</sup>) from relapsing patients (n=6) or patients experiencing graft-versus-host disease (GVHD) (n=7) after alloHSCT. Data are plotted as the mean±SD. Statistical significance from the controls was determined using Mann-Whitney tests. \*P<0.05, \*\*p<0.01. Parts of the figure were drawn by using pictures from Servier medical art (<http://smart.servier.com/>), licensed under a creative commons Attribution 3.0 Unported license (<https://creativecommons.org/licenses/by/3.0/>).

the xenogeneic models (figure 7B). Interestingly, the frequencies of TNFR2-expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells were markedly lower after UTR-1 pretreatment under these experimental conditions. Indeed, the frequency of CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs among CD4<sup>+</sup> T cells was lower in the treated mice (1.01%±0.32%) than the control mice (1.94%±0.55%). At the same time, the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> cells was higher (16.42%±1.93%)

than that of the control group (6.13%±7.87%). These results, obtained in vivo in a short-term xenogeneic stimulation model, are encouraging in terms of the possibility to block human Tregs using an anti-TNFR2 mAb.

We conducted a preliminary preclinical study in a small cohort of grafted patients who were relapsing from their initial blood cancer or suffering from cortico-resistant GVHD (online supplemental table S1) to





reinforce the relevance of a therapeutic strategy based on blocking TNFR2 (figure 7C). In the relapsing-patients, 89.77%±6.51% of 9.44%±5.16% of Tregs highly expressed TNFR2, whereas only 56.68%±16.41% of conventional T cells were TNFR2<sup>+</sup>. Such a difference in expression of TNFR2 between Tregs and conventional T cells not only confirms what we observed in our xenogeneic model in an allogeneic setting, but also validates our therapeutic strategy by suggesting that such an approach will preferentially target Tregs in this clinical setting. An alternative therapeutic approach in alloHSCt suggested by the literature consists of targeting Tregs via TNFR2 expression but using agonist mAbs to activate and expand the Treg population to decrease the allogeneic response and subsequent GVHD.<sup>22</sup> We also studied the differential expression of TNFR2 on Tregs and conventional T cells in the context of cortico-resistant GVHD to assess the versatility of this approach, depending on the therapeutic objective. Although the percentage of TNFR2<sup>+</sup> Tregs was lower (69.72±18.44%) than in healthy volunteers and relapsing patients, it was still higher than that of TNFR2<sup>+</sup> conventional T cells, which in this context dropped to 23.00%±16.65%.

Overall, our results suggest that TNFR2 is a relevant marker for targeting Tregs in humans. In the context of alloHSCt, TNFR2 modulation could serve multiple strategies, from prevention and treatment of relapse (in combination or not with DLI) to the treatment of GVHD.

## DISCUSSION

Here, we demonstrate that anti-TNFR2 treatment directly triggers alloreactivity in a context in which the infused T cells alone are not able. We also establish the proof of concept that anti-TNFR2 treatment can mediate a potent GVL/GVT effect under different experimental models of hematological malignancy relapse after alloHSCt. An antitumor strategy through TNFR2 blockade was initially proposed to treat solid tumors or Sézary lymphoma but the ability of such a strategy to trigger an allogeneic immune response was never developed or tested. Our work makes a decisive contribution to validating a new indication for blockade of the TNF- $\alpha$ /TNFR2 pathway.

Almost all experimental models to study the GVL effect consist of the administration of a large number of T cells with tumor cells and show that an anti-GVHD treatment has no deleterious effect on the GVL effect. Clinical applications for therapeutic strategies tested in such preclinical settings would then be intended for patients who develop GVHD after the graft to treat their GVHD manifestations without jeopardizing the alloreactivity already triggered against their malignancy by the graft. On the contrary, our strategy was conceptually different and designed with the idea of treating patients who relapsed without a previous occurrence of GVHD, a clinical setting that cannot be addressed through the aforementioned experimental models, in which the excess of T cells makes it impossible for tumor cells to develop. Thus, to accurately model

this clinical context in mice required that we cross titrate varying numbers of T cells and tumor cells to find the quantity of injected T cells that does not allow elimination of the tumor cells nor induce GVHD but is sufficient to mediate a strong GVL effect once boosted. This is a finely tuned model, as it is not possible to modify the number of T cells or tumor cells without losing the experimental conditions that allow the question to be addressed. We also successfully defined the experimental conditions that mimic DLI by the postponed administration of donor T cells. The development of such experimental models is an additional original contribution of this study because it makes it possible to precisely model a leukemia relapse in the absence of GVHD in a context in which immune cells, administered at the time of transplantation or later, are present but ineffective. We validated the approach of intensifying alloreactivity by a treatment targeting TNFR2 in each of these experimental models. In the clinical setting, most alloHSCt procedures are indicated to treat patients with acute myeloid leukemia (AML). Here, we used two tumor models that have been widely validated in mice: the very aggressive and difficult to eliminate P815-mastocytoma model, a tumor type clinically not eligible for alloHSCt, and the A20-B cell lymphoma, a more clinically relevant model, but which is easier to eliminate in experimental BMT. In both cases, we observed a strong GVL/GVT effect. In the future, and once human clinical grade anti-TNFR2 is developed, it will be informative to test this approach using a human cell line of AML in a model of xenogeneic GVHD performed in NSG mice.

Our results using these models clearly demonstrate the broader principle that although TNFR2 is expressed by activated T cells, higher TNFR2 expression by Tregs make them preferentially sensitive to anti-TNFR2 treatment *in vivo*. Indeed, we observed that blocking the suppressive effect of Tregs leads to an increase in the percentage of CD8<sup>+</sup> FOXP3<sup>-</sup> T cells. This observation is of prime importance because CD8<sup>+</sup> T cells are essential for the antitumor effect and the Treg/CD8 ratio was previously demonstrated to be a relevant biological marker for several cancers.<sup>40–45</sup> In addition, although the percentage of TNFR2-expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells decreased, it was associated with a marked increase in their proliferation, as shown by an elevated proportion of CD4<sup>+</sup> and CD8<sup>+</sup> KI-67<sup>+</sup> conventional T cells in treated mice. In other words, although the percentage of TNFR2-expressing T cells markedly decreased due to anti-TNFR2 treatment, it occurred without abolishing either the capacity of the treatment to trigger alloreactivity of donor T cells or the associated GVL/GVT effect. This observation provides an additional element in favor of the dual role for TNFR2 in conventional T cells already reported in the literature. Indeed, TNFR2 is predominantly found in activated conventional T cells, in which it acts as a costimulatory molecule, in a unique, non-redundant manner relative to CD28 costimulation.<sup>27, 46</sup> However, its expression is not only essential for optimal proliferation and activation of effector T cells but also for the induction of



activation-induced cell death (AICD), which terminates the proliferative response.<sup>47</sup> Consistent with this dual role, TNFR2<sup>-/-</sup> CD8<sup>+</sup> T lymphocytes exhibit high resistance to AICD, leading to worsened colonic inflammation in a mouse model of colitis.<sup>48</sup> This last observation could partially explain why CD8<sup>+</sup> T cells were more numerous and proliferative in our alloHSCT model following TNFR2 blockade.

CD8<sup>+</sup>FOXP3<sup>+</sup> suppressive T-cells have been previously described in both mouse and human GVHD.<sup>33,34</sup> Although CD4<sup>+</sup>FOXP3<sup>+</sup> CD4<sup>+</sup> Tregs are considered to be major actors of immunosuppression in the field of alloHSCT, whereas CD8<sup>+</sup>FOXP3<sup>+</sup> Tregs are not considered to have the same level of relevance, we studied these cells to better characterize them and eventually use them as a marker for the therapeutic effect of anti-TNFR2 treatment. Here, we show, for the first time, that the CD8<sup>+</sup>FOXP3<sup>+</sup> T-cells that emerge spontaneously after BMT highly express TNFR2 and are also affected by anti-TNFR2 treatment. Despite the general interest in the community in CD8<sup>+</sup>FOXP3<sup>+</sup> Treg cells, to date, there are no specific markers that allow targeting of these cells. Our results conclusively demonstrate that TNFR2 is an excellent candidate, with broad implications in the clinic. Indeed, they validate the dual interest of anti-TNFR2 treatment that targets both CD4<sup>+</sup> and CD8<sup>+</sup> FOXP3<sup>+</sup> Tregs, which may be involved in cancer relapse. In addition, TNFR2 is the only target that makes it possible to block several tolerogenic cell populations: CD4 and CD8 Tregs, in this study, MDSCs,<sup>49–51</sup> or less well-described immunosuppressive populations, such as endothelial progenitor cells,<sup>52</sup> which also express TNFR2.

Over the last few years, we have been strongly committed to establishing a clinically effective strategy for Treg depletion to increase the post-transplant anti-tumor effect. We provided the key proof of concept in humans<sup>12,24</sup> and are currently conducting a confirmatory, randomized, double-blind clinical trial to demonstrate the benefit of Treg-depleted DLI versus standard DLI (DLI-Boost, ClinicalTrials.gov, NCT03236129). The current study constitutes an exciting advance towards this objective by providing a technologically much simpler approach than the ex-vivo elimination of Tregs on CD25 expression upstream of the DLI. In addition, we previously observed that CD25-based depletion of Tregs also resulted in the unwanted partial elimination of antitumor CD8<sup>+</sup>CD25<sup>+</sup> effector T cells.<sup>53</sup> The anti-TNFR2 approach likely targets Tregs without impairing the activation and proliferation of CD8<sup>+</sup> T-cells.

We also tested the advantages and complementarity that an anti-TNFR2 strategy could have relative to immune checkpoint inhibitors that have already reached the clinical stage. In the same model, we observed comparable clinical effects but different biological consequences. Indeed, anti-CTLA-4 treatment led to the almost complete disappearance of Tregs, which did not occur with anti-TNFR2, an interesting difference that could limit the development of side effects in patients relative to

immune checkpoint inhibitors. Furthermore, compared with untreated mice, remaining Tregs following anti-TNFR2 treatments at day 0 or day 10 under-express CTLA-4, CD25, ICOS and NRP, and CD39, KLRG1 and CD103 when mice were treated at day 0, indicating an impaired activation/suppressive function of Tregs. These marked differences compared with anti-CTLA4 therapy suggest that more rapid Treg reconstitution after anti-TNFR2 therapy may occur, thereby limiting the risk of severe GVHD and autoimmune complications.

This approach would have the additional advantage of its great versatility. Indeed, rather than the radical ex vivo elimination of Tregs before T cell infusion, the administration of the therapeutic mAb, and therefore blockade of the suppressive effect of Tregs, could be adapted to the clinical situation of the patient, as modeled here when mice were treated on day 0 or day 10 or in a DLI approach, with comparable efficacy. Indeed, according to the supplier information, which we confirmed in our model, the half-life of anti-TNFR2 in vivo is approximately 48 hours (36 hours in our experimental model of GVHD, data not shown). Early and repeated administration of anti-TNFR2 could correspond to a preventive treatment for patients at high risk of leukemia relapse and low risk of GVHD. Conversely, a short treatment administered well after alloHSCT could trigger alloreactivity in patients at high risk of GVHD and low risk of relapse and only in cases of a documented relapse.

Importantly, this is the first time that in vivo controlled release of an allogeneic immune response has been shown to elicit an antitumor response through the direct targeting of Tregs. Of course, such an antitumor response was associated with strong GVHD in mice, but our previous clinical results have shown that, GVHD, when it occurred in patients, has always been sensitive to conventional immunosuppressive treatments, at least in ex vivo Treg-elimination strategies in DLI.<sup>12,24</sup>

We further highlighted this important translational deliverable by generating essential data showing that Tregs from healthy donors and patients with post-transplant leukemia relapse or GVHD preferentially express TNFR2 relative to effector T cells, as in autoimmune settings.<sup>54,55</sup> These data validate two potential inverse applications of the TNFR2 strategy: blocking Tregs to increase the anti-tumor or allogeneic immune response or inducing Tregs with TNFR2 agonists to dampen the immune response.<sup>13,14</sup> The results reported here represent a major breakthrough on the path to transforming controlled immunomodulation of the allogeneic immune response into clinical reality by targeting Tregs through TNFR2.

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## Annexe 4 : Classification TNM de cancer du sein, 8<sup>e</sup> édition

**Table A1** : Classification TNM anatomique d'après la 8<sup>ème</sup> édition de l'UICC et de l'AJCC (Brierley et al., 2016; Giuliano et al., 2017)

Catégorie	Critères
T – Tumeur primitive clinique (cT) et pathologique (pT)	
Tx	La tumeur primaire ne peut être évaluée
T0	Pas de tumeur primaire
Tis (CCIS)	Carcinome canalaire in situ
Tis (Paget)	Maladie de Paget du mamelon sans tumeur décelable
T1	Tumeur ≤ 20 mm dans sa plus grande diamètre
T1mi	Tumeur ≤ 1 mm
T1a	Tumeur > 1 mm et ≤ 5 mm
T1b	Tumeur > 5 mm et ≤ 10 mm
T1c	Tumeur > 10 mm et ≤ 20 mm
T2	Tumeur > 20 mm et ≤ 50 mm dans son plus grand diamètre
T3	Tumeur > 50 mm dans son plus grand diamètre
T4	Extension directe à la paroi thoracique ou à la peau, quelle que soit la taille
T4a	Extension à la paroi thoracique (atteinte seule du muscle pectoral exclue)
T4b	Extension à la peau (peau d'orange, ulcération ou nodules de perméation)
T4c	T4a + T4b
T4d	Cancer inflammatoire (œdème/érythème ≥ 1/3 du sein)
cN – Ganglion lymphatique régional clinique	

cNx	Évaluation ganglionnaire régionale non réalisable (chirurgie antérieure)
cN0	Absence de métastase ganglionnaire
cN1	Métastase mobile dans les ganglions homolatéraux de niveau I/II
cN1mi	Micrométastase (environ 200 cellules soit > 0,2 mm et ≤ 2 mm)
cN2	
cN2a	Métastase fixée dans les ganglions homolatéraux de niveau I/II
cN2b	Métastase dans les ganglions mammaires internes sans atteinte axillaire
cN3	
cN3a	Métastase dans les ganglions sous-claviculaires homolatéraux
cN3b	Métastase dans les ganglions mammaires internes avec atteinte axillaire
cN3c	Métastase dans les ganglions sus-claviculaires homolatéraux
pN – Ganglion lymphatique régional pathologique	
pNx	Évaluation ganglionnaire régionale non réalisable
pN0	Absence de métastase ganglionnaire ou seule présence de cellules isolées
pN0(i+)	Cellules tumorales isolées (≤ 0,2 mm)
pN0(mol+)	RT-PCR positive sans cellule identifiée microscopiquement
pN1	
pN1mi	Micrométastase (environ 200 cellules soit > 0,2 mm et ≤ 2 mm)
pN1a	Métastase dans 1 à 3 ganglions axillaires dont au moins une > 2 mm
pN1b	Métastase > 0,2 mm dans les ganglions sentinelles mammaires internes
pN1c	Association de pN1a et pN1b
pN2	

pN2a	Métastase dans 4 à 9 ganglions axillaires dont au moins une > 2 mm
pN2b	Métastase mammaire interne clinique sans atteinte axillaire
pN3	
pN3a	Métastase dans $\geq 10$ ganglions axillaires dont au moins une > 2 mm ou métastase ganglionnaire sous-claviculaire (niveau III)
pN3b	Métastase mammaire interne clinique avec atteinte axillaire, pN1a ou pN2a ou pN2a avec pN1b
pN3c	Métastase ganglionnaire sus-claviculaire homolatérale
M – Métastase à distance clinique et pathologique (cM0, cM1, pM1)	
M0	Absence d'évidence clinique ou radiologique de métastase à distance
cM0(i+)	Absence d'évidence clinique ou radiologique de métastase à distance en présence de cellules tumorales $\leq 0,2$ mm ou par techniques moléculaires dans le sang circulant, moelle osseuse ou autre ganglions non régionaux
M1	Métastase à distance détectée par des moyens cliniques et radiologiques (cM1) et/ou métastase prouvée histologiquement > 0,2 mm (pM1)

**Tableau A2** : Stade pronostique dit « anatomique » (Giuliano et al., 2017)

T	N	M	Stade pronostique « anatomique »
Tis	N0	M0	0
T1	N0	M0	IA
T0-1	N1mi	M0	IB
T0-1	N1	M0	IIA
T2	N0	M0	IIB
T2	N1	M0	IIB
T3	N0	M0	IIB
T0-3	N2	M0	IIIA
T3	N1	M0	IIIA
T4	N0-2	M0	IIIB
Tout T	N3	M0	IIIC
Tout T	Tout N	M1	IV



**Tableau A3** : Quelques exemples des révisions de la stadification du cancer du sein utilisant les biomarqueurs et Oncotype DX (Giuliano et al., 2017)

T	N	M	Grade	HER2	RE	RP	Stade (7 <sup>ème</sup> édition)	Stade (8 <sup>ème</sup> édition)
Biomarqueurs								
1	0	0	1	-	-	-	IA	IIA
1	0	0	3	-	+	-	IA	IIA
3	1-2	0	1	+	+	+	IIIA	IB
Oncotype DX RS < 11 pour les tumeurs RE+								
2	0	0	Tout	-	+	Tout	IIA	IB
1-2	1	0	Tout	-	+	Tout	IIA/IIB	IB
0-2	2	0	1-2	+	+	+	IIIA	IB

*Légende : HER2 : récepteur 2 du facteur de croissance épidermique humain, M : classification de métastase à distance, N : classification de ganglion régional, RE : récepteur d'œstrogène, RP : récepteur progestérone, RS (recurrence score) : score de récurrence, T : classification de tumeur primaire.*

## Annexe 5 : Revue sur le rôle émergent des micro-ARNs

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REVIEW



## Radiation therapy for triple-negative breast cancer: emerging role of microRNAs as biomarkers and radiosensitivity modifiers. A systematic review

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### Abstract

**Purpose** Radiation therapy (RT) for triple-negative breast cancer (TNBC) treatment is currently delivered in the adjuvant setting and is under investigation as a booster of neoadjuvant treatments. However, TNBC radioresistance remains an obstacle, so new biomarkers are needed to select patients for any integration of RT in the TNBC therapy sequence. MicroRNAs (miRs) are important regulators of gene expression, involved in cancer response to ionizing radiation (IR) and assessable by tumor tissue or liquid biopsy. This systematic review aimed to evaluate the relationships between miRs and response to radiation in TNBC, as well as their potential predictive and prognostic values.

**Methods** A thorough review of studies related to miRs and RT in TNBC was performed on PubMed, EMBASE, and Web of Science. We searched for original English articles that involved dysregulation of miRs in response to IR on TNBC-related preclinical and clinical studies. After a rigorous selection, 44 studies were chosen for further analysis.

**Results** Thirty-five miRs were identified to be TNBC related, out of which 21 were downregulated, 13 upregulated, and 2 had a double-side expression in this cancer. Expression modulation of many of these miRs is radiosensitizing, among which miR-7, -27a, -34a, -122, and let-7 are most studied, still only in experimental models. The miRs reported as most influencing/reflecting TNBC response to IR are miR-7, -27a, -155, -205, -211, and -221, whereas miR-21, -33a, -139-5p, and -210 are associated with TNBC patient outcome after RT.

**Conclusion** miRs are emerging biomarkers and radiosensitizers in TNBC, worth further investigation. Dynamic assessment of circulating miRs could improve monitoring and TNBC RT efficacy, which are of particular interest in the neoadjuvant and the high-risk patients' settings.

**Keywords** Triple-negative breast cancer · Radiation therapy · Radioresistance · microRNA · Radiosensitization

### Abbreviations

APBI Accelerated partial breast irradiation  
 BC Breast cancer

CSC Cancer stem cells  
 DDR DNA damage repair  
 DFS Disease-free survival  
 DMFS Distant metastasis-free survival  
 EMT Epithelial–mesenchymal transition  
 ER Estrogen receptor

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FFPE	Formalin-fixed paraffin-embedded
IR	Ionizing radiation
HR	Homologous recombination
miR	MicroRNA
NACT	Neoadjuvant chemotherapy
OS	Overall survival
pCR	Pathological complete response
PTEN	Phosphatase and tensin homolog
RT	Radiation therapy
S1P	Sphingosine-1-phosphatase
TCGA	The Cancer Genome Atlas
TME	Tumor microenvironment
TNBC	Triple-negative breast cancer

## Introduction

Breast cancer (BC) is the most common type of cancer affecting women and the second leading cause of cancer-related mortality among females worldwide [1]. Invasive, non-metastatic BC is managed by multimodal therapy, which has become more personalized since the introduction of BC molecular classification [2]. Triple-negative breast cancer (TNBC) accounts for 15–20% of BCs and is the most heterogeneous BC subtype at the histological and biological levels. TNBC usually presents with aggressive clinicopathological features, early recurrence, and poor survival [3, 4]. Neoadjuvant chemotherapy (NACT) significantly improves the survival of the TNBC patients who reached complete pathological response (pCR) [5]. However, most TNBC patients have a post-NACT residual disease and a 6–ninefold higher risk of relapse than patients with pCR [6]. To improve the pCR rates in TNBC, novel approaches have been evaluated, such as PARP inhibitors [7], immunotherapy [8], or ionizing radiation therapy (IR) [9]. Radiation therapy (RT) is being tested in the neoadjuvant setting in TNBC as a booster of the anticancer immune response [10–12]. However, the major obstacle for TNBC RT is inherent and acquired radioresistance. Reliable biomarkers are lacking for the integration of personalized RT in TNBC management. Among numerous biomarkers in development for better TNBC therapy, microRNAs (miRs), members of the small noncoding RNAs, have been drawing increasing attention as tumor tissue and circulating molecules capable of reflecting the tumor response to RT [13]. In addition, modification of miR expression has been shown to radiosensitize TNBC cells [14].

miRs are short (19–25 nucleotides), single-stranded non-coding, regulatory RNAs that modulate gene expression at the post-transcriptional level via translational repression or mRNA degradation [15]. Recent findings revealed that several miRs could effectively modulate cancer response to IR by affecting the regulation of DNA damage repair (DDR), cell cycle control, apoptosis, signal transduction, and tumor

microenvironment (TME) [16, 17]. Furthermore, miRs are liberated into the circulation after radiation-induced tumor tissue destruction, so they can serve as biomarkers to directly and dynamically monitor response to RT by analyzing blood samples. The on-treatment liquid biopsy is particularly useful for adapting anticancer therapies in the neoadjuvant or metastatic setting, as shown by the I-SPY trials [18, 19].

This systematic review will focus on miRs' roles in cellular pathways critical for TNBC response to IR, the possibilities of TNBC radiosensitizing by miR expression modulation, and the capacity of miRs to be biomarkers for personalized TNBC therapy. We will conclude by elaborating on the perspectives of using miRs for patient stratification in clinical trials that specifically evaluate RT efficacy in TNBC.

## Methods

A systematic search for studies on miRs and TNBC response to IR was performed using PubMed, EMBASE, and Web of Science up to January 2021. Keyword combination (*miRNA OR microRNA*) AND (*radioresistance OR radiosensitivity*) AND (*TNBC OR "triple-negative breast cancer"*) was exploited. A manual search was performed for additional relevant articles. The searches were done independently by two authors (NHT and HQN). Only articles in English were selected. Eligible articles satisfied the following criteria: "dysregulation of miRNA in response to IR," "available data on human TNBC cell lines in preclinical models and clinical TNBC patients," and "cellular pathways involved in IR response." Exclusion criteria were "non-English language," "modulatory roles of miRNA in other treatment modalities," "review, editorial, case reports, conference abstracts, and meta-analysis," and "inadequate/unavailable data and duplicated studies."

## Results

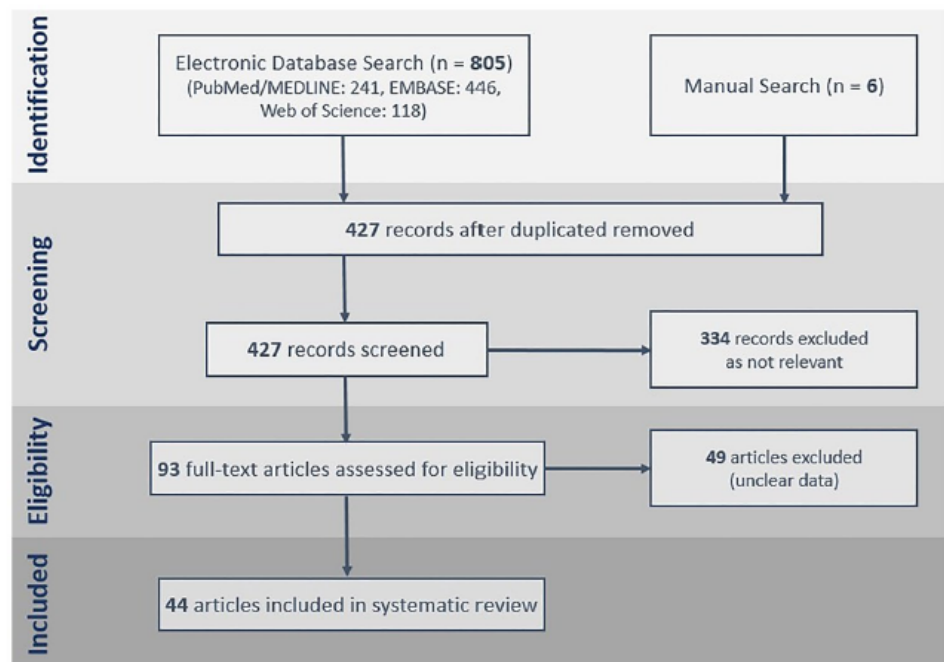
### Studies search and characteristics

We first identified 805 studies from PubMed ( $n = 241$ ), EMBASE ( $n = 446$ ), Web of Science ( $n = 118$ ), and manual search ( $n = 6$ ). After removing duplicate studies, 427 articles were screened. A total of 44 studies fulfilling the inclusion/exclusion criteria were included in the final review. The search strategy and selection are summarized in a PRISMA flow diagram (Fig. 1).

The analyzed studies included *in silico*, *in vitro*, and *in vivo* experiments. The main data source for the *in silico* studies was The Cancer Genome Atlas (TCGA) [20]. Most *in vitro* data on miRs expression under different IR



**Fig. 1** Flowchart of the selection of eligible studies



conditions were obtained using the MDA-MB-231 TNBC cell line. This cell line is derived from a highly aggressive, metastatic, Claudin-low *TP53*-mutated TNBC [21].

The data on miR expression in patient samples were derived from studies using fresh, frozen, or formalin-fixed paraffin-embedded (FFPE) tumor tissues and blood. Overall, 35 miRs or miR signatures were identified. Of these, 21 miRs were downregulated and 13 upregulated in TNBC cell lines, whereas two miRs had double-sided expression depending on TNBC subtype or pathological conditions. The main characteristics of each study are presented in Table 1, emphasizing data related to TNBC radiosensitivity/radioresistance (Fig. 2).

### miRs in TNBC radioresistance

Dysregulation of miRs can be translated to a favorable or unfavorable response to IR in TNBC cells. In general, upregulation of oncogenic miRs (oncomiRs) or downregulation of tumor suppressor miRs confers radioresistance. For example, high expression of miR-21, miR-27a, miR-221, or miR-620, the well-known oncomiRs, is associated with radioresistance. Likewise, reduced expression of powerful tumor suppressor miRs, such as the let-7 family or miR-34, results in radioresistant behavior. Some miRs can play a dual role in TNBC response to IR. miR-122 was demonstrated to have tumor suppressor activity in BC by downregulation of the IGF1R activity and the PI3K/Akt/mTOR pathway [22]. Overexpressing miR-122 in parental MDA-MB-231 cells significantly sensitizes MDA-MB-231 cells to

IR. Conversely, in the radioresistant MDA-MB-231RR cell line, miR-122 functions as an oncomiR and promotes cell survival by regulating several downstream genes involved in proliferation and apoptosis, including *ZNF611*, *ZNF304*, *RIPK1*, *HRAS*, *DUSP8*, and *TNFRSF21* [23]. The associations between miR expression dysregulation and intrinsic responsiveness of TNBC to IR are detailed in Table 1.

Clonogenic assays on TNBC cell lines confirm the strong implication of miRs in response of TNBC cells to IR. TNBC cells are characterized by inherited radioresistance, as demonstrated by several experiments in which TNBC cells were irradiated by doses ranging from the standard 2 Gy to up to 10 Gy and showed delayed and lowered reduction of cell viability [24–26]. In MDA-MB-231 and SUM159 cell lines, reduction of the expression of miR-200c [27], miR-205 [28], and miR-302a [29] was associated with more resistant behavior in response to IR doses from 2 to 8 Gy [4, 27–29]. The MDA-MB-231 line has 100- to 1000-fold higher endogenous expression of miR-221 than other BC cell lines and retains high migration capacity and high proliferation rate after 4 Gy of IR. This resistance was modulated by the miR-211/ZEB1 activity dependent on the oncogenic RAS-RAF-MEK pathway activation and influences the epithelial–mesenchymal transition (EMT) process [30].

### miRs in radiosensitization of TNBC

High-energy IR induces DNA damage and activates a complex network of cellular pathways. We identified ten miRs studied more than once and shown a distinct potential to

**Table 1** Characteristics of included studies

miRNAs	Human TNBC cell lines	IR dose (Gy)	Collection time points (h)	Profiling methods	Endogenous expression	Inherent radiosensitivity	References
let-7a, -7b	MB-231, Bcap-37	2–4	NR	qRT-PCR	Downregulation	Resistance	[55]
let-7d	MB-231, HS587-T, BT-20	2–4–8	12–24	qRT-PCR	Downregulation	Resistance	[54]
20 oncomiRs (miR-216a, let-7e, miR-221, miR-302c, miR-21)	MB-231	2	24	Microarray and qRT-PCR	Downregulation after treated	Sensitivity	[25]
miR-7	MB-468	6	4	RT-PCR	Overexpression after transfected	Sensitivity	[33]
miR-15 family (miR-15a/15b/16)	MB-231	4	Several (3–72)	RT-PCR	Overexpression after transfected	Sensitivity	[81]
miR-16-5p, miR-23b-3p	MB-231	2	24	miRNA-Seq and qRT-PCR	Downregulation	Resistance	[26]
miR-17–92 cluster	MB-361	SD 10 Gy MF 5×2 Gy	15	Illumina HiSeq and RT-PCR	Upregulation <sup>a</sup>	NR	[82]
miR-21	MB-361	0.25–2.5	4–24	qRT-PCR	Overexpression	Resistance	[83]
miR-21	MB-361	5	Several (2–72)	qPCR	Overexpression	Resistance	[65]
miR-22	MB-231	2–4–6–8	NR	qRT-PCR	Downregulation	Resistance	[84]
miR-27a	MB-435, MB-231	8	24	qRT-PCR	Overexpression	Resistance	[85]
miR-27a	MB-231, MB-468	30–50–100	NR	qRT-PCR	Overexpression	Resistance	[34]
miR-33a	MB-231, SUM-159 SUM-149 (IBC)	2–4–6	NR	qRT-PCR and microarray	Overexpression Downregulation	Resistance Sensitivity	[63]
miR-34	MB-231	2.5–5–7.5	3	qPCR	Downregulation	Sensitivity	[42]
miR-95	MB-231	2–4–6–5 (in vivo)	0.5–2–5	SOLiD 5500xl Seq and qRT-PCR	Upregulation	Resistance	[36]
miR-122	MB-231 MB-231RR	4	48	TLD Arrays and qRT-PCR	Low expression Upregulation	Resistance Resistance	[23]
miR-125b	MB-231	2–4–6	4–8–12–16	qPCR arrays and qRT-PCR	Downregulation	Resistance	[60]
miR-129-5p	MB-231, BT549	6	24	qRT-PCR	Downregulation	Resistance	[31]
miR-139-5p	MB-231, MB-157, MB-43, MB-436, MB-468, HCC1937	3	4–8	miRNA microarrays	Overexpression	Sensitivity	[64]
miR-142-3p	MB-468, HCC1806	2–4–6	NR	qRT-PCR	Downregulation	Resistance	[57]
miR-144	MB-231	30–50–100	NR	qRT-PCR	Overexpression	Resistance	[35]
miR-148b	MB-231	2–4–6–8	6–12–24	qRT-PCR	Downregulation	Resistance	[86]
miR-155, miR-10b, miR-21 miR-125a	4T1 (BALB/c mice)	3×4 Gy	Several (1–6wk)	qRT-PCR (plasma + tumor)	Dynamical change <sup>b</sup>	NR	[87]
miR-155	NR	2–4–6–8	Several (1–24)	qRT-PCR	Overexpression	Sensitivity	[45]
miR-155	MB-231	6	24	qRT-PCR	Overexpression	Sensitivity	[88]

Table 1 (continued)

miRNAs	Human TNBC cell lines	IR dose (Gy)	Collection time points (h)	Profiling methods	Endogenous expression	Inherent radiosensitivity	References
High risk miRs (miR-125b-1, miR-181a-2, miR-222, miR-582, miR-874)	NA	NA	NA	Illumina HiSeq	Overexpression	NR	[89]
miR-182	MB-231, MB-453, HCC38, BT549, 21NT	2-4-6	0.5	Taqman miR array and qRT-PCR	Downregulation Overexpression	Resistance Sensitivity	[90]
miR-185-5p	MB-468, MB-231	2-4-6-8	24-48-72	qRT-PCR	Downregulation	Resistance	[91]
miR-185	MB-231, MB-436, MB-361	2-4-6-8-10 6 (in vivo)	1-12-24	qRT-PCR	Downregulation	Resistance	[92]
miR-199a-5p	MB-231	2-4-6, 8	48	qRT-PCR	Downregulation	Resistance	[59]
miR-200c	MB-231	2-4-6-8	6	qRT-PCR	Downregulation	Resistance	[50]
miR-200c	MB-231, BT 549	2-4-6-8	48	qRT-PCR	Downregulation	Resistance	[24]
miR-200c	MB-468	2-4-6-8	4	RT-PCR	Overexpression	Sensitivity	[32]
miR-200c	MB-231, BT 549 MB-231 (in vivo)	2-4-6-8 5×2 Gy	3-12-24 20 days	Microarrays and qRT-PCR	Downregulation	Resistance	[27]
miR-205	SUM159/P0-P2 (RR), MB-231 SUM159-P2 (in vivo)	4-6-10 15	1-24-48	miScript miRNA Array and RT-PCR	Downregulation	Resistance	[28]
miR-218	MB-231	2-4-6	6-12-24	qRT-PCR	Downregulation	Resistance	[93]
miR-221	MB-231	2-4-8	24-48-72	qRT-PCR	Overexpression	Resistance	[30]
miR-210, miR-10b, miR-182, miR-142, miR-221, miR-21, miR-93, miR-15b	MB-231	2-4-6	24	qRT-PCR	Upregulation	Resistance	[53]
miR-223	MB-231 MB-231 (in vivo)	2 2-5	2-4-24 0.5-1.2	miR microarrays and qRT-PCR	Upregulation	Sensitivity	[94]
miR-302a	MB-231, MB-231RR	2-4-6-8-10	24	qRT-PCR	Downregulation	Resistance	[29]
miR-340, miR-429	MB-231 MB-231 (in vivo)	5 3×2.5 Gy	1-4-8-24 4 days after RT	qRT-PCR	Downregulation	Resistance	[62]
miR-499b-5p	MB-231	6-10	NR	qRT-PCR	Downregulation	Resistance	[95]
miR-620	MB-231	2-4-6	24	qRT-PCR	Overexpression	Resistance	[96]
miR-634	MB-234/RR	2-4-6-8	24	qRT-PCR	Downregulation	Resistance	[97]

<sup>a</sup>Differential response to different RT fractionations (upregulation after SD: single dose and downregulation after MF: multi fraction)

<sup>b</sup>Different dynamical changes between oncomiRs (miR-155, miR-10b, miR-21) whose expression was increased with progression and decreased after treatment and tumor suppressor miR-125a which is decreased with progression and increased after treatment

*h* hour, *IR* irradiation, *IBC* inflammatory TNBC, *NR* not reported, *qRT-PCR* real-time quantitative reverse transcription polymerase chain reaction, *RR* radioresistance, *TNBC* triple-negative breast cancer, *wk* week(s)

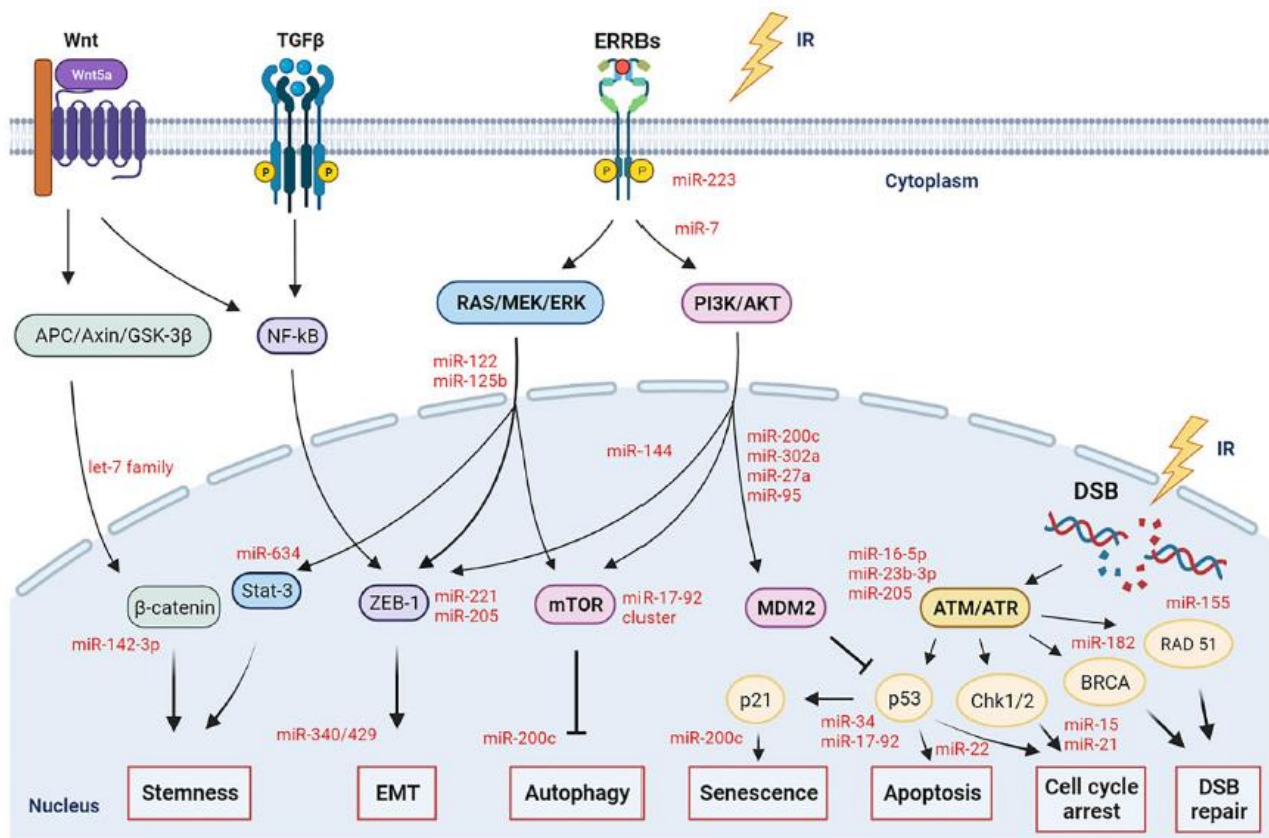
regulate cellular pathways involved in cancer cell response to IR (Table 2).

Apoptosis is the most studied pathway in the context of IR; it involves 18 miRs that regulate at least 15 downstream targets. The second most common pathway activated in response to IR is the double-strand repair system, involving eight miRs and ten downstream targets. Other cellular

processes strongly affected by IR are cell cycle regulation, EMT, cancer stem cell (CSC) survival and renewal, angiogenesis, and autophagy. The intracellular pathways most frequently reported to be targeted by different miRs after cell irradiation are shown in Table 3.

As dysregulation of the miR-regulated cellular pathways has been observed in radioresistant TNBC models, several





**Fig. 2** Overview of radiation-induced signaling pathways in triple-negative breast cancer and involved miRNAs. ATM/ATR signaling activation by IR-induced DSB leads to cell cycle arrest, DNA repair, apoptosis, and senescence. Activation of PI3K/AKT and RAS/MEK/ERK signaling pathways suppresses apoptosis and autophagy induc-

tion or promotes EMT and stemness. Activation of Wnt and TGF $\beta$  signaling pathways can also lead to stemness and EMT, respectively. Multiple miRNAs can regulate the expression of key components in these pathways; therefore, they can modulate the radiation response and radiosensitivity of tumor cells

teams have tried to radiosensitize TNBC by modifying miR expression. In these experiments, 22 miRs were upregulated by TNBC cell transfection with miR mimics or pre-miRs, whereas the expression of 8 miRs was downregulated by transfection of antagomiRs. Radiosensitization of TNBC cellular models was also obtained indirectly by an action on the miR downstream targets, as reported in 15 studies (Table 3). Some authors even tried to block the entire signaling pathway necessary for radioresistance by inhibiting one crucial molecule [24, 30, 31].

### Radiosensitization by miR-induced alterations of the PI3K/AKT pathway

The PI3K/Akt signaling pathway is one of the essential pathways for cancer cell survival and resistance to IR, especially in a hypoxic environment [32–36]. In a panel of human cancer cells with increased EGFR-PI3K-AKT signaling, including radioresistant MDA-MB-468 TNBC cells, ectopic expression of miR-7 attenuated EGFR and AKT expression

and induced radiosensitization by a delayed DDR, shown as prolongation in  $\gamma$ H2AX foci formation 4 h after irradiation with 6 Gy [33]. On the contrary, inhibition of miR-27a [34] and miR-144 [35] was radiosensitizing. These miRs induce TNBC radioresistance by downregulating phosphatase and tensin homolog (*PTEN*) and activating the PI3K/Akt pathway. Transfecting MDA-MB-231 cells with anti-miR-144 successfully rescued *PTEN* expression, downregulated AKT, and resensitized MDA-MB-231 cells to IR [35]. Also, treatment with an anti-miR-27a inhibitor significantly radiosensitized and decreased the migration and invasion of different TNBC cell lines [34]. Another way to inhibit the PI3K/AKT pathway and radiosensitize TNBC cells is by inhibiting sphingosine-1-phosphatase (S1P) signaling, a target of miR-95 [36]. S1P has been reported to activate the AKT pathway and protect from IR-induced cell death [37]. Transfection of human cancer cells with a miR-95 mimic induced increased Akt activation and surviving fractions following 6 Gy of IR, accompanied by a downregulation of the sphingolipid phosphatase SGPP1, an antagonist of S1P. On the other side,

**Table 2** The most studied miRNAs and their targets

miRNA	Possible target	Involved pathway	References
Let-7 family	Lin 28	Cancer stemness	[55]
	Cyclin D1/Akt1/Wnt		[54]
miR-15 family	ATM/TP53/CDK1	Cell cycle	[26]
	Chk1, Wee1		[81]
miR-21	↓subG1 ↑G2 phase	Cell cycle	[65, 83]
	NR	Cancer stemness	[53]
miR-22	Sirt1	Apoptosis	[84]
	MDC1	DSB repair	[98]
miR-27a	CDC27	Apoptosis	[85]
	PTEN/PI3K/Akt		[34]
miR-139-5p	RAD45L, POLQ, TOP2A	DSB repair	[17]
		DSB repair, ROS defense	[64]
miR-155	RAD51	DSB repair	[45]
	LGMN	EMT	[88]
miR-185	LINC00511/STXBP4	Apoptosis	[92]
	PCAT6/TPD52		[91]
miR-200c	TBK1	EMT	[50]
	UBQLN1	Autophagy	[24]
	EGFR	DSB repair	[32]
	LINC02582/USP7/Chk1		[27]
miR-221	ZEB1	EMT	[30]
	NR	Cancer stemness	[25, 53]

DSB double-strand breaks, EMT epithelial–mesenchymal transition, NR not reported

treatment with FTY720, an inhibitor of SIP signaling, sensitized miR-95 overexpressing cells to IR [36].

### Radio sensitization by miR-induced alterations of the DDR pathway

IR induces activation of the DDR pathway, which consists of several important nuclear regulators, such as *ATM*, *BRCA1*, *RAD51*, and *TP53*. The p53 protein is a major transcription factor that lies at the nexus of molecular pathways that monitor cellular disruptions and abnormal mitogenic activations [38]. The miR-34 family houses a consensus p53-binding site, making them the most significantly induced miRNAs as a post-transcriptional target of p53 [38–40]. Mutant p53 downregulates miR-34 expression in several human cancer cell lines and induces overexpression of anti-apoptotic elements, such as Bcl-2, SIRT1, and CDK4/6, thus promoting tumor development [41]. In *TP53*-mutated MDA-MB-231 cells, miR-34 manipulation was shown to alter cell survival post-IR; however, this impact seemed to largely depend on the IR-induced death form intrinsic for the cell: cells that inherently undergo non-apoptotic cell death are sensitized by lowering miR-34a and protected by miR-34a overexpression [42].

RAD51 is a central protein in homologous recombination (HR); dysregulation of RAD51 can lead to impaired

HR and aberrant genome rearrangements often observed in cancer [43]. In silico prediction models identified RAD51 as the direct target of miR-155. miR-155 is one of the best conserved and multifunctional miRNAs, mainly oncogenic, associated with resistance to therapy in various tumors [44]. miR-155 was shown to be a negative regulator of RAD51 foci formation; it enhanced the sensitivity of several BC cell lines to IR doses from 2 to 8 Gy [45]. Thus, miR-155 is another dual-function miR, having both tumor suppressor and oncomiR behaviors, which should be taken into account regarding radiosensitization by this miR.

### Radio sensitization by miR-induced EMT

Several miRNAs were found to target EMT, essential for cancer invasion, metastatic dissemination, and therapeutic resistance [46]. Using an in vitro model and an unbiased miR expression profiling approach, Zhang et al. identified miR-205 as the top downregulated miR in radioresistant mesenchymal TNBC cells [28]. miR-205 and ZEB1, an EMT driver, negatively regulate each other and play opposing roles in DDR and tumor radiosensitivity [28]. ZEB1 increases the expression of matrix metalloproteinases and serves as a central EMT regulator [47]. ZEB1 was shown to be induced after IR alone in radioresistant MDA-MB-231 cells. These cells express very high levels of another miR,



**Table 3** Main pathways implicated in IR response in TNBC

IR-related pathways	Possible downstream target	miRs	IR sensitization method	References
Apoptosis	MAPK, p53, mTOR	miR-17–92 cluster	NR	[82]
	Sirt1	miR-22	miR-22 mimics (Lipofectamine 2000) Knockdown Sirt1	[84]
	CDC27	miR-27a	antagomiR-27a (Lipofectamine 2000) Transfection of CDC27	[85]
	PI3K/Akt, PTEN	miR-27a	anti-miR-27a inhibitor (Lipofectamine 2000)	[34]
	ABCA1	miR-33a	miR-33a inhibitors (Oligofectamine)	[63]
	SGPP1 (S1P/PI3K/Akt pathway)	miR-95	Treatment with FTY720: S1P signaling inhibitor or LY294002: PI3K inhibitor	[36]
	RAS/MAPK (ZNF611, DUSP8, RIPK1)	miR-122	Transfection of antagomiR-122 in RR cells (Lipofectamine 2000)	[23]
	MAPK/c-JUN	miR-125b	Transfection of miR-125b mimics	[60]
	IncCCAT1	miR-148b	si-CCAT1 (Lipofectamine 2000)	[86]
	VDAC1	miR-181a-2	NA	[89]
	RRM2B (TP53 inducible)	miR-222, miR-125b-1		
	PCAT6/miR-185-5p/TPD52	miR-185-5p	si-PCAT6 or miR-185-5p mimics (Lipo- fectamine 3000)	[91]
	LINC00511/miR-185/STXBP4	miR-185	shLNC00511 (Lipofectamine 2000)	[92]
	IncRNA HOTAIR	miR-218	HOTAIR shRNA (Lipofectamine 3000)	[93]
	EGF/EGFR	miR-223	lentiviral miR-223 mimics EGFR inhibitor (Lapatinib) + miR-223 mimics	[94]
	IncHOTAIR/miR-499b-5p/HSPA1A HPGD/PGE2/EP2receptor	miR-499-5p miR-620	miR-499b-5p or si-HSPA1A AH-6809 (antagonism of EP2 receptor) Prospective: COX-2 inhibitor	[95] [96]
	Cell cycle	STAT3	miR-634	miR-643 mimics (Lipofectamine 2000)
ATM/TP53/CDK1		miR-16-5p, miR-23b-3p	NR	[26]
G2/M arrest (Chk1, Wee1)		miR-15 family (miR-15a/15b/16)	miR-15a/15b/16 mimics (Lipofectamine 2000) Co-depletion of Chk1 and Wee1	[81]
DSB repair	↑subG1 ↓G2 phase	miR-21	lentiviral-based anti-miR-21 <sup>a</sup>	[83]
	↑subG1 ↓G2 phase	miR-21	lentiviral anti-miR-21 vector	[65]
	EGFR/PI3K/Akt	miR-7	pre-miR-7 (siPORT NeoFX™)	[33]
	p53	miR-34	anti-miR-34	[42]
	TOP1/2A, XRCC5, RAD45L, POLQ; MAR2A	miR-139-5p	miR-139-5p mimics	[17]
	RAD51	miR-155	lentiviral miR-155 overexpression vector	[45]
	BRCA1	miR-182	miR-182 mimics	[90]
	TBK1	miR-200c	miR-200c mimics (Lipofectamine 2000)	[50]
	EGFR/PI3K/Akt	miR-200c	pre-miR-200c (siPORTNeoFX)	[32]
	LINC02582/USP7/CHK1	miR-200c	LINC02582 siRNA (Lipofectamine 3000)	[27]
Autophagy	ATM/ZEB1/Ubc13	miR-205	nanoliposomal miR-205 mimics	[28]
	AKT1, RAD52	miR-302a	miR-302a mimics (Lipofectamine 2000)	[29]
	HMGB1	miR-129-5p	miR-129-5p mimics (Lipofectamine 2000) Treatment with 3-MA (autophagy inhibi- tor)	[31]
	DRAM1, Beclin-1	miR-199a-5p	miR-199a-5p mimics	[59]
	UBQLN1	miR-200c	miR-200c mimics (Lipofectamine 2000) Treatment with 3-MA (autophagy inhibi- tor)	[24]

Table 3 (continued)

IR-related pathways	Possible downstream target	miRs	IR sensitization method	References
EMT	PTEN/Akt	miR-144	anti-miR-144 inhibitor (Lipofectamine 2000)	[35]
	LGMN/MMP2-MMP9	miR-155	shRNA-miR-155-based lentiviral plasmid	[88]
	ZEB1	miR-221	MEK1 inhibitor (TAK-733)	[30]
	IL4/IL4R $\alpha$ JAK/JNK/ $\beta$ -catenin/Stat6	miR-340/429	IL4 knockdown (shRNA-IL4 or anti-IL4 Ab) miR-340/429 mimics (Lipofectamine 2000)	[62]
Cancer stemness	Lin 28	Let-7	Knockdown Lin 28	[55]
	Cyclin D1/Akt1/Wnt (CSC self-renewal)	Let-7 family	lentiviral-based let-7d vectors	[54]
	CD133/SOX-2/OCT-4	20 oncomiRs	Combined with <i>A. cinnamomea</i>	[25]
	$\beta$ -catenin	miR-142-3p	pre-miR-142-3p (Lipofectamine)	[57]

<sup>a</sup>Differential effect of miR-21 knockdown in TNBC cells and normal epithelial cells

Ab antibody, DBS double-strand breaks, EMT epithelial–mesenchymal transition, IR irradiation, NA not applicable, NR not reported

miR-221 [30]. miR-221 was shown to be dependent on the oncogenic RAS-RAF-MEK pathway activity [48]. Combined therapy using TAK-733, a MEK1 inhibitor, with IR of 4 Gy, successfully downregulated miR-211 expression in MDA-MB-231 cells and inhibited tumor growth and migration activity [30].

ZEB proteins form a reciprocal feedback loop with the miR-200 family. Recent researches have shown that miR-200c inhibited EMT by directly targeting ZEB1, ZEB2, and other EMT regulators [49]. In addition, miR-200c enhanced IR-induced cell death in MDA-MB-231 cells but not in ER-positive MCF-7 cells. This radiosensitization could have happened via downregulation of TBK1, one of the downstream targets of miR-200c [50]. TBK1 knockdown with siTBK1 treatment significantly decreased clonogenic growth after IR, while TBK1 overexpression markedly inhibited miR-200c-induced apoptosis at 8 Gy in MDA-MB-231 cells [50].

### Radiosensitization by miR-induced changes in CSCs

CSCs are a small subpopulation of cancer cells with reduced proliferative potential but with the ability of self-renewal and tumorigenicity, which are not eliminated by anticancer treatment, contribute to residual disease and are the key drivers of cancer relapse [51, 52]. Several studies have demonstrated that IR enhances breast CSC-like phenotype, especially after low to moderate doses [53]. The TNBC CSCs selected by exposure to 4 Gy in vitro were shown to express eight stemness- and radioresistance-related miRs [53]. The tumor-suppressive let-7 miR family has been shown to suppress the self-renewal of CSCs and induce their apoptosis [54,

55]. In combination with let-7d, 2 Gy of IR exhibited markedly more potent inhibition of the CSC self-renewal ability than without let-7d in HS587-T and MM-231 TNBC cells [54]. Similarly, overexpression of let-7a successfully downregulated Lin28, a marker of CSC, and decreased Lin28-induced radioresistance [55]. Transfecting sorted CD44<sup>+</sup>/CD24<sup>-low</sup> human breast CSCs with pre-miR-142-3p significantly repressed  $\beta$ -catenin, the effector protein of the canonical Wnt pathway, which plays a vital role in CSC maintenance [56]. The miR-142-3p overexpressing cells displayed higher sensitivity to all IR doses (2, 4, and 6 Gy) [57].

### Radiosensitization by miR-induced modification of autophagy

Autophagy is a natural cell-destructive mechanism that degrades and recycles cellular components to help the formation of new cells. Autophagy is activated in response to IR-induced stress and contributes to radioresistance in BC [58]. TNBC cell lines MDA-MB-231 and BT549 exhibit lower expression of miR-200c [24] and miR-129-5p [31] and are more radioresistant than normal breast epithelial cells MCF-10A or luminal cells MCF-7. Ectopic overexpression of these two miRs inhibited IR-induced autophagy and decreased radioresistance in MDA-MB-231 cells via targeting *UBQLN1* and *HMGBl*, respectively [24, 31]. Treatment with 3-MA, an autophagy inhibitor that blocks autophagosome formation, could obtain the same radiosensitizing effect. Another miR involved in IR-induced autophagy is miR-199-5p, which plays differential roles depending on the BC subtype [59]. Its ectopic expression inhibited autophagy in irradiated MCF-7 cells but was an



autophagy inducer in the MDA-MB-231 cell line through downstream autophagy-related targets *DRAM1* and *Beclin-1*. This interaction between miR-199-5p and *DRAM1/Beclin-1* had a cell-specific influence on radiosensitivity. Transfection of a miR-199-5p mimic significantly decreased viability of MDA-MB-231 cells after exposure to IR, in a dose-dependent manner, compared to IR only; this radiosensitization was not observed in MCF-7 cell line, indicating that miR-199-5p might be a TNBC-specific radiosensitizer [59].

### miRs and differential radiosensitization effect

Some miRs induce differential effects in malignant and healthy cells. For example, ectopic expression of miR-7 suppressed Akt signaling and increased radiosensitivity in a panel of human cancer cells, including MDA-MB-468 TNBC cells [33]. However, when normal human fibroblasts and astrocytes were transfected with miR-7 and exposed to IR, neither cell type showed decreased survival at 2 Gy than the control-transfected cells without IR. This result demonstrated that miR-7 is not toxic to normal cells exposed to IR [33]. Similarly, the addition of miR-125b and miR-200c presented no observable cytotoxic effect on normal mammary epithelial MCF-10A cells [60] and normal human fibroblasts or astrocytes [32]. This differential effect was also observed in vivo when mice bearing radioresistant SUM159-P2 TNBC cell line xenografts were irradiated by a single dose of 15 Gy, with or without subcutaneous injection miR-205 mimics [28]. Histopathological examination revealed massive necrosis in the tumors treated with both the miR-205 mimics and IR, whereas no necrosis, steatosis, or fibrosis were observed in the livers and the kidneys of the animals. This observation demonstrated that the miR-205 mimic could sensitize malignant cells to IR but did not affect benign cells/organs [28]. These findings are very encouraging since the utmost objective in RT is to efficiently destroy malignant cells with minimal toxicity to the adjacent normal tissues.

### miRNAs as biomarkers for radiotherapy efficacy in TNBC

#### miRs as prognostic biomarkers

More published studies evaluated miRs as prognostic biomarkers in BC patients than as predictors of response to any type of anticancer therapy, including RT. Jang et al. measured miR expression in TNBC surgical specimens and found that miR-9 levels were associated with shorter disease-free survival (DFS) and distant metastasis-free survival (DMFS) of patients treated by adjuvant RT [61]. Similarly, higher levels of miR-16-5p [26], miR-340 [62], and miR-155 [45] were

associated with favorable overall survival (OS), whereas higher expression of miR-33a [63] and lower expression of miR-139-5p [17] correlated with shorter OS. High levels of miR-139-5p [64] and miR-429 [62] were shown to be biomarkers of good DFS and DMFS, respectively. On the other hand, overexpression of miR-21 [65] and miR-33a [63] and downregulation of miR-200c [24, 27] and miR-139-5p [64] were found in patients with poor survival. Upregulation of miR-210 in the serum of BC patients after RT corresponded to higher toxicity and recurrence risk [53]. The correlation between miRNAs dysregulation and clinical outcomes in BC patients treated by adjuvant RT is summarized in Table 4.

#### miRs as predictive biomarkers

Evaluation of miRs as predictive biomarkers started in the studies of NACT for TNBC. In a cohort of TNBC patients treated by a platinum-based NACT, a tenfold increased level of circulating miR-34a was observed post-treatment in responding patients, suggesting that this miR could serve as a reflector of tumor destruction under therapy [14]. Li et al. found that miR-770 was highly expressed in chemosensitive, good prognosis TNBCs [66].

A number of ongoing clinical trials have been testing the biomarker value of miRs for various modalities of BC RT and in different settings. The NCT02186470 trial is studying the role of accelerated partial breast irradiation (APBI) in hormone-responsive, early-stage BC and considers an exploratory evaluation of BC tissue and serum miRNAs before and after APBI. Several studies (REBECCA, NCT02079272; BACCARAT, NCT02605512; EARLY-HEART, NCT03297346) aim to identify the specific miRNA signatures that can be used as biomarkers to predict RT-induced cardiotoxicity.

### Challenges and perspectives

RT for TNBC embarked on an exciting new strategy in recent years. Delivered in the adjuvant setting for decades, RT in TNBC served only to strengthen the effect of NACT without limited personalization. The discovery that RT is a potent inducer of immunogenic cell death highlighted that first-line RT could effectively take an essential step in freeing the human body of cancer—the induction of an intense immune reaction to the malignant tissue, not only at the irradiated site but also at a distance through the so-called abscopal effect. Accordingly, RT could be not only a short-term killer but also a long-term regulator, provided it is well regulated itself.

miRs have been demonstrated to play major roles in the regulation of cancer behavior. The literature search that we performed shows that numerous miRs were involved in the

**Table 4** Prognostic and predictive value of IR-related miRNAs in TNBC

miRNA	Expression	n	Clinical model	Outcomes	References
miR-16-5p	High	1262	BC METABRIC	Longer OS (HR 0.73)	[26]
	High	1061	BC TCGA	Longer OS (HR 0.75)	
miR-21	High	86	FFPE (IDC)	Lower DMFS ( $p=0.029$ )	[65]
miR-33a	High	210	NCBI GEO dataset	Lower DRFS (HR 2.0, $p=0.076$ )	[63]
	High	53	Frozen tissue	Shorter OS in RT patients ( $p=0.065$ )	
miR-95	High	71	BC TCGA dataset	Recurrence ( $p=0.16$ )	[36]
miR-122	High	102	BC TCGA (RT only)	Better RFS (0.041)	[23]
	Loss of function	491	BC TCGA (RT only)	Longer RFS ( $p<0.05$ )	
miR-139-5p	High	1286	5 cohorts (surgery + ART)	Improve survival (DFS, DMFS, BCSS) (HR 0.3–0.5, $p<0.05$ )	[64]
oncomiRs	High	508	BC TCGA dataset	Worse OS ( $p=8.0e^{-08}$ )	[25]
miR-139-5p	Loss of function	1328	5 cohorts (surgery + ART)	Increase risk of LR, poorer RFS, DRFS, DSS and OS	[17]
		458	FFPE EBC patients		
miR-155	High	93	TNBC patients (RT +/– CT)	Better OS (HR 2.24, $p=0.031$ )	[45]
	High	134	TNBC cohort	Increase OS (HR 0.48, $p=0.043$ )	
High risk (8 mRNAs + 5 miRs)	High	73	BC TCGA dataset	Shorter survival (912 vs. 1430 days, $p=0.0039$ ; AUC=0.935)	[89]
		77	GEO dataset	Worse OS ( $p=0.0078$ ; AUC=0.701)	
miR-185-5p	Low	70	Frozen tissue TNBC patients	Correlated with advanced stage	[91]
miR-185	Loss of function	98	Frozen tissue BC patients	Correlated with recurrence ( $p=0.037$ ) and inferior survival ( $p<0.05$ )	[92]
miR-200c	Low	35	FFPE BC patients	Inversely correlated with LN status	[24]
miR-200c	Low	136	FFPE BC patients (surgery + ART)	Worse RFS ( $p=0.003$ )	[27]
miR-205	Low	207	BC Oxford database	Worse DRFS ( $p=0.006$ )	[28]
miR-210	Upregulation after RT	20	Serum samples before, during, and after RT in BC patients	Correlated with recurrence ( $p=0.03$ ) and toxicity ( $p=0.046$ )	[53]
miR-340	High	NR	MIRUMIR BC patients	Better OS	[62]
miR-429	High			Better DFS	
miR-499b-5p	Loss of function	855	GEPIA database (HOTAIR)	Poor OS (HR 1.5, $p=0.041$ )	[95]
			GEPIA database (HSPA1A)	Poor OS (HR 1.25, $p=0.042$ )	

ART adjuvant radiation therapy, BC breast cancer, BCSS: breast cancer-specific survival, CT chemotherapy, DFS disease-free survival, DMFS distant metastasis-free survival, FFPE formalin-fixed paraffin-embedded, GEO Gene Expression Omnibus, GEPIA Gene Expression Profiling Interactive Analysis, IDC invasive ductal cancer, LN lymph node, LR local recurrence, METABRIC Molecular Taxonomy of Breast Cancer International Consortium, NCI National Center for Biotechnology Information, NR not reported, OS overall survival, RFS recurrence-free survival, RT radiation therapy, TCGA The Cancer Genome Atlas

response of TNBC to IR. Regulation of miR expression successfully enhances radiosensitivity of highly radioresistant TNBC cells. miRs are able to show which tumor is radio-sensitive/resistant and to modulate this characteristic. miRs are liberated into the circulation after tumor cell destruction, thereby indicating whether IR produces the desired effect. miRs are druggable [67]. The success of the first miR-based drugs (Patisiran, Miravirsin, Cobomarsen) [68–71], although in non-TNBC indications, reassures us that combinations of IR and miR-based drugs could lead to RT adapted to the situation/patient.

Despite promising potentials of miRs as theranostic biomarkers, there are still challenges to overcome before their applications in routine clinical practice. For the application of miRs in cancer diagnostics, it might

be important to address the origin of tissues from which miRs are released, especially when more tissue-specific exosomal miRs are taken into consideration [72]. MiRs are highly sensitive to every change in sample collecting and processing and, therefore, can lead to bias in the final output of miRs [73], so standardized experimental protocols are urgently needed. Noninvasive circulating miRs are still hard to measure accurately due to their low concentration. Several detection methods are used in parallel, such as qPCR, microarray, and NGS, each with its own strengths and weakness [74]. Efforts to unify the measurement methods and reduce the deviation are then indispensable. Another obstacle to overcome is the globally acceptable standardization of the normalization methods so that the data from different studies can be correctly compared [75].



Furthermore, potential variability in physical and pathological confounding factors must be considered, as they are recognized to modify the miRs expression and therefore question the reliability and reproducibility of miRs in clinical applications [76, 77].

The miR-based therapeutics also hold several major concerns, especially on how to deliver efficiently and precisely the desired miRs to the target [78] and how to deal with the potential off-target effects of the miR therapeutics [79] (since one miR can regulate many genes involving in various developmental and regulatory pathways other than cancer-related pathways), as they can do more harm than benefits. Also, the chemical design of antisense and miR mimics, pharmacokinetic, and pharmacodynamics aspects are important in developing miR-based cancer therapy [80]. In addition, to deal with technical challenges, a better understanding of the complicated network between miR and coding and the effects in upstream/downstream pathways might help develop tailored miR antagonists or mimics and thereby achieve the goal of personalized cancer therapy, including radiosensitizing in TNBC.

The time has come to put both miRs and RT into action for the improvement of TNBC management. For real insight into the usefulness of miRs as radiosensitizers, biomarkers, or monitoring tools in TNBC RT, it is necessary to test various RT schemes in the neoadjuvant setting and various miRs before, during, and after such treatments. Particularly stimulating news is the development of miR signatures reflecting the immune response to cancer, so one can imagine using them to dynamically evaluate changes in tumor immune status during NART. This finding would be beneficial in determining what kind of RT-containing neoadjuvant treatment can be the best long-term TNBC controller. The fact that we can sample patient blood for miR assessment is prodigious, as it allows us to monitor the effect of IR and, eventually, adjust the doses and sequences.

## Conclusion

This review assembled data on the role of miR in TNBC exposed to IR. At present, all these data come from experimental systems. These observations need validation in patients' cohorts treated the same way as in vivo by direct exposure of tumor tissue to IR. Fortunately, an increasing number of trials evaluate neoadjuvant RT in (TN)BC in the context of immunotherapy development for this neoplasm. Which miR would be the best sensitizer or biomarker for which RT? We will know only in several years. This review indicates the shape of things to come in TNBC, in an era in which RT has started a whole new fight toward freedom from cancer.

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## Declarations

**Conflict of interest** All authors declare that they have no conflict of interest.

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## Annexe 6 : Lettre à l'éditeur sur la place de la RTNA dans le cancer du sein



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## LETTER TO THE EDITOR

## Neoadjuvant radiotherapy in triple-negative breast cancer: “the past should not steal the present or hide the future”

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Triple-negative breast cancer (TNBC), with its aggressive features and dismal prognoses, remains a challenge in the optimization of breast cancer (BC) management. Despite significant advances in systemic treatment during the last two decades, approximately half of patients did not achieve a pathological complete response (pCR) and are still faced with an extremely high risk of recurrence [1]. Novel approaches to overcome intrinsic resistance to therapy incorporate ablative strategies, including radiation therapy (RT), a well-established method of inducing tumor local cell death and secondary antitumor immune responses [1]. Furthermore, the development of sophisticated technologies enabling precise delivery of high RT doses to the tumor in the preoperative setting is expected to enhance the therapeutic potential in TNBC as revealed by pCR.

Soares et al. [2] recently published their real-life experience in 127 women with locally advanced TNBC who received neoadjuvant treatment. They stated that neoadjuvant radiotherapy (NART) and the absence of pCR were independent prognostic factors for relapse and 2-year disease-free survival (DFS). Such a biased statement might be misleading and should be taken into consideration with

many cautions. Actually, the 16 (13%) patients who received NART were those who experienced clinically progressive disease and/or grade 3/4 adverse events, depicting a very high-risk subgroup of patients. Yet, it would be very informative to have a detailed presentation of clinicopathological characteristics of the pre- and post-neoadjuvant therapy of these patients to better figure out the true therapeutic resistance. Moreover, no information regarding the NART delivery was reported. We assumed that a normofractionated schedule to the whole breast and/or regional lymph nodes was delivered, acknowledging the absence of adjuvant RT in these patients.

NART is not a novel approach and has been under investigation in BC with encouraging evidence, as summarized in Table 1. When delivered alone, NART can contribute to a pCR rate of 10% among all patients, and of 26% in those with TNBC disease. When sensitizing NART by concomitant administration of weekly paclitaxel, a pCR was achieved in 23% of patients, reaching 54% in patients with hormonal receptor-negative BC subtype. As observed with neoadjuvant chemotherapy (NACT), a pCR obtained from concurrent paclitaxel-RT

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was also translated into superior DFS and overall survival (OS). Moreover, “rescue” chemo-RT in NACT-refractory inoperable BC allowed curative surgery in 71% of patients, in which a pCR was observed in 50% of the histological specimens. In another study, preoperative or adjuvant concurrent chemo-RT was evaluated in 644 patients with locally advanced BC [3]. The neoadjuvant treatment was correlated with better relapse-free survival (RFS) and OS, especially in patients with cT2 tumors. Breast conservation became possible in 50.8% after neoadjuvant chemo-RT with a pCR rate of 29.2% and attained 56% in cN+ patients. This raises the question of an immune-priming phase in the involved lymph nodes and whether the modulation of RT delivery or other radiosensitizers could further improve responses.

Of note, the aforementioned studies employed standard fractionation to the whole breast and/or regional lymph nodes. Despite favorable pCR rates, TNBC patients tend to be at a higher risk of relapse and worse outcomes, especially in the case of residual disease post-neoadjuvant treatment. Every effort must be examined to ameliorate these response rates. Recent technical advancements in RT delivery offer the ability to accurately localize and target the primary disease, enabling the administration of optimal RT doses required to elicit a robust antitumor immune response with the least risk of toxicity. A phase I study tested five dose levels of stereotactic body radiation therapy (SBRT) concomitant with NACT before surgery [4]. This association showed promising results in terms of pCR rate (36%), and the highest pCR rate of 67% was achieved at the level 3 dose (25.5 Gy in 3 fractions). Interestingly, localized irradiation to the intact tumor leaves the possibility of standard-of-care adjuvant RT when indicated, especially in patients with residual disease, with no additional toxicity.

The incorporation of immunotherapy agents into the preoperative setting presents unique advantages for priming antitumor immune response and potential eradication of the disseminated micrometastatic disease. The combination of an immune checkpoint inhibitor (ICI) with hypofractionated RT (8 Gy x 3 fractions) led to increased accumulation of cytosolic DNA damage, activation of cGAS/STING, and

increased type I interferon signaling, necessary for CD8+ T-cell mediated antitumor immune response and regression of non-irradiated lesions, compared to high-dose RT alone [1]. Several ongoing trials are testing this ICI/RT combination to exert the so-called abscopal effect in BC.

In conclusion, the radiation oncology community is excitingly awaiting the upcoming results of ongoing trials implementing modern concepts of NART in the management of BC, particularly, in the TNBC subtype. The matter in these combined therapies is to consider the right treatment for the right patient at the right time, which probably was not achieved in the NART patients reported by Soares et al. [2]. We strongly believe that the major unfavorable prognostic factor in their cohort was the aggressive biology of the tumors treated by NART, but not the NART itself. Therefore, in terms of NART in TNBC, we must not let the past steal the present or hide the future.

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## Résumé :

La radiothérapie (RT) fait partie des principales armes thérapeutiques dans le traitement des cancers. Actuellement en France, on estime à environ 200.000 le nombre de patients qui reçoivent une RT à divers stades de leur maladie chaque année.

L'impact de la RT sur le système immunitaire est reconnu pour son effet "myéloablatif" depuis plusieurs décennies chez les malades candidats aux greffes mais aussi chez les victimes des accidents nucléaires et des explosions aux bombes atomiques ayant induit une insuffisance médullaire mortelle. Au plan thérapeutique, des effets immunostimulateurs de la RT ont été rapportés sous le terme « effet abscopal » correspondant à des réponses antitumorales après la RT dans des sites à distance non irradiés des tumeurs solides.

Mes travaux dans laboratoire (équipe I-BIOT de l'IMRB) se sont focalisés sur la modulation de la réponse immunitaire appliquée aux allogreffes et au cancer dans les deux modèles suivants :

**1. Modèle murin d'allogreffe** : la maîtrise des paramètres de l'irradiation corporelle totale (ICT) est capitale pour la reproductibilité et la fiabilité des résultats expérimentaux dans le modèle murin. Les données de la littérature ont montré une variation considérable dans la reproduction des résultats d'une expérience à l'autre en fonction du dispositif utilisé pour délivrer l'ICT avant allogreffe de moelle osseuse. L'objectif de ce travail était de comparer 2 types d'appareils d'irradiation utilisant différents niveaux d'énergie de photons en termes de résultats post-allogreffe (chimérisme, réaction du greffon contre l'hôte et du contrôle tumoral post-greffe). Nous avons démontré que l'ICT avec des photons de basses énergies induit une inflammation post-ICT plus importante que les photons de hautes énergies et par conséquent une alloréactivité plus importante post-greffe. Cette inflammation peut être modulée par un retard à l'injection de la moelle osseuse programmée en dehors du pic inflammatoire pour réduire ainsi l'alloréactivité post-greffe. Ces données sont à prendre en considération pour l'interprétation des résultats obtenus selon le type d'appareil utilisé dans le modèle expérimental.

**2. Modèle clinique de cancer du sein** : la 2ème partie s'inscrit dans le cadre d'une étude translationnelle d'une recherche clinique prospective évaluant la place d'une radiothérapie (RT) préopératoire dans le cancer du sein triple négatif et luminal B. Ces derniers sont connus pour leur agressivité et leur immunogénicité particulière. Dans ce modèle la RT pré opératoire avait pour objectif d'augmenter les réponses tumorales en agissant, entre autres, sur l'immunité tumorale. Les biomarqueurs immunitaires dérivés du microenvironnement tumoral et du sang périphérique ont été analysés. Sur une analyse préliminaire de 42 patientes, nous avons identifié différents biomarqueurs prédictifs de la réponse pathologique complète selon le traitement pré opératoire administré (avec ou sans RT). Ces résultats permettent de mieux sélectionner les patientes pour une meilleure personnalisation de cette nouvelle approche thérapeutique.