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Louise Rethacker

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Institut de Recherche Saint-Louis – Unité INSERM 1160

Les cellules lymphocytaires innées dans l'immunosurveillance des tumeurs métastatiques

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A ma famille,

A mon Papa.

Résumé

Les cellules lymphocytaires innées (ILC) sont des effecteurs de l'immunité innée et jouent un rôle important dans la régulation de la réponse immunitaire. Les cellules NK (ILC1 cytotoxiques) sont connues pour leurs capacités anti-tumorales. La réponse des ILC auxiliaires (ILC2 et ILC3) face à la tumeur est encore peu décrite chez l'Homme. Par leur résidence tissulaire, les ILC auxiliaires pourraient jouer un rôle important dans la réponse immunitaire face au cancer.

Le développement des thérapies ciblées et des immunothérapies a révolutionné les traitements de nombreux cancers. Les traitements actuels sont « personnalisés », adaptés au profil mutationnel du patient et à la qualité du microenvironnement tumoral. Ces traitements entraînent des réponses rapides et efficaces dans la plupart des cas. Cependant, des mécanismes de résistances sont fréquents, entraînant la rechute de la maladie. Les mécanismes de résistance sont encore mal connus. Les approches actuelles combinent des thérapies ciblant les anomalies spécifiques de la tumeur et un contournement de l'immunosuppression pour restaurer une immunité anti-tumorale effective.

Mon projet de thèse s'inscrit dans cette problématique à travers deux projets : un projet de recherche translationnelle pour analyser l'impact de la résistance aux thérapies ciblées sur l'immunogénicité NK et un projet plus exploratoire qui vise à définir le rôle des cellules lymphoïdes innées (ILC) ganglionnaire dans l'immunosurveillance tumorale.

Le premier projet est centré sur l'étude des ILC cytotoxiques, les cellules NK, et leurs interactions avec les cellules tumorales de mélanome résistantes aux thérapies ciblées, dirigées contre les tyrosines kinases BRAF (Vemurafenib) et MEK (Cobimetinib). Nous avons montré que les cellules résistantes étaient plus sensibles à la lyse par les cellules NK due à des modifications de leur phénotype. L'induction de la résistance induit notamment une augmentation de l'expression des ligands de NKG2D, MICA et ULBP2, qui est impliqué dans l'activation des fonctions NK par les cellules de mélanome. Nous avons également observé une modification de la sensibilité à l'apoptose induite par les récepteurs à domaine de mort. Cette étude démontre donc l'utilité d'une immunothérapie basée sur les cellules NK en combinaison avec des inhibiteurs de BRAF et MEK.

Nous nous sommes ensuite intéressés aux rôles des ILC dans l'immunosurveillance des métastases ganglionnaires du cancer du sein. Ce travail nous a permis d'identifier une population CD127⁺CD56⁺ « ILC3-like », identifiée comme précurseur ILC, diminuée dans les ganglions métastatiques par rapport à des ganglions sains, au profit des cellules NK. Bien que présentant un profil transcriptomique activé, nous observons une diminution de l'expression des NCRs à la surface des

cellules NK dans les ganglions métastatiques, suggérant une diminution de ces récepteurs après contact avec les cellules tumorales. Pour les ILC3, nous observons une augmentation de la production de TNF α , qui dans un contexte de cancer du sein, pourrait participer à la progression tumorale. Cette étude montre l'importance des ILC3 et des cellules NK dans l'immunosurveillance tumorale dans le ganglion. D'un côté, les ILC3 semblent avoir des effets pro-tumoraux, il faudrait donc envisager de bloquer cette population, et d'un autre côté les cellules NK semblent altérées, il faudrait donc les réactiver. Les immunothérapies ciblant les ILC devraient donc être considérées dans les cas d'invasion ganglionnaire dans le cancer du sein.

Mots clés : ILC, cellule NK, cancer, immunothérapie, ganglion

Abstract

Innate lymphoid cells (ILCs) are effectors of innate immunity and play an important role in the regulation of the immune response. NK cells (cytotoxic ILC1) are known for their anti-tumor functions. The immune response of helper-type ILCs (hILC) (ILC1, ILC2 and ILC3) against the tumor is still poorly described in Humans. By their tissue residency, hILCs could play an important role in the immune response to cancer.

The development of targeted therapies and immunotherapies has revolutionized the treatment of many cancers. Current therapies are “personalized”, adapted to the mutational profile of the patient and to the quality of the tumor microenvironment. These treatments result in rapid and effective responses in most of the cases. However, resistance mechanisms are frequent, leading to relapses. The mechanisms of resistance are still poorly understood. Current approaches combine therapies targeting specific tumor abnormalities and bypassing immunosuppression to restore effective anti-tumor immunity.

My thesis project attempts to address this issue through two projects: a translational research project to analyze the impact of resistance to targeted therapies on NK immunogenicity and a more exploratory project which aims to define the role of lymph node (LN) ILC in tumor immunosurveillance.

The first project focuses on the study of cytotoxic ILCs, NK cells, and their interactions with melanoma cells resistant to targeted therapies, directed against the BRAF (Vemurafenib) and MEK (Cobimetinib) tyrosine kinases. We showed that resistant melanoma cells were more sensitive to NK cell lysis due to changes in their phenotype. Resistance notably induces an increase of the expression of the ligands of NKG2D, MICA and ULBP2, which is involved in the activation of NK functions by melanoma cells. We also observed a change in the sensitivity to apoptosis induced by death domain receptors. This study therefore demonstrates the utility of NK cell based immunotherapy in combination with BRAF and MEK inhibitors.

We then studied the role of ILCs in the immunosurveillance of lymph node metastases in breast cancer. This work allowed us to identify a CD127⁺CD56⁺ ILC3-like cells, identified as an ILC precursor, reduced in metastatic LN compared to healthy LN, in favor of NK cells. Although showing an activated transcriptomic profile, we observe a decrease in the expression of NCRs at the surface of NK cells in metastatic LN, suggesting a decrease in these receptors after contact with tumor cells. For ILC3, we observe an increase in the production of TNF α , which in the context of breast cancer, could participate in tumor progression. This study shows the importance of ILC3 and NK cells in tumor immunosurveillance in the LN. On one hand, ILC3s seem to have pro-tumor effects, so we should

consider blocking this population, and on the other hand, NK cells seem to be altered, so they should be reactivated. Immunotherapies targeting ILCs should therefore be considered in cases of LN invasion in breast cancer.

Key words: ILC, NK cells, cancer, immunotherapy, lymph node

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Liste des abréviations

A

ADCC: *Antibody-dependent cell-mediated cytotoxicity*

ADO: Adénosine

APL: Leucémie aiguë pro myélocytaire

AREG: Amphiréguline

B

BiKE: *Bi-specific Killer Engagers*

C

CAR: *Chimeric Antigen Receptor*

CD: Cellule dendritique

CILP: *Common innate lymphoid progenitor*

CLP: *Common lymphoid progenitor*

CMH: Complexe Majeur d'Histocompatibilité

CMV: Cytomégalovirus

CPA: Cellule présentatrice d'antigène

CRTH2 : Récepteur 2 de la prostaglandine D2

CTLA-4: *Cytotoxic T Lymphocyte –Associated protein 4*

D

DAP10/12: *DNAX Activation Protein 10/12*

DISC: *Death-Inducing Signaling Complex*

DNAM-1: *DNAX accessory molecule-1*

E

EGFR: *Epidermal Growth Factor Receptor*

ER: *Estrogen receptor*

ERK: *Extracellular signal-regulated kinase*

F

FasL : Ligand du récepteur Fas

Fc : Fragment constant des immunoglobulines

FLT3: *Fms-like tyrosine kinase 3*

FT: Facteur de transcription

Fv: Fragment variable des immunoglobulines

G

GIST: *Gastro Intestinal Stromal Tumor*

GM-CSF: *Granulocyte-macrophage colony-stimulating factor*

GRB2: *Growth factor receptor-bound protein 2*

GvHD: *Graft versus host disease*

GVL: *Graft versus leukemia*

GZM: Granzyme

H

HA: Hémagglutinine

HER2: *Human Epidermal growth factor Receptor 2*

HLA: *Human Leukocyte Antigen*

I

ICB: *Immune Checkpoint Blocker*

IDO: Indolamine 2,3-dioxygénase

ieILC1: ILC1 intra-épithéliale

IFN: Interféron

Ig: Immunoglobuline

IL: Interleukine

ILC: *Innate lymphoid cell*

ILCP: *ILC precursor*

iSPC: Cellules souches pluripotentes induites

ITAM: Motifs d'Activation des récepteurs Immuns basés sur la Tyrosine

ITGAE: Intégrine alpha E

ITIM : Motif d'inhibition de l'immuno-récepteur à base tyrosine

K

KIR : Récepteurs aux immunoglobulines

KLF2: *Krüppel-like Factor 2*

KLRG1: *Killer cell lectin-like receptor sub-family G member 1*

L

LAG-3: *Lymphocyte Activation Gene 3*

LAM: Leucémie aigüe myéloïde

Lin: *Lineage*

LT: Lymphocyte T

LTi: *Lymphoid tissue inducer*

LT α / β : Lymphotoxine alpha/beta

M

M14R, SK28R : Lignées de mélanome résistantes au vemurafenib

M14S, SK28S : Lignées de mélanome sensibles au vemurafenib

mAb: Anticorps monoclonaux

MAPK: *Mitogen-Activated Protein Kinase*

MDSC: Cellules myéloïdes suppressives

MIC: *MHC class I polypeptide-related sequence*

MMP : métalloprotéases matricielles

N

NCAM: *Neural Cell Adhesion Molecule*

NCR : Récepteur Naturel de cytotoxicité

NF-kb: *nuclear factor-kappa B*

NK: *Natural Killer*

NKCE: *Natural killer (NK) cell engagers*

NKDI: *NK cell developmental intermediates*

NKP: *NK precursor*

O

OLS: *Organe lymphoïde secondaire*

P

PCNA: *Proliferating cell nuclear antigen*

PCR: *Polymerase Chain reaction*

PD-1: *Programmed cell Death-1*

PD-L: *Programmed cell Death-1 Ligand*

PGE2: *Prostaglandine E2*

PI3K: *Phosphatidylinositol 3-Kinase*

PVR: *Poliovirus Receptor*

R

ROR: *récepteur orphelin lié à la protéine RAR*

S

S1P : *Sphingosine-phosphate*

S1PR : *Récepteur de la sphingosine-phosphate*

SDF-1: *facteur stromal dérivé des cellules 1*

SELL: *L-selectine*

SHP-1/2: *Src Homology-containing tyrosine Phosphatase ½*

SI: *synapse immunologique*

SYK : *Spleen tyrosine (Y) Kinase*

T

TAM: *Tumor-associated macrophages*

T-bet : Facteur de transcription T-box

TGF- β : Facteur de croissance transformant β

Th: T helper

TIGIT: *T cell Immunoreceptor with Ig and ITIM domains*

TIM-3: *T-cell Immunoglobulin- and Mucindomain-containing molecule 3*

TKI: Inhibiteur de tyrosine kinase

TLS: Structure lymphoïde tertiaire

TME: microenvironnement tumoral

TNBC : Cancer du sein triple négatif

TNF: *Tumor necrosis factor*

TNM: *Tumor Node Metastasis*

TRAIL: *Tumor-necrosis-factor related apoptosis inducing ligand*

Treg: Lymphocyte T régulateur

TriKE: *Tri-specific Killer Engagers*

TRM: lymphocyte T résident mémoire

U

ULBP: *UL16 Binding Protein*

V

VEGF: *Vascular Endothelial Growth Factor*

Liste des figures

Figure 1 : Nomenclature des ILC.....	23
Figure 2 : Répartition des ILC dans différents tissus.....	24
Figure 3 : Localisation des sous-populations NK.....	25
Figure 4 : Les mécanismes effecteurs des cellules NK.....	29
Figure 5 : Fonctions immunorégulatrices des ILC.....	32
Figure 6 : Les récepteurs exprimés par les ILC et leurs ligands exprimés par les cellules tumorales.....	34
Figure 7 : Plasticité des ILC.....	39
Figure 8 : Modèle de différenciation des ILC humaines.....	42
Figure 9 : Les trois étapes du concept de l'immunosurveillance.....	44
Figure 10 : Fonctions anti-tumorales des cellules NK.....	46
Figure 11 : Mécanismes d'échappement tumoral.....	48
Figure 12 : Immunothérapies augmentant les fonctions des cellules NK.....	49
Figure 13 : Structure et fonction des BiKEs et TriKEs.....	51
Figure 14 : Fonctions pro- et anti-tumorales des ILC auxiliaires.....	61
Figure 15 : Le cancer du sein et ses sous-types.....	88
Figure 16 : Choix thérapeutiques dans le cancer du sein.....	89
Figure 17 : Schéma récapitulatif de l'impact des TKI sur l'immunogénicité NK des cellules de mélanomes.....	129
Figure 18 : Influence du microenvironnement tumoral sur les cellules NK.....	133
Figure 19 : Interactions entre les cellules tumorales et les ILC3.....	135
Figure 20 : Comparaison des ILC dans les ganglions métastatiques de cancer du sein et dans des ganglions sains.....	137

Figures retirées en gras

Liste des tableaux

Tableau 1 : Ligands des récepteurs NCR.....	35
Tableau 2 : Sélection d'essais clinique CAR-NK en cours.....	53
Tableau 3 : Classification de l'AJCC.....	67
Tableau 4 : Expression des ligands (L) des récepteurs NK par les lignées de mélanome sensibles et résistantes au vemurafenib.....	69

Tableau retiré en gras

Liste des figures retirées

Figure 2 : Répartition des ILC dans différents tissus.....	24
Figure 3 : Localisation des sous-populations NK.....	25
Figure 9 : Les trois étapes du concept de l'immunosurveillance.....	44
Figure 10 : Fonctions anti-tumorales des cellules NK.....	46
Figure 13 : Structure et fonction des BiKEs et TriKEs.....	51
Figure 14 : Fonctions pro- et anti-tumorales des ILC auxiliaires.....	61
Figure 15 : Le cancer du sein et ses sous-types.....	88
Figure 16 : Choix thérapeutiques dans le cancer du sein.....	89
Tableau 3 : Classification de l'AJCC.....	67

Table des matières

Résumé.....	1
Abstract	3
Remerciements	5
Liste des abréviations	8
Liste des figures.....	13
Liste des tableaux.....	14
Liste des figures retirées	15
INTRODUCTION	19
Partie 1 : Les cellules lymphoïdes innées (ILC), régulatrices de la réponse immunitaire.....	19
I. Classification des ILC.....	20
II. Localisation et circulation des ILCs.....	22
III. Fonctions effectrices, les ILC1	25
A. Cytotoxicité naturelle	26
B. Cytotoxicité médiée par les anticorps.....	27
C. Les récepteurs à domaine de mort	28
IV. Fonctions immunorégulatrices des ILC	29
V. Les récepteurs NK/ILC et leurs ligands	31
A. Les récepteurs de cytotoxicité naturelle (NCR).....	31
B. Les KIRs.....	34
C. Les récepteurs de type lectine NKG2	34
1. NKG2D	34
2. CD94/NKG2.....	35
D. Les récepteurs accessoires : DNAM-1, CD96, TIGIT	35
E. 2B4 (CD244).....	36
F. CD161	36
G. KLRG1	36
VI. Plasticité	37
VII. Développement et maturation des ILC	39
A. Les stades de différenciation.....	39
B. Le ganglion lymphatique, lieu de maturation des cellules NK	41
Partie 2 : Les ILC dans l'immunosurveillance anti-tumorale	43
I. Les cellules NK dans les tumeurs.....	43
A. Immunosurveillance tumorale	43
B. Echappement tumoral.....	45
II. Immunothérapies NK	47

A. Les anticorps monoclonaux et l'ADCC.....	48
B. Le transfert adoptif.....	50
C. Les inhibiteurs de <i>checkpoints</i> immuns.....	51
D. Combinaisons thérapeutiques	54
E. Thérapies en néo-/adjuvant	56
III. Les ILC auxiliaires dans le cancer	57
OBJECTIFS	60
RESULTATS.....	64
Article n°1 : La résistance des cellules de mélanome aux inhibiteurs de BRAF induit une sensibilité accrue à la lyse médiée par les cellules Natural Killer.....	64
Article 2 : Les lymphocytes innées dans les ganglions métastatiques de cancer du sein.....	85
DISCUSSION	122
CONCLUSION GENERALE	135
BIBLIOGRAPHIE.....	138
Annexe 1: Specific patterns of blood ILC in metastatic melanoma patients and their modulations in response to immunotherapy.....	164
Annexe 2: NKG2D/NKG2-Ligand Pathway Offers New Opportunities in Cancer Treatment.	178
Annexe 3: Two-dimensional dynamic evaluation of natural killer cell-mediated lysis of adherent target cells	187
Annexe 4: Challenges for NK-cell based therapies: what can we learn from lymph nodes?.....	203

INTRODUCTION

INTRODUCTION

Partie 1 : Les cellules lymphoïdes innées (ILC), régulatrices de la réponse immunitaire

Les cellules lymphoïdes innées (ILC) regroupent différentes populations de cellules de l'immunité innée impliquées dans diverses fonctions, incluant l'homéostasie immunitaire tissulaire, la régulation de l'inflammation, en conditions basales et dans des contextes infectieux, d'inflammation chronique ou de cancer. Les premières identifiées sont les cellules Natural Killer (NK) découvertes chez la souris par R. Kiessling, R.B. Herberman et leurs collaborateurs en 1975 (Herberman et al., 1975; Kiessling et al., 1975a). Les cellules NK doivent leur nom à leur fonction de cytotoxicité naturelle. Ce sont de grands lymphocytes granuleux capables de tuer des cellules tumorales sans stimulation préalable. Plus de 20 ans après, les *lymphoid tissue inducer* (LTi) ont été mises en évidence par l'équipe de R.E. Mebius en 1997. Les LTi sont responsables de la formation des organes lymphoïdes chez la souris (Mebius et al., 1997). Il faut ensuite attendre les années 2010 pour que plusieurs équipes montrent l'existence de plusieurs populations lymphocytaires innées de type « *helper* » ou auxiliaires, dont les fonctions et les facteurs de transcription exprimés, les rapprochent des lymphocytes T (LT) CD4. La nomenclature des LT auxiliaires a donc été utilisée pour nommer les différentes populations d'ILC : ILC1 (dont les NK font partie), ILC2 et ILC3 (dont les LTi font partie) (Spits et al., 2013). Du fait de leurs capacités cytotoxiques anti-tumorales et leur présence dans le sang, les cellules NK sont les ILC les plus étudiées et les mieux caractérisées : ce sont les ILC cytotoxiques, productrices de cytokines de types Th1 (IFN- γ , TNF- α), miroir des LT CD8⁺. Les autres ILC sont définies comme étant plutôt immunorégulatrices, via la sécrétion de cytokines, elles sont appelées auxiliaires, miroir des LT auxiliaires CD4⁺.

Les ILC se différencient principalement dans la moelle osseuse puis poursuivent leur maturation dans les organes lymphoïdes secondaires (OLS), comme le ganglion lymphatique. Ce dernier est un site important pour la mise en place de la réponse immunitaire adaptative. Les antigènes et les cellules présentatrices d'antigènes (CPA) arrivent par les vaisseaux lymphatiques, permettant une activation des lymphocytes résidents. Les cellules ILC présentes dans le ganglion peuvent activer les CPA, induisant l'activation des lymphocytes T ou B.

Les ILC auxiliaires sont encore peu étudiées chez l'Humain, mais les données obtenues dans des modèles murins indiquent l'importance de ces cellules dans l'immunosurveillance et soulignent

que l'analyse de ces populations est indispensable, notamment l'étude de leurs fonctions dans un contexte de cancer.

I. Classification des ILC

La classification des ILC est basée sur leur phénotype et leurs fonctions (Figure 1) (Spits et al., 2013).

Les ILC1 sont définies par leur capacité à produire de l'IFN- γ , mais aussi d'autres cytokines de type Th1, Th2 et Th17, et sur l'expression des facteurs de transcription (FT) T-bet et Eomes. Ce groupe ILC1 comprend deux sous-populations : d'une part les cellules NK, cytotoxiques et majoritairement circulantes, d'autre part, les ILC1 auxiliaires, résidant dans les tissus. Ces deux sous-populations nécessitent le FT T-bet pour leur développement, alors que seules les cellules NK expriment Eomes.

Les cellules NK sont caractérisées selon l'expression de la molécule d'adhésion CD56 (NCAM) et l'absence de CD3 (CD3⁻CD56⁺). Elles peuvent également exprimer CD16, récepteur de faible affinité de la partie constante (Fc) des immunoglobulines (Ig) (Fc γ RIII). Selon l'expression de CD56 et CD16, les cellules NK sanguines sont divisées en deux sous-populations : CD56^{bright}CD16⁻, faiblement cytotoxiques et productrices de cytokines, et CD56^{dim}CD16⁺, fortement cytotoxiques et produisant peu de cytokines. Ces deux populations sont présentes en différentes proportions dans les tissus. La population CD56^{dim} est prédominante dans le sang et la rate alors que la population CD56^{bright} est prédominante dans les OLS, comme le ganglion. Dans le ganglion, les cellules NK CD56^{bright} jouent un rôle immunorégulateur, par la production d'IFN- γ , permettant une activation des cellules dendritiques (CD) et favorisant la réponse T de type Th1. Cette population CD56^{bright} est définie comme peu mature, certaines études la considère comme la population précurseur des cellules NK CD56^{dim} (Chan et al., 2007; Huntington et al., 2009).

Les ILC auxiliaires sont définies comme négatives aux marqueurs de lignage (*lineage* ou *lin*) et positives au CD127, récepteur à l'IL-7. Les marqueurs dans le lignage diffèrent parfois entre les équipes. Les plus communs sont le CD3, les TCR $\alpha\beta$ et $\gamma\delta$, afin d'exclure les LT, le CD19, pour exclure les lymphocytes B, le CD14 pour exclure les monocytes. Pour être sûr d'exclure les LT, certaines équipes ajoute le CD4 et le CD5. Cependant, le CD4 et le CD8 et les gènes codant pour le CD3, le CD5 et les TCR peut être exprimé par une population d'ILC1 (Björklund et al., 2016; Roan et al., 2016). Dans la revue de Trabanelli et al., les auteurs tentent de décrire un phénotype universel, et concluent que le lignage devrait au moins contenir les marqueurs suivant : CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD203c et Fc ϵ RI (Trabanelli et al., 2018).

Les ILC1 auxiliaires humaines ne sont pas encore bien définies. Il n'existe pas de marqueurs spécifiques en dehors de l'expression de CD127 et l'absence d'expression de CD117 (c-Kit) et CRTH2 (CD127⁺CD117⁻CRTH2⁻). Une population ILC1 CD127⁺CD117⁻T-bet⁺Eomes⁻Perforine⁻CD56⁻ a été identifiée dans la muqueuse intestinale, capable de produire de l'IFN- γ après activation par l'IL-12, et enrichie chez les patients atteints de la maladie de Crohn (Bernink et al., 2013). En parallèle de ces travaux, une autre étude a défini une population ILC1 intraépithéliale (ieILC1) dans la muqueuse gastro-intestinale et dans les amygdales (Fuchs et al., 2013). Cette population est définie par le phénotype CD127⁺CD56⁺NKp44⁺CD103⁺, elle exprime également Eomes. Les ILC1 intraépithéliales produisent de l'IFN- γ en réponse à l'IL-12 et l'IL-15. De façon intéressante, deux études montrent que les ILC1 peuvent exprimer des marqueurs T. Elles rapportent que les ILC1 sanguines expriment CD3 et CD4 ainsi que les ILC1 résidentes des amygdales expriment CD5 et CD6 (Björklund et al., 2016; Roan et al., 2016).

Les ILC2 produisent des cytokines de type Th-2 (IL-4, IL-5, IL-13) en réponse à l'IL-33 et l'IL-25, et de l'amphiréguline (AREG) (Jeffery et al., 2017; Moro et al., 2010; Neill et al., 2010; Price et al., 2010). Elles dépendent du FT GATA3 et ROR α pour leur développement et leur fonction (Hoyler et al., 2012; Wong et al., 2012). Les ILC2, présentes dans l'intestin, les poumons et le sang, expriment les marqueurs CRTH2 et CD161 (Mjösberg et al., 2011). Elles sont également localisées dans les tissus adipeux (Brestoff et al., 2015). Elles présentent une expression hétérogène de CD117, avec une population CD117^{high} et une CD117^{low} (Hochdörfer et al., 2019). Dans le sang, le FT GATA3 est plus exprimé par la population CD117^{low}, alors que la population CD117^{high} exprime le FT ROR γ t. Elles sont donc définies comme CD127⁺CD117^{+/-}CRTH2⁺.

Les ILC3 sont définies comme Lin⁻CD127⁺CD117⁺CRTH2⁻ et expriment le FT ROR γ t. Elles produisent des cytokines de type Th17 (IL-17 et IL-22). Ce groupe comprend trois sous-populations principales. Les LTI jouent un rôle important dans la formation embryonnaire des ganglions lymphatiques. Très étudiés chez la souris, ils ont également été identifiés chez l'humain dans les ganglions mésentériques fœtaux (Cupedo et al., 2009). Ces LTI sont capables d'induire l'expression des molécules d'adhésion par les cellules mésenchymateuses stromales, via la lymphotoxine, suggérant leur rôle dans le développement des OLS. Des populations d'ILC3 ont été identifiées dans différents tissus adultes. Elles sont divisées en deux sous-populations, en fonction de l'expression des récepteurs à cytotoxicité naturelle (NCR) NKp44 et NKp46 (Glatzer et al., 2013; Hoorweg et al., 2012; Simoni et al., 2017). Ces deux populations produisent de l'IL-17, alors que la production d'IL-22 est décrite comme spécifique des ILC3 NCR⁺. Une des premières population ILC3 a été considérée comme étant une population NK car elle exprime CD56 et le récepteur NKp44 (Cella et al., 2009). Elle a été identifiée dans la muqueuse intestinale et les amygdales. Une population ILC3 NCR⁺ a également été caractérisée au niveau de la

peau (Teunissen et al., 2014). Cette population est plus fréquente chez des patients atteints de psoriasis. Une étude sur les ILC sanguines démontre l'existence d'ILC3 NKp46⁺CD56⁺ (Nagasawa et al., 2019).

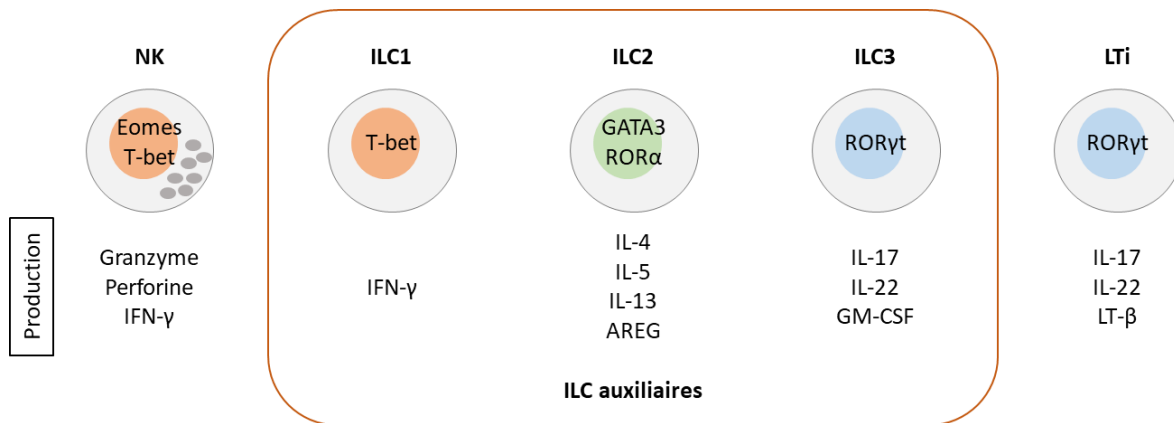


Figure 1 : Nomenclature des ILC. Les ILC sont réparties en 5 groupes : cellules NK (cytotoxiques), les ILC1, ILC2 et ILC3 (ILC auxiliaires) et les LTi. Elles sont définies selon l'expression de facteurs de transcription indispensables à leur différenciation et selon leurs capacités à produire différentes cytokines et leur activité cytotoxique pour les cellules NK.

II. Localisation et circulation des ILCs

Les ILCs auxiliaires ont été décrites comme étant principalement résidentes tissulaires. En effet, dans le sang, les cellules NK sont les ILC majoritaires, représentant 10 à 20% des lymphocytes CD45⁺, contre 0.01% à 0.1% pour les ILC auxiliaires CD127⁺ (Figure 2) (Carrega et al., 2014; Hazenberg and Spits, 2014). Les cellules NK représentent environ 5% des lymphocytes CD45⁺ dans les ganglions lymphatiques, les tissus adipeux viscéraux, les tissus mammaires, le colon et l'intestin. Elles sont présentes en plus grande proportion (95% des ILC) dans la rate, les poumons, les reins, le foie et les tissus adipeux sous-cutané (Yudanin et al., 2019).

Figure 2 : Répartition des ILC dans différents tissus. Les cellules NK sont les ILC majoritaires dans le sang, le sang de cordon, la moelle osseuse, les poumons et la rate. Les ILC3, les ILC1 et les cellules NK sont présentes dans des proportions similaires dans le colon. Dans les amygdales, les ILC3 sont

majoritaires. Les ILC2 sont présentes dans de grandes proportions au niveau de la peau. *Figure tirée de (Simoni and Newell, 2018).*

La distribution des sous-populations NK varie selon le tissu (Figure 3). Dans la moelle osseuse, la rate, les poumons et le sang, les cellules NK sont majoritairement CD56^{dim}CD16⁺ alors qu'elles sont essentiellement CD56^{bright}CD16⁻ dans les ganglions, les amygdales et l'intestin (Dogra et al., 2020). Dans cette étude de Dogra et al., les NK CD56^{bright}CD16⁻ sont décrites comme résidentes tissulaires avec un profil transcriptomique proche des LT résidents mémoires (TRM). En effet, par rapport aux cellules NK CD56^{bright}CD16⁻ du sang et CD56^{dim}CD16⁺ tissulaires, les NK CD56^{bright}CD16⁻ tissulaires présentent une augmentation de l'expression des gènes *ITGAE* (codant pour CD103), *CXCR6* et une sous expression de *S1PR1* et *KLF2*. Le récepteur CD69 est également un marqueur de résidence tissulaire, en plus d'être un marqueur d'activation des lymphocytes. CD69 est plus exprimé par la population NK CD56^{bright}CD16⁻ dans la moelle osseuse, la rate, les poumons et les amygdales alors qu'il est exprimé de façon équivalente entre les deux sous populations de cellules NK dans les ganglions et l'intestin. Dans le foie, une partie des cellules NK résidentes sont, comme chez la souris, CD49a⁺CD56^{bright} exprimant beaucoup de T-bet et peu d'Eomes (Marquardt et al., 2015). Une seconde population NK résidente est décrite dans le foie, exprimant le récepteur de chimiokines CXCR6 ainsi que le marqueur de résidence CD69 (Harmon et al., 2016; Stegmann et al., 2016). Cette population est Eomes^{hi} T-bet^{lo} et a une capacité réduite de production d'IFN- γ par rapport à la population CXCR6⁻ Eomes^{lo} T-bet^{hi}. Les facteurs de transcription Hobit et PLZF ont été associés à la résidence tissulaire dans le foie (Hess et al., 2020; Lunemann et al., 2017). Une population CD69⁺CXCR6⁺ est également décrite dans les tissus lymphoïdes (moelle osseuse, rate, ganglions) (Lugthart et al., 2016).

Figure 3 : Localisation des sous-populations NK. La sous-population NK CD56^{bright} est majoritaire dans les ganglions lymphatiques. Dans le foie, les deux sous-populations sont présentes dans des proportions égales. Dans les autres organes représentés (rate, poumons, moelle osseuse, sang), la sous-population CD56^{dim} domine. *Figure tirée de (Crinier et al., 2020).*

Les ILC auxiliaires prédominantes dans le sang sont les ILC2 et une population de cellules CD117⁺CRTH2⁻ décrites comme précurseur (*Innate lymphoid cell precursor* : ILCP) pouvant donner naissance à toutes les populations ILC matures (Lim et al., 2017). Les muqueuses gastro-intestinales et

orales, les amygdales, les adénoïdes ainsi que la peau sont des tissus dans lesquels la fréquence d'ILC auxiliaires est grande, en particulier ILC3 et ILC2 (Simoni et al., 2017). Les ILC3 NKp44⁺ sont particulièrement nombreuses dans ces tissus. Les ILC2 sont majoritaires au niveau de la peau avec principalement la population ILC2 CD117^{high}, que l'on retrouve également dans le thymus (Meininger et al., 2020). Inversement, les ILC2 CD117^{low} sont les ILC2 majoritaires dans le sang, les amygdales et l'intestin.

Les ILC auxiliaires ont des profils de résidentes tissulaires. Les ILC2 de la peau, des muqueuses et de la rate expriment le marqueur CD69, comme les ILC3 NKp44⁻ de la peau et des muqueuses (Simoni et al., 2017). Les populations ILC3 NKp44⁺ de la rate et des amygdales expriment peu les gènes *SELL* (codant pour CD62L), *CCR7* et *S1PR1* par rapport aux ILC3 NKp44⁻, suggérant une capacité migratoire moindre et donc une résidence tissulaire (Bar-Ephraim et al., 2017).

Les cellules NK expriment des récepteurs de chimiokines leur permettant de migrer dans certains tissus. Au stade immature dans la moelle osseuse, les cellules NK expriment le récepteur CXCR4 induisant leur rétention dans ce tissu (Beider et al., 2003). En effet, de grandes quantités des ligands de CXCR4, CXCL12 et SDF-1, sont présentes dans la moelle osseuse. L'activation des cellules NK par l'IL-2 induit une diminution de l'expression de CXCR4 conduisant à leur sortie de la moelle osseuse. Cette sortie nécessite également le récepteur à la shingosine-1-phosphate 5 (S1PR5), qui guide les cellules vers les vaisseaux sanguins (Mayol et al., 2011; Walzer et al., 2007). Lors de la différenciation des cellules NK, le niveau d'expression de S1PR5 augmente alors que celui de CXCR4 diminue. Les cellules exprimant une plus grande quantité de S1PR5 sont alors attirées dans le sang où le niveau de S1P est le plus élevé. Comme dans la moelle osseuse, la sortie du ganglion dépend du récepteur S1PR5.

Les cellules NK CD56^{bright} du sang migrent vers le ganglion sous l'influence du récepteur CCR7, exprimé à leur surface, et de ses ligands CCL21 et CCL19 produits par les cellules endothéliales des capillaires lymphatiques (Pesce et al., 2016). Les cellules NK CD56^{bright} CCR7⁺ expriment également la molécule d'adhésion CD62L (L-selectine), facilitant l'extravasation des cellules NK. Il a été montré chez la souris que le recrutement des cellules NK au ganglion est dépendant de CD62L et que ce recrutement est activé par des cellules de mélanomes envahissant le ganglion (Chen et al., 2005). Des cellules NK CD56^{bright} perforine^{low} ont été identifiées dans les vaisseaux lymphatiques afférents, démontrant leur circulation des tissus vers le ganglion (Carrega et al., 2014).

Les ILC auxiliaires, bien que majoritairement résidentes tissulaires, sont présentes dans la circulation. Une étude réalisée *in vivo* chez la souris décrit de telles populations circulantes, essentiellement ILC1, attirées au site ganglionnaire via le récepteur CCR7 (Dutton et al., 2019). Une autre étude *in vivo* chez la souris montre que les ILC3 de la muqueuse intestinale sont capables de

migrer vers les ganglions mésentériques, également de façon CCR7 dépendante (Mackley et al., 2015). Dans le sang les ILC humaines expriment le récepteur CD62L, pouvant participer au recrutement des ILC sanguines vers le ganglion (Bar-Ephraim et al., 2019).

Les pathogènes induisent une réponse inflammatoire et une augmentation de facteurs chimio-attractants. Les cellules NK CD56^{dim} du sang expriment les récepteurs CXCR1, CXCR2, CX3CR1, spécifiques de l'inflammation (Castriconi et al., 2018). Par ailleurs, dans l'environnement tumoral la quantité de chimio-attractants des cellules NK CD56^{dim} (CXCL2, CX₃CL1, CXCL1 et CXCL8) est réduite alors que celle des molécules induisant le recrutement des cellules NK CD56^{bright} est augmentée (CXCL9, CXCL10, CXCL19 et CCL5). Ainsi, les cellules NK CD56^{dim} cytotoxiques sont rarement présentes dans le microenvironnement tumoral (TME) alors que les CD56^{bright} immatures et peu actives sont majoritaires. Certaines molécules secrétées par les cellules tumorales, dont le TGF- β , peuvent modifier l'expression de récepteurs de chimiokines et induire ce défaut de recrutement de cellules NK matures et actives. Les ILC auxiliaires peuvent également être recrutées au site inflammatoire et tumoral (Willinger, 2019). Le recrutement de populations ILC3 se fait de façon CCR7 ou CXCR5 dépendante dans des modèles de cancer de sein (Irshad et al., 2017).

Les ILC sont recrutées au site inflammatoire et sont au cœur de la réponse immune innée. Elles exercent deux types de fonctions : des fonctions cytotoxiques, médiées principalement par les cellules NK, et des fonctions immunorégulatrices médiées par les NK et ILC tissulaires.

III. Fonctions effectrices, les ILC1

Les cellules NK sont considérées comme étant les seules ILC exerçant une activité de cytotoxicité dite « naturelle » c'est-à-dire ne nécessitant aucune stimulation préalable, contrairement aux LT. L'activation de la cytotoxicité naturelle des cellules NK peut être notamment induite lors de l'absence de molécules du CMH-I (Complexe Majeur d'Histocompatibilité de classe I), selon la théorie de la reconnaissance du soi manquant (Ljunggren and Kärre, 1990). Cependant, ce mécanisme n'est pas le seul à rentrer en jeu lors de l'activation des fonctions NK. La reconnaissance de la cellule cible et l'activation des cellules NK reposent sur un équilibre entre des signaux activateurs et inhibiteurs transmis par des récepteurs présents à la surface de la cellules NK (Caligiuri, 2008). La mort de la cellule cible par apoptose peut être médiée par différents mécanismes : la cytotoxicité naturelle, la cytotoxicité à médiation cellulaire dépendante des anticorps (ADCC) et enfin par l'engagement des récepteurs à domaine de mort (Figure 4).

Lors de l'interaction entre NK et cellule cible, l'engagement des récepteurs NK avec leurs ligands, induit la formation de la synapse immunologique (SI), la réorganisation du cytosquelette d'actine et des microtubules, permettant la polarisation de granules lytiques au niveau de la SI (Orange, 2008). Ces granules contiennent de la perforine (glycoprotéines cytolytiques formant des pores dans la membrane plasmique) et des granzymes (serine-protéases pro-apoptotiques), libérées lors de la fusion entre la membrane plasmique de la cellule NK et celle de sa cible. La protéine membranaire associée aux lysosomes CD107a est externalisée à la surface des cellules NK après l'exocytose des granules cytotoxiques. L'expression à la membrane de CD107a mesurable par cytométrie en flux est donc un marqueur de l'activité cytotoxique NK (Alter et al., 2004). La perforine forme des pores dans la cellule cible, permettant aux granzymes d'entrer et d'initier l'apoptose via des mécanismes caspase-dépendant ou indépendant.

A. Cytotoxicité naturelle

La cytotoxicité naturelle des cellules NK est régulée principalement par la reconnaissance de soi manquant selon l'hypothèse proposée par Ljunggren et Kärre (Ljunggren and Kärre, 1990). Les cellules NK reconnaissent les molécules de CMH-I des cellules saines, induisant une tolérance de ces cellules du soi. Validant l'hypothèse, plusieurs récepteurs inhibiteurs reconnaissant les molécules de CMH-I ont été identifiés à la surface des cellules NK (KIRs, CD94/NKG2). Les cellules NK ne sont plus inhibées lorsque l'expression des molécules de CMH-I est diminuée ou perdue sur la cellule cible. Une altération de l'expression du CMH-I est fréquente sur des cellules en transformation tumorale ou des cellules infectées par des virus. Cependant l'absence de CMH-I n'est pas toujours suffisante pour activer les fonctions NK, plusieurs récepteurs activateurs ont été mis en évidence, capables d'induire la fonction cytotoxique des cellules NK. Ces récepteurs activateurs sont décrits dans le paragraphe V.

D'autres populations ILC1 ont été décrites comme cytolytiques. Une étude d'un modèle murin de cancer du sein, a identifié une population résidente CD103⁺, de type ILC1, exprimant de la perforine et des granzymes, et induisant la lyse de cellules tumorales (Dadi et al., 2016). Chez l'Homme, la population intra-épithéliale ILC1 (ieILC1) décrite dans les muqueuses, produit des granules lytiques (Fuchs et al., 2013; Simoni et al., 2017). Une étude récente des ieILC1 du duodénum, prouve que cette population est cytotoxique et qu'elle est augmentée chez des patients atteints de maladie cœliaque (Uhde et al., 2020). Chez des patients atteints de leucémie aigüe myéloïde (LAM), une population ILC1 CD127⁺CD56⁺ cytotoxique a été décrite, avec un mécanisme de lyse dépendant des récepteurs KIR mais aussi d'autres récepteurs (NKp80, NKp30, and TRAIL) (Salomé et al., 2019).

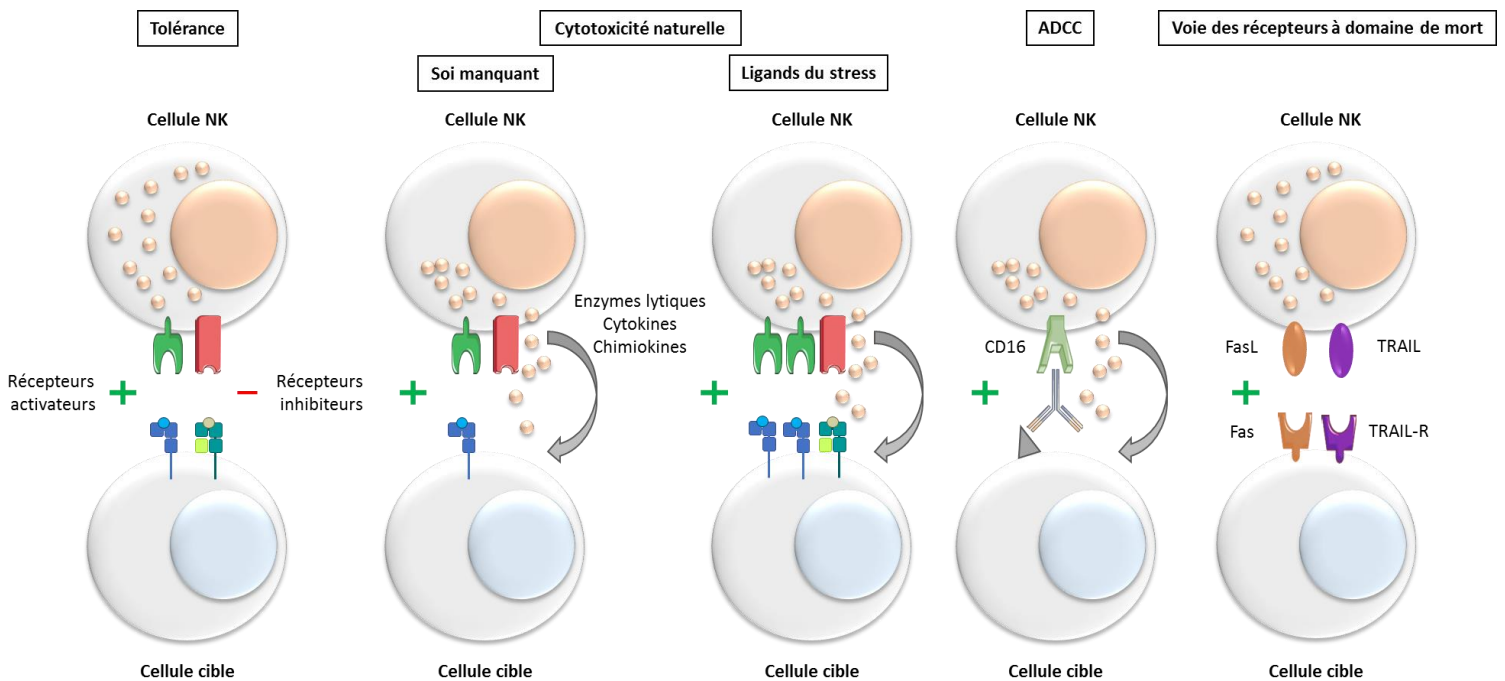


Figure 4 : Les mécanismes effecteurs des cellules NK. L'activation des fonctions NK repose sur une balance entre signaux activateurs et signaux inhibiteurs. Les cellules saines sont tolérées par les cellules NK via la reconnaissance des CMH par les récepteurs inhibiteurs. Les cellules stressées diminuent l'expression des CMH (soi manquant) ou expriment des ligands des récepteurs activateurs, activant la cytotoxicité naturelle des cellules NK. Des anticorps spécifiques d'antigènes tumoraux se lient aux cellules cibles et engagent le CD16, activant l'ADCC médiée par les cellules NK. Certaines cellules NK expriment les ligands FasL et TRAIL qui engagent les récepteurs Fas et TRAIL-R, exprimés par la cellule cible, ce qui entraîne l'activation des voies de l'apoptose. *D'après (Morvan and Lanier, 2016).*

B. Cytotoxicité médiée par les anticorps

En plus de leur fonction de cytotoxicité naturelle, les cellules NK peuvent entraîner la lyse d'une cellule cible par un mécanisme d'ADCC. Ce mécanisme repose sur la reconnaissance du Fc des immunoglobulines sur une cellule cible recouverte d'anticorps par un récepteur à ce Fc, régulant l'exocytose des granules cytotoxiques ou la production de cytokines. Les cellules NK expriment le CD16 (FcγRIIIA), qui s'associe avec les chaînes FcεRI-γ et/ou CD3-ζ, portant des motifs d'activation des récepteurs immuns basé sur la tyrosine (ITAM), phosphorylés après liaison du CD16 au Fc. Les tyrosines kinases ZAP-70 et Syk sont ensuite recrutées et les voies PI3K (*Phosphatidylinositol 3-Kinase*), NF-κb et

ERK sont activées, permettant la dégranulation des cellules NK, la sécrétion de cytokines et, enfin, la mort de la cellules cible (Trinchieri and Valiante, 1993).

c. Les récepteurs à domaine de mort

Les cellules NK peuvent induire l'apoptose des cellules cibles suite à l'engagement des récepteurs à domaine de mort. Les récepteurs à domaine de mort font partie de la famille des récepteurs au TNF, avec notamment les récepteurs TRAIL-R1/R2 (DR4, DR5) et Fas. Les ligands de ces récepteurs, TRAIL et FasL respectivement, sont exprimés à la surface de certaines populations de cellules NK. L'engagement de TRAIL-R1/2 ou Fas active une voie de signalisation intracellulaire dans la cible, commençant par la mise en place du DISC (*Death-Inducing Signaling Complex*), conduisant à l'activation des caspases 8 et 10 conduisant à l'apoptose (von Karstedt et al., 2017; Peter and Krammer, 2003).

Il a été montré *in vitro* que des cellules NK de souris, déficientes en perforine, peuvent lyser des cellules tumorales de lymphome T selon un mécanisme FasL dépendant (Screpanti et al., 2001). Une étude *in vivo* chez la souris montre également un mécanisme indépendant de la perforine et dépendant de FasL, dont l'expression à la surface de la cellule NK est régulée par des récepteurs activateurs (NCR) et inhibiteurs (KIR) (Wallin et al., 2003). FasL est peu exprimé sur les cellules NK activées par IL-2 mais est présent en grande quantité dans des vésicules. La polarisation lors de la dégranulation contrôle le transport de ces vésicules et l'expression de FasL à la surface de la cellule NK (Bossi and Griffiths, 1999). L'expression membranaire de FasL est régulée par des métalloprotéases matricielles (MMP) qui entraînent la libération de FasL sous forme soluble et peu active (Kayagaki et al., 1995).

TRAIL est exprimé par les cellules NK, il peut être utilisé comme marqueur de différenciation et d'activation, et son engagement peut entraîner la mort des cellules cibles (Zamai et al., 1998). Deux études chez la souris ont montré une augmentation de l'expression de TRAIL sur les cellules NK suite à une activation par l'IL-12, et ces NK activées participent à l'élimination des métastases par un mécanisme TRAIL dépendant (Smyth et al., 2001; Takeda et al., 2001). Le transfert adoptif de cellules NK dépourvues de TRAIL dans un modèle de souris n'ayant pas de lymphocytes endogènes, entraîne une diminution de la réponse anti-tumorale médiée par les cellules NK (Pegram et al., 2010).

TRAIL est également exprimé par la population de type ILC1 cytotoxique, décrite chez la souris par Dadi et al. (Dadi et al., 2016). Chez l'Homme, la population type ILC1 CD56⁺ présente chez des patients atteints de LAM, peut induire la mort de cellules cibles via TRAIL (Salomé et al., 2019).

Les ILC auxiliaires jouent un rôle essentiel dans l'homéostasie immunitaire tissulaire, en exerçant un rôle immunorégulateur de l'immunité innée et adaptative, via la sécrétion de cytokines, chimiokines et facteurs de croissance.

IV. Fonctions immunorégulatrices des ILC

Les ILC1 produisent des cytokines de type Th1, principalement IFN- γ , et régulent d'autres cellules de l'immunité, notamment dans les organes lymphoïdes (Figure 5). L'IFN- γ peut stimuler les phagocytes, favorisant une immunité antimicrobienne et le contrôle des infections intracellulaires. La population de cellules NK produisant le plus de cytokines est la population CD56^{bright}. Elle est en contact des LT et des CD, dans les OLS, comme le ganglion (Fehniger et al., 2003). L'IFN- γ produit par les cellules NK leur permet de contrôler l'activation des CPA, dont les CD, et la réponse de type Th1 des LT (Mocikat et al., 2003). Deux types de signaux sont requis pour la production d'IFN- γ par les NK : un signal cytokinique, principalement l'IL-12, mais également d'autres cytokines comme IL-2, IL-15, IL-18, ou un signal induit par l'engagement de récepteurs activateurs comme CD16 ou NKG2D (Parihar et al., 2002). Les cellules NK produisent aussi du TNF- α , du GM-CSF (*granulocyte-macrophage colony-stimulating factor*), de l'IL-10 et IL-13, selon le profil des stimulations cytokiniques reçues (Cooper et al., 2001; Fehniger et al., 1999). Les ILC1 auxiliaires ont aussi été décrites comme étant capables de produire de l'IFN- γ en réponse à l'IL-12, régulant l'immunité tissulaire (Bernink et al., 2013; Fuchs et al., 2013).

Le rôle des ILC2 dans l'inflammation tissulaire a été montré dans différentes études. Dans plusieurs modèles murins, les ILC2 jouent un rôle dans la réponse immunitaire contre les Helminthes (vers parasite), par la production d'IL-13 et IL5, en réponse à l'IL-25 et l'IL-33 (Moro et al., 2010; Neill et al., 2010). Des populations d'ILC2 ont été décrites dans le foie, au niveau de la peau, en fréquence plus importante chez des patients atteints de dermatite atopique. Ces populations produisent de l'IL-13 et de l'AREG, suggérant un rôle des ILC2 dans l'initiation de l'inflammation au niveau de ces organes (Jeffery et al., 2017; Salimi et al., 2013). Les ILC2 jouent un rôle important dans les allergies, notamment via la sécrétion des cytokines IL-5 ou IL-13 (Pasha et al., 2019). Il a été montré chez la souris que la sécrétion de cytokines de type Th2 par les ILC2 permet une activation de la réponse inflammatoire médiée par les éosinophiles. Une étude suggère le même rôle pour les ILC2 humaines, avec une augmentation de cette population chez des patients asthmatiques sévères (Smith et al., 2016). En résumé, ces études sur les ILC2 humaines suggèrent un rôle de ces cellules dans l'inflammation, via la

production de cytokines de type Th2, permettant un recrutement des éosinophiles et une polarisation de type Th2.

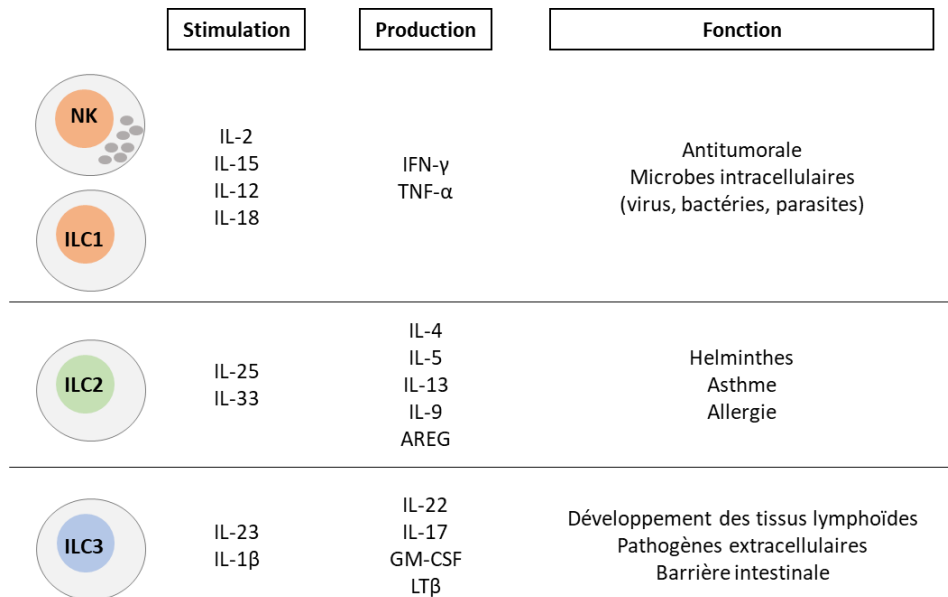


Figure 5 : Fonctions immunorégulatrices des ILC. Les cellules NK et ILC1 produisent de l'IFN- γ et du TNF- α pour lutter contre les pathogènes intracellulaires et les cellules cancéreuses. Les ILC2, stimulées par des Helminthes ou des allergènes, produisent des cytokines de type Th2 (IL-4, IL-5, IL-13, ...). Les ILC3 luttent contre les pathogènes extracellulaires en produisant de l'IL-22 ou IL-17. Elles sont également responsables du développement des tissus lymphoïdes. *D'après (Artis and Spits, 2015; Vivier et al., 2018).*

Les ILC3 sont des cellules productrices d'IL-22, cytokine importante pour son rôle de réparation tissulaire et de barrière contre les pathogènes au niveau des muqueuses (Dudakov et al., 2015). Elles peuvent exprimer les molécules de CMH-II et interagir avec les LT CD4 et ainsi limiter la réponse immune adaptative contre les bactéries commensales de l'intestin (Hepworth et al., 2013). L'IL-18 peut induire la production d'IL-22 par les ILC3. L'IL-18 peut être sécrétée par les CD, présentes à proximité des ILC3 dans les amygdales (Victor et al., 2017). Les ILC3 spléniques sont situées au niveau de la zone marginale, et interagissent avec les lymphocytes B via l'expression de BAFF et CD40L, induisant leur différenciation. Ces populations d'ILC3 peuvent également interagir avec les neutrophiles de la rate via la sécrétion de GM-CSF (Magri et al., 2014). Cette interaction avec les neutrophiles est également décrite dans une étude sur les ILC3 NCR⁺ de la caduque basale, via la sécrétion de GM-CSF mais également d'IL-8 (Croxatto et al., 2016). Deux études suggèrent un rôle des

ILC3 NCR⁺ dans le psoriasis, via la production d'IL-17A et d'IL-22 (Teunissen et al., 2014; Villanova et al., 2014).

L'activation des fonctions des cellules NK repose sur une balance bien caractérisée entre signaux inhibiteurs et activateurs envoyés par des récepteurs exprimés à la surface de ces cellules. Les ILC auxiliaires expriment certains récepteurs NK, suggérant une activation similaire. Les principaux récepteurs NK/ILC sont décrits dans le paragraphe suivant.

V. Les récepteurs NK/ILC et leurs ligands

A. Les récepteurs de cytotoxicité naturelle (NCR)

Les NCRs sont des récepteurs activateurs appartenant à la superfamille des immunoglobulines. Trois récepteurs NCR ont été identifiés: NKp46, NKp30 et NKp44 (Moretta et al., 2000). Les récepteurs NKp46 et NKp30 sont constitutivement exprimés sur les cellules NK alors que le récepteur NKp44 est exprimé après activation par l'IL-2. Ces récepteurs ont été identifiés sur les ILC auxiliaires. Les ILC1 auxiliaires expriment le récepteur NKp46, les ILC2 le NKp30 et les ILC3 les trois NCRs (Figure 6) (Spits et al., 2013).

Les NCRs peuvent reconnaître des ligands exprimés par les cellules tumorales ou infectées par des virus. Certains de ces ligands ont été identifiés et sont listés dans le tableau 1. Ils peuvent notamment reconnaître l'héparine exprimée par les cellules tumorales ou l'hémagglutinine exprimée par les cellules infectées.

Les NCRs possèdent un (NKp30, NKp44) ou deux (NKp46) domaines de type Ig et une région transmembranaire avec un résidu Arginine permettant le recrutement de molécules portant des motifs ITAM, comme CD3 ζ et Fc ϵ RI γ (NKp46, NKp30) (Pessino et al., 1998) ou KARAP/DAP12 (NKp44), qui permettent la cascade de signalisation. Lorsque les NCRs interagissent avec leurs ligands, le motif ITAM est phosphorylé permettant le recrutement et l'activation des protéines kinases Syk et ZAP-70 (Augugliaro et al., 2003). De plus, le récepteur NKp44 possède un motif d'inhibition des récepteurs immuns basé sur la tyrosine (ITIM) fonctionnel qui entraîne l'inhibition des cellules NK, après interaction du récepteur avec le ligand PCNA (*proliferating cell nuclear antigen*), surexprimé à la surface des cellules tumorales (Rosental et al., 2011).

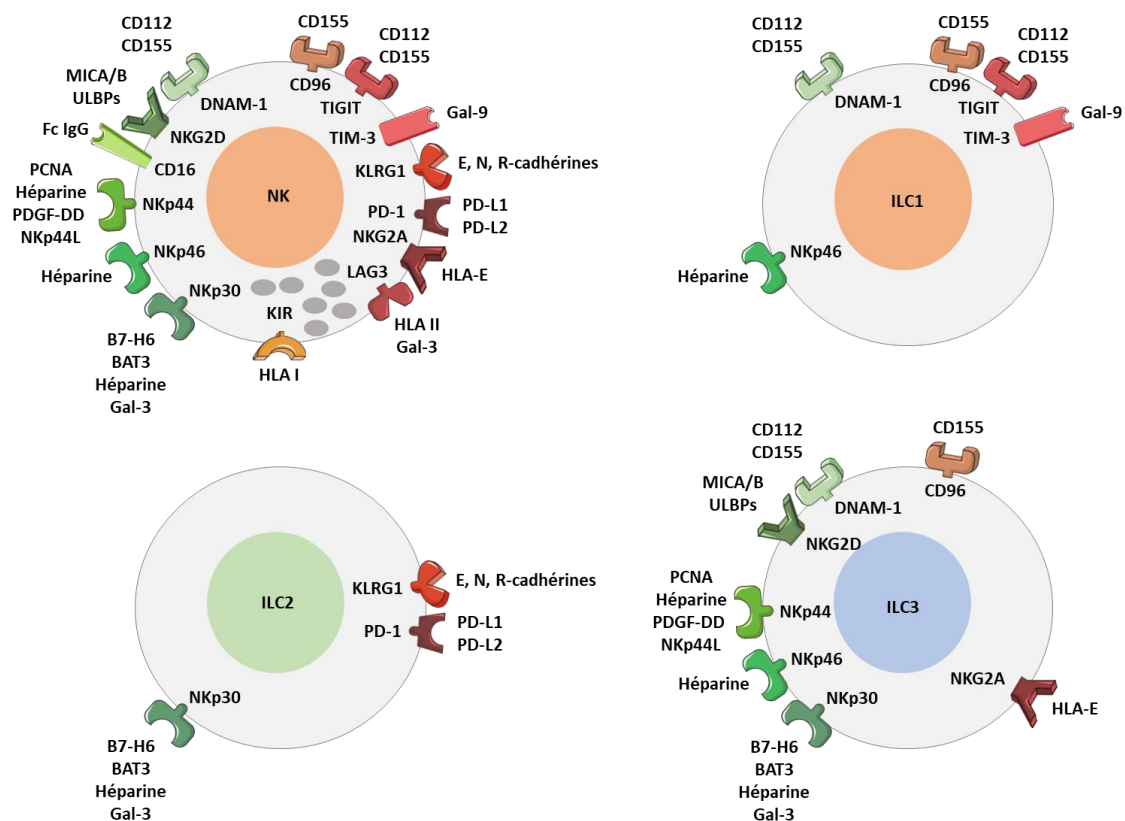


Figure 6 : Les récepteurs exprimés par les ILC et leurs ligands exprimés par les cellules tumorales. Les différents récepteurs NK activateurs (en vert) et inhibiteurs (en rouge) exprimés par les populations d'ILC humaines. Les ligands exprimés par les cellules tumorales sont indiqués. *D'après (Chiossone et al., 2018).*

NKp30 reconnaît des ligands tumoraux et des ligands exprimés par les CD (Ferlazzo et al., 2002; Pende et al., 1999). NKp30 comprend trois isoformes (NKp30a, NKp30b, NKp30c) qui se caractérisent par trois domaines intracellulaires différents identifiés à partir de leur transcrits, induisant différents signaux. Notamment il a été montré que l'isoforme courte NKp30c transduit un signal immunosuppresseur par la production d'IL-10, qui diminue les fonctions NK. La proportion des isoformes régule la fonction du récepteur. Ainsi, dans une étude réalisée sur 80 patients atteints de tumeurs stromales gastro-intestinales (GIST), l'expression prédominante de l'isoforme NKp30c a été

corrélée à une survie réduite, une diminution de la sécrétion de TNF- α , une diminution de dégranulation, et une sécrétion réduite d'IFN- γ en réponse à l'IL-12 (Delahaye et al., 2011).

Récepteur	Ligand (source)	Signal	Référence
NKp46	Héparine (Cellule tumorale)	Activateur	(Hecht et al., 2009)
	Hémagglutinine (HA) et Neuraminidases (Virus Influenza, Vaccine, Newcastle, Sendai, Parainfluenza humain)	Activateur	(Arnon et al., 2004; Jarahian et al., 2011; Mandelboim et al., 2001; McQuaid et al., 2018)
	PfEMP-1 (<i>P. falciparum</i>)	Activateur	(Mavoungou et al., 2007)
	Vimentine (Monocytes infectés par <i>Mycobacterium tuberculosis</i>)	Activateur	(Garg et al., 2006)
	Ligand non identifié (<i>Fusobacterium nucleatum</i>)	Activateur	(Chaushu et al., 2012)
	Adhésines (<i>Candida glabrata</i>)	Activateur	(Vitenshtein et al., 2016)
	Facteur P (voie alterne du complément)	Activateur	(Nami-Mancinelli et al., 2017)
NKp30	BAT3 (Cellule tumorale, DC)	Activateur	(Pogge von Strandmann et al., 2007; Simhadri et al., 2008)
	B7-H6 (Cellule tumorale)	Activateur	(Brandt et al., 2009)
	HA (Virus de la Vaccine, poxvirus)	Inhibiteur	(Jarahian et al., 2011)
	PfEMP-1 (<i>P. falciparum</i>)	Activateur	(Mavoungou et al., 2007)
	Héparine (Cellule tumorale)	Activateur	(Bloushtain et al., 2004; Hecht et al., 2009)
	pp65 (Cytomegalovirus)	Inhibiteur	(Arnon et al., 2005)
	Galectine-3 (Cellule tumorale)	Inhibiteur	(Wang et al., 2014)
	β -1,3-glucan (Champignons)	Activateur	(Li et al., 2018b)
NKp44	PCNA (Cellule tumorale)	Inhibiteur	(Rosental et al., 2011)
	Héparine (Cellule tumorale)	Activateur	(Brusilovsky et al., 2015; Hecht et al., 2009)
	Syndecan-4 (SDC4) (Cellule NK, en cis)	Inhibiteur	(Brusilovsky et al., 2015)
	HA (Virus Influenza, Parainfluenza humain, Newcastle, Sendai)	Activateur	(Arnon et al., 2005; Ho et al., 2008; Jarahian et al., 2009; McQuaid et al., 2018)
	PDGF-DD (Cellule tumorale)	Activateur	(Barrow et al., 2018)
	Nidogen-1 (Membrane Basale)	Inhibiteur	(Gaggero et al., 2018)
	NKp44L (Cellule tumorale, LT infectés par le VIH, chondrocytes)	Activateur	(Baychelier et al., 2013; Białoszewska et al., 2013; Vieillard et al., 2005)
	Protéines d'enveloppe (Virus de la dengue et West Nil)	Activateur	(Hershkovitz et al., 2009)
	Ligands non identifiées (<i>Mycobacterium bovis</i> , <i>Nocardia farcinina</i> , <i>Pseudomonas aeruginosa</i> , <i>Mycobacterium tuberculosis</i>)	?	(Esin et al., 2008, 2013)

Tableau 1 : Ligands des récepteurs NCR. Les NCR reconnaissent différents ligands exprimés par des pathogènes ou par des cellules cancéreuses.

B. Les KIRs

Les récepteurs KIR (*Killer Immunoglobulin-like Receptors*) sont des récepteurs transmembranaires de type I possédant des domaines de type immunoglobuline extracellulaires. Ces récepteurs sont spécifiques des cellules NK (Sagebiel et al., 2019; Spits et al., 2016). La nomenclature des KIR repose sur le nombre de domaines Ig, 2D pour deux domaines et 3D pour trois domaines, et sur la longueur du domaine intracytoplasmique, L pour long et S pour court (Marsh et al., 2003). La famille des KIRs contient des récepteurs activateurs et inhibiteurs. Les KIRs inhibiteurs possèdent une longue queue intracytoplasmique et un ou plusieurs motifs inhibiteurs ITIM. Ces motifs permettent le recrutement de tyrosine kinases, essentielles pour les fonctions inhibitrices de ce récepteur. Les KIRs possédant une courte queue intracytoplasmique s'associent à la protéine adaptatrice DAP12, qui recrute les tyrosines kinases Syk/ZAP-70 via un motif ITAM. Il existe une exception, le récepteur KIR2DL4, qui est un récepteur à longue queue intracytoplasmique, activateur. Il possède un motif ITIM et est relié à la protéine adaptatrice FcεRI-γ, possédant un motif ITAM (Kikuchi-Maki et al., 2005; Rajagopalan et al., 2001).

Les KIRs reconnaissent les molécules HLA de classe I (HLA-A, -B, -C). Les KIRs inhibiteurs ont une plus forte avidité pour leurs ligands. KIR3DL2 reconnaît les molécules HLA-A3 et HLA-A11, KIR3DL1 reconnaît certains allotypes HLA-B, et les KIR2DL1-3 reconnaissent les allotypes HLA-C. Les KIR2DS1-4, activateurs, reconnaissent la molécule HLA-C, KIR2DS4 reconnaît également la molécule HLA-A11. Enfin, le KIR2DL4 s'associe avec la molécule HLA de classe I non classique HLA-G (Campbell and Purdy, 2011).

C. Les récepteurs de type lectine NKG2

Les récepteurs NKG2 sont des protéines transmembranaires de type II appartenant à la famille des lectines de type C. Ils sont exprimés sous forme d'hétérodimère CD94/NKG2, à l'exception du récepteur NKG2D.

1. NKG2D

NKG2D est un récepteur activateur, exprimé par toutes les cellules NK et par les ILC3 de la peau (Dyring-Andersen et al., 2014). NKG2D est un homodimère, il s'associe à la protéine adaptatrice DAP10, recrutant le complexe GRB2/Vav1 et activant la tyrosine-kinase PI3K, à l'origine de l'activation des cellules NK (Bauer et al., 2018).

Les ligands du récepteur NKG2D sont des molécules induites par le stress cellulaire. Certains de ces ligands sont liés au CMH de classe I, ce sont les protéines MICA/B (*MHC class I polypeptide-related sequence A/B*), hautement polymorphes. NKG2D se lie également aux protéines ULBPs (*UL16 binding proteins*). Ces ligands ne sont pas exprimés par les cellules saines, leur expression est induite lors d'une transformation cellulaire, une augmentation de la prolifération ou une infection par un pathogène. Le récepteur NKG2D joue donc un rôle dans l'activation des cellules NK contre les cellules tumorales. Cependant, l'engagement de NKG2D seul, ne permet pas une activation des fonctions NK, sauf après activation par l'IL-2 ou l'IL-15 (Bryceson et al., 2006). D'autres récepteurs, NKp46 ou 2B4, sont nécessaires, envoyant des signaux de « co-stimulations ».

2. CD94/NKG2

Les autres récepteurs du groupe NKG2 s'associent de manière covalente à la molécule CD94 invariante pour former un hétérodimère. Ces hétérodimères sont exprimés par les cellules NK, les LT $\gamma\delta$ et certains LT CD8. NKG2A et NKG2B possèdent un motif ITIM, recrutant les phosphatases SHP-1 and SHP-2, induisant une inhibition des fonctions cytotoxiques (Dréan et al., 1998). NKG2C et NKG2E se lient à DAP12, qui possède un motif ITAM, induisant l'activation des cellules NK (Lanier et al., 1998). Le récepteur NKG2C est impliqué dans la réponse des cellules NK contre le cytomégalovirus humain et est utilisé comme marqueur de définition des cellules NK de type mémoire (Della Chiesa et al., 2015; Lopez-Vergès et al., 2011).

Ces récepteurs se lient aux molécules de CMH-I non classiques HLA-E, qui sont exprimées à la surface des cellules lorsqu'elles ont chargé des peptides dérivés des séquences signal d'autres allotypes HLA (Braud et al., 1998; Lee et al., 1998).

D. Les récepteurs accessoires : DNAM-1, CD96, TIGIT

Les récepteurs « accessoires » sont des récepteurs qui seuls ne peuvent transduire un signal mais qui co-engagés avec des récepteurs activateurs (NCR, NKG2D, CD16) modulent la fonction des NK. Ainsi, DNAM-1, TIGIT et CD96 sont des corécepteurs impliqués dans les fonctions NK. Ils sont également exprimés par les ILC1 et ILC3, et les LT CD8.

DNAM-1 participe à l'activation de la cytotoxicité médiée par les cellules NK (Shibuya et al., 1996). Il reconnaît les molécules CD155 (PVR, *polyo virus receptor*) et CD112 (*Nectin2*), surexprimées

dans plusieurs types de cancers (Bottino et al., 2003). Dans le mélanome, DNAM-1 a été décrit comme essentiel dans la réponse anti-tumorale médiée par les cellules NK (Lakshmikanth et al., 2009).

TIGIT et CD96 reconnaissent les mêmes ligands que DNAM-1. TIGIT reconnaît CD112 et CD155, ce dernier avec une affinité plus forte que DNAM-1 (Yu et al., 2009). CD96 reconnaît uniquement CD155, également avec une affinité supérieure à DNAM-1. Contrairement à DNAM-1, TIGIT et CD96 assurent une fonction inhibitrice de l'activité NK, via des motifs ITIMs (Fuchs et al., 2004; Stanietsky et al., 2009). L'interaction CD155-CD96 a dans un premier temps été caractérisée comme activatrice des fonctions NK (Fuchs et al., 2004). Une étude récente, dans un modèle murin, montre que CD96 a un rôle inhibiteur de la production d'IFN- γ par les cellules NK (Chan et al., 2014).

E. 2B4 (CD244)

Le récepteur 2B4 (CD244) est un membre de la famille des SLAM (*Signaling lymphocytic activation molecule*). Il est exprimé par les cellules NK, les LT, les LT $\gamma\delta$, les monocytes, les basophiles et les éosinophiles (Nakajima and Colonna, 2000). 2B4 possède un domaine intra-cytoplasmique avec un motif de type tyrosine proche d'un motif ITIM. 2B4 est un ligand de grande affinité de CD48 et leur interaction active les fonctions des cellules NK.

F. CD161

Le récepteur CD161 (NKR-P1A) appartient à la famille des lectines de type C. Il est exprimé par les cellules NK, les ILC et les LT (Lanier et al., 1994; Mjösberg et al., 2011; Vivier et al., 2018). CD161 est exprimé par les cellules NK à un stade précoce de leur différenciation, avant l'acquisition de CD56 et CD16, et est moins exprimé par les cellules NK de donneurs positifs au cytomégalovirus (Bennett et al., 1996; Montaldo et al., 2012). L'expression de CD161 est associée à des cellules NK pro-inflammatoires, produisant de l'IFN- γ en réponse aux cytokines IL-12+IL-18 (Kurioka et al., 2018). Il se lie à LTT1 exprimé par les DC et lymphocytes B activés, inhibant la cytotoxicité et la production de cytokines par les cellules NK (Rosen et al., 2005, 2008).

G. KLRG1

Le récepteur KLRG1 appartient également à la famille des lectines de type C et possède un motif ITIM. Il est exprimé par les cellules NK activées, les ILC2, les LT, les mastocytes, les basophiles et

les éosinophiles. KLRG1 est également exprimé par une sous-population ILCP dans le sang qui se différencie en ILC2 et ILC3 (Nagasawa et al., 2019).

KLRG1 se lie aux cadhérines (E, N et R-cadhérines) exprimées par la cellule cible, ce qui bloque la lyse par les cellules NK exprimant KLRG1 (Ito et al., 2006). L'activation des ILC2 est également inhibée par la liaison de KLRG1 avec la E-cadhérine (Salimi et al., 2013).

Les *checkpoint* immuns PD-1, CTLA-4 et TIM 3 sont également exprimés par certaines cellules NK. Leur rôle régulateur des fonctions NK sera traité dans la partie immunothérapie.

Les fonctions décrites précédemment ne sont pas toujours spécifiques d'une sous-population ILC. L'environnement cytokinique peut entraîner un changement fonctionnel et phénotypique, une conversion des différentes populations d'ILC : c'est la plasticité.

VI. Plasticité

Plusieurs études ont montré que les sous-populations ILC définies précédemment sont susceptibles de se convertir en une autre sous-population ILC, sous l'influence de certaines cytokines (Figure 7).

Chez la souris, les cellules NK se différencient en ILC1 sous l'influence du TGF- β (Gao et al., 2017). Les mécanismes à l'origine de cette conversion ne sont pas encore connus, cependant une étude montre que le TGF- β induit une diminution de l'expression de T-bet par les cellules NK (Trotta et al., 2008). Cette plasticité n'a pas encore été mise en évidence chez l'Homme.

Les ILC3 peuvent se différencier en ILC1, productrices d'IFN- γ , cytotoxiques, exprimant des FT et des récepteurs NK, via différentes combinaisons cytokiniques. Une étude a montré que la culture d'ILC3 (appelées NK-22), isolées à partir d'amygdales, avec de l'IL-2 et de l'IL-7, augmente la production d'IFN- γ par ces cellules, sans modifier l'expression du FT ROR γ t. De plus, l'IL-23, avec l'IL-1 β et l'IL-7, augmente également la production d'IFN- γ , s'accompagnant d'une up-régulation des marqueurs NK CD94 et CD56 (Cella et al., 2010). L'IL-12 et/ou l'IL-15 induisent la différenciation des ILC3 NKp44⁺ des amygdales en cellules NK Eomes⁺CD94⁺, possédant des fonctions cytotoxiques (Raykova et al., 2017). Les ILC3 ROR γ t⁺NKp44^{+/+} des amygdales et de l'intestin fœtal, après une culture avec l'IL-2 et l'IL-12, acquièrent un phénotype ILC1, avec une perte de l'expression de ROR γ t, CD117 et NKp44 et un gain

de T-bet (Bernink et al., 2013). La même équipe démontre que la reversion des ILC1 (ex-ILC3) en ILC3 est possible. Les ILC1 des amygdales peuvent se re-différencier en ILC3, cultivées avec de l'IL-1 β , IL-2, et IL-23, elles perdent leur fonction de production d'IFN- γ et produisent de l'IL-22 (Bernink et al., 2015). Le facteur de transcription Aiolos suit l'expression de T-bet, et a été montré comme étant impliqué dans la conversion des ILC3 en ILC1 après culture avec IL-1 β et IL-12 (Cella et al., 2019; Mazzurana et al., 2019).

Les ILC2 ont aussi la capacité de se différencier en ILC1. Différentes équipes ont montré que des ILC2 sanguines, cultivées avec de l'IL-12, avec ou sans IL-1 β , produisent de l'IFN- γ . Leur phénotype bascule vers celui des ILC1, avec une diminution de GATA3, CD117 et CRTH2, et une augmentation de T-bet (Bal et al., 2016; Lim et al., 2016; Ohne et al., 2016; Silver et al., 2016). Une population ILC2 a été caractérisée au niveau de la muqueuse intestinale de patients atteints de la maladie de Crohn, où elles produisent de l'IL-13 mais aussi de l'IFN- γ , cytokine de type ILC1, jouant potentiellement un rôle dans l'inflammation de la muqueuse intestinale (Lim et al., 2016). L'IL-4 peut restaurer le phénotype ILC2, après conversion préalable en ILC1 en présence d'IL-1 β et d'IL-12, avec une augmentation de l'expression de CRTH2 et de CD117 via l'induction de GATA3 (Bal et al., 2016).

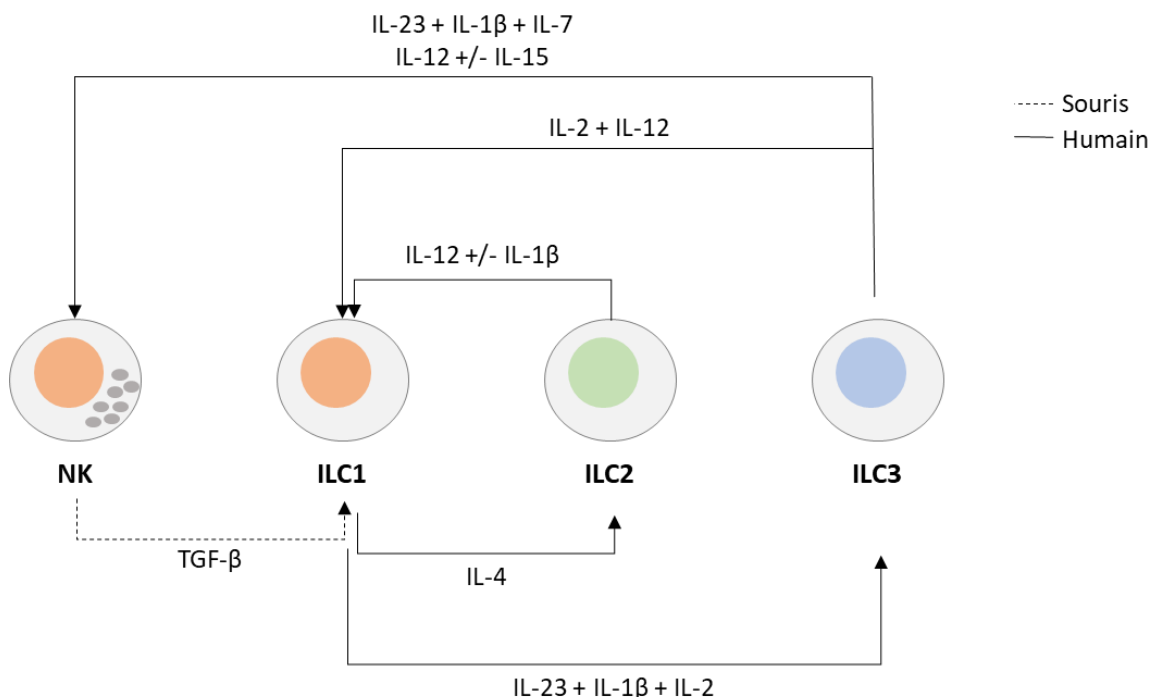


Figure 7 : Plasticité des ILC. Chez l'Homme, Les ILC3 et les ILC2 peuvent se différencier en ILC1 en réponse à différentes cytokines, IL-12, IL-1 β , IL-23, IL-7, IL-2. L'IL-23 avec l'IL-1 β et l'IL-2 entraînent la différenciation des ILC1 en ILC3. L'IL-4 permet aux ILC2 converties en ILC1 de se redifférencier en ILC2. Les ILC3 se différencient en cellules NK en réponse à l'IL-12 avec ou sans IL-15. Chez la souris, les cellules NK se différencient en ILC1 sous l'influence du TGF- β . *Figure adaptée de (Bal et al., 2020).*

VII. Développement et maturation des ILC

Le développement des ILC a bien été étudié chez la souris. Chez l'Homme, malgré l'existence de similitudes entre le développement des ILC murines et humaines, les voies de différenciations et les précurseurs des ILC humaines ne sont pas encore entièrement définis. De plus les données récentes sur les ILC auxiliaires impliquent une révision des modèles proposés sur la différenciation NK. Cette partie fait le point sur les résultats et hypothèses principales de la différenciation des populations ILC humaines.

A. Les stades de différenciation

Les ILC sont issues d'un progéniteur lymphoïde commun (CLP) de la moelle osseuse, défini par le phénotype $\text{Lin}^- \text{CD34}^+ \text{CD45RA}^+ \text{CD10}^+ \text{CD62L}^+ \text{CD117}^-$, également capable de générer les lymphocytes T et B, et les CD (Galy et al., 1995; Kohn et al., 2012).

Avant la découverte des ILC auxiliaires, le développement des cellules NK a été décrit chez l'Homme, et un modèle de différenciation a été proposé par Freud et Caligiuri, caractérisant plusieurs intermédiaires, les NKDI (*NK cell developmental intermediates*), et 5 stades de différenciation NK (Figure 8) (Freud and Caligiuri, 2006). Les NKDI ont été identifiés dans les OLS, les ganglions lymphatiques, la rate et les amygdales (Eissens et al., 2012; Freud et al., 2005, 2006). Ces précurseurs sont également présents dans des organes non lymphoïdes comme le foie, l'utérus et le thymus (Hidalgo et al., 2012; Male et al., 2010; Moroso et al., 2011).

Les intermédiaires du premier stade de différenciation sont définis comme $\text{Lin}^- \text{CD34}^+ \text{CD45RA}^+ \text{CD117}^- \text{CD94}^-$. Ces cellules n'expriment pas CD122, la chaîne beta commune aux récepteurs à l'IL-2 et l'IL-15. Les cellules du deuxième stade sont définies comme $\text{Lin}^- \text{CD34}^+ \text{CD45RA}^+ \text{CD117}^+ \text{CD94}^-$. A ce stade les cellules NKDI expriment CD122 et peuvent répondre à l'IL-15. Les intermédiaires des stades 1 et 2 sont encore capables de donner naissance aux LT ou CD, en plus des ILC.

La suite du développement des ILC n'est pas encore bien caractérisée. Une étude a identifié un progéniteur $\text{Lin}^- \text{CD34}^+ \text{CD45RA}^+ \text{CD117}^+ \text{IL-1R1}^+ \text{ROR}\gamma\text{t}^+$ dans les OLS, pouvant donner naissance à toutes les populations d'ILC (Scoville et al., 2016). Ce nouvel intermédiaire, spécifique des ILC, est appelé CILP (*common innate lymphoid progenitor*).

Au stade suivant, le progéniteur perd l'expression du marqueur CD34. Ce progéniteur, appelé ILCP (*ILC precursor*), a été identifié dans le sang et les tissus, est défini comme

Lin⁻CD34⁻CD7⁺CD127⁺CD117⁺ et peut générer toutes les populations ILC, sous différentes conditions environnementales (Lim et al., 2017).

Par la suite, les précurseurs deviennent spécifiques des différentes populations ILC. Renoux et al. ont identifié un précurseur NK (NKP) présent dans la moelle osseuse fœtale, le foie fœtal, le sang de cordon et dans les amygdales (Renoux et al., 2015). Ce précurseur, défini comme Lin⁻CD34⁺CD38⁺CD123⁻CD45RA⁺CD7⁺CD10⁺CD127⁻, est spécifique des cellules NK, il ne se différencie pas en ILC auxiliaires. Cependant une autre étude caractérise un précurseur ILCP CD34⁻CD117⁺ exprimant le marqueur CD56 et exclusif des ILC1 et ILC3, dans les amygdales (Chen et al., 2018). Dans cette étude, ils montrent également que le « NKP » CD34⁺CD10⁺ précédemment décrit, peut donner naissance aux différentes populations ILC et aux LT, avec certaines conditions de culture.

Les stades postérieurs de différenciation des ILC ne sont pas connus chez l'Homme, à l'exception de celui des cellules NK. En reprenant le modèle de Freud et Caligiuri, le stade 3 de différenciation NK, faisant suite au stade ILCP, est défini par l'acquisition de marqueurs NK tels que CD56, NKp44 ou 2B4. Ces précurseurs NK expriment les FT Eomes et T-bet, indispensables à la suite de leur développement. A ce stade, les cellules NK expriment le marqueur CD127, dont l'expression est perdue par les cellules NK matures.

Le stade 4 est le stade de différenciation des NK immatures en NK CD56^{bright}, dans les OLS. Ce stade est marqué par l'expression de CD94/NKG2A, NKG2D et NKp46 et la diminution d'expression de CD117 et CD127. Les NK CD56^{bright} acquièrent leur capacité de production d'IFN- γ et de cytotoxicité (Freud et al., 2006).

Enfin, les cellules NK acquièrent l'expression des récepteurs CD16 et KIR, exprimés par les NK sanguines CD56^{dim}. Ces récepteurs augmentent leurs capacités cytotoxiques. L'expression de CD117 est perdue et celle de CD94 est diminuée. Le marqueur CD57 est associé avec un stade de maturation terminal, avec une augmentation de l'expression de CD16 et KIR (Lopez-Vergès et al., 2010). Les NK CD56^{dim}CD57⁺ ont des capacités cytotoxiques supérieures aux CD57⁻ lorsqu'elles sont stimulées via le CD16.

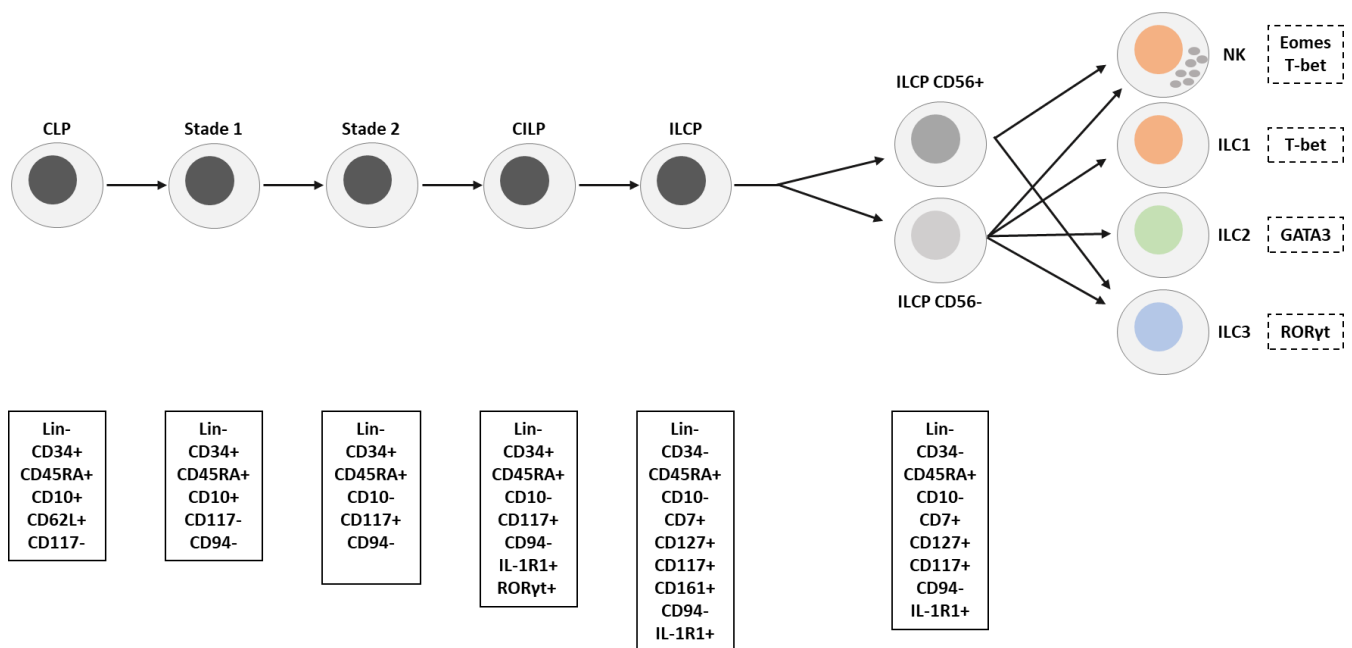


Figure 8 : Modèle de différenciation des ILC humaines. Cette figure présente les différentes étapes du développement des ILC humaines. D'après (Freud and Caligiuri, 2006; Guia and Narni-Mancinelli, 2020; Lim and Di Santo, 2019).

B. Le ganglion lymphatique, lieu de maturation des cellules NK

Le ganglion lymphatique est un OLS, lieu de prolifération et de différenciation des cellules immunitaires et de l'activation de la réponse adaptative via le transport d'antigènes des tissus vers le ganglion. Le ganglion est organisé en plusieurs zones : le sinus capsulaire, où arrive les vaisseaux lymphatiques et sanguins ; le cortex du ganglion, zone des lymphocytes B ; le paracortex, zone des LT et des CD; et le hile, lieu de sortie des vaisseaux lymphatiques. Les lymphocytes T et B, arrivent de la périphérie et sont activés dans le ganglion via les CPA. Les LT y acquièrent leurs fonctions effectrices ou régulatrices et les lymphocytes B se différencient en plasmocytes, cellules productrices d'anticorps, en réponse à la présentation antigénique. Ces lymphocytes circulent ensuite vers la périphérie, vers la source antigénique.

La présence des ILC, et plus particulièrement des LTI, est nécessaire pour le développement des ganglions. De plus, cet organe est connu comme étant un organe clé de la maturation des cellules NK, et donc des ILC. Les précurseurs CD34⁺ sont présents dans les ganglions lymphatiques (Freud et al., 2005). La présence dans les ganglions, des populations d'ILC matures est peu décrite, à l'exception des cellules NK. Ces dernières représentent 1-5% des lymphocytes ganglionnaires et sont majoritairement

CD56^{bright} (Fehniger et al., 1999; Messaoudene et al., 2014). Compte tenu du nombre de ganglions, les NK ganglionnaires représentent la plus grand nombre de cellules NK de l'organisme.

Le ganglion a un rôle majeur dans la réponse immunitaire contre les cellules tumorales. Il intervient dans l'initiation de la réponse immunitaire antitumorale et il représente le premier site d'invasion métastatique lors de la dissémination tumorale. Son envahissement est un élément pronostic important pour différentes tumeurs solides comme l'indique la classification TNM (*Tumor Node Metastasis*). Dans ce contexte, les fluides lymphatiques provenant de la tumeur primaire sont drainés par un ganglion, appelé ganglion sentinelle. Le drainage de différents facteurs tumoraux entraîne une modification du microenvironnement au niveau du ganglion, faisant de cet organe une « niche pré-métastatique » (Sleeman, 2015). Les cellules tumorales peuvent exprimer le récepteur CCR7 et migrer vers le ganglion (Vaahomeri and Alitalo, 2020). Elles peuvent également promouvoir la lymphoangiogenèse via la production de VEGF-C, aussi produit par les macrophages associés à la tumeur (TAM pour *Tumor-associated macrophages*) (Farnsworth et al., 2018). De plus, le VEGF-C induit une augmentation de la production de CCL21 et donc potentiellement une augmentation du recrutement des cellules immunitaires. Cette induction de la lymphoangiogenèse joue donc un double rôle : d'un côté l'augmentation de l'invasion métastatique via la lymphe et de l'autre l'augmentation de l'activation immunitaire. Deux études ont montré que la combinaison de VEGF-C avec des anti-PD-1 augmente la réponse aux inhibiteurs de *checkpoint* immuns (ICB pour *Immune Checkpoint Blocker*) via une augmentation du priming des LT (Fankhauser et al., 2017; Song et al., 2020).

Plusieurs équipes dont la nôtre, ont montré que les cellules NK infiltrent les ganglions drainant la tumeur, leur permettant d'exercer leur rôle d'immunosurveillance lors de la dissémination métastatique. Les données obtenues varient selon le modèle tumoral considéré. Les ganglions drainants les tumeurs de mélanome ou de cancer du sein sont infiltrés par des populations NK CD56^{bright}CD16⁺, présentant un phénotype mature et activé (Frazao et al., 2019; Messaoudene et al., 2014). Ces cellules NK sont capables de lyser des cellules de mélanome ou de cancer du sein, après activation par des cytokines (IL-2/15). D'autre part, Vuletic et al. décrivent une population CD56⁺ présentant une diminution de production d'IFN- γ (Vuletić et al., 2015). Une autre étude décrit une population NK CD56^{dim}CD57⁺ infiltrant les ganglions sentinelles de patients mélanome et ayant des capacités cytotoxiques envers les cellules tumorales (Ali et al., 2014). Dans le cancer colorectal, la présence, au niveau des ganglions, d'une population de cellules NK CD57⁺ a été corrélée à une diminution du nombre des métastases ganglionnaires (Vycital et al., 2018).

Partie 2 : Les ILC dans l'immunosurveillance anti-tumorale

I. Les cellules NK dans les tumeurs

A. Immunosurveillance tumorale

Le système immunitaire joue un rôle important dans le contrôle du développement des cancers. En effet, des patients immunodéficients ou prenant des immunosuppresseurs développent plus de cancers. De plus, de nombreuses études montrent que l'infiltrat lymphocytaire des tumeurs est corrélé à la survie. Ces données sont à la base du concept d'immunosurveillance décrit très tôt par Ehrlich (1909) puis Burnet (1957) et Thomas (1982). En 2004, R Schreiber a émis une théorie rendant compte de l'évolution de la réponse immune au cours du développement d'une tumeur (Figure 9). Le concept d'immunosurveillance tumorale repose sur la règle des trois E qui décrit trois étapes : élimination, équilibre, échappement. La première étape est l'élimination par le système immunitaire des cellules cancéreuses en développement. Par la suite, certaines cellules cancéreuses ne sont pas éliminées par le système immunitaire, les deux types cellulaires se retrouvent en équilibre. Le système immunitaire exerce une pression suffisante sur la tumeur. Enfin, certaines cellules cancéreuses deviennent résistantes et échappent au système immunitaire. Leur prolifération n'est plus contrôlée et la tumeur se développe.

Figure 9 : Les trois étapes du concept de l'immunosurveillance. Figure tirée de (Dunn et al., 2004).

Le rôle anti-tumoral des cellules NK a été démontré *in vivo* dans des modèles murins et *in vitro* avec des lignées cellulaires tumorales ou des cellules tumorales primaires (Figure 10). En 1975, une première preuve de l'activité anti-tumorale des cellules NK est apportée chez la souris dans un modèle

de leucémie (Kiessling et al., 1975b). Les cellules NK ont également un rôle dans le contrôle des métastases avec une croissance tumorale plus agressive et le développement de métastases dans des souris sans cellules NK (Gorelik et al., 1982; Talmadge et al., 1980). Plusieurs études montrent que les cellules NK sont activées par des lignées tumorales, notamment celles sur-exprimant les ligands des récepteurs activateurs et sous-exprimant les molécules de CMH-I. L'expression des ligands des récepteurs NK diffère d'une tumeur à l'autre, ce qui entraîne une variation de la sensibilité à la lyse par les cellules NK. En effet, l'étude de l'interaction de cellules NK humaines avec des tumeurs humaines *ex vivo* montre que l'activité des cellules NK est modifiée en fonction de la cible (Carlsten et al., 2009a). L'activation par les cellules tumorales peut être augmentée en ajoutant des cytokines activatrices, comme l'IL-2, l'IL-15, l'IL-12 ou l'IFN- α/β . Des cellules NK allogéniques ou autologues activées *in vitro* par des cytokines peuvent induire la lyse de cibles tumorales humaines fraîchement isolées.

Démontrer leur rôle anti-tumoral *in vivo* chez l'Homme est plus compliqué, des études cliniques apportent cependant des arguments sur leur rôle dans l'immunosurveillance des tumeurs. Une étude importante, apportant un solide argument, a été réalisée au Japon sur 3625 individus suivis pendant 11 ans, et évaluant la capacité cytotoxique des cellules NK et le développement de tumeurs (Imai et al., 2000). Les résultats de cette étude montrent qu'une forte activité cytotoxique NK est corrélée à une diminution du risque de développer un cancer. Un autre argument concerne le rôle GVL (*graft versus leukemia*) bien établi des NK après une greffe de cellules souches hématopoïétiques haplo-identiques chez des patients atteints de LAM. Dans ce modèle, les cellules NK issues du greffon du donneur, dites allo-réactives, lysent les cellules leucémiques résiduelles et sont associées à une diminution de la rechute des patients (Ruggeri, 2002). Cette fonction anti-tumorale des cellules NK allo-réactives est notamment due à une incompatibilité du HLA I entre donneur et receveur et une activation des cellules NK KIR⁺ spécifiques de molécules HLA-C absentes chez le receveur.

De plus, plusieurs études montrent que des tumeurs hautement infiltrées par les cellules NK sont corrélées à une meilleure survie ou à un risque moins élevé de développer des métastases, notamment chez des patients atteints de cancer de l'estomac (Ishigami et al., 2000), de cancer colorectal (Coca et al., 1997) ou de cancer du sein (Mamessier et al., 2011a). Principalement circulantes, les NK seraient au cœur de la réponse anti-métastatique (López-Soto et al., 2017). Elles ne sont pas majoritaires dans l'infiltrat tumoral, cependant elles peuvent jouer un rôle important dans le recrutement et l'activation d'autres cellules impliquées dans la réponse anti-tumorale. Les cellules NK participent au recrutement des CD au site tumoral par la production de chimio-attractants (CCL5, XCL1, FLT3L) (Barry et al., 2018; Böttcher et al., 2018). L'IFN γ produit par les cellules NK participe à l'activation de la réponse Th1 des LT.

Figure 10 : Fonctions anti-tumorales des cellules NK. Figure tirée de (Bald et al., 2019)

B. Echappement tumoral

Les cellules NK ont donc un rôle important dans la réponse anti-tumorale. Cependant, ces fonctions ne sont pas suffisantes pour stopper le développement des tumeurs. Afin d'échapper à la lyse par les cellules NK, les tumeurs ont développé différents mécanismes d'immunoévasion (Figure 11).

Le TME peut avoir des effets immunosuppresseurs sur les cellules NK. En effet, les cellules tumorales ou les cellules immunes avoisinantes, peuvent sécréter des molécules solubles inhibant l'activité des cellules NK, comme les TFG- β (Wilson et al., 2011), l'adénosine (ADO), le PGE₂ (Holt et al., 2011) ou l'Indoleamine-2,3-Dioxygénase (IDO). Le TFG- β induit une diminution de l'expression des récepteurs NKp30 et NKG2D *in vitro*, pouvant expliquer la diminution d'activation des cellules NK face à des cellules tumorales (Castriconi et al., 2003; Crane et al., 2010). Chez la souris, le TFG- β exprimé à la surface des cellules myéloïdes suppressives (MDSC) présentes dans le TME est responsable de l'inhibition des fonctions NK et de la diminution d'expression de NKG2D (Li et al., 2009). Les TAM inhibent également les fonctions NK via un mécanisme contact dépendant et le TFG- β (Krneta et al., 2017). De plus, les MDSC peuvent induire une diminution de la cytotoxicité et de la production de cytokines par les cellules NK via l'engagement de NKp30 (Hoechst et al., 2009).

Une seconde stratégie d'échappement à la réponse anti-tumorale des cellules NK, a pour cible le récepteur NKG2D. Les ligands de NKG2D, exprimés à la surface des cellules tumorales, peuvent être clivés par des MMP (Salih et al., 2002). Ces ligands, sous forme soluble, peuvent inhiber le récepteur NKG2D (Groh et al., 2002). Les patients atteints de leucémies présentent des taux élevés de ligands NKG2D solubles associés à une diminution de l'expression du récepteur à la surface des cellules NK et

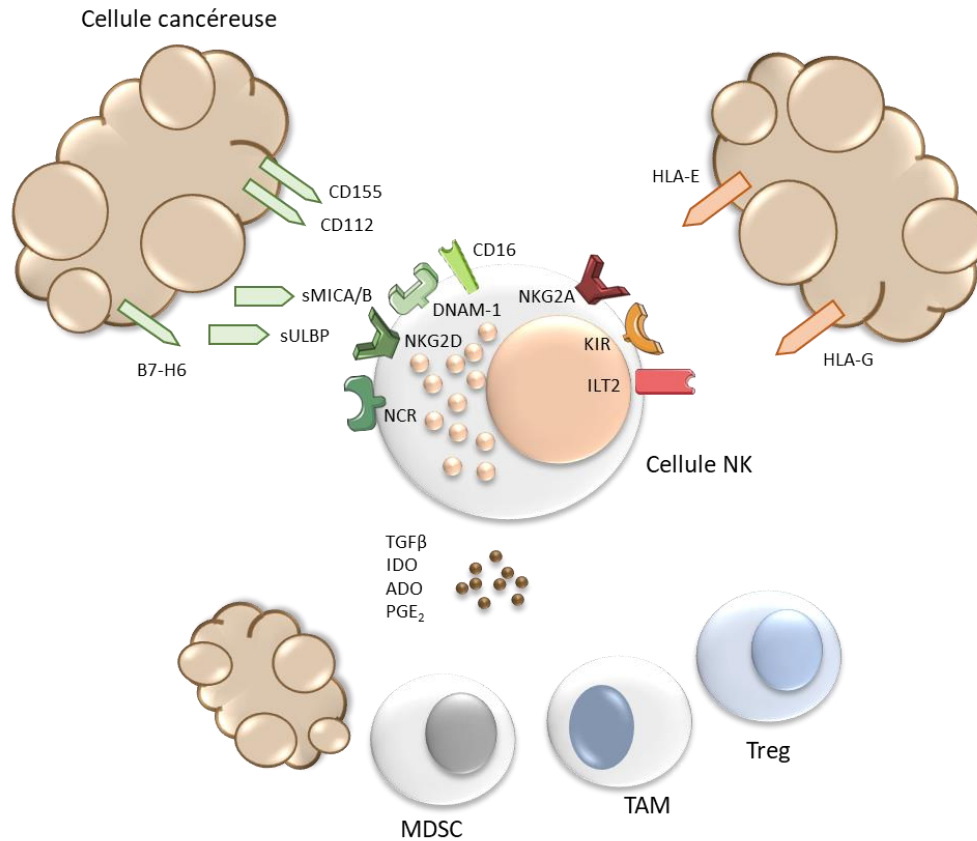
d'une diminution de leurs fonctions anti-tumorales (Hilpert et al., 2012). La concentration de MICA/B solubles (sMICA) est considérée comme un biomarqueur pronostique potentiel, avec des concentrations élevées associée à un mauvais pronostic (Zhao et al., 2017). Enfin, il a été montré, dans le mélanome métastatique, que les ULBPs solubles (sULBP) sont associés à une survie réduite (Maccalli et al., 2017). De plus, les cellules tumorales peuvent induire l'expression des ligands de NKG2D, à la surface des cellules myéloïdes, via la sécrétion de facteurs solubles, comme le lactate déshydrogénase (Crane et al., 2014). Les ligands exprimés par les cellules myéloïdes provoquent une diminution de l'expression de NKG2D à la surface des cellules NK et donc de leurs fonctions anti-tumorales.

Les cellules tumorales expriment d'autres ligands engageant des récepteurs activateurs NK, dont le contact prolongé peut également induire l'inhibition des fonctions NK. Dans le carcinome ovarien, les cellules tumorales exprimant CD155 induisent une down-régulation de l'expression de DNAM-1 sur les cellules NK (Carlsten et al., 2009b). Une corrélation inverse a été montrée entre l'expression de CD112 sur les cellules tumorales de patients atteints de LAM et l'expression de DNAM-1 sur les cellules NK de ces patients (Sanchez-Correa et al., 2012). Le récepteur NKp30 est également sous-exprimé sur les cellules NK infiltrant des tumeurs de carcinome ovarien, par rapport aux NK sanguines, induisant une diminution des fonctions NK (Pesce et al., 2015). Cet article suggère que cette sous-expression de NKp30 est due à un engagement prolongé du récepteur avec son ligand B7-H6, exprimé par les cellules tumorales de carcinome ovarien.

D'un autre côté, les cellules cancéreuses expriment à leur surface des ligands des récepteurs inhibiteurs. Par exemple, les molécules HLA-E, ligands du récepteur CD94/NKG2A, sont surexprimées dans plusieurs types de cancers (Derré et al., 2006; Guo et al., 2015; McWilliams et al., 2016; Nguyen et al., 2009). Les molécules HLA-G ont également été associées à un mécanisme d'échappement tumoral (Rouas-Freiss et al., 2005). En effet, les molécules HLA-G sont reconnues par des récepteurs inhibiteurs exprimés par les cellules NK (ILT2 et KIR2DL4). Il a été montré que l'expression de HLA-G par des lignées tumorales inhibe les fonctions NK et que son blocage permet de les réactiver (Lin and Yan, 2018). Enfin certaines cellules NK peuvent exprimer les récepteurs PD-1, CTLA-4, LAG-3 ou TIM-3, connus pour leur rôle dans l'inhibition des fonctions des LT. Ces récepteurs sont ciblés par des immunothérapies, développées afin de réactiver les LT, décrites dans la partie suivante.

1. Diminution des signaux activateurs

2. Augmentation des signaux inhibiteurs



3. Molécules immunosuppressives

Figure 11 : Mécanismes d'échappement tumoral. Les cellules tumorales expriment des ligands activateurs et produisent des ligands solubles qui peuvent entraîner une diminution de l'expression des récepteurs activateurs (1). Les cellules tumorales sur-expriment également des ligands inhibiteurs qui entraînent une augmentation des signaux inhibiteurs au niveau des cellules NK (2). Enfin les cellules tumorales et les cellules du microenvironnement (MDSC, TAM, Treg) sécrètent des molécules immunosuppressives (TGF- β , IDO, ADO, PGE₂) (3). *D'après (Chauhan et al., 2020; Li and Sun, 2018)*

II. Immunothérapies NK

Les données sur la régulation des cellules NK et l'essor des immunothérapies ciblant les récepteurs immuns favorisent l'intérêt croissant de manipuler ces effecteurs pour contrôler la croissance tumorale (Figure 12).

A. Les anticorps monoclonaux et l'ADCC

De nombreux anticorps monoclonaux (mAbs) ont été développés en clinique, ciblant spécifiquement un antigène tumoral. L'un des mécanismes d'action de ces mAbs est l'ADCC médiée par les cellules NK. Le récepteur CD16 reconnaît le fragment Fc des mAbs qui sont liés aux cellules tumorales.

1. Ciblage des cellules tumorales

2. Blocage des signaux inhibiteurs

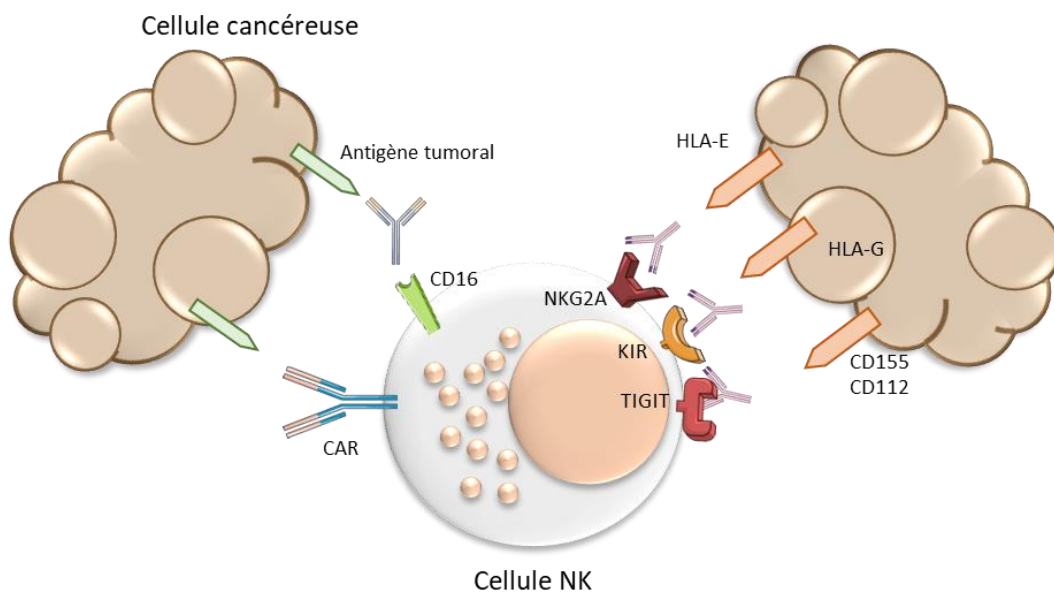


Figure 12 : Immunothérapies augmentant les fonctions des cellules NK. Les cellules NK peuvent être orientées vers les cellules tumorales via des anticorps thérapeutiques, bi ou tri-spécifiques ou via des CAR augmentant l'activation des cellules NK par les cellules cancéreuses (1). Les récepteurs inhibiteurs sont bloqués par des anticorps réduisant les signaux inhibiteurs et favorisant l'activation des fonctions NK (2). *D'après (Li and Sun, 2018).*

Le rituximab ciblant l'antigène CD20 exprimé par les lymphocytes B tumoraux, dans le lymphome non-Hodgkinien est un des premiers mAb thérapeutiques développés. L'implication de l'ADCC dans la réponse aux anti-CD20 a mis en évidence notamment par le rôle du polymorphisme du gène codant pour le fragment Fc dans la réponse clinique (Weng and Levy, 2003).

Dans les tumeurs solides, le cetuximab cible le récepteur EGFR, surexprimé dans plusieurs types tumoraux, comme le cancer colorectal. Il a été montré que cet mAb augmente l'activité des

cellules NK *in vivo*, via le CD16 (Veluchamy et al., 2016). De plus, un essai clinique de phase II montre que l'activité ADCC des cellules NK est corrélée à la stabilisation de la maladie et à la survie sans progression (Taylor et al., 2015). Dans les tumeurs mammaires HER2 (positives), le trastuzumab, un mAb anti-HER2, active l'ADCC médiée par les cellules NK *in vitro* (Carson et al., 2001). Dans une étude clinique chez des patientes atteintes de cancer du sein métastatique, la réponse au traitement a été associée à une forte activité ADCC des cellules NK (Beano et al., 2008). Le développement des mAb thérapeutiques capables d'engager avec une grande affinité l'isoforme CD16 exprimée par les NK est une approche intéressante pour certaines tumeurs.

Pour favoriser le contact entre cible et NK, des anticorps bispécifiques et tri-spécifiques, appelés BiKEs et TriKEs, engageant une réponse NK, ont été développés (Figure 13). Ces anticorps reconnaissent un antigène sur la cellule tumorale (CD20, CD33, CD19) et sur la cellule NK (CD16), permettant la formation d'une synapse immunologique. Ils sont composés d'un fragment variable (Fv) reliés à un (BiKE) ou deux (TriKE) autres Fv, reconnaissant des antigènes différents. Une cytokine NK (notamment l'IL-15) peut éventuellement remplacer un des Fv dans la structure du TriKE, augmentant la prolifération et la survie des cellules NK (Schmohl et al., 2016; Vallera et al., 2016). Les études précliniques *in vitro* et *in vivo* chez la souris, montrent que les BiKEs et les TriKEs induisent la lyse de tumeurs hématologiques et solides (Hodgins et al., 2019). Un autre type d'anticorps trispécifique est développé, appelé NKCE pour « *natural killer (NK) cell engagers* » (Gauthier et al., 2019). Il cible deux récepteurs activateurs NK, CD16 et Nkp46, en plus d'un antigène tumoral, le CD20. L'engagement de deux récepteurs NK permet une meilleure activation des cellules NK. En effet, l'étude préclinique dans le modèle murin démontre que le NKCE entraîne une réponse supérieure par rapport à un anticorps anti-CD20. Les premiers essais cliniques utilisant ces molécules débutent.

Figure 13 : Structure et fonction des BiKEs et TriKEs. Les anticorps bispécifiques BiKEs sont composés d'un fragment variable (Fv) reconnaissant un antigène tumoral relié à un autre Fv reconnaissant le CD16 exprimé par les cellules NK. Les anticorps tri-spécifiques TriKEs sont composés d'un Fv supplémentaire reconnaissant un autre antigène tumoral. L'engagement du CD16 et la reconnaissance de la cible tumorale active les fonctions NK. *Figure tirée de (Felices et al., 2016).*

B. Le transfert adoptif

Le transfert adoptif est une approche visant à transférer des cellules immunes fonctionnelles pour potentialiser une réponse anti-tumorale. Les cellules NK sont une source d'effecteurs cytotoxiques pouvant être amplifiées efficacement *ex vivo*. Le transfert de cellules NK peut se faire soit en condition autologue, avec des cellules NK venant du patient, soit allogénique, avec des cellules NK venant de donneurs sains. Le transfert de cellules NK autologues est peu efficace en clinique, en raison des altérations fonctionnelles fréquentes des NK de patients et de leurs capacités prolifératives plus faibles. Le transfert allogénique est lui beaucoup plus efficace. Comme mentionné précédemment, lors d'une greffe de cellules souches hématopoïétiques l'incompatibilité HLA-I/KIR augmente l'activité anti-tumorale des cellules NK allogéniques sans causer de maladie du greffon contre l'hôte (GvHD pour *Graft versus Host Disease*).

Afin d'améliorer les réponses et d'augmenter l'efficacité de ces transferts adoptifs, les cellules NK génétiquement modifiées, exprimant un récepteur antigénique chimérique (CAR), sont actuellement développées. Le CAR est composé d'un fragment d'anticorps (fragment variable scFv) se liant à l'antigène tumoral, et en intracellulaire, la chaîne CD3 ζ et un ou plusieurs domaines de protéines de costimulation, comme 4-1BB. Les LT exprimant un CAR (CAR-T) sont actuellement en clinique et ont induit des taux de réponses impressionnants notamment dans le lymphome non-Hodgkinien en ciblant le récepteur CD19 (Kallam and Vose, 2019). Par rapport à ces CAR-T, les CAR-NK ont l'avantage de pouvoir attaquer des cellules tumorales qui ont perdu l'expression du CMH-I. Elles seraient également moins toxiques pour le patient n'induisant pas ou peu de tempête cytokinique. Plusieurs essais cliniques sont actuellement en cours pour tester l'efficacité et la tolérance, dans des tumeurs hématologiques et solides (Tableau 2) (Zhang et al., 2020). Les cellules NK utilisées pour la fabrication des CAR-NK proviennent de plusieurs sources : des lignées cellulaires NK (NK-92), des NK fœtales du sang de cordon, des NK sanguines ou bien des cellules souches pluripotentes induites (iSPC).

Des études *in vitro* et *in vivo* dans des modèles murins ont montré l'efficacité de CAR-NK contre des cellules tumorales. Comme les CAR-T, les CAR-NK anti-CD20 et anti-CD19 entraînent une augmentation de la lyse de cellules tumorales CD20 ou CD19 positives *in vitro* (Imai et al., 2005; Müller et al., 2008; Romanski et al., 2016). Des cellules NK sanguines avec un récepteur chimérique comportant le récepteur NKG2D et les protéines activatrices DAP10 et CD3 ζ ont une activité cytotoxique supérieure pour lyser différentes lignées tumorales, par rapport à des cellules NK non modifiées (Chang et al., 2013). Il a été montré que ces CAR-NK-NKG2D pouvaient également lyser les MDSC, présentes dans le TME et surexprimant les ligands de NKG2D (Parihar et al., 2019). Les CAR-NK

sont également efficaces contre des tumeurs solides. Par exemple, des CAR-NK ciblant le récepteur HER2 sont capables de lyser des cellules tumorales HER2+, *in vitro* et *in vivo* (Schönfeld et al., 2015).

Numéro d'identification	Statut	Phase clinique	Cancer	Cible	Source de cellules NK
NCT03415100	En recrutement	Phase I	Tumeurs solides métastatiques	Ligands NKG2D	NK sanguines
NCT03940820	En recrutement	Phase I/II	Tumeurs solides ROBO1+	ROBO1	NK-92
NCT03941457	En recrutement	Phase I/II	Cancer du pancréas ROBO1+	ROBO1	NK-92
NCT03056339	En recrutement	Phase I/II	Tumeurs lymphocytes B	CD19	NK-92
NCT04245722	En attente de recrutement	Phase I	Tumeurs lymphocytes B	CD19	iPSC
NCT03056339	En recrutement	Phase I/II	Tumeurs lymphocytes B	CD19	Sang ombilical et de cordon
NCT03692767	En attente de recrutement	Phase I	Lymphomes B réfractaires	CD22	Inconnue
NCT03383978	En recrutement	Phase I	Glioblastome HER2+	HER2	NK-92
NCT03692663	En attente de recrutement	Phase I	Cancer de la prostate	PSMA	NK-92
NCT03692637	En attente de recrutement	Phase I	Cancer des ovaires épithélial	Mésothéline	NK sanguines
NCT03940833	En recrutement	Phase I/II	Myélome multiple	BCMA	NK-92

Tableau 2 : Sélection d'essais clinique CAR-NK en cours.

C. Les inhibiteurs de *checkpoints* immuns

Les anticorps ciblant les *checkpoints* inhibiteurs immuns ont révolutionné la prise en charge des cancers. Dans un premier temps, de tels mAbs développés pour réactiver les LT, les anticorps inhibiteurs de *checkpoint*, sont actuellement étudiés pour réactiver les cellules NK. En effet, les cellules NK, dans un contexte tumoral, expriment certains *checkpoints* initialement décrit sur les LT, comme

PD-1 ou TIM-3 (Benson et al., 2010; Hadadi et al., 2019; Pesce et al., 2017; Wang et al., 2015). Comme pour les LT, l'expression de ces récepteurs entraîne une diminution des fonctions anti-tumorales des cellules NK. Des récepteurs inhibiteurs, plus spécifiques des cellules NK, sont également ciblés par des ICB.

Anti-KIR

Les récepteurs KIR inhibiteurs sont importants dans la réponse anti-tumorale. L'un des mécanismes régulateur important des cellules NK est l'expression des molécules HLA-I par les cellules cibles, inhibant les cellules NK via les KIR. Pour les tumeurs hématologiques (LAM, lymphome T) qui expriment des ligands des récepteurs NK inhibiteurs, des anticorps bloquant les KIR présentent un intérêt. Des anticorps inhibiteurs de ces récepteurs ont donc été développés. Des essais cliniques de phase I, dans des tumeurs hématologiques et solides, ont démontré que le lirilumab (anti-KIR2D) n'entraîne pas d'effets secondaires sévères (Vey et al., 2018). Plusieurs essais de phase II sont en cours, dans différents types de cancer, seul ou en combinaison (anti-PD-1, anti-CD20, 5-azacytidine).

Anti-NKG2A

L'expression du récepteur inhibiteur NKG2A est élevée à la surface des cellules NK infiltrant de nombreux cancers (André et al., 2018). Son ligand, HLA-E, est surexprimé par différents types de tumeurs, ce qui rend cette voie d'inhibition des NK particulièrement intéressante pour potentialiser l'immunosurveillance tumorale NK. De plus, NKG2A est exprimé par certains LT infiltrant les tumeurs et un mAb bloquant ce récepteur pourrait également réactiver ces cellules. L'inhibition de NKG2A par le Monalizumab (mAbs), en combinaison avec un anti-PD-L1, entraîne une augmentation des fonctions NK contre les cellules tumorales HLA-E⁺, *in vitro* et *in vivo*. Le Monalizumab en combinaison avec le cetuximab (anti-EGFR) augmente l'ADCC médiée par les cellules NK contre des lignées cellulaires de carcinome squameux de la tête et du cou. Cette combinaison a donc été testée dans un essai de phase II, entraînant un taux de réponse supérieur à celui du cetuximab seul. D'autres essais cliniques sont en cours dans des tumeurs solides (cancer de l'ovaire, cancer colorectal métastatique).

Anti-TIGIT, anti-CD96

De nombreuses études décrivent le rôle de TIGIT et l'intérêt de son inhibition pour réactiver les LT CD8. L'expression de TIGIT sur les cellules NK infiltrant des tumeurs est associée à une diminution de leurs fonctions (Zhang et al., 2018). Dans cette étude, le blocage de TIGIT permet de restaurer l'activité des cellules NK contre les tumeurs de plusieurs modèles murins. Des essais cliniques sont en cours, testant les anti-TIGIT seuls ou en combinaison avec des anti-PD-1 ou PD-L1.

La présence de cellules NK sanguines exprimant CD96 et DNAM-1 a été associée à un mauvais pronostic chez des patients atteints de cancer du pancréas (Peng et al., 2016). De même, la fréquence de cellules NK CD96⁺ est corrélée à un mauvais pronostic dans le cancer du foie (Sun et al., 2019). Cette population CD96⁺ présente une diminution des fonctions de production de TNF- α . *In vitro*, le blocage de CD96 augmente la lyse de cellules tumorales par les cellules NK. Dans une étude préclinique, le traitement de souris avec des anti-CD96 permet de diminuer le développement de métastases (Blake et al., 2016). Cette diminution est dépendante des cellules NK, de la sécrétion d'IFN- γ et elle est plus importante chez des souris qui n'expriment pas TIGIT. La combinaison anti-CD96 et anti-PD-1 permet d'augmenter les fonctions anti-tumorales des cellules NK. Les anti-CD96 ne sont pas encore testés en clinique.

Anti-PD-1, anti-CTLA-4

Les anticorps inhibiteurs des *checkpoints* immuns CTLA-4 et PD-1 ont été les premiers à démontrer leur efficacité dans le traitement du cancer. Essentiellement étudiés pour corriger l'activité des LT, ces deux récepteurs sont également exprimés par certaines populations NK et ces dernières pourraient participer à la réponse clinique des patients traités.

Le récepteur CTLA-4 est exprimé chez les cellules NK de souris après activation par l'IL-2 et comme chez les LT, son engagement induit l'inhibition des fonctions NK (Stojanovic et al., 2014). L'expression de CTLA-4 par les cellules NK humaines n'a pas été démontrée. Le rôle des cellules NK dans la réponse aux inhibiteurs de CTLA-4 a été étudié chez la souris. Dans un modèle de cancer colorectal, la thérapie anti-CTLA-4 induit une activation des cellules NK du TME, pouvant être expliquée par l'inhibition des Treg CTLA-4⁺ (Sanseviero et al., 2019). Les cellules NK infiltrant les tumeurs n'expriment pas CTLA-4 mais elles exercent principalement une fonction ADCC contre les cellules tumorales. De plus, l'ajout d'IL-15 à cette thérapie augmente le contrôle de la tumeur. Cette étude montre également que chez les patients recevant de l'ipilimumab, la réponse au traitement corrèle avec la présence d'une population de cellules NK CD56⁺ intra-tumorale. Dans un modèle murin de mélanome, l'effet de la combinaison thérapeutique anti-CTLA-4 avec de l'IL-2 est dépendante des LT CD8 et des cellules NK (Kohlhapp et al., 2015).

L'expression et le rôle de PD-1 par les cellules NK a été montré. Une étude chez des patients atteints de myélome multiple démontre que les NK de patients expriment PD-1 et que son blocage active les fonctions NK contre des cellules de myélome autologues (Benson et al., 2010). Le récepteur PD-1 est exprimé par les cellules NK matures chez ¼ de la population et cette expression est strictement corrélée à une infection CMV (Pesce et al., 2017). Cette population NK PD-1⁺ est augmentée dans le carcinome ovarien. Les cellules NK PD-1⁺ présentent une diminution de leurs

fonctions cytotoxiques et cytokiniques, qui sont réactivées après blocage par des anti-PD-1. L'augmentation d'une population NK PD-1⁺, présentant une diminution de ses fonctions, a également été reportée dans le sarcome de Kaposi (Beldi-Ferchiou et al., 2016).

Anti-TIM-3

TIM-3 a pour ligand la galectine-9, souvent surexprimée dans les tumeurs. Les cellules NK expriment TIM-3 et son expression est augmentée après activation (Gleason et al., 2012; Ndhlovu et al., 2012). Le récepteur TIM-3 est décrit par Gleason et al. comme activateur, augmentant la production d'IFN- γ par les cellules NK. D'un autre côté Ndhlovu et al. montrent que l'engagement de TIM-3 diminue la cytotoxicité des cellules NK. Dans certains contextes tumoraux, les cellules NK périphériques présentent une augmentation de l'expression de TIM-3 associée à une diminution de l'activation des fonctions anti-tumorales (da Silva et al., 2014; Wang et al., 2015; Xu et al., 2015). Le blocage de TIM-3 permet la restauration des fonctions NK. La présence de cellules NK TIM-3⁺ dans le sang est associée à un mauvais pronostic et à un stade avancé dans les cancers du poumon, les tumeurs gastro-intestinales et le mélanome métastatique. Wang et al. montrent que le développement de la tumeur chez la souris est corrélé à l'augmentation de l'expression de TIM-3 par les cellules NK, expliquant la fréquence plus élevée chez les patients de stades avancés. Les cellules NK TIM-3⁺ peuvent également infiltrées les tumeurs gastro-intestinales (Komita et al., 2015). Plusieurs essais cliniques testant les anti-TIM-3 sont actuellement en cours dans des tumeurs solides avancées.

Anti-LAG-3

LAG-3 est exprimé par les LT et les cellules NK et reconnaît les molécules de CMH-II (Baixeras et al., 1992). Les anticorps anti-LAG-3 utilisés chez la souris permettent une restauration des fonctions des LT. L'implication des cellules NK dans la réponse aux anti-LAG-3 n'a pas été étudiée. Une étude réalisée chez la souris montre que l'absence de LAG-3 diminue la lyse de cibles tumorales par les cellules NK (Miyazaki et al., 1996). A l'inverse, chez l'Homme le blocage de LAG-3 n'augmente pas la cytotoxicité des cellules NK (Huard et al., 1998). Plus d'études sont nécessaires afin de préciser le rôle de LAG-3 dans les fonctions anti-tumorales des cellules NK.

D. Combinaisons thérapeutiques

Les cellules NK exercent un rôle anti-tumoral important. Cependant elles ne représentent qu'une petite proportion des cellules immunitaires infiltrant les cancers et leurs fonctions sont fréquemment diminuées dans un contexte tumoral. Une monothérapie visant les cellules NK n'a été

que rarement montrée comme étant suffisamment efficace pour induire une régression tumorale. Les thérapies anti-cancéreuses sont de plus en plus administrées en combinaison avec d'autres immunothérapies, des chimiothérapies, radiothérapie ou thérapies ciblées. L'intérêt de combiner des traitements est de viser plusieurs acteurs du développement tumoral. De plus, la chirurgie, la chimiothérapie, la radiothérapie ou les thérapies ciblées peuvent avoir des effets immunomodulateurs et ainsi augmenter l'efficacité des immunothérapies.

En effet, la radiothérapie et certaines chimiothérapies peuvent induire l'immunité anti-tumorale via notamment un mécanisme appelé « mort cellulaire immunogène ». Les cellules cancéreuses traitées vont libérer des signaux de danger qui activent les CD puis les LT (Emens and Middleton, 2015; Yu et al., 2019). Les fonctions des cellules NK peuvent également être augmentées par chimio ou radiothérapie. Notamment, ces traitements entraînent une augmentation de l'expression de B7-H6, ligand de NKp30, à la surface des cellules cancéreuses qui s'accompagne d'une augmentation de la lyse de ces cellules par les cellules NK (Cao et al., 2015). La chimiothérapie peut être utilisée en combinaison avec le transfert adoptif de cellules NK. Une étude montre que cette combinaison thérapeutique chez des patients atteints de cancer colorectal augmente la survie et diminue la récurrence (Li et al., 2018).

Les thérapies ciblées, notamment les inhibiteurs de tyrosine kinase (TKI), peuvent également moduler la réponse immunitaire. Plusieurs études dans des hémopathies malignes montrent que certaines de ces thérapies agissent directement ou indirectement sur l'activité des cellules NK (Krieg and Ullrich, 2013). Les TKI sont également utilisés dans les tumeurs solides. Le Cabozantinib (TKI) utilisé dans un modèle murin de cancer rénal humain augmente l'expression d'EGFR à la surface des cellules tumorales augmentant la réponse aux CAR-NK spécifiques de EGFR et leur infiltration intra-tumorale (Zhang et al., 2017). Des lignées de cancer de l'ovaire dans un premier temps résistantes au cetuximab (mAb EGFR) deviennent plus sensibles à la lyse NK et à l'ADCC après un pré-traitement avec un TKI dirigé contre EGFR (Mallmann-Gottschalk et al., 2019). Mon équipe a montré que le vemurafenib, un TKI inhibant la kinase BRAF, diminue l'expression des ligands activateurs B7-H6 et MICA/B et augmente celle de ligands inhibiteurs HLA-I, à la surface de lignées de mélanome (Frazao et al., 2017). Cette modulation des ligands NK induit une diminution de la lyse induite par les cellules NK des cellules de mélanome traitées par le vemurafenib.

Enfin, les immunothérapies NK peuvent être administrées en combinaison avec des immunothérapies ciblant d'autres types cellulaires. En effet, les anti-NKG2A et anti-KIR sont actuellement testés en combinaison avec les inhibiteurs de *checkpoint* CTLA-4 et PD-1, permettant en parallèle une réactivation des LT. Le transfert adoptif de cellules NK peut être associé à une

administration de cytokines (IL-2, IL-15) (Shimasaki et al., 2020). Le transfert adoptif de NK peut être couplé avec un traitement par mAbs anti-tumoraux. Un essai clinique de phase I a montré l'efficacité d'un transfert adoptif de cellules NK en combinaison avec le trastuzumab ou le cetuximab dans les cancers gastriques et colorectaux (Ishikawa et al., 2018). Les patients de cette étude présentaient une augmentation de la production d'IFN- γ et pour certains une régression tumorale.

Certaines de ces thérapies sont données après une chirurgie, en adjuvant, pour prévenir la réapparition de la tumeur ou avant un traitement chirurgical, en néo-adjuvant pour diminuer la masse tumorale.

E. Thérapies en néo-/adjuvant

Dans la prise en charge du cancer, la chirurgie reste un acte préconisé dans la plupart des cas. Elle peut être suivie d'une thérapie pour éliminer les éventuelles cellules tumorales résiduelles et éviter la rechute, la thérapie est dite adjuvante. En effet la moitié des patients après un acte chirurgical rechute et développent des métastases. La thérapie donnée en adjuvant peut être de la chimiothérapie, la radiothérapie, une thérapie ciblée ou plus récemment une immunothérapie. Plusieurs essais cliniques ont été réalisés pour évaluer l'efficacité des immunothérapies en thérapie adjuvante, notamment celles pouvant impacter les cellules NK. Dans le mélanome, différentes études montrent que les inhibiteurs de *checkpoint* immuns PD-1 et CTLA-4 sont efficaces en adjuvant (Eggermont et al., 2018; Weber et al., 2017). En effet, les mAbs anti-PD-1 (nivolumab ou pembrolizumab) et les anti-CTLA-4 (ipilimumab) augmentent la survie sans récurrence, avec une efficacité supérieure pour les anti-PD-1. Les thérapies induisant le mécanisme d'ADCC médié par les cellules NK sont aussi testées en adjuvant. Dans le cancer du sein HER2 positif, le trastuzumab (anti-HER2 mAbs) après une chimiothérapie, en adjuvant, permet d'augmenter la survie (Piccart-Gebhart et al., 2005).

Une thérapie avant la chirurgie peut également être prescrite, elle est dite néo-adjuvante. Elle permet de réduire la taille de la tumeur avant la chirurgie. La chimiothérapie peut être prescrite en néo-adjuvant. Cependant une étude clinique montre que chez des patients atteints de cancer du rectum la chimiothérapie en néo-adjuvant a un effet délétère sur l'activité des cellules NK et augmente le risque de développer des métastases (Koda et al., 2003). Des immunothérapies en complément ou en remplacement de la chimiothérapie lors de traitement néoadjuvant pourraient présenter des avantages. L'immunothérapie en néo-adjuvant permettrait une activation du système immunitaire plus efficace. En effet, la tumeur primaire étant encore présente, il y a une plus grande quantité d'antigènes tumoraux pouvant induire une meilleure activation du système immunitaire adaptatif. Il

existe actuellement plus de 100 essais cliniques pour tester les anti-PD(L)-1 en néo-adjuvant, seuls ou en combinaison (Topalian et al., 2020). Dans le mélanome, les anti-PD-1 ou les anti-CTLA-4 augmentent l'infiltrat lymphocytaire dans le tissu tumoral (Amaria et al., 2018). Plusieurs études montrent que les LT intra-tumoraux et périphériques ont un phénotype activé après une thérapie anti-CTLA-4 (Carthon et al., 2010; Tarhini et al., 2014). Dans un modèle murin de cancer du sein triple négatif (TNBC), une thérapie néo-adjuvante anti-PD-1 présente une efficacité thérapeutique supérieure en comparaison à la même thérapie donnée en adjuvant (Liu et al., 2016). Cette étude montre également que cette augmentation de la survie des souris recevant le traitement en néo-adjuvant dépend des LT CD8, des cellules NK et de la production d'IFN- γ . De plus, le rôle des cellules NK dans la réponse aux anti-PD-1 en néo-adjuvant est montré dans un modèle de souris transgénique de cancer du pancréas (Brooks et al., 2018). L'utilisation d'inhibiteurs du récepteur CD96, exprimé principalement par les cellules NK, après les anti-PD-1 augmente la survie à long terme.

Les ganglions drainant les tumeurs pourraient jouer un rôle important dans les réponses aux immunothérapies administrées en condition néo-adjuvante. Ainsi, ces dernières permettraient d'augmenter la présentation antigénique par les CD aux LT au niveau du ganglion. Elles pourraient également agir sur l'activation des cellules NK ganglionnaires, cet organe étant un lieu de maturation privilégié de ces effecteurs anti-tumoraux. Plus d'études sont nécessaires afin d'évaluer les effets des thérapies néo/adjuvantes sur le système immunitaire et afin de tester d'autres immunothérapies en néo/adjuvant, notamment celles ciblant les cellules NK.

III. Les ILC auxiliaires dans le cancer

Le rôle des ILC auxiliaires dans le cancer est moins connu que celui des cellules NK. Quelques études chez la souris et chez l'humain se sont intéressées à ces populations (Figure 14).

Les ILC1 auxiliaires sont les moins étudiées dans le cancer. Chez la souris il a été montré que les cellules NK, dans un contexte tumoral et sous l'influence du TGF- β , étaient converties en ILC1, n'exerçant plus de fonctions anti-tumorales (Gao et al., 2017b). Chez l'humain, les ILC1 auxiliaires sont encore difficilement identifiables, expliquant le manque d'études spécifiques de cette population chez des patients atteints de cancer. Une population CD56⁺ « ILC1-like » est identifiée chez des patients atteints de LAM, elle présente une diminution d'expression de récepteurs activateurs (NKp30, NKp80, TRAIL) qui s'accompagne d'une diminution de ses capacités cytotoxiques (Salomé et al., 2019).

Les ILC2 seraient impliquées dans une augmentation de l'environnement immunosuppresseur. En effet, dans différentes études portant sur des patients atteints de cancer gastrique, leucémies ou

encore cancer de la vessie et de la prostate, la fréquence d'ILC2 sanguines est augmentée par rapport à des individus sains (Bie et al., 2014; Chevalier et al., 2017; Trabanelli et al., 2017). Dans la leucémie aiguë pro myélocytaire (APL) les ILC2 peuvent être activées via les ligands de CRTH2 ou NKp30 (Trabanelli et al., 2017). L'activation des ILC2 entraîne la sécrétion d'IL-13, activant les MDSC, augmentant le microenvironnement immunosuppresseur. D'un autre côté, une étude montre que l'IL-33 est associée à une augmentation de l'infiltration tumorale par les ILC2 dans des tumeurs de pancréas humaines enrichies en T CD8 (Moral et al., 2020). Dans ce modèle, l'infiltrat ILC2 est associé à un bon pronostic. Dans la même étude, les anti-PD-1 utilisés dans un modèle murin de cancer du pancréas diminuent l'inhibition des ILC2 exprimant PD-1, augmentant leur activité anti-tumorale.

Le rôle des ILC3 dans le processus tumoral a été décrit dans différentes études, présentant ces cellules comme pro-tumorales pour certaines et anti-tumorales pour d'autres. En effet, l'IL-23, connue pour activer les ILC3, est sécrétée par certaines cellules tumorales, entraînant la sécrétion d'IL-17 qui peut induire la prolifération tumorale (Chan et al., 2014; Koh et al., 2019; Liu et al., 2019). Chez des patients atteints de cancer du sein, la fréquence d'ILC3 ROR γ t⁺ intra-tumorales corrèle avec l'invasion métastatique des ganglions lymphatiques (Irshad et al., 2017). Les ILC3 induisent le développement des métastases via la production, dans le TME, de chimiokines et cytokines pro-tumorales. Dans un modèle murin de cancer colorectal, l'IL-22 produit par les ILC3 induit favorise le développement tumoral (Kirchberger et al., 2013). Une population CD3⁻IL-22⁺, considérée ILC3, a été identifiée chez des patients atteints d'un cancer colorectal. De même, dans le cancer du pancréas, une augmentation des ILC3 sanguines et intra-tumorales et de l'expression d'IL-22 a été décrite, par rapport à des individus sains (Xuan et al., 2020). *In vivo*, l'IL-22 produit par les ILC3 induit une augmentation de la prolifération, de l'invasion et de la migration de cellules de cancer du pancréas, via la voie AKT. D'autres études indiquent que les ILC3 peuvent jouer un rôle anti-tumoral. Ainsi, une population ILC3 NCR⁺, ayant des fonctions LTi, a été associée à la formation de structures lymphoïdes tertiaires (TLS), ces dernières étant connues pour être associées à un bon pronostic (Carrega et al., 2015).

Figure 14 : Fonctions pro- et anti-tumorales des ILC auxiliaires. Les ILC1, ILC2 et ILC3 ont des fonctions anti-tumorales. Les ILC1 produisent de l'IFN γ et du TNF α quand elles sont activées par l'IL-15. Les ILC2 produisent de l'IL-5 après activation par l'IL-33, participant au recrutement des éosinophiles. Elles peuvent également activer les LT. Les ILC3 NCR⁺ contribuent à la formation d'organes lymphoïdes tertiaires au niveau de la tumeur. D'un autre côté, les ILCs auxiliaires peuvent avoir des fonctions pro-tumorales. Le TGF β peut induire la différenciation des cellules NK en ILC1 possédant des fonctions anti-tumorale moindre. Les ILC2 produisent de l'IL-13 recrutant les MDSC ayant un effet immunosuppresseur sur les LT. Les ILC2 produisent également de l'IL-9, activant les LT, de l'amphiréguline (AREG) et de l'IL-4, activant la croissance tumorale et la dissémination. Les ILC3 activées par l'IL-23 produisent de l'IL-17 et de l'IL-22 pouvant induire une immunosuppression des fonctions LT. *Figure tirée de (Crinier et al., 2019)*

OBJECTIFS

Le développement des thérapies ciblées et des immunothérapies a révolutionné les traitements de nombreux cancers. Les avancées faites grâce au séquençage à haut débit ont permis de mieux appréhender les anomalies génétiques impliquées dans le développement et la progression des cancers, et conduit à la découverte de nouvelles cibles thérapeutiques. Les études sur la présence et qualité du stroma inflammatoire dans le TME des cancers, corrélées à la réponse naturelle ou sous traitement des patients, ont placé les immunothérapies comme une alternative thérapeutique incontournable. Les traitements actuels tiennent compte de ces avancées et sont maintenant « personnalisés », adaptés au profil mutationnel et à la qualité du TME de la tumeur de chaque patient. Les thérapies ciblées, comme les TKI ou les anticorps monoclonaux dirigés contre des molécules de surface, entraînent des réponses rapides et efficaces dans la plupart des cas. Cependant des mécanismes de résistances sont fréquents, entraînant la rechute de la maladie. Les immunothérapies, reposant sur des anticorps bloquant des récepteurs immuns inhibiteurs, permettent d'avoir des régressions tumorales plus durables pour certains patients. De nombreux patients résistent à ces immunothérapies, selon des mécanismes mal connus. Les approches actuelles combinent des thérapies ciblant les anomalies spécifiques de la tumeur et un contournement de l'immunosuppression pour restaurer une immunité anti-tumorale effective. Mon projet de thèse s'inscrit dans cette problématique à travers deux projets : un projet de recherche translationnelle pour analyser l'impact de la résistance aux thérapies ciblées sur l'immunogénicité NK et un projet plus exploratoire qui vise à définir le rôle des cellules lymphoïdes innées (ILC) ganglionnaires dans l'immunosurveillance tumorale. Ces projets sont développés dans deux modèles tumoraux d'intérêt, le mélanome métastatique porteur de la mutation *BRAFV600E* pour le projet 1 et les cancers du sein pour le projet 2.

Le premier projet est centré sur l'étude des ILC cytotoxiques, les cellules NK, et leurs interactions avec les cellules tumorales de mélanome résistantes aux thérapies ciblées, dirigées contre les tyrosines kinases BRAF (vemurafenib) et MEK (cobimetinib). Des travaux antérieurs du laboratoire ont montré que les cellules NK de patients atteints de mélanome métastatique étaient altérées après traitement par chimiothérapies et que le potentiel de dégranulation de ces cellules NK était corrélé au stade de la maladie. Au début de ma thèse, le laboratoire venait de publier une étude montrant que le traitement au vemurafenib des cellules de mélanome altère l'expression de certains ligands des récepteurs NK et diminue l'activité cytotoxique des cellules NK (Frazao et al., 2017; Fregni et al., 2011, 2013). Dans la continuité de cette étude, le laboratoire a développé des lignées de mélanome

métastatique résistantes au vemurafenib et à la combinaison thérapeutique vemurafenib/cobimetinib. Mon objectif a été d'étudier l'immunogénicité de ces lignées de mélanome métastatique, sensibles versus résistantes, et leur sensibilité aux fonctions cytotoxiques NK. Ces travaux font l'objet d'un premier article en co-première autrice, publié en 2020 dans le journal *Journal for Immunotherapy of Cancer*.

Le projet n°2 visait la caractérisation des ILC, cytotoxiques et auxiliaires, infiltrant le ganglion lymphatique, l'un des premiers sites métastatiques de plusieurs tumeurs dont les cancers du sein. Notre laboratoire a précédemment mis en évidence l'implication des cellules NK ganglionnaires (ILC1c) dans l'immunosurveillance des tumeurs métastatiques. Ces travaux ont identifié les sous-populations NK présentes dans les ganglions, notamment des cellules CD56^{bright}CD16⁺ plus abondantes dans les ganglions drainant la tumeur. Les cellules NK infiltrant les ganglions métastatiques de cancer du sein ont un phénotype activé et exercent des fonctions cytotoxiques élevées contre les cellules tumorales après stimulation par les cytokines (Frazao et al., 2019; Messaoudene et al., 2014). Cependant, les populations ILC auxiliaires n'avaient pas été prises en compte dans ces travaux. Le projet d'étude des ganglions métastatiques de cancer du sein, en collaboration avec le Dr. Eliane Piaggio (Institut Curie), se poursuit donc sur l'étude de l'ensemble des populations ILC, en comparaison avec les tumeurs et des ganglions lymphatiques de donneurs sains (collaboration service de réanimation de l'Hôpital Saint-Louis). Cette étude a pour but de déterminer les caractéristiques des ILC des ganglions et de comparer les ganglions de donneurs et ceux drainant la tumeur dans la perspective d'optimiser les traitements (néo-)adjuvant et éventuellement trouver de nouvelles cibles. Ces résultats sont en cours de finalisation et feront l'objet d'un deuxième article que je signe en premier auteur.

Mes travaux de thèse ont donc pour objectif principal d'étudier le rôle des ILC dans l'immunosurveillance de tumeurs métastatiques. Pour répondre à cette question j'ai étudié :

1/ La résistance de lignées de mélanomes métastatiques aux thérapies ciblées et l'effet de cette résistance sur les fonctions des cellules NK

2/ Les caractéristiques des ILC dans les ganglions de donneurs et les ganglions métastatiques de patientes atteintes de cancer du sein

Ces résultats apportent des données originales sur les ILC ganglionnaires en distinguant les ILC cytotoxiques et les ILC auxiliaires. Ils précisent les fonctions et caractéristiques des ILC dans un contexte tumoral, permettant des avancées thérapeutiques, par la compréhension du microenvironnement et par la mise en évidence de nouvelles cibles. Ce projet apporte également de nouveaux arguments pour le développement des immunothérapies basées sur les cellules NK.

RESULTATS

RESULTATS

Article n°1 : La résistance des cellules de mélanome aux inhibiteurs de BRAF induit une sensibilité accrue à la lyse médiée par les cellules Natural Killer

BRAF inhibitor resistance of melanoma cells triggers increased susceptibility to natural killer cell-mediated lysis

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Le mélanome est le cancer de la peau le plus grave car il a une forte capacité métastatique, dont l'incidence augmente depuis une dizaine d'années. Le mélanome se développe à partir des mélanocytes, cellules pigmentaires de la peau. Il existe différents facteurs augmentant les risques de développer un mélanome : l'exposition au soleil et aux UV artificiels, les coups de soleil durant l'enfance, la présence de nombreux grains de beauté (ou *nævi*) (plus de 50) et des antécédents familiaux de mélanome.

Au diagnostic d'un mélanome, le stade de la maladie est déterminé selon la classification réalisée par l'AJCC (*American Joint Committee on Cancer*) en 2009 (Balch et al., 2009) (Tableau 3). Les mélanomes de stade 0, I et II sont des tumeurs localisées. Les sous-types sont ensuite définis selon l'épaisseur de la tumeur (épaisseur de Breslow ou le niveau de Clark), le taux mitotique et l'ulcération. Au stade III la tumeur primaire est disséminée et des métastases dans les ganglions régionaux sont détectées. Les patients présentant un mélanome de stade IV ont des métastases à distance qui peuvent concerner différents organes.

En France, 25% des mélanomes sont diagnostiqués à un stade tardif, stade III ou métastatique. A ce stade plusieurs options de traitement sont proposées : la chirurgie, la chimiothérapie, la radiothérapie, et plus récemment les immunothérapies et enfin les thérapies ciblées. Les thérapies ciblées ont été développées suite à la découverte qu'environ 50% des mélanomes métastatiques portent une mutation au niveau du gène *BRAF* qui est dans 90% des cas une mutation V600E. La kinase BRAF est impliquée dans la voie des MAP kinases (MAPK pour *Mitogen-Activated Protein Kinase*)

jouant un rôle dans la survie et la prolifération des cellules. La mutation V600E active constitutivement BRAF entraînant une survie et une prolifération incontrôlée des cellules cancéreuses. Les inhibiteurs de BRAF, tel que le vemurafenib (Roche), entraîne une réponse rapide avec une diminution de la tumeur et une amélioration de la survie des patients. Cependant ces réponses sont souvent limitées dans le temps et certains patients rechutent. La résistance aux inhibiteurs de BRAF implique différents mécanismes qui mènent à la réactivation de kinases en aval de BRAF dans la voie des MAPK ou dans la voie PI3K (Fedorenko et al., 2011). La kinase MEK peut notamment être responsable de la réactivation de la voie des MAPK. Le traitement actuel repose donc sur une combinaison inhibiteur de BRAF avec inhibiteur de MEK, comme le cobimetinib (Roche), qui augmente la survie sans progression (Long et al., 2014). Malgré cette combinaison thérapeutique, certains patients rechutent et deviennent résistant à ces deux inhibiteurs.

Tableau 3 : Classification de l'AJCC. M : métastase. N : ganglion. P : stade déterminé par l'examen anatomo-pathologique. T : tumeur initiale. *Tableau tiré de Pharmacie Clinique Pratique en Oncologie – Mélanome par I. Madeline.*

Par ailleurs, l'immunothérapie a révolutionné la prise en charge des patients atteints de mélanome. Le mélanome est connu pour être une tumeur immunogène. Ainsi l'administration d'IL-2 à haute dose a été la première immunothérapie utilisée dans le mélanome, cependant celle-ci induit de nombreux effets secondaires et un effet anti-tumoral limité (Atkins et al., 1999). Depuis 2005, le développement clinique des ICB, anti-CTLA-4 et anti-PD-1, a changé la prise en charge des patients à un stade avancé, et dans une impasse thérapeutique avant l'arrivée de ces anticorps. Les ICB anti-CTLA-4 et anti-PD-1 ont rapidement montré leur efficacité dans des essais incluant de grands nombres de patients et induisent une réponse à long terme, avec 50% de survie à cinq ans. Cependant seuls 20% des patients développent des réponses complètes et durables. Les mécanismes de réponse et résistance aux ICB ne sont pas encore totalement élucidés.

Les cellules NK par leur rôle dans la réponse anti-tumorale du mélanome pourraient avoir une influence sur la réponse aux ICB. Plusieurs équipes, dont la nôtre, se sont intéressées aux fonctions anti-tumorales de ces cellules NK chez les patients atteints de mélanome. Ainsi, notre équipe a montré que les cellules NK circulantes de patients atteints d'un mélanome avancé présentent une faible expression des récepteurs NKp46 et NKG2A (Fregni et al., 2011, 2013). Une autre équipe observe une diminution des récepteurs NKG2D et DNAM-1 à la surface des cellules NK circulantes (Mirjačić Martinović et al., 2014). Ces modifications d'expression des récepteurs NK s'accompagnent d'une diminution de la lyse, de la production de perforine, de la dégranulation et de la production d'IFN- γ par les NK sanguines des patients. Notre équipe a également étudié les cellules NK dans les ganglions métastatiques, identifiant des populations NK particulières (CD56^{bright}CD16⁺). Comme les cellules NK sanguines, les cellules NK ganglionnaires présentent des fonctions altérées mais peuvent être réactivées par les cytokines IL-2 ou IL-15 (Ali et al., 2014; Messaoudene et al., 2014). La fréquence de NK CD56^{bright} circulantes est inversement corrélée à la survie et aux métastases à distances (de Jonge et al., 2019). Les traitements des patients mélanomes modifient l'activité des cellules NK. Notre équipe a montré que la chimiothérapie augmente l'expression de NKG2A et NKp46 par les cellules NK circulantes de patients altérant leur fonctions (Fregni et al., 2011). Nous avons également observé que le vemurafenib (inhibiteur de BRAF) modifie l'expression des ligands des récepteurs NK à la surface des cellules de mélanome entraînant une diminution de la lyse par les cellules NK (Frazao et al., 2017).

Ayant montré l'impact des thérapies ciblées sur l'immunogénicité NK des mélanomes, confirmé par d'autres études (Krieg and Ullrich, 2013; Mallmann-Gottschalk et al., 2019), nous avons poursuivi cette observation en étudiant l'effet de la résistance aux thérapies ciblées sur l'activité anti-tumorale des cellules NK.

A partir de deux lignées de mélanome porteuses d'une mutation homozygote *BRAF* V600E (SK28S, M14S) et modulant leur expression des ligands NK lors du traitement par le vemurafenib, des variants résistants ont été générés (SK28R et M14R). La résistance à l'inhibiteur de BRAF, le vemurafenib, a été induite par la culture prolongée avec des doses croissantes d'inhibiteur (de 0.1µM à 10µM).

La prolifération cellulaire des variants n'est pas inhibée par le vemurafenib, contrairement à celles des lignées parentales. Les mécanismes de résistance des deux lignées modèles sont différents. La résistance de la lignée SK28 implique la réactivation de la voie AKT alors que celle de la lignée M14 est due à la réactivation de MEK et ERK.

L'expression des ligands des récepteurs NK par les cellules sensibles/résistantes au vemurafenib est modifiée. L'expression de MICA et ULBP2 est supérieure dans la lignée SK28R par rapport à la sensible. A l'inverse, l'expression d'ULBP2 par la lignée M14R est diminuée par rapport à la lignée sensible. Nous avons mesuré la quantité de ligands solubles dans les surnageants de culture des lignées de mélanome. Dans la lignée M14R la diminution d'ULBP2 et de B7-H6 membranaire s'accompagne d'une augmentation d'ULBP2 et B7-H6 solubles. Ces niveaux altérés d'expression de ligands NK ont une influence sur le statut fonctionnel des cellules NK. En effet, la dégranulation, la lyse et la production d'IFN-γ des cellules NK face aux cellules de mélanomes résistantes (SK28R) au vemurafenib est augmentée en comparaison aux cellules sensibles.

Les cellules NK peuvent induire la mort cellulaire via les récepteurs à domaine de mort. Nous avons observé une augmentation de l'expression de TRAIL-RII par les lignées SK28R et une diminution de Fas par les lignées M14R. L'apoptose induite par le traitement des lignées avec des protéines TRAIL recombinantes est supérieure chez les lignées SK28R par rapport aux sensibles. Au contraire, l'apoptose induite par TRAIL est forte chez les M14 sensibles et diminue fortement chez les résistantes.

Nous avons ensuite évalué l'effet du traitement combinant un inhibiteur de BRAF et un inhibiteur de MEK, donné actuellement en clinique aux patients portant la mutation *BRAF* V600E. La combinaison inhibe la prolifération des deux lignées sensibles ainsi que celle de la lignée M14R. La lignée SK28R est résistante à cette combinaison thérapeutique. Le traitement combiné diminue l'expression de MICA, ULBP2 et TRAIL-RII par les lignées SK28S alors qu'elle n'est pas altérée chez les résistantes. L'expression d'ULBP2 est diminuée par le double traitement des lignées M14S alors qu'elle augmente faiblement sur les cellules résistantes. Enfin, la combinaison entraîne une diminution de l'expression de TRAIL-RII à la surface des lignées M14S et R. Les changements d'expression des ligands NK entraînent une diminution de la dégranulation et la production d'IFN-γ par les cellules NK cependant celles-ci restent supérieures face aux lignées résistantes. Enfin, la lyse des cellules cibles par


les cellules NK n'est pas modifiée par le double traitement combiné des lignées SK28S/R et M14R. On observe une diminution de la lyse des cellules M14S par les cellules NK après le double traitement.

L'analyse des taux de ligands NK solubles présents dans le sérum de 60 patients mélanome traités par TKI ou ICB, montre une augmentation des concentrations sériques de MICA et CEACAM1 à 6 mois de traitement chez les patients ne répondant pas aux traitements. Chez les patients mutés *BRAF* et résistants aux thérapies ciblées, la concentration de PD-L1 solubles diminue à 6 mois de traitement. Chez des patients recevant des immunothérapies, les non répondeurs présentent des taux de MICA et B7-H6 solubles supérieures avant le traitement. Ces résultats indiquent que les ligands NK sériques pourraient représenter des biomarqueurs pronostiques utiles pour suivre les patients traités par TKI et/ou immunothérapies. Ils soulignent l'intérêt des cellules NK dans l'immunosurveillance des mélanomes et des stratégies thérapeutiques ciblant ces effecteurs.

	SK28	SK28R		M14	M14R
NKG2D-L	MICA ++ ULBP2 ++ sULBP2 +	MICA +++ ULBP2 +++ sULBP2 +		MICA +/- ULBP2 +++ sULBP2 +	MICA + ULBP2 ++ sULBP2 ++
CEACAM1-L	CEA-CAM1 +++ sCEA-CAM1 +++	CEACAM1 +/- sCEACAM1 +		CEACAM1 + sCEACAM1 ++	CEACAM1 +/- sCEACAM1 +
NKp30-L	B7H6 ++ sB7H6 +	B7H6 +++ sB7H6 +++		B7H6 ++ sB7H6 +/-	B7H6 + sB7H6 +
KIR-L	HLA-A,B,C +++	HLA-A,B,C +++		HLA-A,B,C +++	HLA-A,B,C +++
NKG2A-L	HLA-E +	HLA-E ++		HLA-E +	HLA-E ++
LAG-3-L	HLA-DR +/-	HLA-DR +/-		HLA-DR +	HLA-DR +++
PD-1-L	PD-L1 ++ PD-L2 +	PD-L1 ++ PD-L2 ++		PD-L1 + PD-L2 +	PD-L1 ++ PD-L2 +
LFA-1-L	ICAM1 +++	ICAM1 ++		ICAM1 +++	ICAM1 ++
TRAIL	TRAIL-RI + TRAIL-RII ++	TRAIL-RI + TRAIL-RII +++		TRAIL-RI + TRAIL-RII +++	TRAIL-RI + TRAIL-RII +++
Fas-L	Fas +	Fas +		Fas +	Fas +++

Tableau 4 : Expression des ligands (L) des récepteurs NK par les lignées de mélanome sensibles et résistantes au vemurafenib. +/- : pas d'expression ou faible expression (0-10%). + : faible expression (10-20%). ++ : expression moyenne (20-60%). +++ : forte expression (60-100%).

BRAF inhibitor resistance of melanoma cells triggers increased susceptibility to natural killer cell-mediated lysis

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ABSTRACT

Background Targeted therapies and immunotherapies are first-line treatments for patients with advanced melanoma. Serine–threonine protein kinase B-RAF (BRAF) and mitogen-activated protein kinase (MEK) inhibition leads to a 70% response rate in patients with advanced melanoma with a *BRAF*^{V600E/K} mutation. However, acquired resistance occurs in the majority of patients, leading to relapse. Immunotherapies that activate immune cytotoxic effectors induce long-lasting responses in 30% of patients. In that context, combination of targeted therapies with immunotherapy (IT) is a promising approach. We considered boosting natural killer (NK) cell tumor immunosurveillance, as melanoma cells express stress-induced molecules and activate NK cell lysis.

Methods Here we have generated vemurafenib (a BRAF inhibitor)-resistant (R) cells from *BRAF*^{V600E} SK28 and M14-sensitive (S) melanoma cell lines and investigated how resistance interferes with immunogenicity to NK cells. We determined the levels of several soluble molecules including NK ligands in 61 melanoma patients at baseline and 6 months M post-treatment with targeted therapies or immunotherapies.

Results Vemurafenib resistance involved activation of p-AKT in SK28R and of p-MEK/p-ERK in M14R cells and was accompanied by modulation of NK ligands. Compared with S cells, SK28R displayed an increased expression of natural killer group 2 D (NKG2D) receptor ligands (major histocompatibility complex class I chain-related protein A (MICA) and UL16-binding protein 2 (ULBP2)) whereas M14R exhibited decreased ULBP2. SK28R and M14R cells induced higher NK degranulation and interferon gamma secretion and were more efficiently lysed by donor and patient NK cells. SK28R showed increased tumor necrosis factor-related apoptosis-inducing ligand receptor II (TRAIL-RII) expression and TRAIL-induced apoptosis, and TRAIL-induced apoptosis of M14R was decreased. Combined BRAF/MEK inhibitors abrogated the growth of SK28S, M14S, and M14R cells, while growth of SK28R was maintained. BRAF/MEK inhibition attenuated NK activity but R cell lines activated polyfunctional NK cells and were lysed with high efficiency. We investigated the relationship of soluble NK ligands and response to treatment in a series of melanoma patients. Soluble NKG2D ligands known to regulate the receptor function have been associated to cancer progression. Serum analysis of patients treated with targeted therapies or IT indicates that soluble forms of

NK ligands (MICA, B7H6, programmed cell death ligand 1, and carcinoembryonic antigen cell adhesion molecule 1) may correlate with clinical response.

Conclusion These results support strategies combining targeted therapies and NK-based immunotherapies.

INTRODUCTION

Over the past 7 years, immunotherapy (IT) has become a reference treatment for a growing number of solid tumors. Most immunomodulatory strategies focus on enhancing T cell responses, but there has been a recent surge of interest in harnessing the underexplored natural killer (NK) cell compartment for therapeutic interventions. NK cells show cytotoxic activity against diverse tumor cell types, and some of the clinical approaches originally developed to increase T cell cytotoxicity may also activate NK cells.

NK cells are potent cytolytic effectors involved in tumor immunosurveillance. NK cells represent a major source of interferon gamma (IFN γ) in vivo and NK-derived IFN γ is crucial in priming T helper 1 responses.¹ Human CD3⁺CD56⁺ NK cells represent 5%–20% of peripheral blood mononuclear cells (PBMCs) and comprise CD56^{dim} and CD56^{bright} subsets. The cytotoxic CD56^{dim} NK cell subset predominates in blood (90% of NK cells) while CD56^{bright} NK cells, considered as precursors of CD56^{dim} NK cells and producing cytokines, represent 5%–10% of blood NK cells.² NK activation depends on an intricate balance between activating and inhibitory signals that determine whether the target will be susceptible to NK-mediated lysis.³ Three natural cytotoxicity receptors (NCRs) involved in NK cell activation were identified: NKp46 and NKp30 that bind to B7H6, expressed by resting NK cells, and NKp44, induced after stimulation by cytokines. Activation of NK cells is triggered by additional receptors. Natural killer group 2 D (NKG2D), expressed by most NK cells, binds



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major histocompatibility complex (MHC)-related antigens (MIC)-A/B molecules, and UL16-binding proteins (ULBP1–4), induced on stressed cells. NK cell activation is regulated by inhibitory receptors that recognize different human leukocyte antigen class I (HLA-I) molecules, including the killer immunoglobulin receptors, CD94/NKG2A, CD85j, and leukocyte-associated immunoglobulin-like receptors.^{4–6} Activated NK cells express tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and Fas-L and induce target cell apoptosis through engagement of death domain receptor Fas and TRAIL receptor II (TRAIL-RII).^{7,8}

Tumor cells expressing stress-induced molecules and displaying frequent alterations of HLA-I expression trigger NK cell activation. However, cancer cells have the ability to escape from NK cell recognition through the modulation of NK activating receptors, the secretion of immunosuppressive factors, the expression of inhibitory checkpoint, or the altered migration/recruitment of NK cells to the tumor.⁹ NK cells infiltrate various tumors including melanoma¹⁰ and their frequencies have been related to favorable clinical outcome.¹¹ Numbers of tumor infiltrating NK were positively correlated with regression of melanocytic lesions.¹² Thus, NK cells enter the arsenal of immunotherapies and new approaches NK-based immunotherapies are evaluated.^{13,14}

Metastatic melanoma is an aggressive disease with a high metastatic potential. For more than 20 years, therapies approved by the Food and Drug Administration for metastatic melanoma patients were high-dose interleukin-2 (IL-2) and dacarbazine (DTIC). These treatments were associated with response rates of 10%–20%, rare complete responses, and no improved overall survival. Better understanding of the genetic changes associated with melanoma development identified activating mutations in the genes encoding serine/threonine kinases BRAF and NRAS,¹⁵ establishing mitogen-activated phosphokinase (MAPK) signaling pathways as drug targetable in melanoma. Up to 50% of melanomas carry an activating *BRAF* mutation and 90% of reported *BRAF* mutations result in a substitution of glutamic acid for valine at amino acid 600 (the V600E mutation). This *BRAF* mutation constitutively activates BRAF and downstream signal transduction in the MAPK pathway. In melanoma patients bearing a V600E/K mutation, BRAF inhibitors induce rapid responses, improved overall survival, and progression-free survival but responses lack durability.^{16,17} The efficiency of BRAF inhibitors is limited by resistance mechanisms leading to disease relapse or progression in a majority of treated patients after an initial documented clinical response. Several mechanisms of resistance to BRAF inhibitors have been identified as leading to the reactivation of the downstream kinases in the MAPK pathway and the phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) pathway.¹⁸ Actual treatment relies on the combination of specific BRAF^{V600E} inhibitors with mitogen-activated protein kinase (MEK) inhibitors (either vemurafenib plus cobimetinib or dabrafenib plus

trametinib or encorafenib plus binimetinib) resulting in higher responses rates and improved survival than BRAF inhibitor alone.^{19,20}

There is growing evidence that targeted therapies likely modulate the immunogenicity of cancer cells, indicating the interest of their combinations with immunotherapies. In a previous report, we showed that treatment with vemurafenib of sensible *BRAF*-mutated melanoma cell lines was associated with change in the expression of NK ligands and decreased susceptibility to NK-mediated lysis.²¹ In the present study, we have evaluated the susceptibility to NK-mediated lysis of *BRAF*-mutated melanoma cell lines resistant to vemurafenib (BRAF inhibitor) and/or cobimetinib (MEK inhibitor). We have determined how this resistance modulates the immunogenicity of melanoma cells and discuss the consequence for combined therapies.

MATERIALS AND METHODS

Cell culture and generation of vemurafenib resistant cell lines

Vemurafenib (PLX4032/RO5185426; Genentech, South San Francisco, CA, USA), obtained from Institut Roche (Roche Scientific Partnership), was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Saint-Louis, Missouri, USA). DMSO was used as vehicle control. M14 and SK28 cell lines were provided by other laboratories (SK28 by F. Faure; M14 by H. Benlalam). Melanoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute (RPMI) media (Life Technologies, Bleiswijk, the Netherlands) supplemented with 10% Fetal calf serum (FCS) (Eurobio, Les Ulis, France) and 1% penicillin–streptomycin (Life Technologies). To generate vemurafenib-resistant variants (R), exponentially growing sensible (S) SK28 and M14 cells were initially treated with 0.1 μM of vemurafenib. At confluence, cells were subcultured and treated with an increased concentration of vemurafenib, until reaching a concentration of 10 μM. Batches of S and R cells were frozen. Tumor cells were thawed every 2 months and cultured 1 week before use in experiments. The vemurafenib-resistant cell lines, SK28R and M14R, were maintained in culture medium without vemurafenib.

Melanoma patients and serums

Sixty-one consecutive patients with advanced melanoma, receiving a first-line systemic treatment (table 1) between June 2013 and November 2017 in the Department of Dermatology of the Dijon University Hospital, and agreeing to be included in MelBase, a French multicentric cohort dedicated to the prospective follow-up of patients with advanced melanoma, were included. Serum samples were collected at baseline (T0) and 6 months after the onset of treatment and were stored at the Centre de Ressources Biologiques in Dijon. Patients were treated by either first-line targeted therapy (BRAF/MEK inhibitors) or IT (ipilimumab or anti-programmed cell death 1 (PD-1)), according to investigator's choice. Soluble (s) intercellular adhesion molecule 1 (ICAM1),

Table 1 Characteristics of the patients included and treatment (clinical stage evaluated to American Joint Committee on Cancer (AJCC) 7th edition)

Patients			
Men	27	10	1
Women	13	8	2
Median age (years)	69	61	74
Clinical stage			
IIIB	1	2	0
IIIC	13	4	0
IV M1a	4	0	2
IV M1b	11	3	0
IV M1c	11	9	1
Therapeutic treatment			
	Immunotherapy	Targeted therapy	Chemotherapy
Vemurafenib	0	2	0
Dabrafenib	0	3	0
Vemurafenib+cobimetinib	0	5	0
Dabrafenib+trametinib	0	8	0
Ipilimumab	4	0	0
Nivolumab	15	0	0
Pembrolizumab	15	0	0
Ipilimumab+nivolumab	6	0	0
Deticene	0	0	2
Fotemustine	0	0	1
Clinical responses at the time of sampling (6M)			
Complete response	7	0	0
Partial response	13	6	0
Stabilization	7	6	1
Progression	13	6	2

carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), and PD ligand 1 (PD-L1) were measured in addition to sMICA/B, sULBP1-6 (NKG2D ligands), and sB7H6 (NCR3 ligand). PBMCs from six metastatic melanoma patients (stage IV M1c, three women and three men) before treatment with immunotherapies were obtained to assess NK cell degranulation.

Sequencing

DNA purification was performed with the DNA easy blood and tissue Kit (Qiagen, Hilden, Germany). DNA concentration was measured with a NanoDrop device (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The Department of Genetics and Molecular Biology at Cochin Hospital (Paris, France) performed sequencing of BRAF and 22 known genes in the cell lines, using Ion Torrent Next-Generation Sequencing (Life Technologies) and Ion AmpliSeq Colon and Lung Cancer Research Panel (v2).

Expression of p-MEK, p-ERK, and p-AKT by western blot

Cells treated 30 min with medium containing DMSO or vemurafenib (10 μ M) were lysed and proteins were extracted using NP-40 Buffer (Invitrogen, Carlsbad,

California, USA) supplemented with sodium orthovanadate and proteases and harvested by scraping. The amount of proteins was measured by Pierce Micro BCA Protein Assay Kit (Life Technologies). Ten micrograms of proteins of each sample were electrophoretically segregated and transferred onto a nitrocellulose membrane. The primary antibodies to detect total and phosphorylated forms of ERK, MEK, and AKT (online supplementary table S1) (Cell Signaling Technology, Leiden, the Netherlands), diluted following the manufacturer's instructions, were incubated overnight at 4°C. Then the membrane was incubated with anti-rabbit IgG secondary antibody for 1 hour at room temperature. Pierce ECL Plus (Life Technologies) was used for visualization of the protein bands.

Cell proliferation using the xCELLigence system and MTS assay

Cell proliferation was evaluated with the xCELLigence System (ACEA Biosciences, San Diego, California, USA). After background measurement, cells were seeded for a 6-hour adhesion in E-plates in a volume of 100 μ L/well

at 1.3×10^4 cells/well (M14) or 1×10^4 cells/well (SK28). Then, increasing concentrations of vemurafenib and cobimetinib in medium with DMSO (0.0001%) were added. Cell proliferation was monitored every 15 min for up to 3 days by measuring impedance values, expressed as cell index (CI), normalized to the addition of the drug. CI are self-calibrated values generated by the system, calculated as $CI = (Z_i - Z_0)/15$ ohm, where Z_i is the impedance at an individual time point during the experiment and Z_0 is the impedance at the beginning of the experiment. Alternatively, cell proliferation was monitored by MTS assay. Briefly, 8×10^3 cells/well for SK28 and 10^4 cells/well for M14 were seeded in a 96-well plate for 6 hours and treated as above for 24–72 hours. Then, 20 μ L/well of combined MTS/PMS solutions (Promega, Madison, Wisconsin, USA) were added and the plate was incubated for 4 hours at 37°C. Absorbance was recorded at 490 nm.

Transcriptomic analysis

Melanoma cells were stored as dry pellets at -80°C prior to transcription analysis by GeneChip Human Transcriptome Array (Affimetrix, Santa Clara, California, USA). Transcriptomic data were analyzed with the Gene Set Enrichment Analysis (GSEA) software.

PBMC isolation and NK cell immunoselection

Blood samples from healthy donors were obtained from EFS (Etablissement Français du Sang, Hôpital St Louis). PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. NK cells were purified by negative immunoselection using the NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), leading to $>95\%$ of $CD3^-CD56^+$ cells. Purified NK cells ($1 \times 10^6/\text{mL}$) were cultured in RPMI 1640 medium (Life Technologies), supplemented with 10% human serum AB (Biowest, France) and activated with IL-2 (100 IU/mL, Miltenyi Biotec) for 6 days with medium renewal every other day.

Flow cytometry analyses

Multicolor flow cytometry assays were performed on a FACSCanto II cytometer (BD Biosciences, San Jose, California, USA) and analyzed with FlowJo software (V.10.6.2). Melanoma cells treated or not with vemurafenib and cobimetinib in medium containing DMSO (0.0001%) were labeled with antibodies listed in online supplementary table S1.

CD107a degranulation and IFN γ secretion by NK cells

Donor NK (1×10^5) cells were stimulated with 1×10^5 melanoma cells in the presence of the CD107a-FITC antibody in 200 μ L of medium containing monensin (BD Biosciences). Patient PBMCs (10^6) activated overnight with IL-2 (100 IU/mL) were stimulated with 10^5 melanoma cells in the presence of CD107a-FITC antibody. After 5 hours of coculture, cells were labeled for 30 min at 4°C with CD45, CD16, CD3, and CD56 antibodies (online supplementary table S1) and washed. Cells were analyzed on a FACSCantoII flow cytometer and CD107a degranulation

was evaluated on gated $CD45^+CD3^-CD56^+$ NK cells. Baseline NK cell CD107a degranulation was determined in the absence of target cells. Additional cocultures of 5×10^4 donor NK cells and 5×10^4 K562 or melanoma cells were conducted for 24 hours to measure IFN γ secreted by NK cells. Cell-free supernatants were harvested and an ELISA assay was carried out following the manufacturer's instructions (OptiEIA Set Human IFN γ ; BD Biosciences). In some experiments, melanoma cells were treated for 20 hours with medium DMSO (0.0001%) alone or with vemurafenib (5 μ M) and cobimetinib (10 nM) before the coculture for 5 hours with donor IL-2 + IL-12 (50 IU/mL)-activated NK cells. Then, CD107a degranulation and IFN γ production were measured by intracellular staining in the presence of brefeldin A (Sigma Aldrich), using the Foxp3 staining buffer set (Invitrogen) and IFN γ -APC antibody (Miltenyi Biotec).

NK cell-mediated lysis assay using the xCELLigence system

NK cell-mediated lysis of adherent targets was assessed using the xCELLigence system.²¹ SK28S and SK28R cells (1×10^4), and 1.3×10^3 M14S and M14R cells were seeded in the E-plate and their adhesion was monitored for 6 hours, before addition of cytokine-activated NK cells (at 1:1 ratio). In some experiments, NK cells were incubated for 20 min with mAbs to block NKG2D, NKp30, NKG2A receptors (10 μ g/mL) or control isotypes before being added to targets. To assess the impact of inhibitors, adherent targets were treated for 20 hours with medium containing DMSO (0.0001%) alone or with vemurafenib (5 μ M)+cobimetinib (10 nM) before the addition of NK cells (at 1:1 ratio). CI was measured every 15 min for 6 hours. Results are expressed as percentage of lysis determined from normalized CI (nCI, to the addition of NK cells) with the RTCA software, with the following equation: % of lysis = $[nCI(\text{no effector}) - nCI(\text{effector})]/nCI(\text{no effector}) \times 100$. Percentages of lysis were calculated taking as reference the CI from targets without NK cells.

Apoptosis assay

Apoptosis was measured by Annexin-V staining. SK28 and M14 cell lines (4×10^4 and 5×10^5 cells, respectively) were plated in 24-well plates, treated with TRAIL recombinant protein (200 μ g/mL, R&D System, Minneapolis, Minnesota, USA) or Staurosporin (1 μ M, Sigma) for 12 hours and 24 hours. Cells were washed with cold Phosphate Buffered Saline (PBS) and resuspended in staining buffer containing Annexin-V-PE (BD Biosciences), according to the manufacturer's instructions. Cells were analyzed using a FACSCantoII flow cytometer.

Soluble ligands in supernatants and serum

Cells were seeded in 12-well plates and treated for 24 or 48 hours with medium DMSO (0.0001%) alone or with vemurafenib (5 μ M) and cobimetinib (10 nM). Soluble (s) MICA, sULBP2, and sB7H6 were quantified in cell-free supernatants by ELISA following the manufacturer's instructions (Human MICA, ULBP2 or B7H6 DuoSet

ELISA; R&D Systems). For patient serum analysis, a magnetic Luminex assay (immunoassay kit, R&D systems) was devised to measure the concentrations of sICAM1, sCEACAM1, sMICA and sMICB, sULBPs (1, 2/5/6, 3, 4), and sPD-L1. The operations were performed according to the instructions of the manufacturer. The detection was done on a Luminex xMAP system (Luminex Corporation, Austin, Texas, USA) and analyzed using the ProcartaPlex software (Thermo Fisher Scientific). sB7H6 were quantified by ELISA.

Statistical analyses

Analyses of significance were performed with the Wilcoxon rank test, a non-parametric paired t test, and Mann-Whitney non-parametric unpaired t test for clinical data, using the GraphPad Prism V.7.0 software (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

RESULTS

Characterization of the paired vemurafenib S and R melanoma cells

BRAF inhibitor-resistant variants were obtained after several months of continuous culture of the cells with increasing doses of vemurafenib up to 10 μM . The genotyping of the cell lines was performed by next-generation sequencing, analyzing 22 gene transcripts. M14 bore homozygous *BRAF*^{V600E}, *TP53*, and *PIK3CA* mutations, and SK28 bore homozygous *BRAF*^{V600E}, *TP53*, *EGFR*, and *PTEN* mutations. SK28R and M14R cells displayed the same mutational status than the corresponding SK28S and M14S cell lines (online supplementary table S2).

The acquisition of resistance to vemurafenib induced distinct modulation of ERK/MEK/AKT signaling pathway. Phosphorylated levels (p-) of MEK, ERK, and AKT molecules were analyzed by western blotting (figure 1A) in cells incubated for 30 min with medium containing 10 μM of vemurafenib or DMSO. The activation of each protein (phospho/total form) ratio was calculated and the results were expressed taking as reference S cells in DMSO. The modulation of the signal in R cells and in response to vemurafenib was determined (figure 1A, right panel). Levels of p-MEK and p-ERK were not affected in SK28R cells, but SK28R cells displayed high amounts of p-AKT compared with SK28S. In M14R, amounts of p-MEK and p-ERK were increased, and similar p-AKT levels were found M14S and M14R cells (figure 1A, right panel). Treatment with vemurafenib inhibited p-MEK and p-ERK in all cells.

Cell growth of S and R cells was monitored in the presence of different concentrations of vemurafenib (0.4–50 μM). In a dose-dependent manner, vemurafenib abrogated the growth of M14S and strongly inhibited that of SK28S (figure 1B) while growth of R cells was less affected. SK28R and M14R cells exhibited increased IC50 of at least 25 μM as determined with the MTS assay, and resistance was maintained in the absence of the inhibitor in the culture for 8 weeks (figure 1C).

The comparison of the transcription profiles in S and R cells suggested an increased immunogenicity to NK cells in R cells. The gene expression study included 30905 genes. GSEA was performed to determine concordant differences between differentially expressed genes and selected gene sets from the MSigDB database. Based on comparison with curated genomic databases (Kyoto Encyclopedia of Genes and Genomes, Biocarta, Reactome, and Gene Ontology sets), a relative enrichment of pathways related to TNF α signaling, inflammatory responses, IFN γ response was significantly increased in R cells. R cell profiles were associated with higher expression of adhesion molecules like ICAM1 or VCAM1 and pro-inflammatory molecules (TNFAIP6, IL-1, and IL-6). Genes involved in migration and epithelial–mesenchymal transition, such as MCL1, SERPINE1, and TIMP1, were upregulated in R cells. Interestingly, genes related to apoptosis pathway were increased in SK28R but not in M14R cells (figure 1D, online supplementary figure S1).

Phenotypic modulation of NK ligands in vemurafenib R cells activates NK-mediated lysis

SK28R and M14R cells displayed a modified phenotype compared with their sensitive counterparts. As for signaling, the phenotype was distinctly modulated in SK28 and M14 cells. Compared with parental, SK28R exhibited strongly increased expression of MICA and ULBP2. M14R exhibited reduced ULBP2 and MICA was slightly increased but remained low. B7H6 expression maintained in SK28 S and R cells was slightly decreased in M14R cells (figure 2A). HLA-I expression was maintained and HLA-E molecules were increased in SK28R. In contrast, HLA-E was reduced in M14R, and class-II HLA-DR molecules expressed by M14S cells increased in M14R cells. SK28S and R cells exhibited high expression of PD-L1 and PD-L2. PD-L1 was induced in SK28R cells. Acquired resistance was associated with reduced expression of adhesion molecules ICAM1 and CEACAM1 by R cells (figure 2A). As shedding of MICA and ULBP molecules regulates NKG2D function, we quantified the levels of sMICA and sULBP in cell supernatants (figure 2B). Higher ULBP2 membrane expression by SK28R cells was not associated with significant increase in sULBP2 levels. In M14R cells, decreased ULBP2 and B7H6 membrane expression was associated with increased sULBP2 and sB7H6, indicating a shedding-dependent regulation of the molecule in M14R cells. Very low amounts of sMICA were detected. Strongly reduced sCEACAM1 levels accompanied the decreased membrane expression of the molecule in R cells (figure 2B, online supplementary figure S2).

To determine the effect of drug resistance on NK cell activation, we measured degranulation (evaluated by CD107a membrane expression) and IFN γ production in response to stimulation with S and R cell lines. In eight independent experiments, percentages of NK cell degranulation were increased in response to SK28R and M14R compared with SK28S and M14S, respectively (figure 2C).

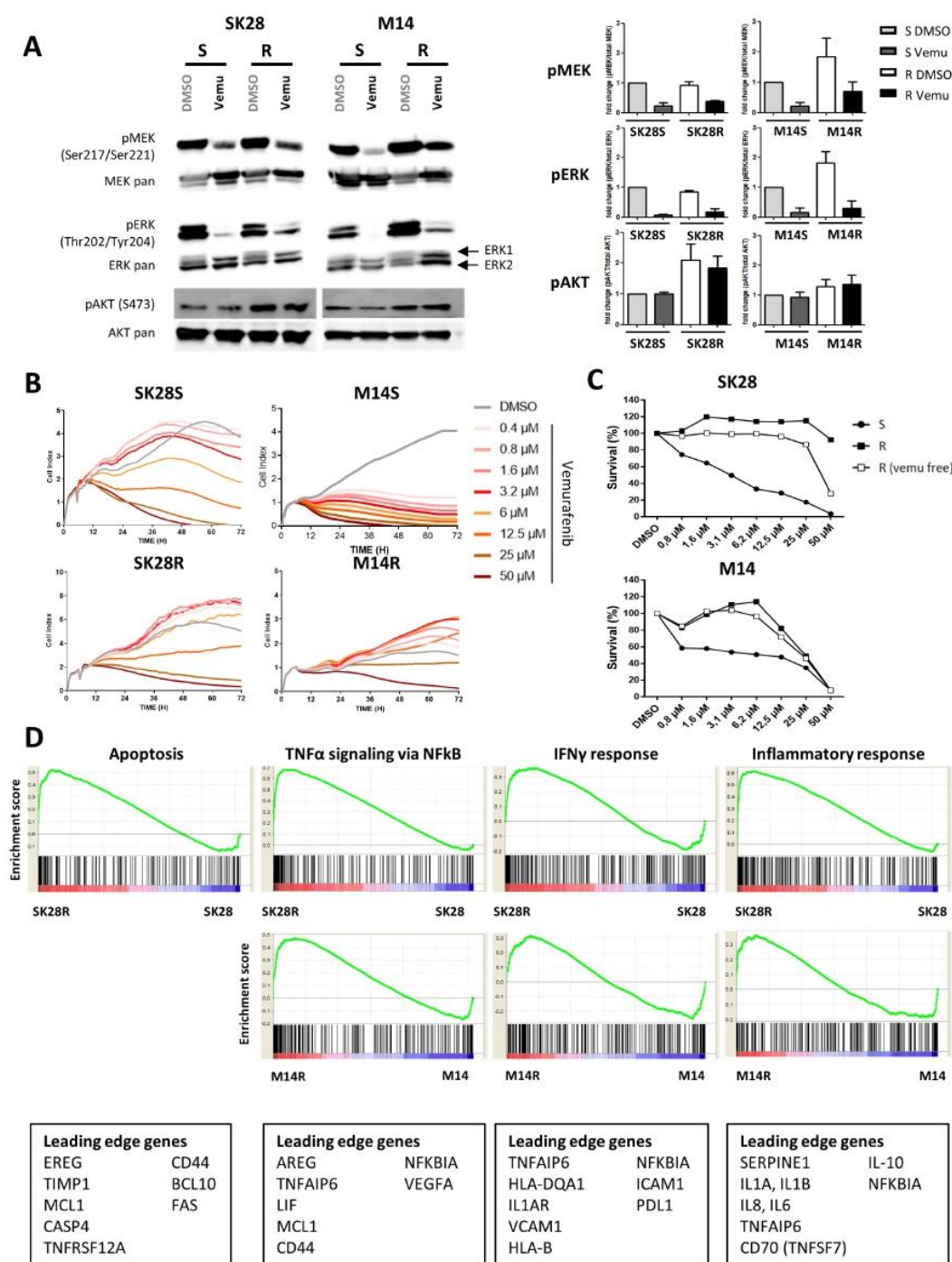


Figure 1 Characterization of vemurafenib-sensitive (S) and vemurafenib-resistant (R) cells. Activation of MAPK (ERK, MEK) and AKT pathways was determined by western blotting. Cells were incubated in medium with DMSO alone or with vemurafenib (10 μ M) for 30min to assess basal and drug-induced kinase activation (A). Signals were quantified and ratio to basal condition (S cells in medium DMSO) was calculated to express variations in R cells and/or following treatment (A, right panel). Proliferation and survival of S and R cell lines treated by increased concentrations of vemurafenib (0.4 μ M–50 μ M) for 72 hours. Cell growth was evaluated by dynamic measure of cell index by the xCELLigence technique: one representative experiment of three (B). R cells were cultured 2 months with or without drug and cell survival and IC50 values were measured by MTS assay (C). GSEA in transcription analysis of R versus S cells. GSEA enrichment plots in R cells for selected pathways related to NK immunogenicity. Each plot shows the ES of upregulated leading edge genes of each pathway. Each black bar depicts one individual gene and green line the ES of this gene (D). AKT, protein kinase B; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinases; ES, enrichment score; GSEA, Gene Set Enrichment Analysis; IFN γ , interferon gamma; MEK, mitogen-activated protein kinase; NK, natural killer.

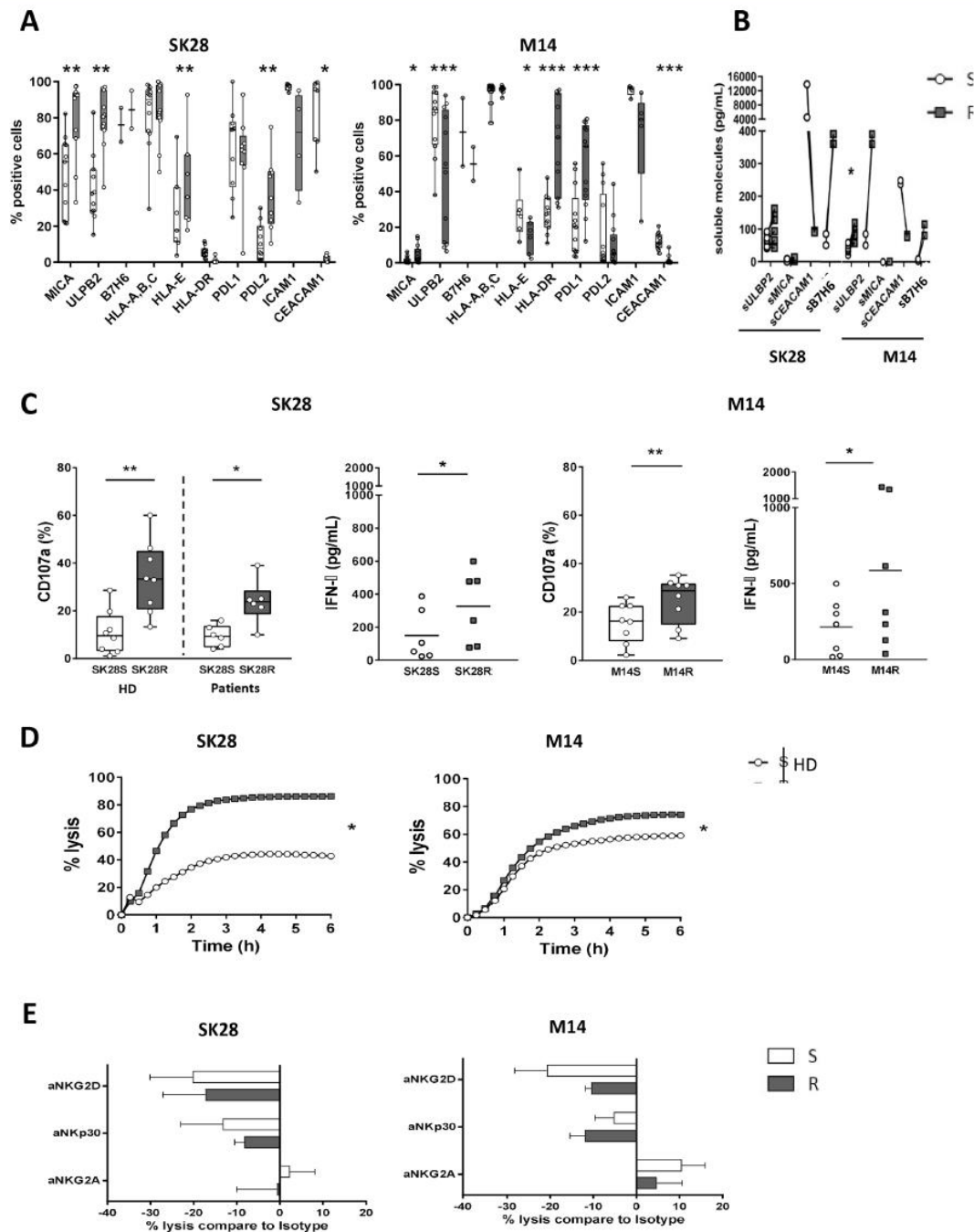


Figure 2 Phenotype of S and R melanoma cell lines is associated with increased NK cell activation. Percentages of expression of NKG2D, NKp30, and PD-1 ligands, HLA and adhesion molecules (mean values of 7–12 experiments) by S and R cells (A). Levels of sNK ligands (sMICA, sULBP2, and sB7H6) in supernatants were quantified by ELISA and sCEACAM levels by Luminex assay: levels are normalized for 10^5 cells. Functional capacities (degranulation, IFN γ production) of activated NK cells. IL-2-activated NK cells were labeled with CD107a and cocultured for 6 hours with melanoma cell lines (left panels). IFN γ production in supernatants of NK/target cocultures (24 hours) was quantified by ELISA (right panels) (C). NK-mediated lysis was assessed by dynamic measure of cell index by the xCELLigence system. Targets were seeded and let to adhere 6 hours before the addition of NK cells (ratio 1/1). Results are mean values of seven independent experiments (D). In some experiments, NK cells were incubated with anti-NKR mAbs (NKG2D, NKp30, NKG2A: $10 \mu\text{g/mL}$) for 20 min before addition to the targets: results are expressed as percentage of lysis inhibition calculated at T=300 min in the presence NKR mAbs (E). Statistical analyses were performed with the Wilcoxon rank test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). CEACAM, carcinoembryonic antigen cell adhesion molecule; HLA, human leukocyte antigen; IFN γ , interferon gamma; NK, natural killer.



The production of IFN γ by IL-2-activated NK cells was higher in response to stimulation by SK28R (mean value 350 pg/mL) than SK28S (mean value 150 pg/mL), and in M14R (mean value 600 pg/mL) compared with M14S (mean value 200 pg/mL) (figure 2C). Moreover, blood NK cells from six metastatic melanoma patients showed a higher activation toward SK28 R cells (figure 2C, right panels).

NK-mediated lysis was monitored by the dynamic decrease CI of adherent targets incubated with NK cells.^{21 22} The results from seven independent experiments showed that NK cells induced the lysis of 80% of SK28R cells 120 min post-addition of NK cells, compared with 40% of lysis of SK28S (figure 2D). There was a mean increase of 15% of M14R lysis compared with M14S at 180 min (figure 2D). Blocking of NKG2D induced a 20% reduction of lysis of SK28S, SK28R, and M14S while the inhibition of M14R NK lysis by NKG2D reached 10%. Blocking Nkp30 also reduced NK-mediated lysis of targets by 15%–20%. Blocking NKG2A increased the lysis of M14S by 10%–15% and by 5%–10% for M14R but did not affect the lysis of SK28 cell lines. Resistance was accompanied by increased NK cell activation and higher NK-mediated lysis of BRAF-resistant melanoma cells involving NKG2D, Nkp30, and NKG2A receptors (figure 2E).

Drug and TRAIL-induced apoptosis in S and R cell lines

NK activation through death domain receptor engagement also contributes to tumor cell lysis. Investigating this pathway, we first showed that vemurafenib resistance was associated with increased TRAIL-RII expression by SK28R cells. Fas was not expressed. The low expression of Fas by M14S cells was increased in M14R cells and TRAIL-RII was strongly expressed by M14S and M14R cells (figure 3A).

To assess the death domain receptor functionality, receptors were engaged by agonist TRAIL and Fas-L molecules and apoptosis measured by Annexin-V staining. Low TRAIL-induced apoptosis of SK28S was increased in SK28R cells (mean 15%–35%, n=6). Staurosporin, activating caspase-independent apoptosis, induced higher apoptosis in SK28R than in SK28S cells. In contrast, the high TRAIL-induced apoptosis of M14S (mean 65%) dropped significantly to a mean value of 25% in M14R cells (n=6) (figure 3B). Moreover, Fas did not trigger apoptosis in M14R cells (data not shown) despite increased Fas expression and staurosporin apoptosis were maintained (figure 3C). SK28R cells exhibited higher susceptibility to apoptosis than SK28S cells while M14R acquired resistance to death domain receptors apoptosis.

Phenotype of S and R cell lines treated with combined vemurafenib and cobimetinib

As the treatment of melanoma patients with a BRAF^{V600E} tumor now involves combined BRAF and MEK inhibitors, we treated S and R cell lines with 5 μ M of vemurafenib in the presence of increasing doses of cobimetinib

(5 nM–2.5 μ M) for 72 hours. Combined inhibitors abrogated the growth of SK28S and M14S cells. The growth of M14R cells exhibiting high levels of p-ERK and p-MEK (see figure 1A) was abrogated with high doses of cobimetinib (0.1 and 2.5 μ M) (figure 4A). SK28R cells in which p-ERK and p-MEK were not induced were resistant to the combination (figure 4A).

We determined the impact of combined inhibitors on phenotype of S and R cells. In SK28S cells, there was a decreased expression of MICA and ULBP2, whereas ULBP2 was induced and MICA was maintained in treated SK28R cells (figure 4B, online supplementary figure S3). Treatment abrogated TRAIL-RII in SK28S cells but not in SK28R cells. HLA-I maintained in SK28S was increased in SK28R, and HLA-E was augmented in SK28S and SK28R cells. PD-L1/PD-L2 expression was decreased in SK28S and augmented in SK28R cells. High CEACAM1 expression was maintained in SK28S and remained abrogated in treated SK28R.

BRAF+MEK inhibition decreased ULBP2 expression in M14S cells whereas it was slightly increased in M14R-treated cells (figure 4B, online supplementary figure S3). Combined inhibitors reduced TRAIL-RII expression in M14S and M14R cells. The expression of HLA-I and HLA-E was increased prominently in treated M14R cells (figure 4B). PD-1 ligands expression was not affected by the treatment (figure 4B, lower panels).

Resistant cells treated with both BRAF and MEK inhibitors maintained high NK cells immunogenicity

When the targets were treated with vemurafenib and cobimetinib (5 μ M+10 nM) for 20 hours before their coculture with NK cells, the percentages of degranulating NK cells were reduced but still higher toward R than S cell lines (figure 5A). Furthermore, SK28R and M14R cells treated or not with inhibitors induced higher percentages of CD107⁺/IFN γ ⁺ polyfunctional NK cells than SK28S and M14S cells, when cultured with IL-2 +IL-12-activated NK cells (figure 5B). Finally, the lysis of cells treated for 20 hours with BRAF+MEK inhibitors was assessed. Inhibitors reduced the NK-mediated lysis of M14S cells, while lysis of SK28S, SK28R, and M14R cells was not affected by the treatment further supporting NK immunotherapies for resistance to targeted therapies (figure 5C).

Soluble NKG2D and Nkp30 ligands in melanoma patients treated by BRAF inhibitors or immune checkpoint blockers

Resistance to BRAF/MEK inhibition is accompanied by NK ligand modulations impacting NK cell functions. We investigated whether soluble NK ligands represent surrogate markers for clinical evolution and/or response to treatment. Sixty-one patients bearing advanced melanoma (IIC to IVM1) were included: 18 BRAF-mutated (mut) patients received tyrosine kinase therapies (TKI), 40 BRAF wild-type (wt) patients received IT, and 3 received chemotherapy (table 1). Serum was harvested at baseline (T0) and 6 months (M6) after the beginning of the treatment. sMICA, sMICB, sULBPs and sPD-L1, sICAM1 and sCEACAM1 were

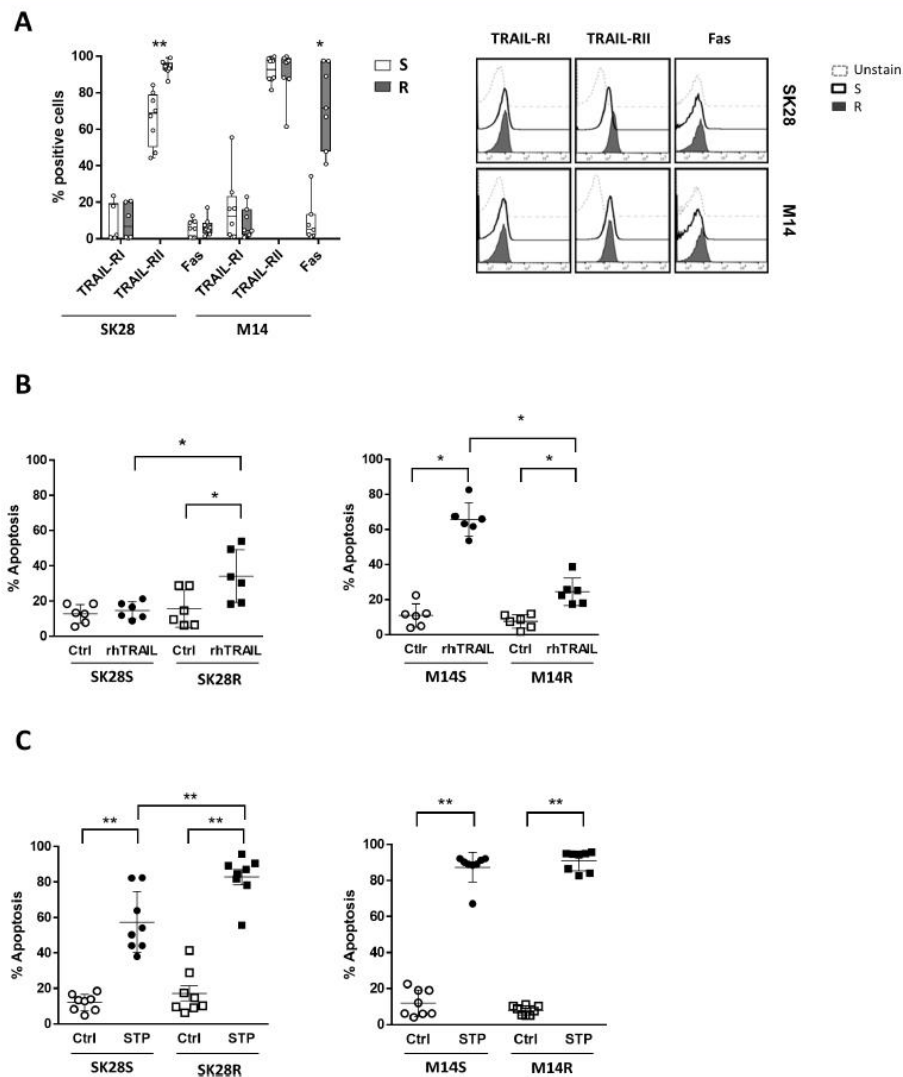


Figure 3 Apoptosis in S and R melanoma cell lines. Percentages of expression (mean values of eight experiments) and FACS plots (one representative experiment) of death domain receptors by S and R cell lines (A). TRAIL-triggered apoptosis in S and R cell lines incubated with TRAIL agonist (200 $\mu\text{g}/\text{mL}$) for 12 hours (B) and staurosporin (1 μM)-induced apoptosis (pool of 12 hours and 24 hours) (C). Apoptosis was assessed by Annexin-V staining. Statistical analyses were performed with the Wilcoxon rank test (* $p < 0.05$ and ** $p < 0.01$). FACS, fluorescence-activated cell sorting; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-RI, TRAIL receptor I; TRAIL-RII, TRAIL receptor II.

quantified in serum by Luminex and sB7H6 (NKp30 ligand) measured by ELISA. sMICA was detected in all but one patient, sMICB in 11 patients while sULBPs were present in few patients (<20). Levels of sB7H6 were detected at baseline in all but four patients showed high levels compared with donors (600 pg/mL vs 86 pg/mL). Serum sPD-L1 was detected in 52 patients, and sICAM1 and sCEACAM1 were present in all patients. At baseline, levels of sICAM1 (590 ng vs 727 ng), sCEACAM1 (54 ng vs 45 ng) were comparable in BRAFmut and BRAFwt patients (data not shown). A trend for higher sMICA was found in BRAFwt patients (192 pg vs 78 pg), while sPD-L1 and sB7H6 levels were comparable (figure 6A). We found increased levels of sCEACAM1 and sMICA levels at 6M in non-responder (stabilization and progression) patients but not in responder (complete or

partial) patients (figure 6B). In BRAFmut-TKI-treated patients, lower sPD-L1 were found at 6M in non-responders (figure 6B, left panel). In IT-treated patients, higher baseline and M6 levels of sMICA and sB7H6 were found in non-responders (figure 6B, right panel) indicating that these sNK ligands may be prognostic in response to IT.

DISCUSSION

Specific inhibitors of oncogene-driven mutations in cancer cells induce unprecedented high tumor response rates. BRAF and MEK inhibitors are widely used since BRAF mutation is present in almost 50% of cutaneous melanoma, and they induce clinical response in most of the patients. Unfortunately, resistance consequent from

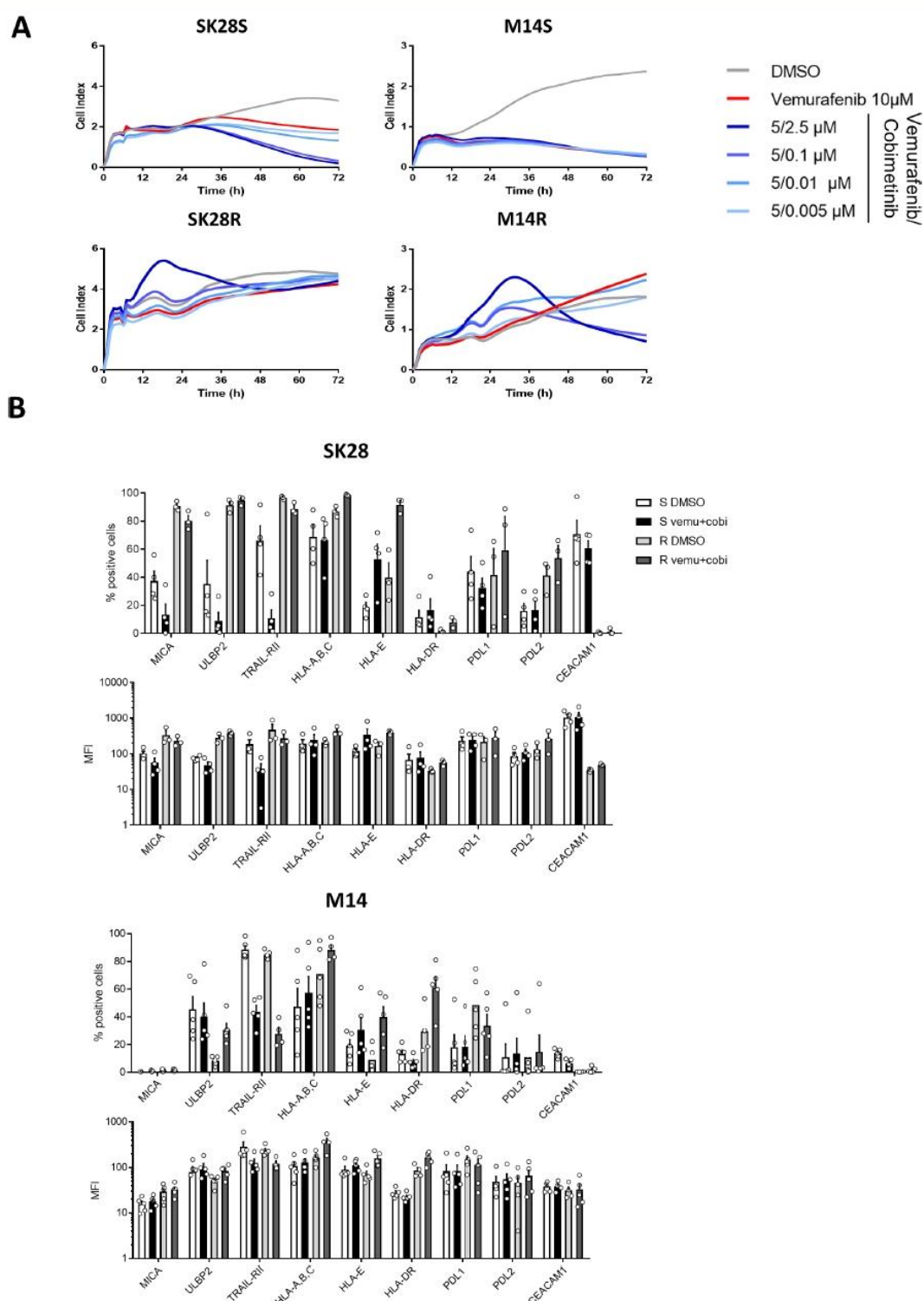


Figure 4 Growth and phenotype of S and R cells treated by combined BRAF and MEK inhibitors. Cells were cultured in medium containing DMSO (5 μ M) or vemurafenib (5 μ M) combined with growing concentrations of cobimetinib (5 nM–2.5 μ M) for 72 hours. Cell growth was assessed by the dynamic measure of cell index (A). Expression of ligands involved in NK cell/target interactions was determined in cells treated for 48 hours in medium containing DMSO (5 μ M) or vemurafenib (5 μ M)+cobimetinib (0.5 μ M): box plots summarized four experiments (B). CEACAM, carcinoembryonic antigen cell adhesion molecule; DMSO, dimethyl sulfoxide; HLA, human leukocyte antigen; MEK, mitogen-activated protein kinase; MICA, major histocompatibility complex (MHC) class I chain-related protein A; PD-L, programmed cell death ligand; TRAIL-RII, tumor necrosis factor-related apoptosis-inducing ligand receptor II; ULBP, UL16-binding protein.

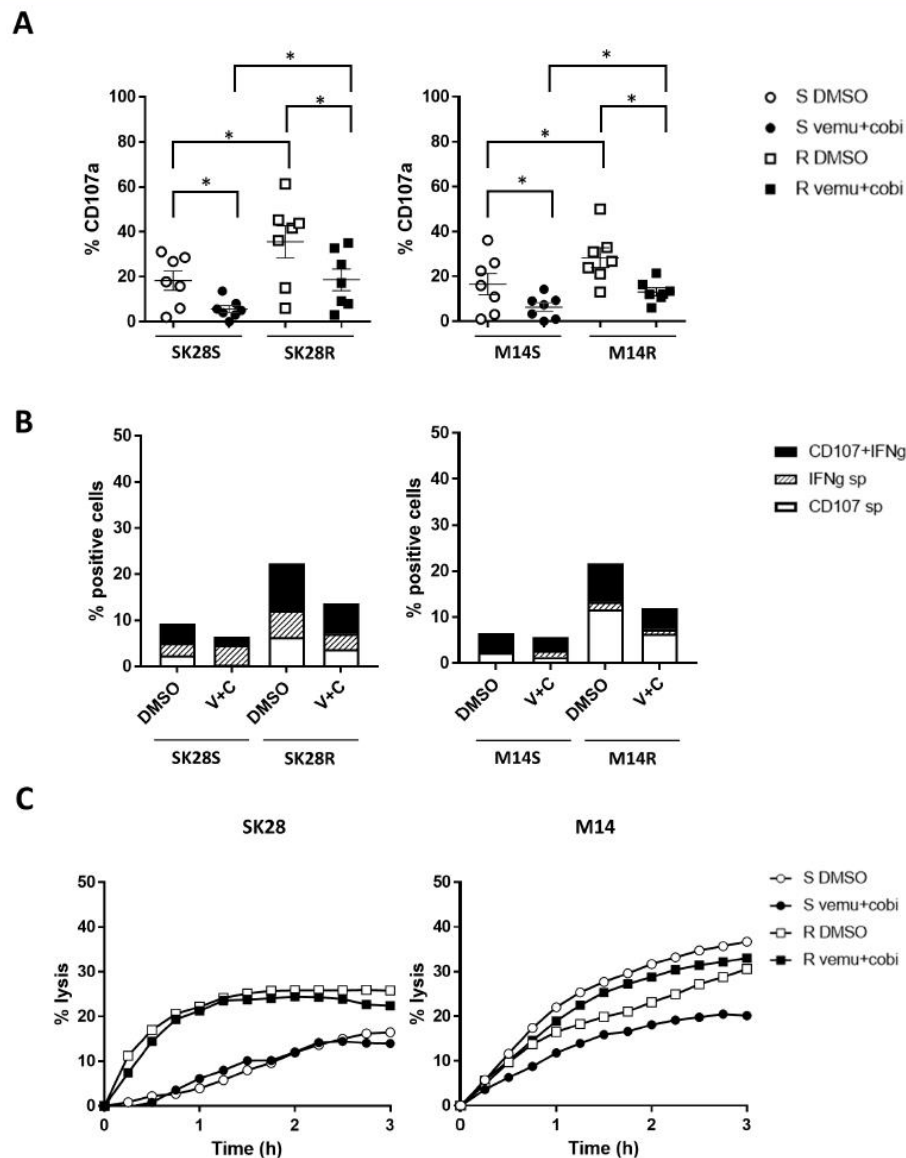


Figure 5 Impact of vemurafenib and cobimetinib treatment of S and R targets on NK cell activation and NK-mediated lysis. Targets were incubated for 20 hours with medium containing DMSO (5 μ M) or vemurafenib (5 μ M) and cobimetinib (10 nM), washed, detached, and cocultured for 6 hours with IL-2-activated NK cells. Percentages of degranulating CD107a NK cells toward S and R targets treated or not with inhibitors (n=7) (A). Detection of polyfunctional (CD107a and IFN γ) NK cells in response to targets treated with DMSO or inhibitors: IL-2 +IL-12-activated NK cells were cultured for 6 hours with targets treated or not with inhibitors (B). NK cell-mediated lysis of targets treated or not with inhibitors measured by xCELLigence assay. Targets were cultured 20 hours in medium containing DMSO (0.0001%) alone or with vemurafenib (5 μ M) and cobimetinib (10 nM). One representative experiment of three (C). Statistical analyses were performed with the Wilcoxon rank test (*p<0.05). DMSO, dimethyl sulfoxide; IFN γ , interferon gamma; IL, interleukin; NK, natural killer.

additional mutations or non-genomic changes provides alternate growth of tumor cells leading to patient relapse. A major goal, therefore, is to develop rational combinatorial approaches that exploit the specific benefits of oncogenic pathway disruption using targeted agents with the unique ability of IT to mediate long-term responses in metastatic cancers.^{23–25} Despite the fact that oncogene-driven therapeutic approaches does not take into account tumor immunology, emerging

experiences suggest that targeted therapies can turn immune-resistant tumors into immune-sensitive ones.²⁶ The impact of targeted therapies on the expression of immune regulators may directly influence antitumor immune responses. We here demonstrate that resistance of melanoma cells to BRAF (and MEK) inhibitors was accompanied by an increased immunogenicity to donor and metastatic patient NK cells. Modulation of NK ligands and sensitivity to death domain receptors

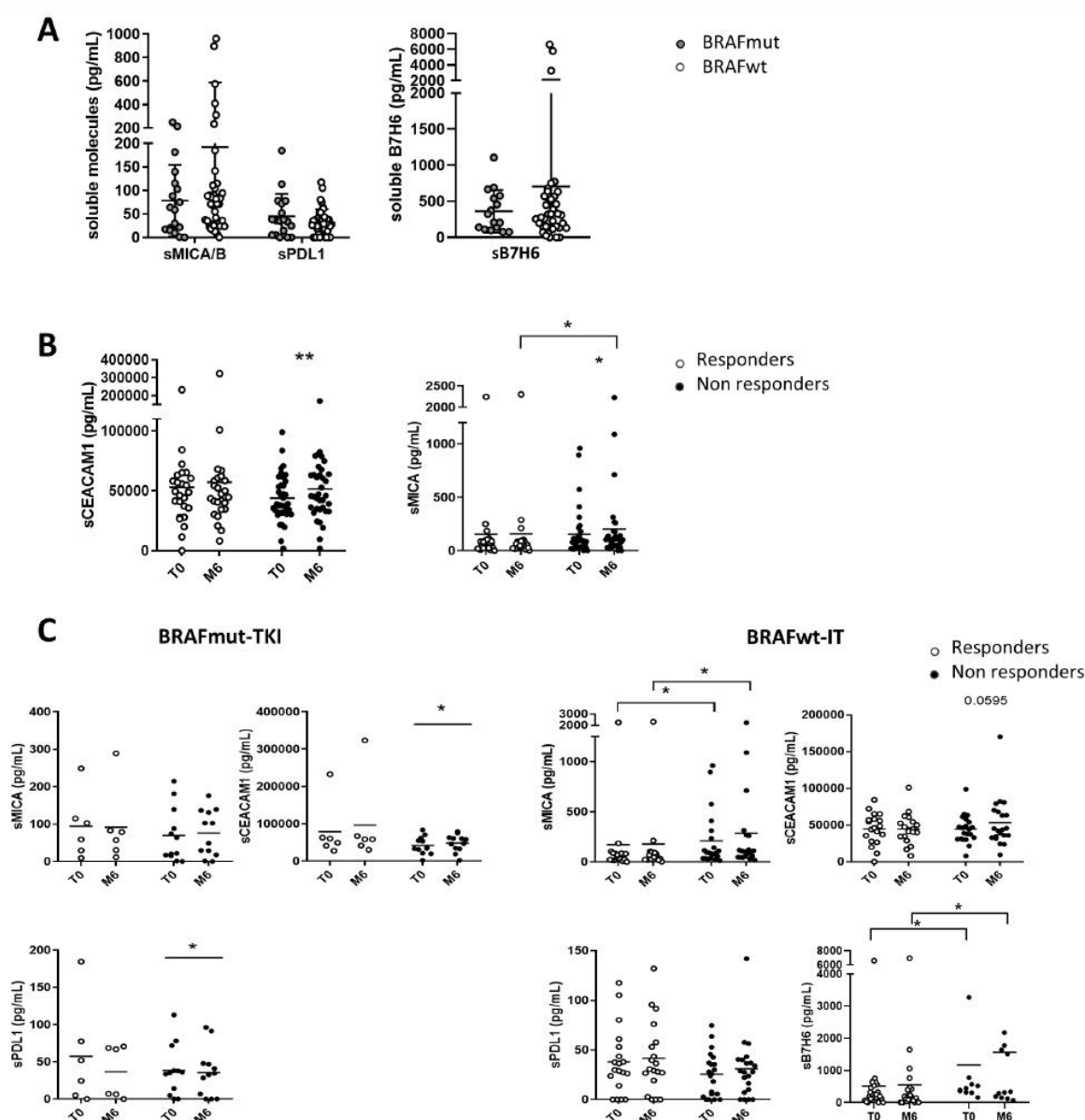


Figure 6 Quantification of soluble markers in the serum of 61 melanoma patients received target therapies or IT. Serum samples at baseline (T0) and 6 months (M6) after the onset of treatment were obtained. Soluble molecules MICA and PD-L1 were measured by a magnetic Luminex immunoassay (R&D) and sB7H6 was measured by ELISA in BRAF-mutated (mut) patients receiving TKI and BRAF wild-type (wt) patients receiving IT. Baseline (T0) levels of sMICA, sPD-L1, and sB7H6 in BRAFmut and BRAFwt patients (A). Comparison of sCEACAM1, and sMICA measured in responders and non-responders at T0 and M6 in all patients (B). Comparison of sMICA, sPD-L1, sB7H6, and sCEACAM1 at T0 and M6 in TKI (left) and IT (right)-treated patients (C). Clinical response at 6M was evaluated according to RECIST V.1.1 criteria. Analyses of significance were performed with the Mann-Whitney rank test (* $p < 0.05$). CEACAM, carcinoembryonic antigen cell adhesion molecule; IFN γ , interferon gamma; IT, immunotherapy; MICA, major histocompatibility complex (MHC) class I chain-related protein A; PD-L, programmed cell death ligand; TKI, tyrosine kinase inhibitors.

favor activation of NK cells and tumor cell lysis. However, frequent relapses in patients indicate that tumor may escape NK cells in vivo and that boosting NK cells may be require in combination with targeted therapies to potentiate the antitumor response.

First, we show that vemurafenib-resistant variants from *BRAF*^{V600E} SK28 and M14 cell lines, characterized

by different additional mutations, exhibited distinct signaling. There was an increased MEK/ERK activation in M14R, and cobimetinib inhibited their growth. In contrast, the EGFR-mutated/*BRAF*^{V600E} SK28R cells that displayed high activation of PI3K-AKT signaling (but no ERK/MEK activation) were resistant to combined BRAF/MEK inhibitor, confirming the role of EGFR mutation

in MEK co-resistance.^{27–29} Moreover, EGFR mutation was associated with higher PD-L1 expression.³⁰

Vemurafenib resistance is accompanied by a modulation of the NKG2D/NKG2DL interactions. This pathway, sensing oncogenic stress and cell transformation, is critical for tumor immunosurveillance.^{31–32} It is regulated by several mechanisms. ERK and AKT influence NKG2DLs expression on stressed cells.^{33–34} Furthermore, negative regulation of NKG2D function involves shedding of soluble NKG2DLs by tumor-derived matrix metalloproteinases (MMP), leading to downregulation of NKG2D and hampering its signaling.³⁵ In SK28R cells, resistance was associated with increased MICA and ULBP2 membrane expression that favored their NKG2D-mediated lysis by NK cells. Additionally, high expression of PD-1 ligands in SK28 cells suggests that immunotherapies targeting NKG2D and PD-1 for patients with BRAF/EGFR-mutant tumors represent an interesting option. In contrast, shedding of ULBP2 leading to increased sULBP2 may hamper NKG2D function in M14R cells, indicating that MMP may control proteolytic shedding of ligands involved in negative feedback signaling and affect NK receptors activity.³⁶

Immunogenicity of M14R cells relied on additional pathways, with augmented HLA-DR and PD-L1 expression. While HLA-DR may be considered as a progression marker,^{37–38} it was reported that HLA-class II-positive melanoma cells presented antigenic peptides, stimulated CD4⁺ T cells, and associated with response to immune checkpoint blockade, increased progression-free and overall survival, as well as CD4⁺ and CD8⁺ T cells tumor infiltrates.³⁹ Furthermore, PD-1/PD-L1 interaction score and IDO-1/HLA-DR co-expression were strongly associated with anti-PD-1 response.⁴⁰ The increased PD-L1 in M14R is in agreement with previous reports⁴¹ and it suggests that immune checkpoint blockers may be relevant for such genetic profiles.

The data from the cellular models showed that changes in membrane and soluble forms of NKL accompanied resistance to treatment and may constitute useful prognostic biomarkers. Consequently, we have conducted analyses of soluble ligands in a series of melanoma patients. We observed that sMICA/B and sB7H6 and sCEACAM1 were negatively correlated with response in melanoma patients who received target therapies or immune checkpoint inhibitors. A meta-analysis of the literature⁴² indicated that serum sMICA/B represents a prognostic marker in various cancers. In melanoma patients, sULBP2 was associated with disease progression, and sMICA was correlated with reduced survival.⁴³ Absence of sMICB and sULBP1 in baseline serum was correlated with improved survival in patients treated with IT.⁴⁴ Targeting shedding of NKG2D and NKp30 ligands improved cell-based IT.⁴⁵ Our results further indicate that baseline sMICA/B and sB7H6 values may predict response to IT.

NK cell antitumor function also involved caspase-dependent death domain receptor-induced apoptosis. Interestingly, we showed that resistance to vemurafenib promoted apoptosis in SK28R cells but not in M14R cells.

Increased TRAIL-RII expression in SK28R cells correlated with higher TRAIL-induced apoptosis. Staurosporin-induced apoptosis was also augmented in SK28R, indicating that granzyme-mediated lysis and caspase-dependent apoptosis might be involved in their increased NK immunogenicity. In contrast, death domain receptor-induced apoptosis was reduced in M14R cells. High p-ERK and MCL-1 transcripts in M14R cells (online supplementary figure S4) may protect them from TRAIL-induced apoptosis by inhibition of SMAC/DIABLO release from mitochondria.⁴⁶ The increase in anti-apoptotic molecules, XIAP and MCL-1, may be involved in their resistance to Fas-mediated apoptosis.⁴⁷ Mutation of p53 in M14R cells may concur to their death domain receptor resistance.⁴⁸ The comparative analysis of the apoptotic pathway in SK28 and M14 cell models (online supplementary figure S4) outlines their distinct regulation of TNF receptor (TNFR) pathway: increased proapoptosis TRAF3 and NFKBIA in SK28R and TNFR/TRAF1 prosurvival signaling in M14R.

We described an additional impact on immunosurveillance induced by BRAF/MEK inhibitors, showing that resistance to inhibitors favored recognition and lysis by NK cells. The above observations confirm that the immune system exert a critical contribution to antitumor responses even in the context of non-immunotherapeutic treatments.⁴⁹

CONCLUSIONS

We evidenced that beside the mainstream BRAF^{V600E}-altered pathway, the different additional mutations present in melanoma cells interfere with immunogenicity to NK cells, acquisition of resistance to targeted therapies. Thus, it may be useful to take into account accompanying mutations in addition to oncogene-driven mutations to design appropriate combinations between target therapies and immunotherapies.

Combining BRAF/MEK inhibition with immunotherapies to increase response rates is actively investigated in preclinical models and clinical trials.^{50–51} The present results also favor the interest of NK-based immunotherapies that may offer a way to balance toward immunostimulation.

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Article 2 : Les lymphocytes innées dans les ganglions métastatiques de cancer du sein

Innate lymphoid cells in breast cancer metastatic lymph node

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En préparation

Le cancer du sein est un des cancers les plus fréquents chez la femme. Les tumeurs mammaires sont très hétérogènes. Une première classification est basée sur l'expression des récepteurs à l'oestrogène (ER pour *estrogen receptor*), à la progestérone (PR pour *progesterone receptor*) et du récepteur HER2 par les cellules tumorales. Cette classification définit quatre sous-types: les positifs aux récepteurs hormonaux (70% des cancers du sein), les HER2⁺ (20-25%), les triples négatifs (TNBC) ER⁻ PR⁻ HER2⁻ (15%) et triples positifs ER⁺ PR⁺ HER2⁺. L'évolution clinique et la réponse aux traitements des patients varient dans un même sous-type. Afin d'avoir une classification plus précise, Perou et al. ont étudié l'expression génique des cancers du sein et ont identifié quatre nouveaux sous-types : ER⁺/Luminal, HER2⁺, *basal-like* et *normal-like*. Sorlie et al. divisent par la suite le sous-type luminal en deux catégories : A et B. Ainsi la classification moléculaire nommée PAM50 distingue quatre sous-types définis en fonction de l'expression de 50 gènes (Figure 14). Le sous-type luminal A, le plus fréquent, est caractérisé par une forte expression des gènes de l'épithélium luminal et les gènes ER et PR. Il est associé à un bon pronostic et une bonne réponse aux thérapies hormonales. Le sous-type luminal B présente une plus faible expression d'ER et PR en comparaison au luminal A et est associé à un pronostic intermédiaire. Les cellules tumorales HER2⁺ sont hautement prolifératives et ces tumeurs sont de mauvais pronostic. Des anticorps ciblant HER2 et des inhibiteurs spécifiques ont été développés pour traiter ces tumeurs. Le sous-type basal est caractérisé par des cellules tumorales exprimant certains gènes typiques des cellules basales. Ces tumeurs sont très agressives et de mauvais pronostic, associées à de fréquentes rechutes. Enfin, les cancers du sein TNBC sont aussi associés à un pronostic défavorable et une résistance aux traitements. En plus de la caractérisation du sous-type, le stade de la tumeur mammaire est déterminé selon la classification TNM : T pour la taille de la tumeur, N pour l'atteinte des ganglions lymphatiques drainant la tumeur et M pour les métastases à distance. Les stades vont de 0 à 4, 0 correspondant à la tumeur primaire et 4 à une tumeur ayant induit des métastases dans d'autres organes.

Figure 15 : Le cancer du sein et ses sous-types. Les sous-types intrinsèques définis par Perou et Sorlie sont basés sur l'expression de 50 gènes (PAM50). Les sous-types intrinsèques de substitution sont utilisés en clinique et sont définis par l'expression de protéines testée par histologie et immunohistochimie : les récepteurs aux œstrogènes (ER), les récepteurs à la progestérone (PR), le récepteur du facteur de croissance épidermique humain 2 (HER2) et le marqueur de prolifération Ki67. GES : signature d'expression génique. NST : Pas de type spécial. *Figure tirée de (Harbeck et al., 2019).*

Le statut ganglionnaire est un facteur pronostique de survie des patients atteints de cancer du sein et la présence de métastases ganglionnaires est corrélée à une diminution de la survie à 5 ans (To et al., 2020). Le processus de migration des cellules tumorales de cancer du sein vers le ganglion se fait via les récepteurs CXCR4 et CCR7 (Shields et al., 2007). Le ganglion est un site important pour la réponse immunitaire dans le cancer du sein et la présence de métastases dans cet organe est responsable d'un changement du microenvironnement altérant la réponse immunitaire.

La classification permet de déterminer le traitement le plus adapté au sous-type et au stade tumoral (Figure 16). Le traitement de référence est la chirurgie afin de retirer un maximum de la masse tumorale. Lors de cette opération un prélèvement du ganglion sentinelle est effectué pour évaluer la dissémination de la tumeur. En fonction du stade et de l'extension tumorale, plusieurs traitements peuvent accompagner la chirurgie : la radiothérapie, la chimiothérapie. Ces traitements peuvent être administrés en néo-adjuvant pour diminuer la taille de la tumeur avant l'opération ou en adjuvant pour éviter l'apparition de nouveaux sites métastatiques. Certaines thérapies sont spécifiques d'un sous-type donné : l'hormonothérapie pour les cancers ER⁺ et PR⁺ et les thérapies ciblées pour les cancers HER2⁺. Enfin, de nombreuses études montrent que l'infiltration de lymphocytes au niveau de la tumeur est associée à des évolutions cliniques favorables (Ali et al., 2016; Li et al., 2021; Oshi et al., 2020). Les inhibiteurs de *checkpoint* ont donc été testés dans certains sous-types. Les anticorps ciblant la voie PD-1/PD-L1 entraînent une réponse clinique chez des patients atteints de cancer du sein de type TNBC, métastatiques (Emens, 2018). D'autres immunothérapies sont actuellement en phases cliniques dans d'autres sous types de cancer du sein.

Figure 16 : Choix thérapeutiques dans le cancer du sein. La décision thérapeutique se fait en fonction du sous-type. Tous les patients ER+ reçoivent une thérapie hormonale après chirurgie. La chimiothérapie est recommandée en combinaison pour les patients présentant un risque de récurrence (envahissement des ganglions drainant). Pour les sous-types TNBC et HER2+, les patients reçoivent des thérapies néo-adjuvantes avant la chirurgie. *Figure tirée de (Harbeck et al., 2019).*

Parmi les effecteurs cytotoxiques anti-tumoraux, le rôle des ILC contre les cellules de cancer du sein est rapporté dans différentes études. Elles indiquent que les ligands des récepteurs des cellules NK sont exprimés de façon hétérogène dans les différents sous-types de cancer du sein et que des ligands NK solubles sont sécrétés par les cellules cancéreuses du sein inhibant la réponse NK (Mamessier et al., 2011a). La même équipe identifie deux sous-populations de cellules NK sanguines CD56^{bright}CD16⁺ et CD56^{dim}CD16⁻ immatures et inactives, augmentées chez les patients atteints de cancer du sein et également présentes au site tumoral (Mamessier et al., 2013). Notre équipe a montré que des populations NK activées CD56^{bright}CD16⁺NKG2A⁺ sont présentes en grandes proportions dans les ganglions drainant les cancers du sein (Frazao et al., 2019).

Les données limitées et pas toujours concordantes de la littérature sur les populations ILC dans l'invasion ganglionnaire selon le type tumoral ou le stade tumoral nous ont incitées à approfondir la caractérisation des ILC dans cet organe important de la réponse immunitaire, en tenant compte des avancées récentes sur les populations ILC auxiliaires.

Les objectifs de notre étude sont de définir les populations ILC présentes dans les ganglions mésentériques de donneur, et de comparer ces populations à celles présentes dans les ganglions axillaires métastatiques de patientes atteintes d'un cancer du sein. Nous souhaitons, avec ce projet, évaluer le rôle des ILC, cytotoxiques et auxiliaires, dans l'immunosurveillance tumorale des ganglions drainants afin d'améliorer les traitements actuels. Pour réaliser cette étude, j'ai obtenu des ganglions mésentériques de donneurs grâce à une collaboration avec le service de réanimation de l'Hôpital Saint-Louis. En parallèle, grâce à une collaboration avec l'équipe du Dr. Eliane Piaggio à l'Institut Curie j'ai obtenu des prélèvements de ganglions métastatiques et des prélèvements tumoraux de patientes atteintes de cancer du sein.

Dans un premier temps, j'ai caractérisé les ILC dans les ganglions de donneurs sains. A l'aide d'analyses par cytométrie en flux multicolores, d'analyse non supervisée de type t-SNE (pour *Visualization of t-distributed stochastic neighbor embedding*), trois populations ILC majoritaires ont été identifiées et caractérisées dans les ganglions mésentériques de donneurs : les cellules NK, les ILC3 et une population CD127⁺CD56⁺ « ILC3-like ». Le profil d'expression génique de ces populations a été

défini par qPCR multiplexe. La population CD127⁺CD56⁺ « ILC3-like » possède un profil transcriptomique proche des ILC3, mais exprime également des FT et des gènes de fonctions de type NK. De plus, après activation par des cytokines et stimulation par la cible tumorale K562, cette population « ILC3-like » est capable de dégranuler et produit de l'IFN- γ , comme les cellules NK.

A partir des prélèvements de patientes, j'ai analysé les ILC des ganglions métastatiques de patientes atteintes de cancer du sein. En comparaison aux ganglions de donneurs, la proportion de cellules CD127⁺CD56⁺ « ILC3-like » est diminuée dans les ganglions métastatiques, en faveur des cellules NK. Ces dernières présentent une diminution de l'expression des NCR et de NKG2A mais un profil transcriptomique de cellules activées. Les ILC3 sont également modulées par le TME dans le ganglion métastatique, avec une augmentation d'expression du gène du TNF- α . Le profil de résidence tissulaire observé dans les ganglions de donneurs (CD69⁺, CXCR6⁺) n'est pas retrouvé dans les ganglions métastatiques, suggérant une circulation de ces cellules en cas de processus tumoral.

Cette étude montre qu'il est nécessaire d'étudier le rôle des ILC dans l'immunosurveillance tumorale des ganglions. Ces populations interagissent avec les différents acteurs de la réponse anti-tumorale et de la réponse aux immunothérapies. Nous montrons ici qu'elles sont impactées par le TME du ganglion. La diminution des récepteurs activateurs à la surface des cellules NK pourraient favoriser la croissance tumorale. A cela s'ajoute la production de TNF- α par les ILC3 en contact avec les cellules tumorales. Les nouvelles combinaisons thérapeutiques devront prendre en compte ces données, afin de réactiver les cellules NK et peut-être inactiver les ILC3.

Innate lymphoid cells in breast cancer metastatic lymph node

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ABSTRACT

Innate lymphoid cells (ILC) are important immune regulators including Natural Killer cells and helper-type ILC involved in tissue homeostasis. Their presence and functions in human lymph nodes are still unclear, but they most likely contribute to immunosurveillance of solid tumors prone to disseminate locally. As immunotherapies become a first line treatment for several types of cancers, investigation of the innate immune cells in tumor draining lymph nodes is useful. In the present studies, we first characterized in depth innate lymphoid cells present in donor lymph nodes (HD-LNs). ILC including Natural killer (NK) cells that infiltrate LN were characterized by flow cytometry in a series of mesenteric LNs, in steady state. Mesenteric LNs contained NK cells, ILC3 and a unique CD127⁺CD56⁺ ILC subset with intermediate features of NK-cells. Tumor-draining lymph nodes (TD-LNs) are the first site of metastasis of breast cancer (BC) and an important relay of immunosurveillance that may be targeted during adjuvant or neo adjuvant therapies. We thus investigated the ILCs in tumor draining lymph nodes from breast cancer patients. Compared to HD-LNs, TD-LN exhibited decreased CD127⁺CD56⁺ ILCs and increased proportions of NK cells. The TD-LNs are infiltrated by specific CD56⁺NCR⁻ NK cells that displayed an activated transcriptomic profile. The tumor microenvironment also affected ILC3 that showed high levels of TNF α transcripts.

INTRODUCTION

Innate lymphoid cells are important players in the immune homeostasis of tissues. They include NK cells or cytotoxic ILC1, endowed with cytotoxic functions and involved in lysis of transformed cells, and helper-type ILC (hILC) that were classified in 3 groups according to helper T cells. ILC1 producing IFN γ and TNF α , ILC2 producing IL-5 and IL-13 and ILC3 producing IL-17 and IL-22 (Hazenberg and Spits, 2014). Like NK cells, ILC1 express the transcription factor T-bet, but not Eomes. ILC2 express GATA3 and ILC3 ROR γ t. While NK cells display an established role in tumor immunosurveillance, the involvement of hILC is not known. The lymph node (LN) is the primary site of tumor spread in some solid tumors, and in particular in breast cancer (BC). LN status is a prognostic factor in breast cancer patients and the presence of LN metastases is correlated with decreased 5-year survival (To et al., 2020). The LN is an important site for the immune response in BC and the presence of metastases in this organ is responsible of a change in the microenvironment that can alter the immune response. Knowing the role of tumor draining lymph nodes (TD-LN) in the dissemination of cancer cells and metastasis, we investigated the ILC populations in healthy donor LN (HD-LN) and TD-LN of BC patients. This tumor model is justified by the development of immunotherapies for BC patients with their use in adjuvant or neoadjuvant conditions.

ILC are found in secondary lymphoid organs (SLO) but few reports have described the presence of hILC in human LN. LN represents a major site of NK cell maturation. They differentiate in the bone marrow and in peripheral LN and reach circulation where they represent 5–15% of the blood lymphocytes. Two major NK subpopulations are classically described in humans: CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻ cells. The CD56^{dim} population predominates in blood (90% of NK cells), as well as at the site of inflammation, and exhibits a high cytotoxic potential, whereas CD56^{bright} NK cells are more abundant in SLO, such as LN (95% NK cells), have the ability to produce cytokines upon activation and are considered precursors of the mature CD56^{dim} counterpart (Fehniger et al., 2003). In the mouse, ILC3 is the major population in LN (Mackley et al., 2015).

NK cell activation is regulated by a balance between activating and inhibitory signals originating from activating and inhibitory receptors present at the membrane of NK cells. Inhibitory receptors recognize ligands present on healthy cells and activating receptors interact with ligands specifically upregulated on tumor cells or infected cells. Three natural cytotoxicity receptors (NCRs) are involved in activation of NK cell functions: NKp46 and NKp30, expressed by resting NK cells, and NKp44, induced after cytokine activation (Lanier, 2003; Moretta and Moretta, 2004). The NCRs are involved in the activation of NK cell lysis of BC cells (Moretta et al., 2001). ILC3 can express NCRs, and the engagement of NKp44 has been shown to activate their production of IL-22 and GM-CSF (Glatzer et al., 2013; Hoorweg et al., 2012). On the other

hand, NK cell activation is controlled by HLA class I–specific inhibitory receptors. The CD94/NKG2A receptor interacts with HLA-E molecules upregulated on tumor cells. The inhibitory receptors CD96 and TIGIT are in competition with CD226/DNAM-1 for CD155 binding and limit NK cell activation (Andrade et al., 2014; Chan et al., 2014).

NK cells can infiltrate a variety of healthy and tumor tissues (Carrega et al., 2014; Shi et al., 2011). Moreover, they can control metastasis in experimental tumor models and human tumors (Glasner et al., 2018; Guillerey et al., 2016; López-Soto et al., 2017; van der Weyden et al., 2017). However, tumor cells avoid recognition and elimination by NK cells with different mechanisms, and tumor-infiltrating NK cells often display phenotypic alterations such as NCR or NKG2D downregulation (Dahlberg et al., 2015). Therapies targeting NK cells are required to restore the control of the tumor and metastasis (Ma et al., 2013). The role of hILC in the immune response to cancer is less clear, with anti- and pro-tumor responses described in different tumor types (Crinier et al., 2019).

ILC represent an interesting targets for the immunotherapy of solid tumors. hILC can be targeted by immune checkpoint blockers (ICB). ILC2 express CTLA-4 and PD-1 in breast cancer and ILC2 and ILC3 express PD-1 in gastrointestinal cancer (Salimi et al., 2018). Immunotherapies targeting NK cells are developed and tested in clinical phase. The tumor microenvironment (TME) can have an effect on the efficacy of these therapies, in particular the immune-regulatory hILC.

In that context, we investigated ILC from HD-LN and TD-LN to improve understanding of their implication in BC tumor control and lead to development of new ILC–based strategies. ILC3 were activated in TD-LN, producing the pro-inflammatory cytokine TNF α . NK were also activated but presented a downregulation of membrane NCR expression.

MATERIALS AND METHODS

Samples and patients

This study involved 27 breast cancer (BC) patients undergoing primary tumor surgery and or LN curettage as standard-of-care surgery at Institut Curie Hospital (Paris, France). All patients were included after obtaining written informed consent. The study protocol was approved by an ethic committee "Ile de France," and the Declaration of Helsinki protocols were followed. Fragments from TD-LNs selected by the pathologist (to allow the histological analysis for the validation of the resection quality) were obtained from the operating room. Diagnosis of tumor metastasis in TD-LNs was done by the pathologist following macroscopic examination of the samples, confirmed by the core facility that performed histological examination and immunohistochemistry using anti-cytokeratin to detect isolated cancer cells. Nodal invasion by tumor cells was confirmed by CD45/epithelial cell adhesion molecule (EpCAM, clone 9C4, Biolegend) staining by flow cytometry. Patients did not receive any prior treatments other than surgery. Supplementary Table 1 summarizes the mutational status of the patients.

21 organ donor–derived mesenteric LNs were obtained from the reanimation department at Saint Louis Hospital (Paris, France) following ethical procedures and analyzed as non-cancer controls (healthy donor, HD-LNs).

Tissues were mechanically dissociated and incubated one hour at 37°C in collagenase IV, hyaluronidase and DNase I. Dissociated tissue were stored in liquid nitrogen.

Flow cytometry staining and cell sorting

Samples were first stained with Flexible Viability Dye eFluor 506 (eBioscience) for 30 min and then stained with surface antibodies (Supplementary Table 2) in brilliant Stained Buffer (BD) at 4°C for 30 min. Cells were fixed and acquired on LSRFortessa (BD).

For cell sorting, samples were depleted of lin^+ cells using the EasySep FITC positive selection Kit II (Stemcell). 100 cells per subset were sorted according to the gating strategy shown in Figure 2A and 6A.

viSNE and CITRUS analysis

Pre-gating of lin^-CD7^+ cells was done with FlowJo10. Samples were analyzed using viSNE in Cytobank, first separately and then concatenated. A clustering of the viSNE map was done using FlowSOM (Cytobank).

CITRUS algorithm (Cytobank) was used to compare LN ILC subsets abundance and markers expressions between donors and patients. CITRUS does a hierarchical clustering map based

on similarity of expression of markers. The minimum cluster size of events of the total data use was 1% and the events sampled per file were 171. The analysis was based upon abundance of events using Significance Analysis of Microarrays (SAM), with a false discovery rate of 0.01.

CD107a degranulation and IFN γ assays

Cells from LN cell suspensions were stimulated overnight in medium containing IL-2 (10ng/mL) or IL-7 (20ng/mL) with IL-12 (5ng/mL) (Miltenyi Biotec) and then co-cultured for 5 hours with K562 cells at a 1:1 effector:target ratio in U-bottom plates in presence of Golgi Stop (BD Biosciences), BrefeldinA (BD) and CD107-FITC antibody (Table S2). Cells were then stained with Flexible Viability Dye eFluor 506 (eBioscience) for 30 min and stained with surface antibodies (Table S2). For IFN γ staining, cells were fixed and permeabilized for 30 min in FoxP3 buffer (eBioscience) and stained with anti-IFN γ -APC (BD) in perm buffer for 30 min.

RT-qPCR

Cells are directly sorted in the reaction mix of the The SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). In one-step, RNA was reverse transcribed to cDNA and cDNA was pre-amplified 19 cycles using target gene Taqman primer pool, according to the manufacturer's instructions. List of Taqman probes is shown in Supplementary Table 3. After pre-amplification, reactions were loaded on a Fluidigm microfluidic chip using the Juno system (Fluidigm) and the chip was run on the BioMark real-time PCR reader (Fluidigm). All expression data are normalized to *GAPDH* and the relative quantification is calculated.

Statistics

Statistical tests were generated by Prism version 8 (GraphPad Software Inc). Nonparametric Wilcoxon matched-pairs signed-rank test was used to compare the mRNA expression between different ILC subsets. The Mann-Whitney test was used to compare the mRNA between donors and patients. Principal component analysis (PCA) was performed in R, to visualize and organize the multivariate data from donors. Correlations were done with R studio using the non-parametric Spearman correlation test. A p value < 0.05 was considered significant. Hierarchical clustering was done online at <http://www.heatmapper.ca/expression/> with Spearman rank correlation test.

RESULTS

Phenotype of innate lymphoid cells in lymph node

We assessed the proportions of ILC populations in HD-LN. ILCs are defined as lin^-CD7^+ cells and represented 1 to 2% of alive cells in the LN. To distinguish the different ILC populations we performed a viSNE analysis. Based on the viSNE map, we performed a FlowSOM clustering (Fig. 1A). The previously described combination of the backbone markers, CD127, CRTH2, CD117, CD56 and CD16 allowed the distinction of NK cells (cytotoxic ILC1) from helper-type ILCs (ILC1, ILC2, ILC3) on the viSNE map (Hazenberg and Spits, 2014; Lim et al., 2017) (Fig. 1A-B). We found four main ILC populations in the HD-LN. The NK cells defined as $CD56^+CD127^-$ cells included two sub-populations: the $CD16^+$ (10%) and the $CD16^-$ (23%). The helper-type ILCs (hILC) defined as $CD127^+CD56^-$ (24%). Within the hILCs, ILC3 ($CD127^+CD56^-CD117^+CRTH2^-$) subset were predominant (68%), while ILC1 ($CD127^+CD56^-CD117^-CRTH2^-$) constituted 32% and rare ILC2 ($CD127^+CD56^-CD117^{+/}CRTH2^+$) were detected. Finally, a unique population expressing the ILC markers CD56, CD127 and CD117 but not CD16 nor CRTH2 was identified as $CD127^+CD56^+$ (39%) in mesenteric LN. As shown on the viSNE map, NK cells from the HD-LN expressed the NCR NKp46, NKp30 and NKp44 (Fig. 1B). A fraction of NK cells also expressed NKG2D, NKG2A. Concerning hILC, a subset of ILC3 (9%) expressed NKp44 and low level of NKp46. The $CD127^+CD56^+$ cells did neither express the activating receptors NKp30, NKG2D nor the inhibitory receptor NKG2A. A subset of the $CD127^+CD56^+$ population (15%) expressed NKp44 and low levels of NKp46.

The marker of residency CD69 is highly expressed by all ILC populations in the HD-LN. CXCR6 was expressed exclusively by NK cells from HD-LN and around 52% of NK cells co-expressed CD69 and CXCR6 (Fig. 1C). A small population within the $CD127^+CD56^+$ ILC cells co-expressing CD103 with CD69 and NKp44 was detected.

NK cells, ILC3 and $CD127^+CD56^+$ ILC, expressed CD161 and DNAM-1, and only NK cells expressed TIGIT.

A specific $CD127^+CD56^+$ ILC3-like subset is present in mesenteric lymph nodes

To further characterize the unique $CD127^+CD56^+$ population, expressing some receptors in common with NK cells, the three major ILC subsets were sorted from HD-LN (n=5/6) and transcriptomic analyses were performed. Data from molecular analyses were submitted to a principal component analysis (PCA) to assess the distribution of the ILC subpopulations (Fig.

2A-B). In the PCA, CD127⁺CD56⁺ cells were more closely related to ILC3 than NK cells. The mRNA expression of the marker of definition was confirmed (Fig. 2C).

Considering the main variables contributing to PC1 and PC2, we observed that compared to ILC3, CD127⁺CD56⁺ ILC3-like cells expressed higher levels of *EOMES* and *TBX21* (coding for T-bet), two transcription factors implicated in the differentiation of NK cells (Fig. 2D). CD127⁺CD56⁺ ILC3-like cells expressed lower levels of *RORC* and *ZBT16* (coding for PLZF), implicated respectively in the differentiation of ILC3 and hILC. ILC3 and CD127⁺CD56⁺ ILC3-like cells were characterized by high levels of *AHR*, *ID2*, *JUN* and *FOS*, less expressed by NK cells.

Considering function related genes, CD127⁺CD56⁺ ILC3-like cells resembled to NK cells (Fig. 2E). They expressed the genes *PRF1* and *GZM (A, B, K and H)*, at lower levels than NK cells. Moreover, CD127⁺CD56⁺ ILC3-like cells expressed high level of transcripts coding for Th1 type cytokines *IFNG* and *TNFA*. For some samples, they also expressed the genes coding for the cytokines produced by ILC3, *CSF2* (coding for GM-CSF) and *IL22*.

In addition, we looked at the expression of some receptors involved in ILC development (Fig. 2F). *IL2RB*, *KLRD1* (coding for CD94), *KLRC1* (coding for NKG2A) and *CD244* are more expressed by CD127⁺CD56⁺ ILC3-like cells than ILC3 but less than NK cells. In contrast, CD127⁺CD56⁺ ILC3-like cells from all samples analyzed expressed the genes *IL23R* and *IL1R1*, required for ILC3 activation. Based on these data, CD127⁺CD56⁺ ILC3-like cells were considered as hILC type.

CD127⁺CD56⁺ ILC3-like cells have NK cells functional capacities

We assessed the capacity to degranulate and to produce IFN γ by NK cells, ILC3 and the CD127⁺CD56⁺ ILC3-like cells (Fig. 3). As depicted in Fig.2, ILC3 and CD127⁺CD56⁺ ILC3-like cells differentially expressed the *IL2RB* gene and may respond to IL-2 stimulation. The activation of ILCs by the target K562 following overnight stimulation with two cytokine combinations, ie IL-2+IL-12, or IL-7+IL-12, was evaluated. Stimulated with IL-2+IL-12, NK cells and CD127⁺CD56⁺ ILC3-like cells degranulated and produced IFN γ against K562 whereas ILC3 were not (Fig. 3A). With IL-7 plus IL-12, all three subsets degranulated with K562 stimulation and only NK cells and CD127⁺CD56⁺ ILC3-like cells are producing IFN γ (Fig. 3B).

These results let us hypothesize that this CD127⁺CD56⁺ ILC3-like cells population is an ILC precursor with ability to differentiate into NK cells or ILC3 depending on the microenvironment.

ILC populations infiltrating metastatic lymph node

Taking as reference the ILC populations described in mesenteric lymph nodes, we have studied the phenotype of ILC infiltrating TD-LN of breast cancer patients. We analyzed the ILC frequencies and their profiles within invaded lymph nodes obtained after surgical resection from patients with luminal BC.

In axillary TD-LN, the percentages of ILC largely varied among the samples constituting 0.5% up to 6% of $\text{lin}^- \text{CD7}^+$ cells (data not shown). viSNE analysis on both HD-LN and TD-LN, outlined significant differences in the distributions of the proportions ILCs populations in the two types of samples (Fig. 4A). In TD-LN, the percentages of $\text{CD127}^- \text{CD56}^-$ cells were increased, while the proportions of $\text{CD127}^+ \text{CD56}^+$ ILC3-like cells were decreased. The percentages of hILC were similar in TD-LN and HD-LN. We performed correlation analyses to determine the relations between the percentages of the different ILC subsets (Supplementary Fig. 2). In HD-LN, percentages of $\text{CD127}^+ \text{CD56}^+$ ILC3-like cells were inversely correlated with percentages of CD16^- and CD16^+ NK cells and percentages of hILC were inversely correlated with CD16^- NK cells (coefficient correlation > 0.7). In TD-LN, the percentages of $\text{CD127}^- \text{CD56}^-$ cells were inversely correlated with CD16^- NK cells. In TD-LN, the relationships between NK cells subsets and $\text{CD127}^+ \text{CD56}^+$ ILC3-like cells are altered, suggesting a change in the homeostatic regulation.

The viSNE map allowed the identification of the same three main populations of ILC in donor and patient LN but these populations presented different phenotype in the two types of samples (Fig. 4B). From the viSNE map, the major changes that occurred in ILCs from TD-LN was a decreased NKp46 (NKp30) expression and a dramatic decrease of CD69 expression. Additional analyses performed in few samples revealed a trend for decreased expression of DNAM-1, TIGIT and CD161 in NK cells from TD-LN (Supplementary Fig. 1). DNAM-1 seemed to be less express in hILC and $\text{CD127}^+ \text{CD56}^+$ from TD-LN as well.

In order to better characterize in depth the ILC subsets in healthy versus tumor TD-LN we performed the CITRUS (cluster identification, characterization, and regression) analysis. CITRUS algorithm is design for the discovery of statistically significant stratifying biological signatures within single cell datasets containing numerous samples across multiple known endpoints (here healthy vs tumor draining LN). First, CITRUS realize the unsupervised hierarchical clustering of the cells phenotypically similar and then identify changes in the frequencies of this clusters between healthy and tumor draining lymph nodes.

With a selection of the data obtained with a false discovery rate (FDR) of 0.01 (Fig. 5A), analysis by CITRUS identified in TD-LN a significant decrease of 6 groups of clusters and a significant increase of 4 groups of clusters. Among the decreased clusters in TD-LN, the resident NK cell subsets expressing CD56^{bright}, NCRs, NKG2A and CD69 (cluster n°2) were identified. In contrast, proportions of CD56^{bright} NCR^{low}, CD69⁻ and CD56^{dim} NCR^{low} cells (clusters n°8-9) were increased (Fig. 5B). A subset of NK cells CD16⁺ expressing NKp46, NKG2A, CTLA-4 and CD69 (cluster n°3) is decreased in TD-LN. A cluster of CD127⁺CD56⁺ ILC3-like resident subset expressing CD117, NKp44 and CD69 (cluster n°1) was decreased in TD-LN. The ILC3 resident subset CD127⁺CD117⁺CD69⁺ (clusters n°5-6) was also drastically reduced whereas a subset of NKp44⁺CTLA-4^{low}CD69^{neg} ILC3 (cluster n°10) was augmented in TD-LN.

Transcriptomic profile of ILC infiltrating metastatic lymph nodes

To further study the ILC subsets present in the TD-LN and to determine their functional status, transcriptomic analyses in purified CD56⁺CD16⁻ NK cells (comprising CD127⁺CD56⁺ ILCs), CD56⁺CD16⁺ NK cells and ILC3 (CD127⁺CD56⁻CD117⁺CRTH2⁻) from TD-LN and HD-LN were performed (Fig. 6A). The genes differentially expressed between TD-LN and HD-LN were determined. In TD-LN, CD16⁺ NK cells expressed higher levels of *NCAM1* (CD56) and *IL7RA* (CD127) (Fig. 6B). The expression of the transcription factor genes *TBX21* (T-bet), *EOMES*, *ZBTB16* (PLZF), *ID2* and *FOS*, was also increased in TD-LN CD16⁺ NK cells, indicating an activation of these cells (Fig. 6C). In TD-LN, ILC3 also presented high levels of transcription factor genes *RORC*, *ZBTB16* and *FOS*. The last one was also more expressed in CD16⁻ NK cells of TD-LN.

Concerning activating NK receptors, their expressions were higher in CD16⁻ and CD16⁺ NK populations from TD-LN, with high levels of the transcripts of the activating receptors DNAM-1, CD94, NKG2D, NKp46 and NKp30 (Fig. 6D). ILC3 didn't express high levels NK receptors genes in HD-LN. The expression of *NCR3* (NKp30) and *CD226* (DNAM-1) were higher in TD-LN. For inhibitory receptors, *TIGIT* transcript were less produced in CD16⁻ NK cells from TD-LN than in HD-LN and the opposite was found for *KLRC1* (NKG2A) transcripts. In CD16⁺ NK cells from TD-LN, *KLRG1* and *KLRC1* genes were highly expressed. The gene coding for the marker of activation CD44 was more expressed by CD16⁺ NK cells in TD-LN but was less expressed by ILC3 in the patient.

Next, to evaluate the functional capacities of ILC in TD-LN we analyzed the expression of genes involved in cytotoxicity, genes coding for cytokines and cytokines receptors known to activate ILCs functions (Fig. 6E). TD-LN CD16⁻ NK cells expressed higher levels of *PRF1*,

suggesting a higher cytotoxic capacity. This population and the TD-LN ILC3 population expressed lower levels of the gene encoding for pro-inflammatory cytokine CXCL8 (IL-8). *IFNG* was expressed by NK cells (CD16⁺ and CD16⁻) at the same levels in HD-LN and TD-LN. In TD-LN, ILC3 produced less transcripts of *IL22* and more of *TNFA* compare to HD-LN ILC3. ILC3 from HD and TD-LN expressed same amount of the pro-inflammatory cytokines genes *CSF2* (GM-CSF) and *IL32* (data not shown). ILC3 and CD16⁺ NK cells from TD-LN expressed higher levels of *IL2RB*, suggesting an advanced maturation stage. Moreover, CD16⁺ NK cells expressed higher levels of *IL18R1* in the TD-LN.

As the cytometry analyses outlined residency markers modulation in TD-LN ILC, the genes involved in the tissue residency and the migration were evaluated (Fig. 6F). Interestingly, we found that NK cells and ILC3 in TD-LN expressed higher levels of *CD69* and *SELL* (CD62L) whereas the surface expression of CD69 was lower in TD-LN. The expression of *ITGAE* (CD103) was higher in CD16⁺ NK cells from patients. Concerning CD16⁻ NK cells, the level of *S1PR5* transcripts was lower in TD-LN compare to HD-LN.

Taking this results altogether, the transcriptomic profiles of ILCs in TD-LN reflect the presence of activated cells endowed with potential antitumor capacities.

ILC in tumor compare to metastatic lymph node

We compared ILC present in the breast tumor tissues and in the TD-LN. We observed a higher frequencies of lin⁻CD7⁺ ILC in the tumor compare to the lymph node, from HD or from patients (Fig. 7A). Moreover, viSNE analyses allowed to compare the phenotype of ILCs and to confirm the possibility of the migration of these cells from the tumor to the draining lymph node. The prominent NK cell subset found in the TD-LN compare to the HD-LN (NKp46⁻CD69⁻) was present in the tumor, suggesting a migration to the LN (Fig. 7B). Tissue resident NK cell, CD127⁺CD56⁺ ILC3-like and ILC3 subsets were present in the tumor, expressing high levels of CD69.

DISCUSSION

In the present studies we investigated the ILC populations in donor LN and tumor draining LN from breast cancer patients. First, we described three main ILC populations in the lymph node at steady state. These populations correspond to NK cells, ILC3 and an original CD127⁺CD56⁺ ILC3-like population. This latter presented a transcriptomic profile sharing similitudes with ILC3 and NK cells.

In TD-LN these three populations were present but in different proportions than in donor LN. Moreover, ILC populations, presenting an activated transcriptomic profile, were impaired in TD-LN, particularly the NK cells that exhibited downregulation of the surface expression of NCRs.

Lymph nodes are important sites of maturation and differentiation of ILC. In SLO and in particular in LN, NK developmental intermediates are present. Stage 3 NK cells have been reported in human SLO as CD34⁻CD117⁺CD127⁺CD56⁺CD94⁻, producing IL-22 and expressing the transcription factor AHR (Hughes et al., 2009, 2010). Some studies showed that this population include ILC3 (Freud et al., 2014). Stage 3 NK cells and ILC3 indeed display phenotype similarities. In our studies, we described CD127⁺CD56⁺ ILC3-like cells of the mesenteric LN that expressed both the genes coding for the transcription factors required for NK cell maturation (T-bet and EOMES) and the ones implicated in ILC3 maturation (ROR γ t and AHR). Moreover, this population expressed the genes of the cytotoxic molecules perforin and granzymes, and Th1 type cytokines and the ILC3 signature cytokines GM-CSF and IL-22. In response to stimulation with K562, CD127⁺CD56⁺ ILC3-like cells degranulated and produced IFN γ as NK cells. CD127⁺CD56⁺ ILC3-like cells expressed higher transcripts levels for *KLRD1* (CD94), *IL2RB* and *CD244* (2B4) compared to ILC3, but less than NK cells. Thus CD127⁺CD56⁺ ILC3-like cells are related to both NK cells and ILC3. A similar population has been described in tonsils and demonstrated some NK functionalities, such as production of IFN γ , TNF α and moderate cytotoxic capacities (Crellin et al., 2010). In steady state, AHR has been identified as a transcription factor preventing the differentiation of stage 3 CD34⁻CD117⁺CD94⁻ NK cells or IL-1R1^{hi} ILC3 to mature NK cells, producing IFN γ and having lytic capacities (Hughes et al., 2009). It noteworthy that the high expression of *AHR* could prevent the differentiation of CD127⁺CD56⁺ ILC3-like cells from the LN into NK cells or ILC3.

Interestingly, the frequencies of ILC populations were dramatically changed in TD-LN. The CD127⁺CD56⁺ ILC population was shrunked in the TD-LN in favor of NK cells. The tumor microenvironment can induce phenotypic or functional changes in the ILC population through different mechanisms. The CD127⁺CD56⁺ ILC could differentiate into NK cells in response to cytokine produced in a metastatic environment. In particular, several cytokines are known to influence the ILC3 to ILC1 plasticity. IL1- β , produced by DC, expands and preserves IL-1R^{hi} IL-22⁺ AHR⁺ immature NK cells in tonsils (Hughes et al., 2010). On the other hand, NKp46⁺CD127⁺ROR γ t⁺ cells can produce IFN γ in response to IL-2 and IL-12 (Bernink et al., 2013; Cella et al., 2010). In the mouse, lymphoid RORC⁺ ILC are able to suppress tumor growth after IL-12 activation and acquired an NK-like phenotype with an up-regulation of T-bet and NK cell functions (Nussbaum et al., 2017; Vonarbourg et al., 2010).

A switch toward NK cells may indicate an ongoing tumor immunosurveillance in the TD-LN as NK cells are key players in the control of metastasis. High numbers and functions of NK cells is correlated with the absence of metastasis in some solid tumors (López-Soto et al., 2017). However, NK cells were likely altered by the tumor microenvironment with altered activating receptor expression and alteration of their functions. Blood and intra-tumor NK cells from breast cancer patients presented decrease of activating receptors and were non-cytotoxic (Mamessier et al., 2011, 2013). In mice, low NKp46 expression was found in intra-tumor NK cells in a breast cancer model (Krneta et al., 2016). Our present results indicated an alteration of the CD56⁺CD16⁻ NK cells from TD-LN with low expression of NCR and NKG2A at the surface despite high levels of the corresponding transcripts. Higher levels of *PRF1* in TD-LN than HD-LN NK cells was also found. As this results may reflect the presence of higher proportions CD127⁺CD56⁺ ILC3-like in HD-LN than TD-LN, the sorting of the CD127⁺CD56⁺ ILC3-like cells in donors and patients will allow their comparative analyses.

We previously showed that NKp46 was reduce on NK cells from metastatic lymph nodes compare to non-invaded LN of breast cancer patients (Frazao et al., 2019). This previous findings emphasized the infiltration by CD16⁺NKG2A^{high} NK cells in the TD-LN not noticed in the present studies with the CITRUS analysis. This discrepancy may have different explanations. NK cells were defined as CD45⁺CD3⁻CD56⁺ cells without taking into account other ILC populations and thus the different gating strategy of NK cells explained the differences observed between the two studies. Nevertheless, the transcriptomic results of our study showed that TD-LN CD16⁺ NK cells highly expressed the genes of the activating receptors (NCR, NKG2D, DNAM-1), the inhibitory receptors (KLRG1, NKG2A), the markers of activation (CD44, CD69) and the cytokines receptors (IL2RB, IL18R1) demonstrating an activation of these cells and a potential anti-tumor response.

Helper-type ILC residing in the LN are mostly ILC3 (CD127⁺CD56⁻CD117⁺). In the HD-LN, a subset of ILC3 expressed NKp46 and NKp44. Interestingly, we noticed a low expression of *PRF1* and *IFNG* by HD-LN ILC3. As resident in the LN, these ILC3 may participate to the local anti-tumor immune response and the control of metastasis. The changes observed (increased *TNFA* and decreased *IL22*) in the ILC3 in the patients LN may reflect the dissemination of the tumor cells in the LN. The production of TNF α by ILC3 has been shown to be activated by lung tumor cell lines and NKp44 is the receptor involved in this activation (Carrega et al., 2015). NKp44 is particularly implicated in the production of TNF α and not IL-22 or GM-CSF for which the production is activated by cytokines (IL-23, IL-1 β) (Glatzer et al., 2013). TNF α is a pro-inflammatory cytokines found in breast cancer patient and known to promote tumor growth, to enhance migration and invasion of BC cells, and to participate to chemotherapy resistance

(Cruceiru et al., 2020). Anti-TNF α therapies are tested in breast cancer and the blockade of ILC3 could synergize with these current therapies.

In HD-LN, tissue resident ILC expressed high levels of CD69, and CXCR6 for NK cells. The CD69⁺CXCR6⁺ NK cell subset was found in lymphoid tissue, producing IFN γ , perforin and granzyme B after activation (Lugthart et al., 2016). In the TD-LN there was a dramatic decrease of these CD69⁺ resident ILCs, confirming our previous report (Frazao et al., 2019). Interestingly, the gene *CD69* is highly expressed by TD-LN ILC contrasting with the membrane expression. CD69 is involved in the retention of lymphocytes in the LN with S1PR1 and the regulation of the surface expression of CD69 is associated with S1PR1 (Cyster and Schwab, 2012). There was no difference in *S1PR1* gene expression in favor of a post-transcriptional regulation. In TD-LN, the expression of CD69 might be prevented from the surface by S1PR1 implying a possible circulation of ILCs (Shiow et al., 2006). It may be hypothesized that ILC populations in TD-LN may represent circulating from the tumor to the TD-LN. CD16⁻ NK cells from the TD-LN expressed low level of *S1PR5* compare to HD-LN, arguing against the migrating of this population in the tumor context. The expression of S1PR5 in the surface can also be regulated after transcription. NK cells are known to traffic from the tissues to the lymph node in a CD62L, CCR7-dependant manner (Castriconi et al., 2018). In the mouse, ILC3 are able to traffic from the intestine to the draining mesenteric lymph node in a CCR7 dependent way (Mackley et al., 2015). The NKp46⁻CD69⁻ NK cells identified at the tumor site and in the TD-LN may correspond to such tumor derived ILC migrating to TD-LN. Alternatively, the tumor cells invading the LN could induce the same phenotype than in the primary tumor.

The TD-LNs are one of the first site of metastasis in breast cancer. The dissemination of tumor cells in the LN modify the microenvironment (Sleeman, 2015). Our findings are in favor of the activation of NK cells and ILC3 and their phenotypic changes induced by metastatic invasion of the TD-LN. Current immunotherapies have been developed to restore mostly T cells in at the tumor site. These therapies now administered as adjuvant or neo adjuvant conditions, can impact on T cells and other cells in lymphoid tissue and especially in the LN, key organ in the activation of the immune response. In mice, TD-LN are essential in the response to PD1/PDL1 blockade therapy (Fransen et al., 2018). Moreover, loco-regional administration of ICB, targeting delivery to the TD-LN, enhances the efficacy of the treatment (Francis et al., 2020). In the LN, ILC are localized at the T-B interface and can interact with T cells, possibly influencing their responses to immunotherapies. Tumor invasion of the LN may impair NK cells, decreasing their NCR membrane expression and their functions. However they presented an activated transcriptomic profile implying that with the right signal they could be activated, if the local immunopressive factors are bypassed. NK-based immunotherapies should therefore be

considered in combination with other immunotherapies. On the other hand, ILC3 activation in the TD-LN may induce a pro-tumor immune response with the production of $\text{TNF}\alpha$, in agreement with a report showing that $\text{ROR}\gamma\text{t}^+\text{ILC3}$ correlated with LN metastasis in BC (Irshad et al., 2017). Blockade of ILC3 activity should also be consider in combination with current therapies.

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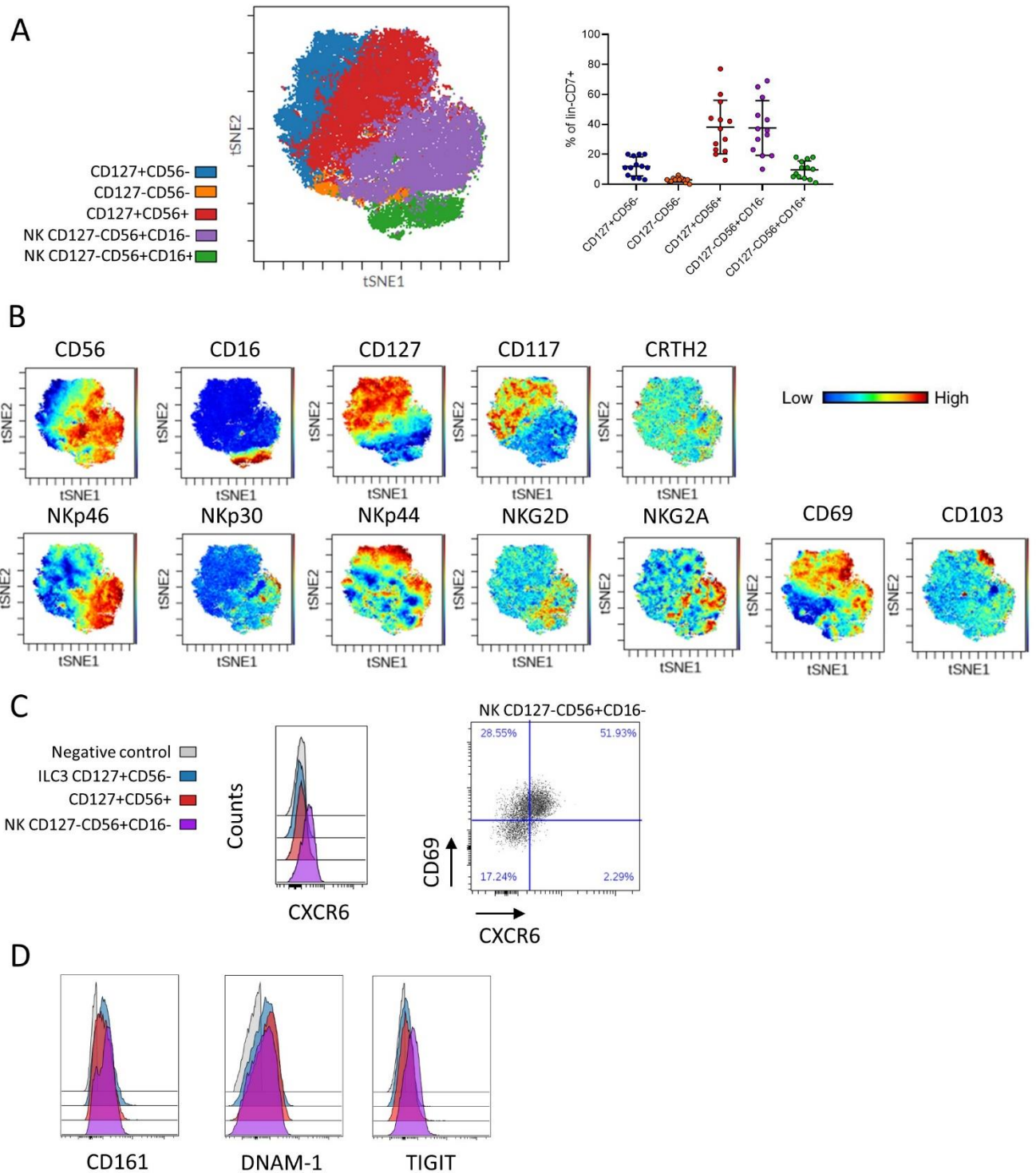


Figure 1. Innate lymphoid cells in donor lymph nodes. viSNE map of lin-CD7⁺ cells from donor lymph node, showing the FlowSOM clustering of ILC subsets, each color representing a subset. Percentages of ILC populations in HD-LN (n=13) (A). viSNE map with expression of different markers using a color scale, from blue (low) to red (high) (B). Histograms representing expression of CXCR6 and dot plot representing CD69 and CXCR6 expression in NK cells (C). Histograms representing the expression of CD161, DNAM-1 and TIGIT in all donors (D).

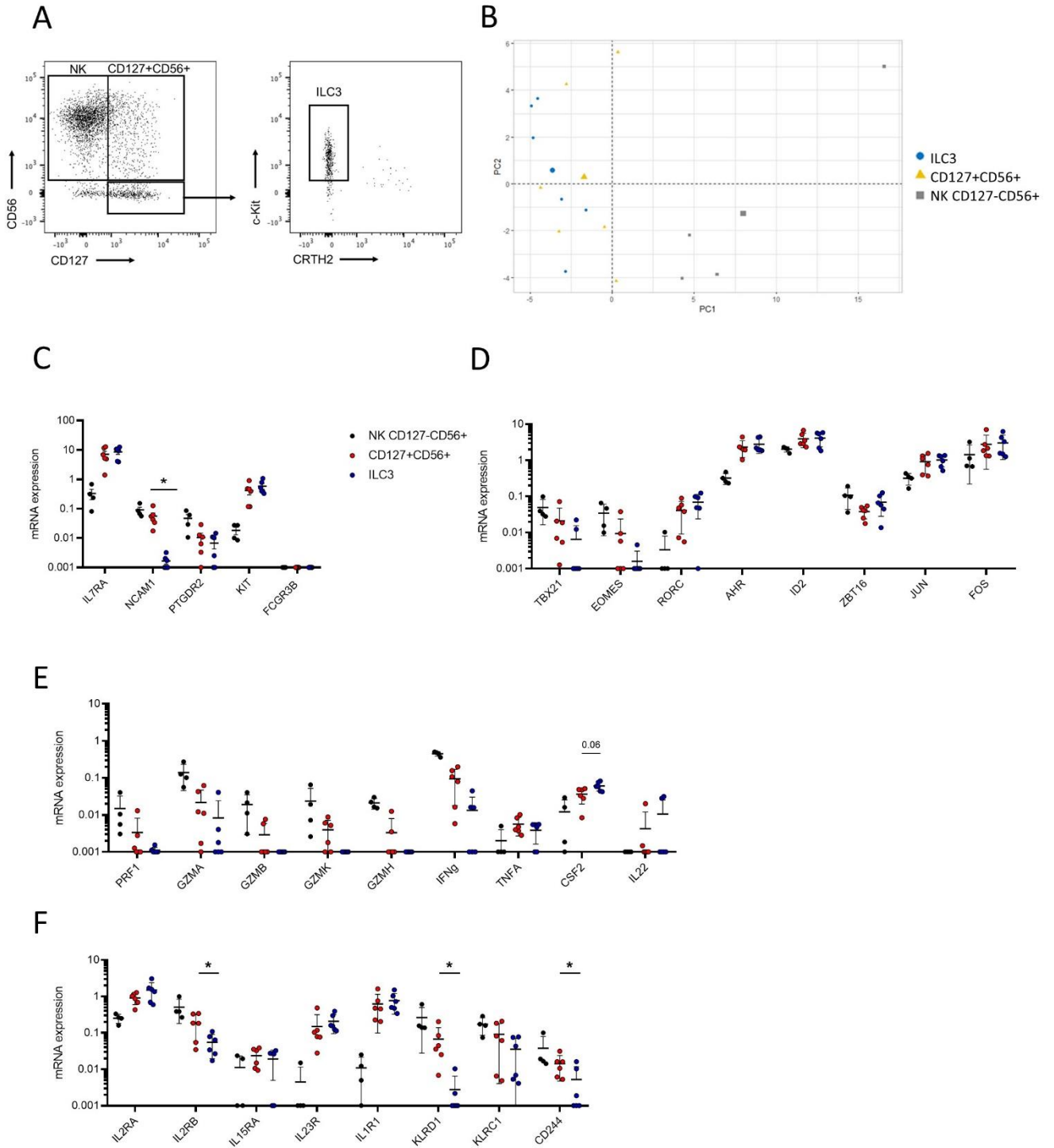


Figure 2. The transcriptional signature of ILC in the donor lymph node. Gating strategy for cell sorting of ILC subsets (A). Principal component analysis (PCA) of ILC subsets (n=5/6) (B). mRNA expression of the principal variables contributing to PC1 and PC2 including definition markers of the ILC subsets, transcription factors, cytotoxic molecules, cytokines, cytokines receptors and markers involved in NK differentiation. Wilcoxon matched-pairs test was used to compare the expression between two populations (* p<0,05)(C-F).

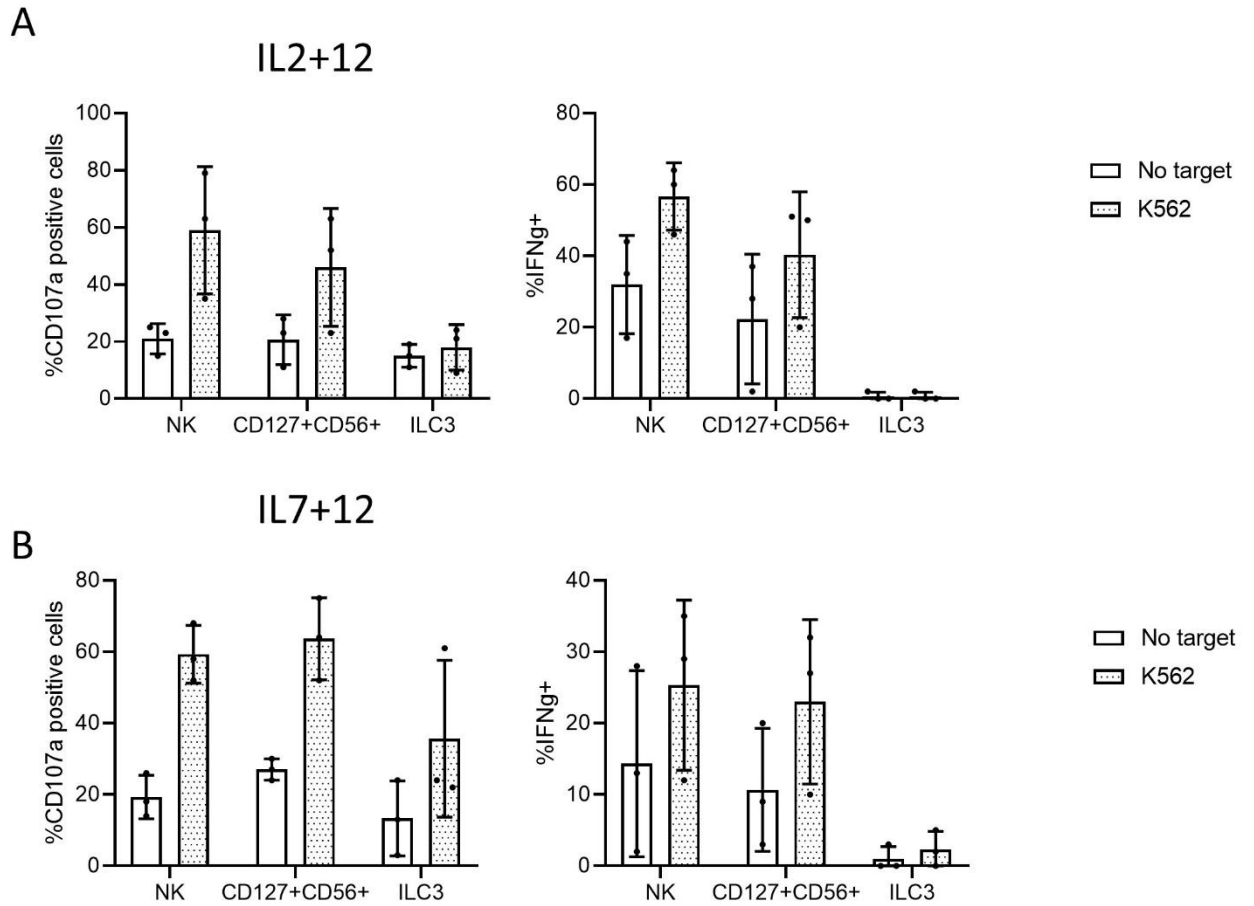


Figure 3. Degranulation potential and IFN γ production of ILC in HD-LN. Percentages of degranulation (CD107a positive cells) and intracellular IFN γ of ILC subsets without target and with K562. ILC were activated overnight with IL-2 and IL-12 (A) or IL-7 and IL-12 (B) (n=3).

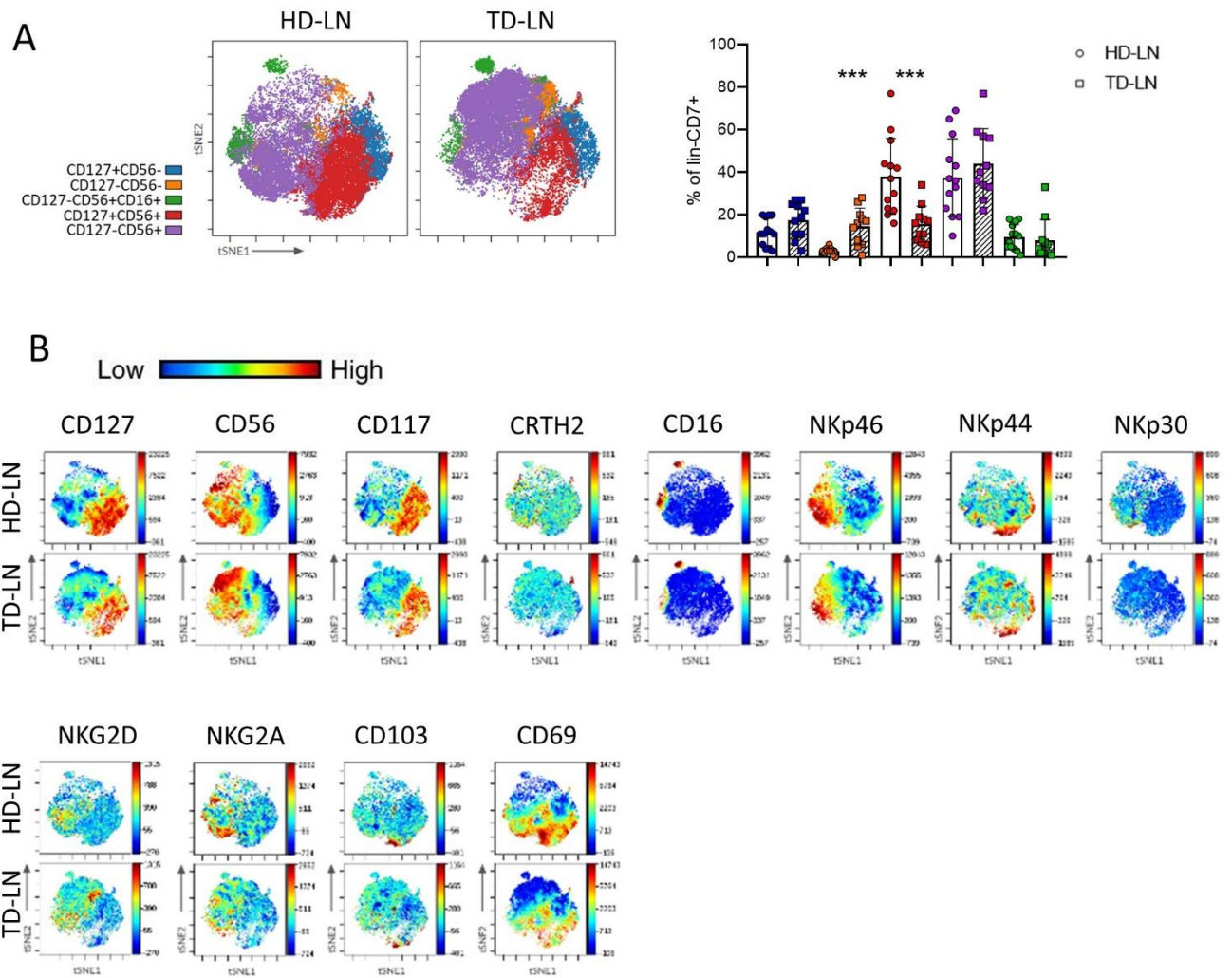


Figure 4. ILC in TD-LN compare to HD-LN. viSNE map of lin⁺CD7⁺ cells concatenated from HD-LN (n=13) and TD-LN (n=11), showing the FlowSOM clustering of ILC subsets, each color representing a subset. Percentages of each subsets in HD and in TD-LN. Mann Whitney test was used to compare the percentages between HD-LN and TD-LN (***) p<0,005 (A). viSNE map comparing ILC from HD-LN and TD-LN, with expression of different markers using a color scale, from blue (low) to red (high) (B).

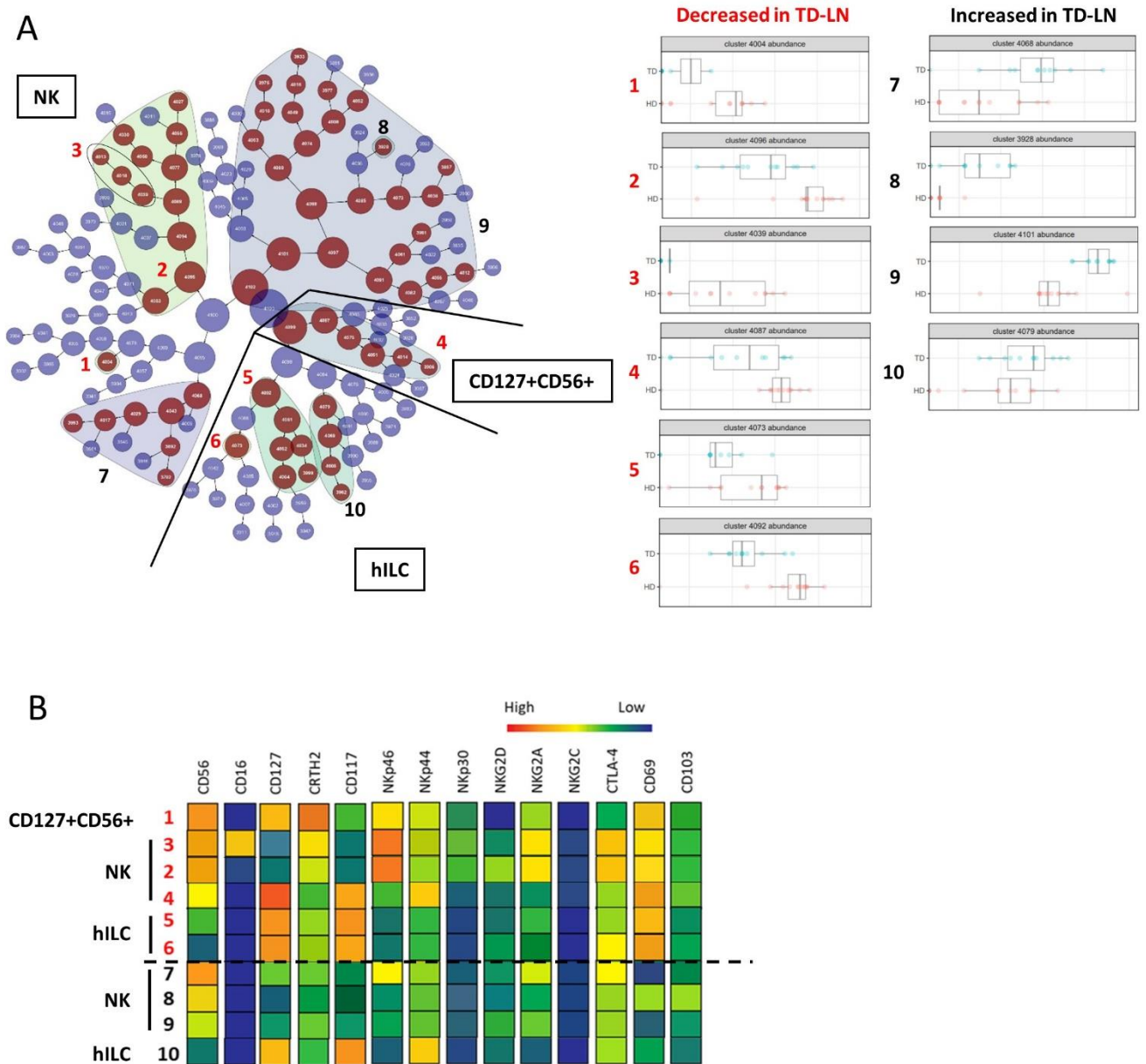


Figure 5. Citrus analysis of ILC abundance in TD-LN compare to HD-LN. Hierarchical clustering map of the CITRUS analysis comparing TD-LN (n=11) and HD-LN (n=12). In dark red, clusters whose cell abundances are different between the two conditions. Ten groups of clusters are noted. Cell abundance in the different clusters is represented on the left side and clusters for which the cell abundance is lower in TD-LN are in red (A). Heatmap presenting the phenotype of the ten clusters (B).

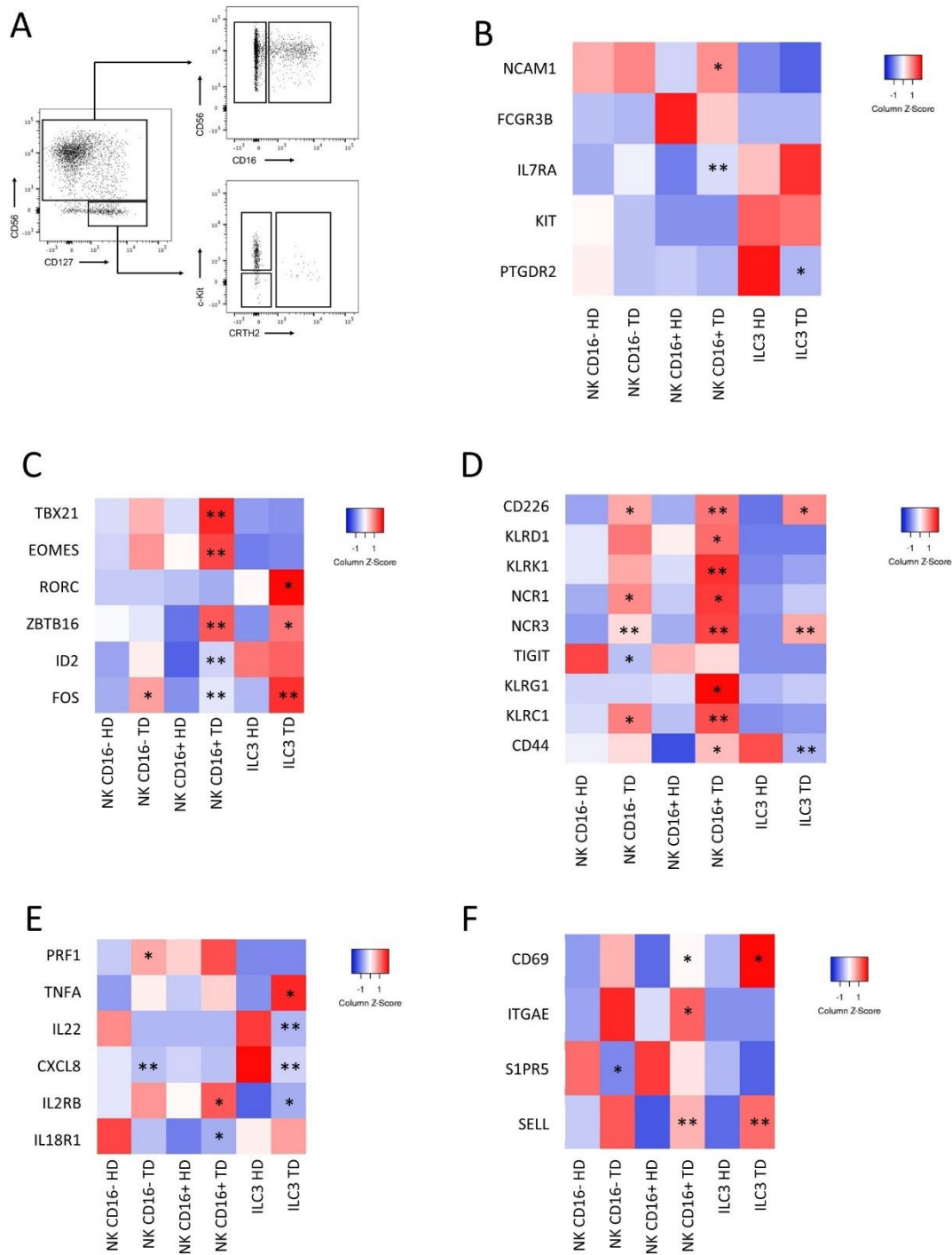


Figure 6. Transcriptomic profile of ILC in TD-LN compare to HD-LN. Gating strategy for cell sorting of ILC subsets (A). Heatmaps of genes differentially expressed between TD-LN (n=5) and HD-LN (n=8), including definition markers of the ILC subsets, transcription factors, NK receptors, cytotoxic molecules, cytokines, cytokines receptors and residency/migration markers (B-F). Mann-Whitney test was used to compare HD-LN and TD-LN (* p<0,05 ; ** p<0,01)

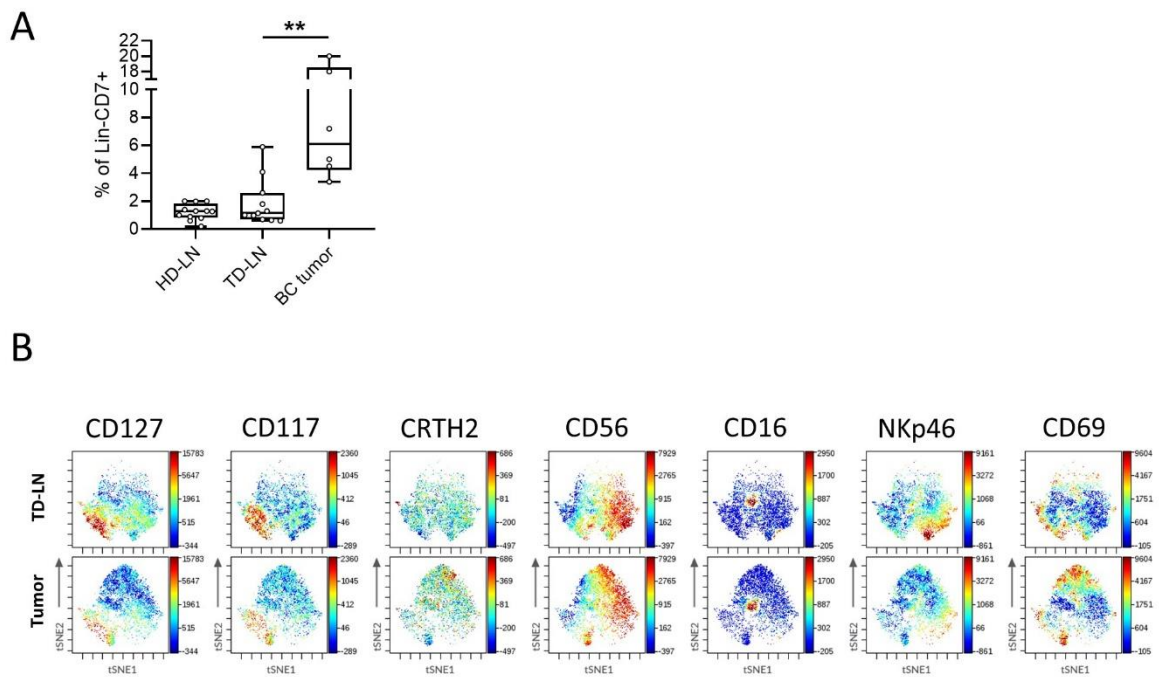


Figure 7. ILC in breast cancer tumor. Percentages of lin-CD7+ cells in alive cells in HD-LN (n=12), TD-LN (n=11) and BC tumor (n=6). Mann-Whitney test was used to compare different tissues (** p<0,01) (A). viSNE map comparing ILC from TD-LN and BC tumor, with expression of different markers using a color scale, from blue (low) to red (high) (B).

Mutational status		
Lymph node		
ER		3
ER/PR		12
ER/HER2		2
NA		1
Tumor		
ER/PR		9

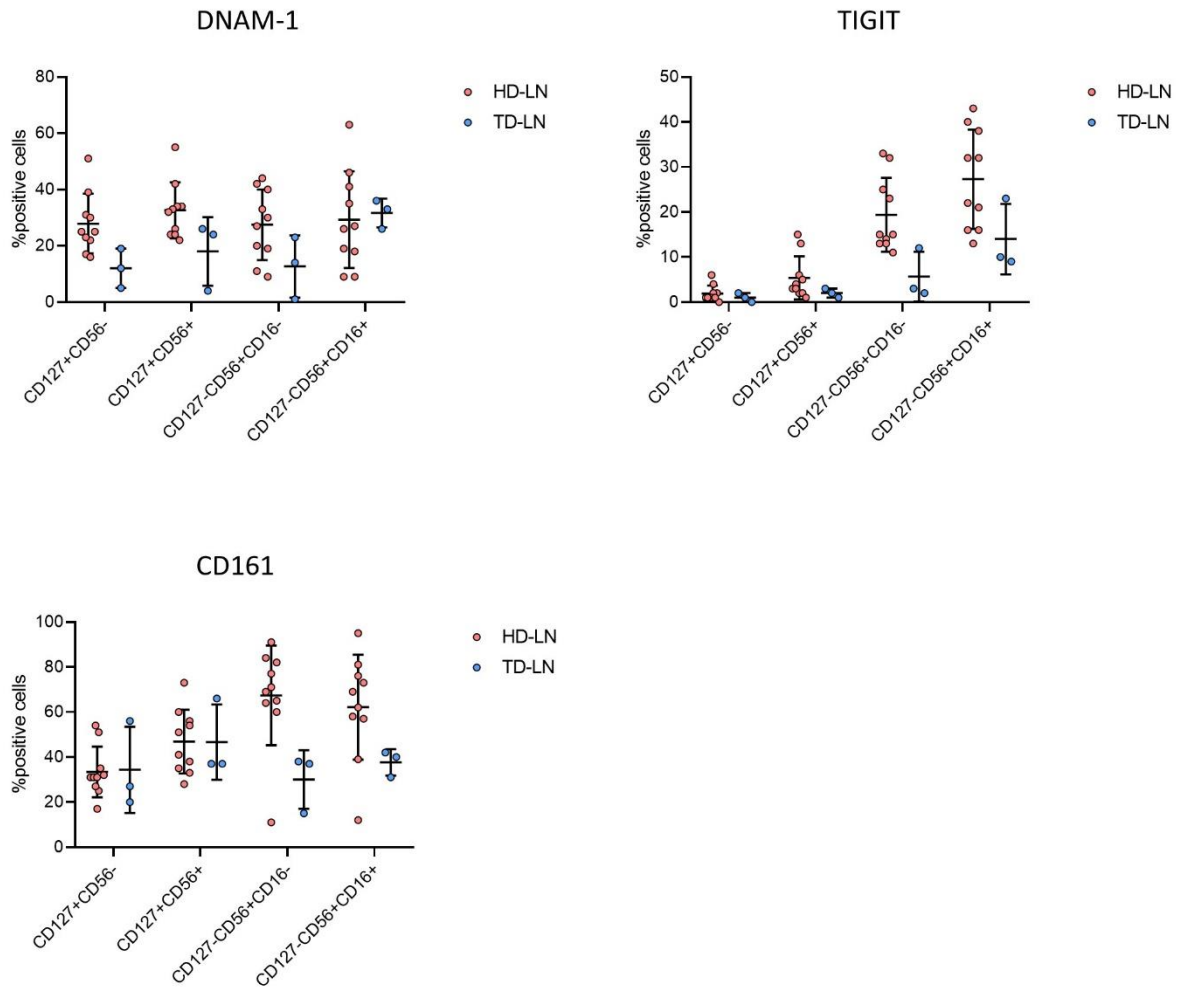
Supplementary Table 1. Mutational status of BC patients included in the study.

MARKER	CLONE	SOURCE	IDENTIFIER
CD3	OKT3	Biolegend	317306
CD4	RPA-T4	BD Bioscience	555346
CD5	UCHT2	Biolegend	300606
CD33	HIM3-4	Biolegend	303304
CD14	M5E2	BD Bioscience	555397
CD19	HIB19	Biolegend	302206
TCRAB	IP26	Biolegend	306706
TCRGD	B1	Biolegend	331208
CD235a	HI264	Biolegend	349104
CD7	M-T701	BD Bioscience	562541
CD127	MB15-18C9	Miltenyi Biotec	130-113-410
CD56	BD159	BD Bioscience	557919
CD16	3G8	BD Bioscience	560195
CD16	3G8	BD Bioscience	563785
CD117	104D2	BD Bioscience	562687
CRTH2	BM16	Biolegend	350104
CRTH2	BM16	BD Bioscience	562992
NKp46	9E2/NKp46	BD Bioscience	562101
NKp46	9E2	Biolegend	331950
NKp30	P30-15	BD Bioscience	563385
NKp44	44.189	Invitrogen	46-3369-42
NKG2D	1D11	BD Bioscience	563408
NKG2C	134591	BD Bioscience	748163
DNAM-1	DX11	Miltenyi Biotec	130-099-966
NKG2A	131411	BD Bioscience	747917
TIGIT	741182	BD Bioscience	747840
CTLA4	BNI3	BD Bioscience	555854
CD69	FN50	BD Bioscience	612817
CD103	Ber-ACT8	BD Bioscience	563162
CD62L	145/15	Miltenyi Biotec	130-113-619
CD96	NK92.39	Biolegend	338411
CD161	HP-3G10	BD Bioscience	748280
CXCR6	K041E5	Biolegend	356012
CD107a	H4A3	BD Bioscience	561343
IFNg	B27	BD Bioscience	562017

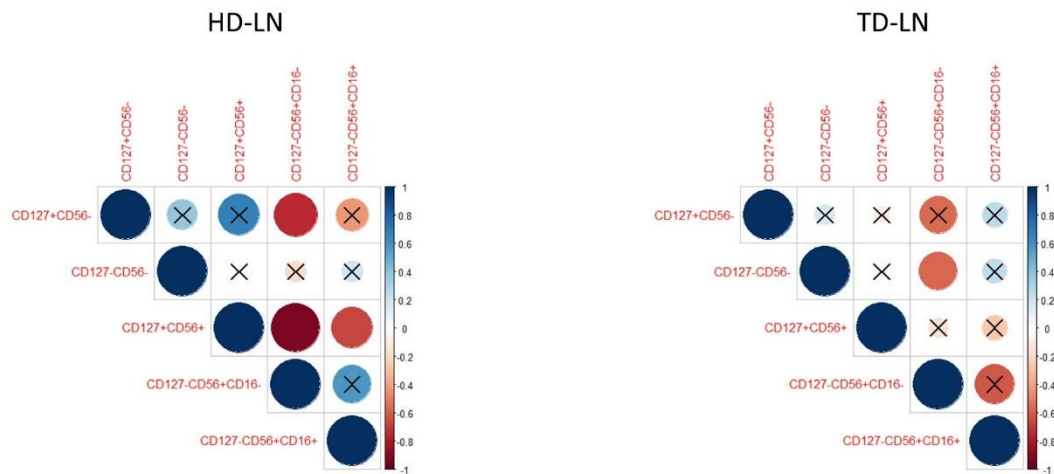
Supplementary Table 2. List of antibodies for flow cytometry.

Gene	Probe id	Gene	Probe id
PRF1	Hs00169473_m1	FCGR3B (CD16)	Hs04334165_m1
GZMA	Hs00989184_m1	CD34	Hs02576480_m1
GZMB	Hs00188051_m1	PTGDR2 (CRTH2)	Hs01867513_s1
GZMK	Hs00157878_m1	KIT (CD117)	Hs00174029_m1
GZMH	Hs00277212_m1	CD44	Hs01075864_m1
IFNG	Hs00989291_m1	IL2RA	Hs00907777_m1
TNFA	Hs00174128_m1	IL2RB (CD122)	Hs01081697_m1
IL13	Hs00174379_m1	IL12RB1	Hs01106578_m1
IL5	Hs01548712_g1	IL12RB2	Hs00155486_m1
IL4	Hs00174122_m1	IL18R1	Hs00977691_m1
IL9	Hs00174125_m1	IL23R	Hs00332759_m1
IL17A	Hs00174383_m1	IL1R1	Hs00991010_m1
IL22	Hs01574154_m1	IL15RA	Hs00542602_g1
CSF2 (GM-CSF)	Hs00929873_m1	IL1RL1 (IL33R)	Hs00249384_m1
LTA	Hs06633590_s1	CXCR6	Hs01890898_s1
LTB	Hs00242739_m1	CXCR3	Hs01847760_s1
IL32	Hs00992441_m1	S1PR1	Hs01922614_s1
KIR2DL1	Hs04961778_gH	S1PR3	Hs00245464_s1
KIR2DL2	Hs04961776_uH	S1PR4	Hs02330084_s1
KIR2DL3	Hs04961777_gH	S1PR5	Hs00928195_s1
KIR2DL4	Hs00427106_m1	SLAMF1	Hs00234149_m1
KIR3DL1	Hs01592437_m1	XCL1	Hs00751481_s1
KIR3DL2	Hs00601497_gH	SELL (CD62L)	Hs00174151_m1
KIR3DL3	Hs00601067_m1	CXCL8	Hs00174103_m1
CD96	Hs00976975_m1	CCL4	Hs99999148_m1
TIGIT	Hs00545087_m1	CCR7	Hs01013469_m1
CD226 (DNAM-1)	Hs00170832_m1	TBX21 (T-BET)	Hs00203436_m1
KLRG1	Hs00195153_m1	IKZF3 (Aiolos)	Hs05037772_s1
CTLA4	Hs00175480_m1	ZBTB16 (PLZF)	Hs00232313_m1
HAVCR2 (TIM-3)	Hs00958618_m1	JUN	Hs01103582_s1
PDCD1 (PD-1)	Hs01550088_m1	FOS	Hs99999140_m1
LAG3	Hs00958444_g1	DUSP	Hs00244839_m1
KLRD1 (CD94)	Hs00233844_m1	EOMES	Hs00172872_m1
KLRC1 (NKG2A)	Hs00970273_g1	RORC (ROR γ t)	Hs01076112_m1
KLRC1 (NKG2D)	Hs00183683_m1	GATA3	Hs00231122_m1
KLRC2 (NKG2C)	Hs02379574_g1	RORA (ROR α)	Hs00536545_m1
NCR2 (NKp44)	Hs00183113_m1	ID2	Hs04187239_m1
NCR3 (NKp30)	Hs00394809_m1	AHR	Hs00169233_m1
NCR1 (NKp46)	Hs00183118_m1	CD3e	Hs01062241_m1
CD244 (2B4)	Hs00175569_m1	CD4	Hs01058407_m1
KLRB1 (CD161)	Hs00174469_m1	CD8 β	Hs00174762_m1
CD69	Hs00934033_m1	CD33	Hs00233544_m1
ITGAE (CD103)	Hs01025372_m1	CD5	Hs00204397_m1
B3GAT1 (CD57)	Hs01024500_m1	CD7	Hs00196191_m1
IL7R α (CD127)	Hs00902334_m1	GAPDH	Hs99999905_m1
NCAM1 (CD56)	Hs00941830_m1	ACTB	Hs99999903_m1
		RPL27	Hs03044961_g1

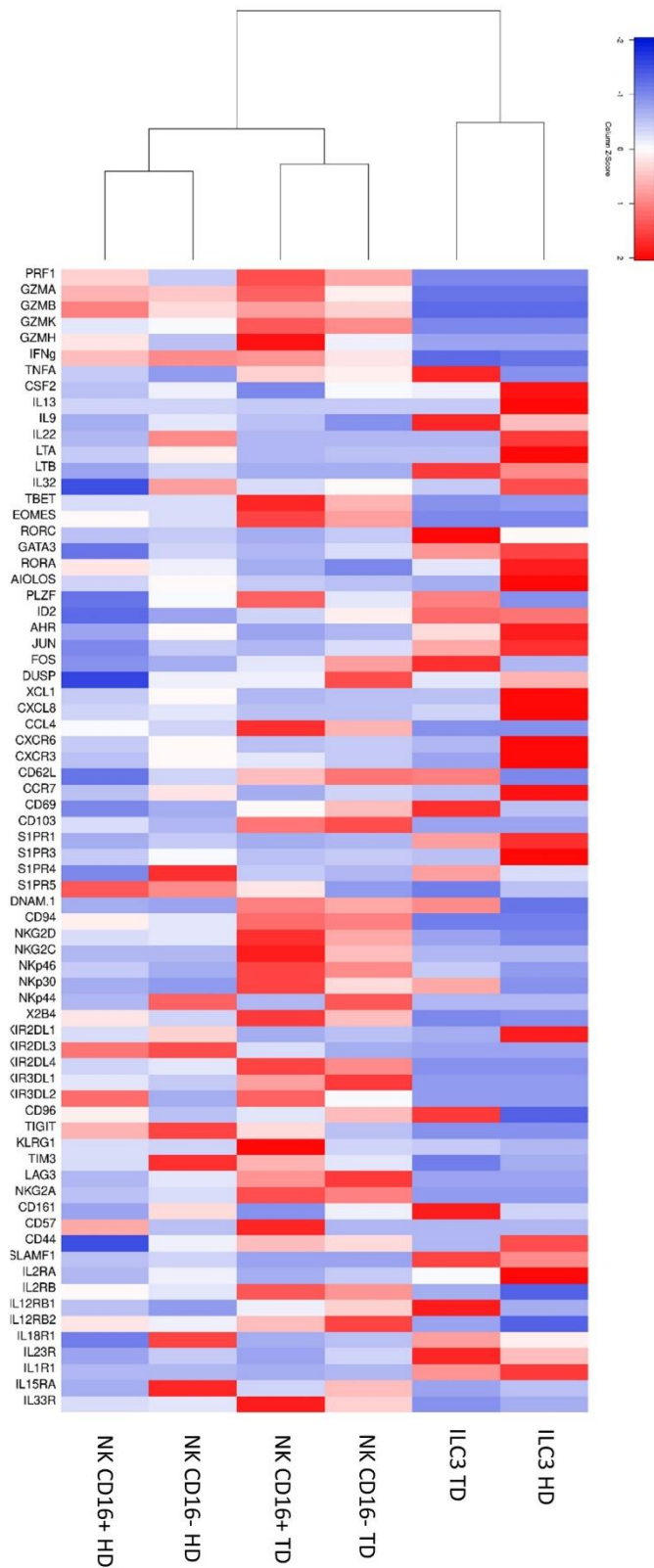
Supplementary Table 3. List of Taqman probes for RT-qPCR.



Supplementary figure 1. Percentages of positive cells for DNAM-1, TIGIT and CD161 in ILC subsets in HD-LN (n=10) and TD-LN (n=3).



Supplementary figure 2. Correlation analysis of percentages of ILC subsets in HD-LN and TD-LN.



Supplementary figure 3. Hierarchical clustering of ILC subsets in HD-LN and TD-LN.

DISCUSSION ET PERSPECTIVES

DISCUSSION

L'immunothérapie a révolutionné les traitements anti-cancéreux. Une étape importante a été la mise en évidence d'une corrélation entre la présence d'un infiltrat et l'évolution clinique des patients, permettant de définir la contexture immunitaire comme un critère pronostique (Bruni et al., 2020). Par ailleurs, la caractérisation fonctionnelle des cellules immunes présentes dans le microenvironnement tumoral a abouti à de nouvelles thérapies telles que les inhibiteurs de points de contrôle immuns. Ces immunothérapies qui revigorent les LT anti-tumoraux peuvent entraîner des réponses durables cependant encore beaucoup de patients y sont résistants. Les raisons de cette résistance innée ou des résistances secondaires aux immunothérapies observées chez une proportion notable des patients ne sont pas bien comprises. Les immunothérapies actuelles sont pour la plupart focalisées sur l'activité anti-tumorale des LT cytotoxiques. Cependant, d'autres cellules immunes lymphoïdes sont impliquées dans la réponse anti-cancéreuse, notamment les cellules lymphoïdes innées (ILC), première ligne de défense contre le cancer.

L'activité de notre équipe est focalisée sur les rôles des ILC dans l'immunosurveillance des cancers. Il est maintenant admis que les cellules NK ont des capacités anti-tumorales, par leur fonction de cytotoxicité naturelle ou par la production de cytokines immuno-régulatrices. Cependant, les cellules NK sont souvent altérées après un contact chronique avec les cellules tumorales. Elles présentent des altérations de l'expression de leurs récepteurs activateurs et inhibiteurs entraînant une réduction de leurs fonctions. Une autre observation importante dans l'immunosurveillance tumorale par les cellules NK a été de montrer que les traitements anti-cancéreux ciblant la tumeur modifient également les fonctions des cellules NK. Notre équipe a notamment montré que la fonction cytotoxique des cellules NK est altérée après un traitement par chimiothérapie (Fregni et al., 2011). De plus des thérapies sont en développement afin de restaurer les capacités anti-tumorales des cellules NK (Chiossone et al., 2017). Le rôle des ILC auxiliaires dans l'immunosurveillance tumorale est moins connu. Des études reportent leur présence dans le microenvironnement tumoral et leurs fonctions anti- ou pro-tumorales (Crinier et al., 2019).

Mon travail de Thèse a donc porté sur l'immunosurveillance tumorale par les ILC avec deux objectifs principaux :

1. Comprendre l'impact de la résistance aux thérapies ciblées sur l'immunogénicité NK des mélanomes
2. Caractériser les populations d'ILC ganglionnaires de donneurs et celles infiltrant les ganglions métastatiques.

Les résultats obtenus sur les deux projets sont discutés séparément.

Résistance aux thérapies ciblées et impact sur les fonctions des cellules NK

Les inhibiteurs de BRAF et MEK sont la première ligne de traitement des mélanomes métastatiques présentant la mutation BRAFV600E. Ils entraînent une réponse clinique rapide chez les patients dont la tumeur porte la mutation cependant beaucoup rechutent au bout de quelques mois (Chapman et al., 2011). Dans un travail récent, le laboratoire a montré que traitées par un inhibiteur de BRAF, les cellules de mélanomes mutées *BRAF* présentent des modulation d'expression des ligands NK (Frazao et al., 2017). J'ai poursuivi ce travail et me suis intéressée au statut des cellules NK stimulées par des cellules de mélanomes résistantes aux inhibiteurs de BRAF (Vemurafenib) et de MEK (Cobimetinib) dans le mélanome métastatique. Nous avons montré que la résistance au Vemurafenib et au Cobimetinib induit une modulation de l'expression de ligands de récepteurs NK et une augmentation de l'activation des fonctions NK, démontrant le potentiel d'une immunothérapie ciblant les cellules NK (Figure 17).

Mes résultats montrent que les mécanismes de résistance aux inhibiteurs de la voie BRAF/MEK impliqués diffèrent en fonction de la lignée considérée (SK28 ou M14). Pour la lignée SK28R, nous observons une augmentation de la voie PI3K/AKT, parallèle à la voie des MAPK, ce qui lui confère une résistance à la combinaison d'inhibiteurs de BRAF et MEK. La lignée M14R présente une augmentation de la voie MEK/ERK, sa prolifération est donc inhibée par l'inhibiteur de MEK.

Concernant l'immunogénicité des lignées, la résistance induite sur deux lignées de mélanomes (SK28 et M14) entraîne une modification de l'expression des ligands de NKG2D, MICA et ULBP2.

Pour la lignée SK28R, nous avons observé une augmentation de ces ligands membranaires par rapport à la lignée sensible, et de faible taux de ligands solubles dans les surnageants de culture. Cette modulation est associée à l'augmentation de l'activation des cellules NK de donneurs et de patients. L'activation de la voie PI3K/AKT peut être à l'origine de l'augmentation de l'expression de MICA et ULBP2, car il a été montré que l'expression de MICA sur les cellules K562 est régulée par la voie PI3K/AKT (Boissel et al., 2006).

Pour la lignée M14R, l'expression de MICA est augmentée. En revanche, l'expression d'ULBP2 est diminuée par rapport à la lignée sensible, et les taux d'ULBP2 solubles sont augmentés, suggérant une régulation par clivage de la protéine membranaire. Il a été montré que le clivage des ligands de NKG2D est un des mécanismes d'échappement tumoral aux cellules NK (Schmiedel and Mandelboim, 2018). Les ligands membranaires des cellules tumorales sont clivés par des métalloprotéases et les

ligands solubles se lie à NKG2D entraînant l'internalisation de ce récepteur et donc la diminution de l'activation des cellules NK. La réactivation de la voie des MAPK chez M14R, via ERK et MEK, peut, comme AKT, jouer sur l'expression des ligands de NKG2D (Ogbomo et al., 2008). Nous observons une plus faible activation des cellules NK par la lignée M14R par rapport à SK28R, possiblement liée au clivage d'ULBP2. De plus, le blocage de NKG2D induit une réduction de la lyse des cellules SK28R de 20%, supérieure à celle de M14R qui est seulement de 10%. Ce résultat indique que la production d'ULBP2 solubles par les cellules M14R diminue l'expression de NKG2D par les cellules NK et donc que la lyse des cellules M14R fait intervenir un autre récepteur.

L'augmentation de la sensibilité à la lyse par les cellules NK des cibles SK28R et M14R peut impliquer la diminution de l'expression de CEACAM1 par rapport aux lignées sensibles. L'isoforme CEACAM1-L, possédant un motif ITIM, est exprimé par les cellules NK. Cette isoforme se lie à CEACAM1 exprimée par les cellules tumorales et inhibe les fonctions activatrices de NKG2D (Hosomi et al., 2013). De surcroît, l'expression de CEACAM1 par les cellules tumorales entraîne la rétention des ligands de NKG2D dans le compartiment intracellulaire (Chen et al., 2011). L'inactivation du gène de CEACAM1 augmente l'expression des ligands de NKG2D à la surface de la cellule et donc la lyse par les cellules NK.

Les ligands de NKG2D solubles ont été associés à l'évolution tumorale dans différents cancers, dont le mélanome. Nous avons évalué les taux de MICA/B et ULBP chez des patients atteints de mélanome métastatique. Nous avons montré qu'un taux élevé de MICA solubles est associé à une réponse moindre aux immunothérapies. Il a été montré précédemment que l'absence de MICB et ULBP-1 solubles est associée à une augmentation de la survie chez des patients recevant des immunothérapies et que les taux d'ULBP-1 et -2 solubles étaient positivement corrélés à la réponse aux ICB (Maccalli et al., 2017). De plus, les ULBP2 solubles sont indicateurs de mauvais pronostic, associés à la progression tumorale, la charge tumorale et une survie réduite chez des patients atteints de mélanome métastatique (Paschen et al., 2009). Des anticorps anti-MICA diminuant le clivage ont été évalués et montrent une inhibition de la croissance tumorale, du développement de métastases associés à l'activation des cellules NK par les récepteurs NKG2D et CD16 (Ferrari de Andrade et al., 2018). Les MMP du TME peuvent également cliver le ligand de NKp30, B7-H6, inhibant les fonctions NK (Schlecker et al., 2014). Nous avons donc évalué la présence de B7-H6 solubles dans notre cohorte de patients et nous avons observé un niveau plus élevé chez les non-répondeurs aux immunothérapies.

Le récepteur NKG2A est un *checkpoint* immun important, des cellules NK et de certaines populations de LT. Des anticorps bloquants sont actuellement testés en clinique (van Hall et al., 2019). Dans notre étude, la lignée M14R exprime moins de HLA-E, ligand du récepteur inhibiteur NKG2A, et

est plus sensible à la lyse par les cellules NK que la lignée parentale M14. HLA-E est exprimé à la surface des tumeurs de mélanomes primaires et métastatiques (Derré et al., 2006; Tremante et al., 2014). De plus, les taux de HLA-E solubles sont plus élevés chez les patients atteints de mélanome par rapport à des donneurs sains (Allard et al., 2011). Des anticorps anti-NKG2A pourraient donc activer la lyse médiée par les cellules NK des patients atteints de mélanomes métastatiques. Les cellules M14R présentent une augmentation de l'expression de PD-L1, confirmant les résultats d'une autre équipe (Jiang et al., 2013). Il existe donc une relation entre la résistance aux inhibiteurs de BRAF et l'augmentation de PD-L1, ce qui suggère l'intérêt d'évaluer la combinaison thérapeutique anti-PD1/anti-NKG2A.

Les cellules NK peuvent induire l'apoptose des cellules tumorales par la voie des récepteurs à domaine de mort en exprimant TRAIL et FasL se liant à TRAIL-RI/RII ou Fas sur les cellules cibles (Prager and Watzl, 2019). Les cellules SK28R expriment plus de TRAIL-RII que les SK28 parentales et sont plus sensibles à la lyse induite par un agoniste de TRAIL et par la staurosporine. En étudiant le transcriptome de SK28R, nous avons observé des différences d'expression des gènes jouant un rôle dans l'induction de l'apoptose, notamment une augmentation de l'expression du gène de la caspase 4 chez les cellules SK28R par rapport aux sensibles. La caspase 4 active la caspase 3 qui est impliquée dans l'induction de l'apoptose par TRAIL et la staurosporine (Chae et al., 2000; von Karstedt et al., 2017). Pour les patients présentant ce profil, des agonistes de TRAIL ou d'autres agents pro-apoptotiques pourraient être administrés avec des inhibiteurs de BRAF.

Les lignées M14S/R présentent une forte expression de TRAIL-RII et nous observons une augmentation de l'expression de Fas chez les cellules M14R. De façon intéressante, nous notons une diminution de l'apoptose induite par TRAIL chez les M14R par rapport aux M14S, alors que les deux lignées sont sensibles à l'apoptose induite par la staurosporine, ces deux composés étant des inducteurs d'apoptose différents. La diminution de sensibilité à TRAIL peut s'expliquer par la modification de l'expression de plusieurs gènes. En effet, nous observons une diminution de l'expression de gènes codant pour des protéines pro-apoptotiques (*CASP1*, *TP53*, *MYC*, *RIPK1*) et une augmentation de gènes codant pour des protéines anti-apoptotiques (*MCL1*, *MDM2*, *TRAF1*). Dans certains cas, TRAIL-RII peut être un facteur pro-tumoral, associé à la survie et à la prolifération. En effet, TRAIL-RII peut activer les kinases MEK et ERK ce qui entraîne une diminution du relargage de SMAC/DIABLO par la mitochondrie et donc une diminution de l'apoptose induite par TRAIL (Belyanskaya et al., 2008; Zhang et al., 2003). L'augmentation de l'activation de MEK/ERK dans les cellules M14R peut donc expliquer la diminution de la sensibilité à TRAIL.

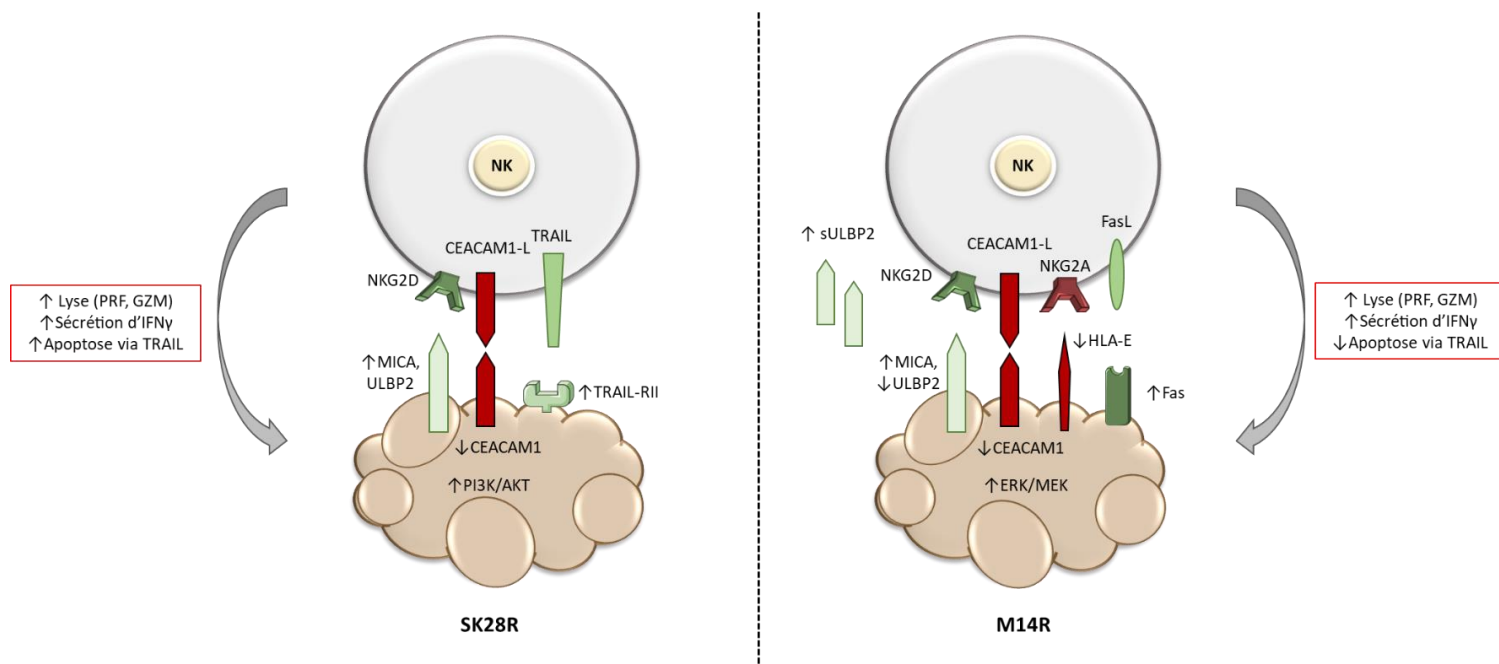


Figure 17 : Schéma récapitulatif de l'impact des TKI sur l'immunogénicité NK des cellules de mélanomes. Les cellules tumorales SK28R, par rapport aux SK28S, présentent une augmentation de l'expression des ligands du récepteur activateur (en vert) NKG2D, MICA et ULBP2, et une diminution du ligand inhibiteur (en rouge) CEACAM1. Le récepteur TRAIL-RII est également augmenté. Ces modifications causent une augmentation de la lyse, de la sécrétion d'IFN- γ par les cellules NK et de l'apoptose induite par TRAIL. Les cellules M14R, par rapport aux M14S, présentent une augmentation de MICA et une diminution d'ULBP2, CEACAM1 et HLA-E. La quantité d'ULBP2 solubles, dans le milieu de culture de la lignée M14R, est supérieure à celle des M14S. Le récepteur Fas est augmenté chez les M14R. Ces modifications du phénotype entraînent une augmentation de la lyse, de la sécrétion d'IFN- γ par les cellules NK en réponse aux M14R par rapport aux M14S. En revanche, l'apoptose induite via TRAIL est diminuée par rapport aux M14S.

Ayant montré une association entre les ligands NK solubles et la réponse aux traitements dans une série de patients atteints de mélanome métastatique, nous avons caractérisé les ILC sanguines de patients à un stade avancé, traités par des anticorps bloquants CTLA-4 (Ipilimumab) (voir Annexe 1). Les cellules NK sont les ILC majoritaires du sang, les ILC auxiliaires représentant 5-10% des ILC. Avant traitement, les patients sont caractérisés par une diminution des populations NK activées (CD56⁺CD16⁺NCR⁺) par rapport à des donneurs sains. L'ipilimumab entraîne une diminution du pourcentage d'ILC circulantes (lin⁻CD7⁺). En comparant les patients qui contrôlent ou non la maladie (évaluation à 16 semaines), nous avons notés une plus grande proportion de cellules NK avec un

phénotype de cellule activées, CD56^{dim}CD16⁺DNAM-1⁺TIGIT⁺ chez les patients contrôlant la maladie. La présence de ces cellules NK serait donc un marqueur de bon pronostic pour les patients recevant des anti-CTLA4. Cette étude indique que les populations ILC (et notamment les cellules NK) sont impliquées dans la réponse aux ICB. Elle souligne l'intérêt des immunothérapies visant les NK en combinaison avec les immunothérapies conventionnelles. Par exemple, une combinaison anti-CTLA4 avec des CAR-NK qui permettrait aux patients non-répondeurs d'avoir des cellules NK fonctionnelles. Dans cette étude, les ILC auxiliaires étaient rares et nous n'avons pas observé de modifications significatives de ces cellules sanguines.

Ces travaux font l'objet d'une publication dans *Cancers* (éditions MDPI), pour un numéro spécial sur les cellules NK et les cancers.

Les ILC auxiliaires étant majoritairement résidentes tissulaires, il est important de les étudier au niveau de la tumeur primaire ou au niveau des sites métastatiques, notamment le ganglion drainant.

Immunosurveillance des métastases ganglionnaires par les ILC

Les cellules NK jouent un rôle clé dans l'immunosurveillance et le contrôle des métastases (López-Soto et al., 2017). Le choix du modèle du ganglion lymphatique pour l'étude des ILC repose sur les arguments suivants. Le ganglion est le premier site de dissémination dans de nombreux cancers et particulièrement dans le cancer du sein. Cet organe est également important pour la mise en place de la réponse immunitaire anti-tumorale. Nous avons caractérisé les populations NK ganglionnaires dans une étude publiée en 2019 et montré que des cellules NK CD56^{bright}CD16⁺NKG2A⁺ activées infiltraient les ganglions drainant des tumeurs de cancer du sein (Frazao et al., 2019). Cette étude était focalisée sur les ILC cytotoxiques, les cellules NK, sans analyser les ILC auxiliaires, en raison de la difficulté à les identifier par des marqueurs spécifiques. Depuis, plusieurs études ont défini des combinaisons de marqueurs pour distinguer les populations NK et ILC auxiliaires humaines (Hazenbergh and Spits, 2014; Lim et al., 2017; Trabanelli et al., 2018). La définition des populations reste cependant difficile et la comparaison avec d'autres projets peut être difficile en raison de la diversité des marqueurs de définition utilisés, notamment pour les mix "lineage". Plusieurs revues ont été publiées afin d'harmoniser l'identification de ces populations (Trabanelli et al., 2018; Vivier et al., 2018). Pour notre analyse multiparamétrique en cytométrie en flux 17 couleurs, nous nous sommes basées sur l'étude de Lim et al. (Lim et al., 2017). A cette difficulté s'ajoute celle des données sur la plasticité des ILC. En effet, suivant le contexte environnemental, une population ILC peut se trans-différencier en une autre (Bal et al., 2020). De plus, nous continuons de découvrir l'existence de précurseurs ILC, avant intégrés

à la population des ILC3 (Lim et al., 2017). Il est donc indispensable de progresser dans la caractérisation de ces populations dans différents tissus chez l'humain.

La présence des ILC auxiliaires dans les ganglions a été mise en évidence chez la souris (Mackley et al., 2015). Chez l'homme, leur présence dans les ganglions est moins documentée. Nous nous sommes donc intéressés aux populations ILC, cytotoxiques et auxiliaires, présentes dans les ganglions métastatiques de patientes atteintes de cancer du sein, en comparaison de celles présentes dans les ganglions lymphatiques de donneurs sains et de celles présentes dans les tumeurs mammaires.

Nous avons analysé les ILCs de deux sites ganglionnaires différents, le ganglion mésentérique drainant l'intestin chez les donneurs, et le ganglion métastatique axillaire drainant les tissus mammaires. Les résultats observés dans notre étude pourraient refléter cette différence de localisation des ganglions analysés. Chez la souris, la proportion des ILC auxiliaires n'est pas la même dans les ganglions drainant les muqueuses et les ganglions périphériques (Mackley et al., 2015). Chez l'homme, quelques études montrent des populations ILC distinctes en fonction du site anatomique. Des précurseurs NK et des cellules NK immatures seraient présentes dans les ganglions mésentériques et les intestins, et absentes dans les ganglions drainant les poumons (Dogra et al., 2020). Dans une revue de Trabanelli et al., il est mentionné que la proportion d'ILC auxiliaires peut être différente entre le ganglion axillaire et le mésentérique (Trabanelli et al., 2018). Cependant, l'identification de populations ILC identiques dans les ganglions métastatiques et dans les tumeurs primaires, sont en faveur d'un impact de la tumeur sur les ILC ganglionnaires.

Dans le ganglion sain, les populations ILC majoritaires sont les cellules NK, les ILC3 et une population CD127⁺CD56⁺ de type ILC3 ou « ILC3-like ». Cette dernière population correspondrait à celle qui a d'abord été décrite comme étant des cellules NK à un stade 3 de leur développement mais a été par la suite considérée comme une population ILC3 (Freud et al., 2014; Hughes et al., 2009, 2010). Une étude décrivant les différents stades de différenciation des cellules NK dans plusieurs tissus montre que les cellules NK du ganglion sont majoritairement des précurseurs NK présentant un phénotype CD56^{bright}CD127⁺CD161⁺ (Dogra et al., 2020). Nous montrons que la population CD127⁺CD56⁺ « ILC3-like » ganglionnaire présente un profil transcriptomique proche de celui des ILC3 et des cellules NK. En effet, les cellules CD127⁺CD56⁺ « ILC3-like » expriment des facteurs de transcription en commun avec les cellules NK (T-bet, Eomes) et d'autres en commun avec les ILC3 (RORγt, AHR). D'un point de vue fonctionnel, elles expriment les gènes des molécules cytotoxiques (PRF, GZM), des cytokines IFN-γ, TNF-α mais aussi du GM-CSF et de l'IL-22. Après activation par des cytokines et stimulation par la cible K562, les cellules CD127⁺CD56⁺ « ILC3-like » dégranulent et produisent de l'IFN-γ, comme les cellules NK, alors que les ILC3 n'en produisent pas et dégranulent uniquement après activation par l'IL-7. La

population ganglionnaire CD127⁺CD56⁺ « ILC3-like » serait donc à un stade intermédiaire entre ILC3 et cellule NK. Dans les amygdales un tel précurseur ILC existe, exprimant CD127, CD117 et CD56 et ayant la capacité de se différencier en ILC3 ou en cellule NK (Chen et al., 2018). Afin de confirmer cette hypothèse, nous voudrions tester le potentiel de différenciation de cette population, avec différentes cytokines pouvant induire une différenciation NK ou ILC3. Nous souhaitons également approfondir sa caractérisation, en évaluant l'expression d'autres récepteurs et en testant ses fonctions *in vitro* (lyse, production de cytokines de type Th1 ou Th17). Il serait aussi intéressant de réaliser une étude du transcriptome complet de ces cellules.

La comparaison des ILC ganglionnaires de donneurs et de patientes apportent des données originales. Dans les ganglions métastatiques, nous observons une diminution de la population CD127⁺CD56⁺ « ILC3-like », en faveur des cellules NK. Le TME pourrait donc entraîner la différenciation de la population immature CD127⁺CD56⁺ en population NK mature. Il a été montré que dans un contexte non inflammatoire la production d'IL1- β par les cellules dendritiques préserve les cellules NK IL-1R^{hi} IL-22⁺ AHR⁺ immatures (Hughes et al., 2010). En revanche, dans un contexte inflammatoire, l'IL-2 et l'IL-12 induisent la production d'IFN- γ par les cellules NKp46⁺CD127⁺ROR γ t⁺ (Bernink et al., 2013; Cella et al., 2010). Chez la souris, plusieurs études montrent que l'activation par l'IL-12 et l'IL-15 des populations ILC ROR γ t⁺ induit une augmentation de l'expression de marqueurs NK, de facteurs de transcription impliqués dans la différenciation NK et des fonctions types NK (Nussbaum et al., 2017; Raykova et al., 2017; Vonarbourg et al., 2010). Cette différenciation en cellule NK pourrait donc avoir lieu dans le ganglion métastatique.

Les fonctions anti-tumorales des cellules NK ont été prouvées par de nombreuses études *in vitro* et *in vivo* chez la souris. Dans le cancer du sein, les cellules NK sanguines et intra-tumorales sont décrites comme non-cytotoxiques, sous-expriment certains récepteurs activateurs (NKp30, NKG2D, DNAM-1 et CD16) et sur-expriment des récepteurs inhibiteurs (NKG2A) (Mamessier et al., 2011b, 2011a). Par l'utilisation d'un nouveau type d'analyse non-supervisée, l'algorithme CITRUS, nous avons mis en évidence une altération de l'expression des NCR (NKp46 et NKp30) et de NKG2A par les cellules NK CD16⁻ des ganglions métastatiques des patientes avec un cancer du sein. Le contact continu des cellules NK avec les cellules tumorales exprimant les ligands des NCRs pourrait être à l'origine de la diminution d'expression de ces récepteurs, comme montré chez des patients atteints de carcinome ovarien (Pesce et al., 2015) (Figure 18). Plusieurs molécules solubles pourraient aussi être à l'origine de ce mécanisme d'échappement tumoral. Le TGF- β est une molécule retrouvée en grande quantité chez les patientes atteintes de cancer du sein, qui exerce des effets pro-tumoraux dans les stades avancés, induisant notamment la migration des cellules tumorales (Desruisseau et al., 2006; Zhao et al., 2018). Le TGF- β peut également agir sur les cellules immunes du TME. Il induit, *in vitro*, une

diminution de l'expression de NKp46 et NKp30 chez les NK et ILC1 CD56⁺ (Castriconi et al., 2003; Han et al., 2018; Salomé et al., 2019). L'IDO est une autre molécule soluble du TME, connue pour ses effets immunosuppresseurs, favorisant la progression tumorale. L'expression d'IDO par les CPA est reportée dans différentes tumeurs et dans les ganglions drainant des tumeurs (Prendergast et al., 2018). IDO induit la dégradation du tryptophane et la production de kynurénine, ce qui inhibe les LT. La kynurénine, via la voie STAT, diminue l'expression des NCRs et de NKG2D, diminuant les fonctions NK contre des cellules cancéreuses de la thyroïde (Park et al., 2019). Le blocage de ces molécules pourrait donc restaurer l'expression des NCR à la surface des cellules NK ganglionnaires. Nous avons réalisé des expériences préliminaires pour évaluer l'effet des cellules tumorales sur les cellules NK en faisant des expériences de co-culture avec des lignées de cancer du sein. Nous observons une diminution de l'expression de NKp46 suite à une co-culture avec la lignée MCF-7. Nous souhaiterions également évaluer l'effet des molécules du TME (TGF- β , IDO) sur le phénotype des cellules NK.

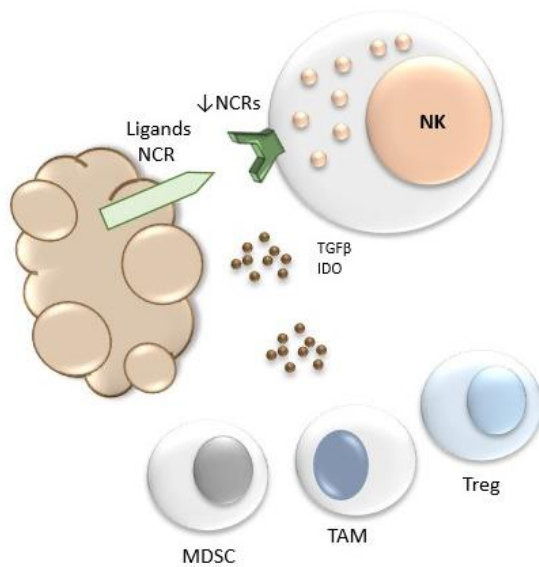


Figure 18 : Influence du microenvironnement tumoral sur les cellules NK.

Une population NK CD16⁺NKp46⁺NKG2A⁺ est fortement diminuée dans les ganglions métastatiques par rapport aux sains. Ces résultats sont contradictoires avec ceux de notre étude précédemment publiée en 2019, dans laquelle nous observions une infiltration de NK CD16⁺NKG2A^{high} dans les TD-LN de patientes (Frazao et al., 2019). Cette discordance pourrait être due à la nouvelle définition des cellules NK qui tient compte des ILC auxiliaires. En effet, les cellules NK étaient définies comme CD45⁺CD3⁻CD56⁺ sans tenir compte du marqueur définissant les ILC auxiliaires, les récepteurs

CD127. Dans cette nouvelle étude, nous sélectionnons les ILC suivant l'expression du marqueur CD7 et sur l'absence d'expression de plusieurs marqueurs (lin), excluant les cellules T, B, DC, monocytes et érythrocytes. Cette définition plus précise pourrait écarter des populations ILC, dont les cellules NK CD16⁺NKG2A^{high} observées dans la première étude. Cependant, nous ne voyons pas de populations lin⁺ exprimant fortement le CD16. Il faudrait réaliser des analyses comparatives des cellules NK avec les deux stratégies différentes pour confirmer cette hypothèse. Une autre hypothèse est l'effet de la cryopréservation sur l'expression du CD16. Il a été montré qu'une population CD56^{dim}CD16⁻ pouvait être induite par une cryopréservation dans l'azote liquide (Lugthart et al., 2015). Dans notre étude tous les échantillons ont été congelés dans l'azote liquide alors que dans l'étude de 2019 ils étaient directement analysés après dissociation.

L'étude du profil d'expression génique par qPCR multiplexe apporte de nouvelles données sur les populations ILC dans le ganglion métastatique. Les cellules NK CD16⁻, présentant un phénotype de cellules anergiques (NCR⁻NK2GA⁻), expriment fortement les gènes codant pour les NCRs et NKG2A. Nous notons également une augmentation de l'expression du gène du corécepteur DNAM-1 et du gène *PRF1*. Ces cellules NK CD16⁻ ont donc un profil transcriptomique de cellules activées mais qui présentent une diminution de l'expression de récepteurs à sa surface. Comme discuté précédemment, cette diminution d'expression à la surface peut être post-transcriptionnelle, due au TME. L'analyse CITRUS montrant la diminution d'une population NK CD16⁺ dans le ganglion métastatique par rapport au ganglion de donneur, nous avons également comparé l'expression des transcrits dans cette population. Nous avons observé une expression élevée des gènes des récepteurs activateurs (NCR, NKG2D, DNAM-1), inhibiteurs (KLRG1, NKG2A), de marqueurs d'activation (CD44, CD69) et de récepteurs de cytokines (IL2RB, IL18R1) dans les NK de ganglions tumoraux. Ce profil révèle une activation des cellules NK CD16⁺ dans les ganglions métastatiques. Les cellules NK sont donc activées par le TME mais l'expression membranaire des récepteurs activateurs NK est diminuée. Il faudrait évaluer les conséquences de ces changements phénotypiques sur les capacités anti-tumorales des cellules NK. Les fonctions NK de lyse et de production de cytokines pourraient être testées face à des cibles tumorales de cancer du sein *in vitro*.

A contrario, les ILC3 pourraient exercer des fonctions favorisant la croissance tumorale. Dans le ganglion métastatique de cancer du sein, notre étude révèle une forte expression du gène du TNF- α et une plus faible expression du gène de l'IL-22 que dans le ganglion sain. Des ILC CD56⁺CD3⁻ immuno-régulatrices, infiltrant les cancers ovariens, produisent de l'IL-22 et limitent la croissance des LT et leur production de cytokines (Crome et al., 2017). Dans les ganglions métastatiques, l'absence de production d'IL-22 par les ILC3 pourrait être en faveur d'une croissance et d'une activation des LT ganglionnaires. Les ILC3 des ganglions de donneurs et de patients expriment également les gènes des

cytokines pro-inflammatoires GM-CSF et IL-32. Chez la souris, la production de GM-CSF par les ILC3 intestinales est dépendante de l'IL-1 β sécrétée par les macrophages, et permet d'activer les DC et les macrophages afin de réguler l'homéostasie des LT régulateurs (Mortha et al., 2014). Dans le ganglion, le GM-CSF sécrété par les ILC3 induirait une activation des DC et monocytes, favorisant l'activation des LT effecteurs et des LT régulateurs.

La production de TNF- α par les ILC3 peut être activée par l'engagement de NKp44 par son ligand présent sur des cellules de cancer du poumon (Carrega et al., 2015) (Figure 19). Une seconde étude montre que le récepteur NKp44 est impliqué dans la production de TNF α mais n'active pas la production d'IL22 ou de GM-CSF (Glatzer et al., 2013). Dans notre étude, le ligand de NKp44 pourrait être présent sur les cellules tumorales de cancer du sein et induire la production de TNF- α par les ILC3. Or, le TNF- α dans le cancer du sein est associé à la croissance tumorale, l'augmentation de la migration et de la dissémination des cellules tumorales (Cruceriu et al., 2020). Les ILC3 seraient donc pro-tumorales dans ce contexte et il faudrait envisager de bloquer leurs fonctions.

Dans la suite du projet, il serait important d'évaluer la production de TNF- α par les ILC3 face à des cibles de cancer du sein, par cytométrie en flux. Nous pourrions également évaluer l'effet des ILC3 et du TNF- α produit par ces cellules sur la croissance tumorale et tester l'effet d'un blocage de NKp44. En clinique ce blocage devra être spécifique des ILC3, à l'aide d'un anticorps bi ou tri-spécifique, afin de ne pas bloquer les activités anti-tumorale des cellules NK qui peuvent exprimer ce récepteur.

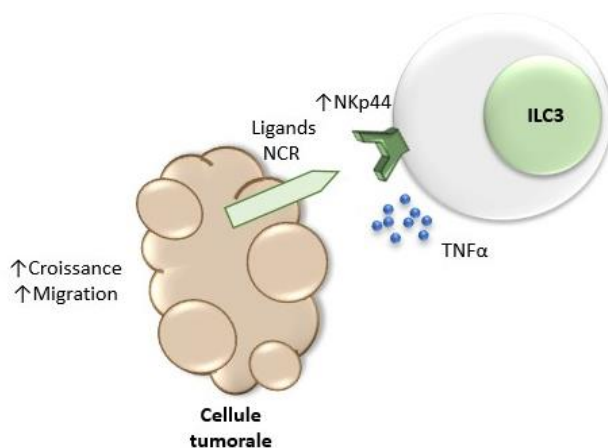


Figure 19 : Interactions entre les cellules tumorales et les ILC3.

Les ILC auxiliaires sont essentiellement résidentes tissulaires. Nous montrons que dans un ganglion de donneur elles expriment fortement le marqueur de résidence tissulaire CD69. Les cellules NK ganglionnaires expriment également ce marqueur de résidence et co-expriment CXCR6. Ce profil a été associé à la résidence tissulaire dans le foie et dans les organes lymphoïdes secondaires (Harmon et al., 2016; Lugthart et al., 2016; Stegmann et al., 2016). Lors de l'invasion métastatique, nous observons la diminution des ILC présentant une expression membranaire de CD69. En revanche, ces populations expriment fortement le gène *CD69*, ce qui suggère une régulation post-transcriptionnelle de l'expression de la protéine à la surface des cellules. Il a été décrit que l'expression de CD69 à la surface était régulée par la protéine S1PR1 (Cyster and Schwab, 2012). Nous n'avons pas pu déterminer l'expression des S1PR à la surface des ILC car il n'existe pas d'anticorps fonctionnels. Cependant au niveau génique, nous n'avons pas observé de différence d'expression de *S1PR1* entre les deux contextes. La migration des cellules NK a été décrite comme étant plutôt S1PR5 dépendante (Drouillard et al., 2018; Jenne et al., 2009). Nous notons une diminution de l'expression de *S1PR5* dans les NK CD16⁻ des ganglions métastatiques, suggérant une diminution de leurs capacités de migration. Toutefois, ici encore, nous n'avons pas pu mesurer son expression à la surface. La résidence tissulaire a été associée à des populations NK peu différenciées (Dogra et al., 2020). Les cellules NK du ganglion métastatique seraient donc plus matures, à un stade de différenciation plus avancé par rapport à celles des ganglions de donneurs.

Les protéines impliquées dans la migration des cellules NK vers le ganglion sont CD62L et CCR7 (Castriconi et al., 2018). L'expression de *CD62L* est plus élevée dans les ILC du ganglion métastatique, suggérant une migration de la tumeur vers le ganglion. Il a été montré chez la souris que les ILC3 étaient capables de migrer de l'intestin vers les ganglions mésentériques, de façon CCR7 dépendante (Mackley et al., 2015). Les ILC pourraient donc migrer de la tumeur vers le ganglion drainant puis vers la périphérie. Un dernier élément appuyant cette hypothèse est la présence d'une population au phénotype similaire, NKp46⁻CD69⁻, dans la tumeur et dans le ganglion envahi. Dans la suite du projet, la capacité de migration des ILC ganglionnaires pourraient être évaluée à l'aide de Transwell ou par imagerie avec la technologie Incucyte.

Les résultats de cette étude sont récapitulés dans la figure suivante (Figure 20).

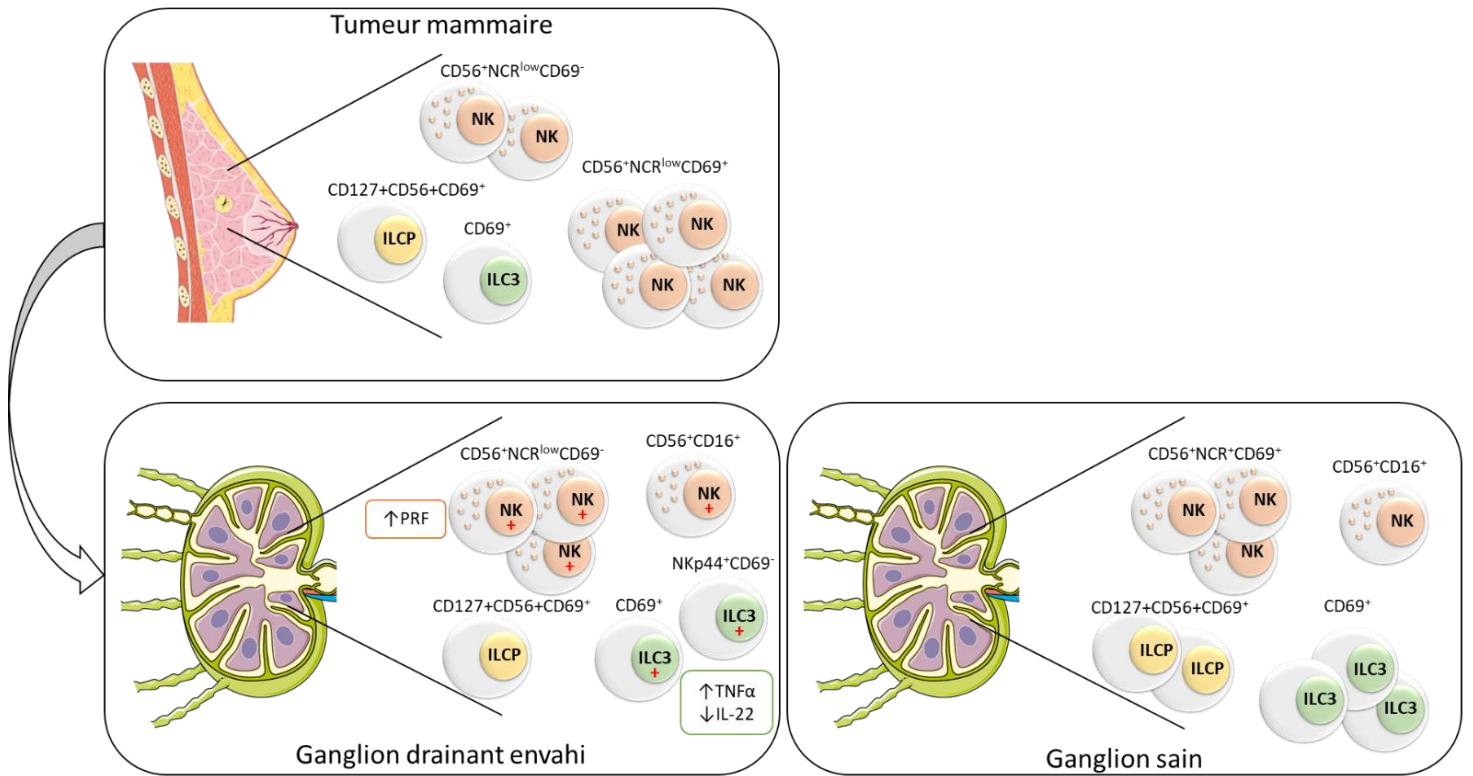


Figure 20 : Comparaison des ILC dans les ganglions métastatiques de cancer du sein et dans des ganglions sains. + : transcrits de cellules activées.

CONCLUSION GENERALE

Mon projet de thèse avait pour objectif d'étudier le rôle des ILC, incluant les ILC auxiliaires, dans l'immunosurveillance tumorale des cancers métastatiques.

Mes travaux ont généré des données nouvelles et originales qui peuvent avoir des retombées en immunothérapie anti-tumorale. Ils ont permis de mettre en évidence l'augmentation de la sensibilité à la lyse par les cellules NK des cellules de mélanomes métastatiques résistantes aux inhibiteurs de BRAF et MEK. Ces données et la détection de biomarqueurs NK associés à la réponse aux traitements des patients mélanomes sont en faveur de stratégies thérapeutiques combinées impliquant les cellules NK. Restaurer les fonctions anti-tumorales des cellules NK pourrait être bénéfique chez certains patients résistants aux thérapies ciblées. Nous avons observé une diminution d'expression des molécules HLA-E lors d'une résistance induite par la voie MEK/ERK, participant à l'augmentation de la sensibilité à la lyse NK. Il serait donc envisageable d'utiliser une thérapie avec des anticorps bloquant NKG2A, qui ont prouvé leur efficacité en étude préclinique et qui sont actuellement testés en clinique (van Hall et al., 2019). Combiner des anticorps anti-ICB avec des anticorps anti-NKG2A pourrait aussi potentialiser une réponse anti-tumorale. Potentialiser les fonctions NK par des anticorps trispécifiques activant les voies d'ADCC et de cytotoxicité naturelle, et favorisant les conjugués tumeur/effecteurs pourrait être bénéfique (Gauthier et al., 2019). Des approches de thérapies cellulaires utilisant des CAR-NK avec un CAR NKG2D pourraient être évaluées compte tenu du rôle important de cette voie d'activation dans les fonctions anti-tumorales des cellules NK (et LT) (voir Annexe 2, revue sur NKG2D). L'augmentation de l'expression des ligands de NKG2D à la surface des cellules tumorales résistantes aux thérapies ciblées est en faveur de ces approches.

Pour des traitements en condition adjuvante ou néoadjuvante, dans des cancers du sein (mélanome) présentant un envahissement local, mes travaux indiquent que le TME du ganglion induit une modification du phénotype et des fonctions des ILC. Les cellules NK du ganglion métastatique sont activées et présentent une augmentation de l'expression de gènes impliqués dans les interactions avec le TME et des gènes de fonctions. Cependant, le contact continu des cellules NK avec les cibles tumorales et/ou les facteurs solubles produits localement altérerait l'expression de récepteurs activateurs à leur surface. La compréhension du processus impliqué dans cette diminution de l'expression des NCRs est nécessaire afin de mettre en place une thérapie bloquant cet effet et permettant l'activation des fonctions NK.

L'activation des cellules NK semble donc nécessaire dans le contrôle des métastases ganglionnaires. En revanche le rôle des ILC3 ne semble pas être associé à un effet anti-tumoral. En effet, ces cellules

produisent des cytokines pro-inflammatoires qui, dans le cancer du sein, ont été associées à un mauvais pronostic. Des analyses complémentaires sont nécessaires pour déterminer le mécanisme d'activation de cette population dans ce contexte afin de développer une thérapie bloquante.

Ces travaux démontrent à nouveau l'intérêt des immunothérapies basées sur les cellules NK pour des patients atteints de mélanome ou cancer du sein métastatiques, traités par TKI et/ou ICB. Ils révèlent l'existence d'une population ILC3 ganglionnaire produisant des cytokines pro-inflammatoires en réponse à l'invasion métastatique, mettant en évidence l'importance d'étudier le rôle des ILC auxiliaires dans l'immunosurveillance tumorale.

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ANNEXES

Annexe 1: Specific patterns of blood ILC in metastatic melanoma patients and their modulations in response to immunotherapy.

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Article

Specific Patterns of Blood ILCs in Metastatic Melanoma Patients and Their Modulations in Response to Immunotherapy

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Simple Summary: Anti-CTLA-4 and anti-PD-1 immune checkpoints inhibitors (ICI) have revolutionized the treatment of metastatic melanoma patients, leading to durable responses. However, some patients still not respond to this clinically used immunotherapies and there is a lack of biomarkers leading to the choice of first-line therapies. Innate lymphoid cells (ILC) express immune checkpoint receptors and are involved in anti-melanoma immune response. The aim of this article is to study ILCs from peripheral blood of melanoma patients receiving Ipilimumab, an anti-CTLA-4 treatment, and their association with clinical responses to this therapy. Our results show an impact of Ipilimumab on ILCs proportions and phenotype in blood. Moreover, the presence of anergic CD56^{dim}CD16^{DNAM-1} NK cells were associated with progression of the disease. These findings demonstrate the important role of ILC in the response to ICI.

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Abstract: Immunotherapy targeting immune checkpoint receptors brought a breakthrough in the treatment of metastatic melanoma patients. However, a number of patients still resist these immunotherapies. Present on CD8⁺T cells, immune checkpoint receptors are expressed by innate lymphoid cells (ILCs), which may contribute to the clinical response. ILCs are composed of natural killer (NK) cells, which are cytotoxic effectors involved in tumor immunosurveillance. NK cell activation is regulated by a balance between activating receptors that detect stress molecules on tumor cells and HLA-I-specific inhibitory receptors. Helper ILCs (h-ILCs) are newly characterized ILCs that secrete cytokines and regulate the immune homeostasis of tissue. We investigated the modulation of blood ILCs in melanoma patients treated with ipilimumab. Circulating ILCs from metastatic stage IV melanoma patients and healthy donors were studied for their complete phenotypic status. Patients were studied before and at 3, 6, and 12 weeks of ipilimumab treatment. A comparison of blood ILC populations from donors and melanoma patients before treatment showed changes in proportions of ILC subsets, and a significant inverse correlation of CD56^{dim} NK cells and h-ILC subsets was identified in patients. During treatment with ipilimumab, percentages of all ILC subsets were reduced. Ipilimumab also impacted the expression of the CD96/TIGIT/DNAM-1 pathway in all ILCs and increased CD161 and CTLA-4 expression by h-ILCs. When considering the response to the treatment, patients without disease control were characterized by higher percentages of CD56^{bright} and ILC1. Patients with disease control displayed larger populations of activated CD56^{dim}CD16^{DNAM-1} NK cells, while anergic CD56^{dim}CD16^{DNAM-1} NK cells were prominent in patients without disease control. These results provide original findings on the distribution of ILC subsets in advanced melanoma patients and their modulation through immunotherapy. The effects of ipilimumab on these ILC subsets may critically influence therapeutic outcomes. These data indicate the importance of considering these innate cell subsets in immunotherapeutic strategies for melanoma patients.

Keywords: melanoma; innate lymphoid cells; ipilimumab; natural killer cells

1. Introduction

Melanomas are highly metastatic tumors for which the first-line treatment is immunotherapy. Immune checkpoint blockers targeting CTLA-4 and PD-1 restore the T lymphocytes antitumor reactivity and induce long-lasting responses [1–4]. However, the vast majority of patients develop either primary or secondary resistances [5]. Natural killer (NK) cells are potent cytolytic effectors that play a role in the innate and adaptive antitumor immune responses. NK cells are endowed with the capacity to kill tumor cells without prior sensitization, and NK cells are considered highly sophisticated detectives of harmful changes in cells themselves and are pivotal catalyzers of adaptive T cell responses [6,7]. For instance, NK cells are considered the major source of IFN γ in vivo, and NK-derived IFN γ is crucial for priming T helper 1 responses [8]. NK cells originate from bone marrow and mature in the lymph nodes. Though they mainly circulate, NK cells also infiltrate lymphoid organs and tissue by migrating through blood and lymphatics.

Blood NK cells represent 5 to 20% of circulating lymphocytes and contain two major sub-populations according to the expression level of the adhesion molecule CD56 (NCAM) and the expression of CD16 (Fc γ R): CD56^{dim}CD16⁺ and CD56^{bright}CD16⁺ subsets. The CD56^{dim} population predominates in blood (90% of NK cells) and at sites of inflammation, exhibits a high cytotoxic potential, and broadly expresses MHC-I-specific inhibitory receptors. In contrast, the CD56^{bright} subset predominates in lymph nodes (95% of NK cells), produces cytokines upon activation, displays a low cytotoxic potential, and is considered to be a precursor stage of terminal CD56^{dim} NK cells [8,9].

NK activation depends on an intricate balance between activation and inhibitory signals, which determine whether the target will be susceptible to NK-mediated lysis [10,11]. Three main natural cytotoxicity receptors (NCRs) involved in NK cell activation were identified: NKp46 and NKp30, expressed by resting NK cells, and NKp44, induced after stimulation by cytokines. Activation of NK cells is triggered by additional receptors. NKG2D, which is expressed by a majority of peripheral NK cells, binds MHC-related antigen (MIC)-A/B molecules and UL16-binding proteins (ULBP1-4), which are induced on the membranes of stressed cells. The DNAX accessory molecule 1 (DNAM-1), an adhesion molecule belonging to the immunoglobulin superfamily, promotes many of these functions in vitro. NK cells require DNAM-1 for the elimination of tumor cells that are comparatively resistant to the NK-cell-mediated cytotoxicity caused by the paucity of other NK-cell-activating ligands [12]. Simultaneous engagement of NKp46 and DNAM-1 induces cytotoxicity and cytokine secretion by resting NK cells [13]. NK cell activation is controlled by inhibitory HLA-I-specific NK receptors, KIR (killer Ig-type receptor), which are present in the NK CD56^{dim} subset, and the ubiquitous C lectin-type CD94/NKG2A receptor, which binds to HLA-E molecules [14]. The KIR receptors CD158a and b recognize HLA-Cw4 (C2 type) and HLA-Cw3 (C1 type) molecules, respectively [15,16].

Activation of endogenous NK cells with interleukin (IL)-2 and adoptive transfer of in vitro activated autologous NK cells mediate antitumor activity in experimental and clinical settings [17]. NK cells can cure human melanoma lung metastases in nude mice treated with chronic indomethacin therapy combined with multiple rounds of IL-2 [18]. Studies of immune infiltrates in melanoma from The Cancer Genome Atlas (TCGA) indicate that patients with brisk tumor infiltrating lymphocytes (TIL) scores had improved melanoma-specific survival. They further outlined an association between cytokine/chemokine expression and immune tumor infiltrates, suggesting that the expression of specific tumor cytokines (IFN γ , IL10, TGF β) represents important biomarkers of melanoma immune response [19].

Recently, NK cells were classified in the family of innate lymphoid cells (ILCs), which gather cytotoxic effectors (NK cells) and helper-type ILCs (h-ILCs) [20]. The h-ILCs arise

from a common lymphoid progenitor [21,22] and are located in mucosal barriers and various tissues [23], including the intestine, skin, lung, and adipose tissue [24]. ILCs are heterogeneous subsets of lymphocytes that have emerging roles in orchestrating immune response and contribute to maintaining homeostasis and regulating tissue inflammation [25]. Compared to NK cells, h-ILCs are rare in blood relative to other immune cell populations. They are classified into distinct subsets—ILC1, ILC2, and ILC3—which mirror the subsets of CD4⁺ helper T cells. ILC1 produces IFN γ , ILC2 produces IL5 and IL13, and ILC3 produces IL17 and IL-22. ILC subsets have distinct cytokine and transcription factor profiles that align with their biological functions. However, it has emerged that ILC subsets are not phenotypically fixed, but exhibit a considerable heterogeneity and plasticity in different environments [26,27].

The role of h-ILCs in disease pathogenesis has been reported, and their involvement in tumor immunosurveillance is suspected in growing numbers of studies [28]. Several distinct subsets of h-ILCs at skin barriers are involved in the complex regulatory network in local immunity, potentiating adaptive immunity and the inflammatory response [29]. Recent research progress in h-ILC biology has outlined abnormal functions and potential pathogenic mechanisms in autoimmune-related skin diseases. The diversity and plasticity of ILCs and their unique functions in disease conditions suggest their potential value in immunotherapy [30].

A prerequisite for the intelligent implementation of NK cells in an antitumor regimen is a thorough understanding of the changes in the different ILC subsets in tumor patients compared to donors and monitoring in response to immunotherapy. Here, we investigated the phenotype and function of peripheral ILCs in a series of metastatic melanoma patients before and during the course of immunotherapy.

Discrete blood h-ILCs are found in humans, and taking into account their proximity to NK cells, it is interesting to consider these different subsets in cancer patients receiving immunotherapy. The present study assesses the circulating innate lymphoid populations in a new manner, revealing significant changes in the different ILC subsets in blood from melanoma patients and the impact of ipilimumab on these populations.

2. Patients and Methods

2.1. Patient Characteristics and Study Design

Patients with unresectable stage III or IV melanoma were eligible for ipilimumab treatment if they had previously failed in one or more systemic therapies. Ipilimumab is a fully humanized anti-CTLA-4 IgG1k monoclonal antibody approved by the food and drug administration (FDA) for late-stage melanoma after having been demonstrated to extend overall survival in two pivotal phase III trials in a subset of patients [1,31]. Patients received intravenous ipilimumab in doses of 3 mg/kg every 3 weeks for an expected total course of four doses. Treatment was discontinued if either a related adverse event that could not be tolerated occurred or rapidly progressive disease was noted. Blood samples from 16 patients were collected before each injection of ipilimumab at weeks 0, 3, 6, 9, and 12. All patients gave their informed consent for this study in accordance with institutional ethical guidelines. Blood samples from 12 healthy donors were collected from the blood donor center (Etablissement Français du Sang, Hôpital Saint-Louis).

2.2. Definition of Tumor Assessment and Main Clinical Outcomes

Tumors were staged with total-body computed-tomography (CT) scans and cerebral magnetic resonance imaging (MRI) at the baseline, week 12, and week 16. The overall response was assessed according to the immune-response-related criteria (irRCs) derived from time-point response assessments (based on tumor burden).

2.3. Flow Cytometry and Monoclonal Antibodies

Absolute lymphocyte counts were determined using the TruCount system (BD Biosciences) with CD45 PerCPb mAbs (BD Multitest, BD Biosciences, Franklin Lakes, NJ, USA).

Immunophenotyping and ILC analyses were performed on frozen peripheral blood mononuclear cell (PBMC) samples. PBMCs were first stained with Flexible Viability Dye eFluor 506 (eBioscience, San Diego, CA, USA) for 30 min and then stained with surface antibodies (Table S1) in brilliant Stained Buffer (BD) at 4 °C for 30 min. Cells were fixed and acquired on LSRFortessa (BD) and analyzed with FlowJo10.

Total blood ILCs were identified as lin⁻ CD7⁺ cells. CD56 and CD16 further distinguished NK cells, while CD127, CD117, and CRTH2 were used to recognize helper ILCs. Markers in lin included CD3, CD4, CD8, CD5, TCR, CD33, CD19, CD14, and CD235a to exclude T cells, B cells, erythrocytes, and myeloid cells. NK cells were gated as CD56⁺CD127^{low}CD16⁺, ILC1 as CD127⁻CD56⁺CD117⁻CRTH2⁻, ILC2 as CD127⁻CD56⁺CD117⁺CRTH2⁺, and ILC3 as CD127⁻CD56⁺CD117⁻CRTH2⁻. In addition to this backbone, the expression of NK receptors (NCR, NKG2D, CD161, NKG2A, NKG2C, DNAM-1, CD96), markers of activation (CD69, CD62L), and immune checkpoints (CTLA-4, PD-1, TIGIT) was determined in ILC subpopulations. A comparison between donors' and patient's blood ILCs was analyzed. In addition, we compared blood ILC subsets in patients before and after 3, 6, and 12 weeks of ipilimumab treatment.

2.4. CITRUS Analysis

The CITRUS (cluster identification, characterization, and regression) algorithm (Cytobank) was used to compare the abundance of ILC (lin-CD7⁺) subsets and expression of markers between donors and patients, as well as between disease controller and non-controller patients. CITRUS uses a hierarchical clustering map based on similarities in the expression of markers (Table S1). The minimum cluster size of events of the total data used was 2%, and the number of events sampled per file was 5000. The analysis was based upon the abundance of events using significance analysis of microarrays (SAM), with a false discovery rate of 0.01.

2.5. Statistical Analyses

The data are described using means and standard deviations for quantitative variables and counts and percentages for qualitative variables. Comparisons between patients' and donors' (HD) immunological statuses at baseline were performed using a Mann-Whitney test (Prism 8, GraphPad Software Inc., San Diego, CA, USA). Comparisons between different times of treatment for the same patient were performed using a Wilcoxon rank sum test. Correlations were performed with R studio using the non-parametric Spearman correlation test. A *p* value < 0.05 was considered significant.

3. Results

3.1. Melanoma Patients Exhibit a Specific Distribution of Blood ILCs

The proportions of ILC subsets were comparable in donors and patients before treatment (Figure 1A). The NK cells constituted the major subsets, including >80% of CD56^{dim} and 1–12% of CD56^{bright} NK cells. Among the h-ILCs, which represented 2 to 12% of the CD7⁺ cells, there was a major subset of ILC1 (2 to 6%) and 2–4% of ILC2; ILC3 cells were rare (mean value of <0.5% of lin⁻CD7⁺ cells). There was a trend of increasing percentages of ILC2 in patients compared to donors (Figure 1B). We performed correlation analyses to determine the relations between the proportions of the different ILC subsets. In melanoma patients, the percentages of CD56^{dim} cells were inversely correlated with the percentages of ILC1, ILC2, and ILC3 (coefficient correlation > 0.7). In patients, the three h-ILC subsets were positively correlated with each other, while ILC2 and ILC3 did not correlate in do-

nors (Figure 1C). These data suggest that in patients with metastatic melanoma, the relationships between the NK subsets and h-ILC subsets were altered, suggesting a change in the homeostatic regulation of these populations in the blood.

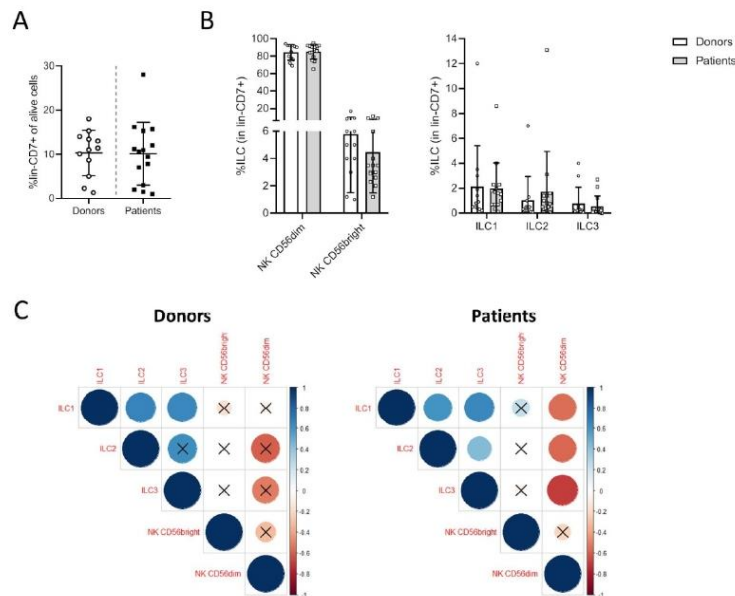


Figure 1. Proportions of innate lymphoid cell (ILC) subsets among the global lin-CD7⁺ population. Percentages of lin-CD7⁺ ILCs in live peripheral blood mononuclear cell (PBMC) from donors and patients (A). Percentages of the different ILC populations in lin-CD7⁺ cells (B). Correlations between the proportions of ILC populations in lin-CD7⁺ cells. A Spearman test was used to calculate the correlation coefficient (X: p value > 0.05) (C).

3.2. Phenotypic Analysis of ILC Subsets in Donors and Patients

A comparison of the ILC populations in donors and patients before treatment was performed with the CITRUS (cluster identification, characterization, and regression) algorithm, which was designed for the fully automated discovery of statistically significant stratifying biological signatures. Using multiparametric flow cytometry data, it allows the identification of ILC subsets that are significantly different in donors and patients before treatment (Figure 2A). CITRUS identified two NK populations that were less present in patients' blood. These populations included activated CD56⁺CD16⁺NKp46⁺NKG2D⁺NKG2A⁻ and CD56⁺CD16⁺NKp46⁺NKG2D^{low} NK cells. Supervised analyses of discrete h-ILC populations showed that blood ILC1 cells had a phenotype that resembled the one of NK cells expressing NK-activating receptors, NKp30 and NKG2D, and inhibitory TIGIT and CTLA-4. These NK-activating receptors were not expressed by ILC2 and ILC3. ILC2 expressed DNAM-1 and CTLA-4; ILC3 was NKR negative and exhibited high CTLA-4 expression (Figure 2B). A comparison of donors' and patients' ILCs showed similar phenotypes of ILC subsets before ipilimumab treatment. There were modest differences, which included an increase in CD69 and decreased CD62L expression by all ILC subsets in melanoma patients (Figure 2B).

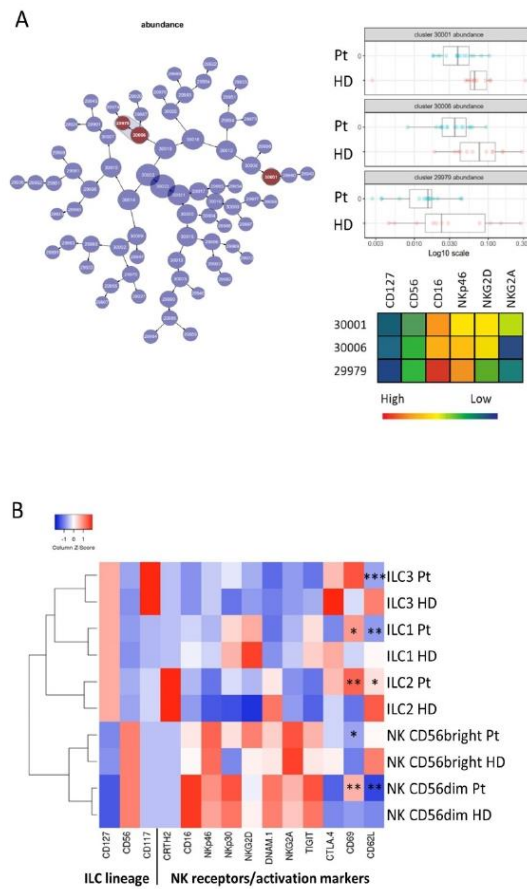


Figure 2. Phenotypic characteristics of blood ILC subsets in donors' (HD) and patients' (Pt) blood. Hierarchical clustering map of the CITRUS (cluster identification, characterization, and regression) analysis for comparing donors and patients before treatment. Clusters whose cell abundances are different between the two conditions are in dark red. Cell abundance in the three clusters is represented on the left side with a heatmap presenting the phenotypes of these clusters (A). Heatmap presenting the markers expressed by ILC populations in HD and in Pt. Mann–Whitney test was used to compare HD and Pt (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$) (B).

3.3. Ipilimumab Induces Early Changes in ILC Proportions

Ipilimumab induced early changes in the proportions and the phenotypes of the ILC subsets. First, the percentages of $\text{lin}^+ \text{CD7}^+$ cells were significantly reduced at 6 weeks (W6) after the beginning of treatment (Figure 3A). The percentages of the ILC1, ILC2, $\text{CD56}^{\text{bright}}$, and CD56^{dim} subsets were decreased through the treatment with ipilimumab (Figure 3B).

Phenotypic analyses of ILC subsets were performed at W12 of the treatment (Figure 3C), revealing modest phenotypic changes. The $\text{CD96}/\text{DNAM-1}/\text{TIGIT}$ pathways were altered in patients. In treated patients, CD96 expression by all ILCs was significantly reduced, except for ILC3 ($p = 0.08$). There was a trend of decreased expression of DNAM-1,

whereas TIGIT was not changed. In addition, there was a higher expression of CD161 by ILC1 and ILC2 in the treated patients. The low CTLA-4 expression in ILCs was increased in ILC1 cells and reduced in ILC3. Expression of Nkp46 was reduced and increased in the ILC3 subsets from ipilimumab-treated patients (Figure 3C).

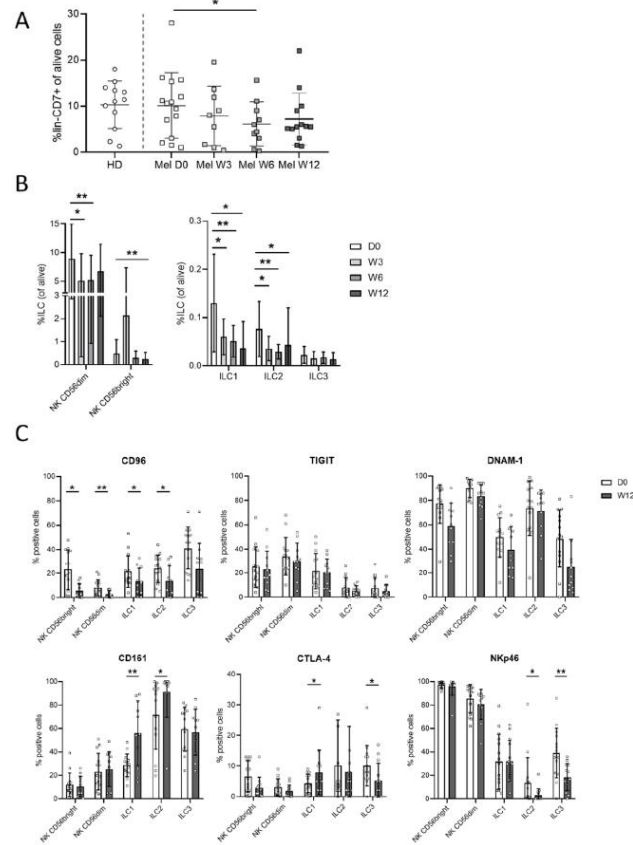


Figure 3. Phenotypic assessment of the immune checkpoint in ILCs from patients treated with ipilimumab. Percentages of linCD7⁺ ILCs in live PBMCs from donors and patients following ipilimumab treatment at day 0, weeks 3, 6 and 12 (D0, W3, W6, W12) (A). Percentages of the different ILC populations in linCD7⁺ cells in patients following treatment (B). Expression of receptors in ILC populations in patients at D0 and at W12 of treatment (C). A Wilcoxon matched-pair signed rank test was used to compare percentages between D0 and other times of treatment (* $p < 0.05$, ** $p < 0.01$).

3.4. Correlation with Clinical Status and Response to Treatment

The clinical status of the 16 patients included was evaluated at W16 of treatment; eight patients had progressed, corresponding to patients without disease control (w/oDC), and eight had stable or reduced tumors, corresponding to patients with disease control (DC).

First, we characterized absolute lymphocyte counts (ALCs), as lymphopenia is a frequent feature of cancer patients with advanced disease associated with poor prognosis. In a previous study [32] including a larger series of patients, peripheral T cell lymphopenia was associated with poor disease control, and $ALC > 10^9/L$ before treatment was correlated with survival and clinical response to ipilimumab. Moreover, T cell counts increased early (W3) following ipilimumab treatment and were maintained at W12. In the present study, none of the 8 DC patients were lymphopenic, and all had increased ALC in response to ipilimumab (Figure 4A). In contrast, three out of seven of the patients w/oDC were lymphopenic, and ipilimumab had no effect on the ALC. We found no correlation between absolute ILC numbers and disease control and no change in ILC counts following treatment with ipilimumab (Figure 4A, right).

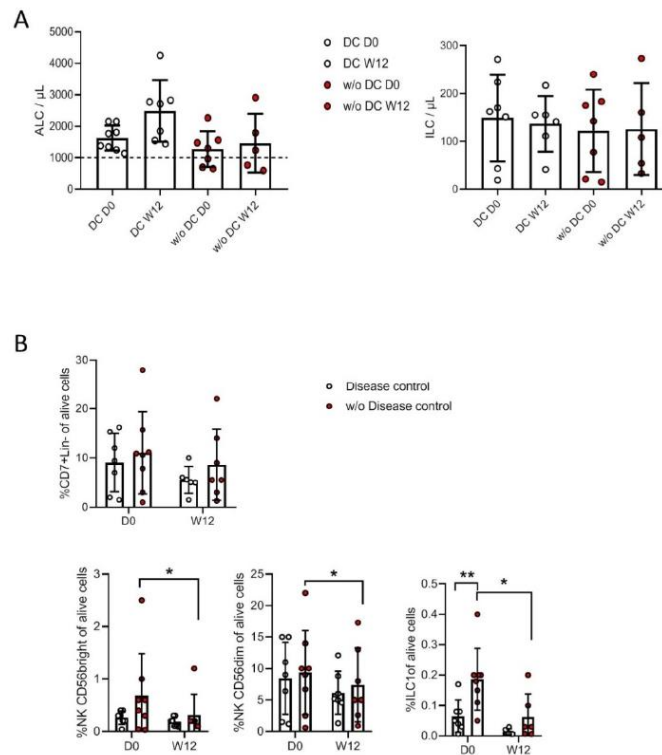


Figure 4. Assessment of ILC subsets in patients with disease control (DC) and in patients without disease control (w/oDC). Absolute lymphocyte counts (ALC) and absolute ILC counts per μL in DC and w/oDC patients (A). Percentages of lin^+CD7^+ ILC, $CD56^{bright}$, $CD56^{dim}$, and ILC1 in live PBMCs from DC and w/oDC patients (B). A Wilcoxon matched-pair signed rank test was used to compare the percentages in patients with DC between D0 and other times of treatment. A Mann-Whitney test was used to compare the percentages between DC and w/oDC patients (* $p < 0.05$, ** $p < 0.01$).

The proportions of blood ILC subsets differed between the two groups of patients (Figure 4B, lower panels). Before treatment, we found a trend of higher percentages of $CD56^{bright}$ NK cells and a significant increase in ILC1 percentages in w/oDC patients than

in patients with DC, whereas the CD56^{dim} NK cell percentages were comparable. Ipilimumab affected the proportions of ILC subsets in patients w/oDC, reducing the percentages of CD56^{bright}, CD56^{dim} NK cells, and ILC1 at W12 compared to at D0. Ipilimumab did not induce any significant changes in h-ILC proportions in patients with DC (Figure 4B).

A comparison of the ILC populations in patients with DC and in w/oDC patients was performed with CITRUS, thus allowing the identification of ILC subsets that distinguished the two groups of patients (Figure 5). Before (Figure 5A) and at W12 of treatment (Figure 5B), patients with DC were characterized by elevated abundance of CD56^{dim}/CD16⁺/DNAM-1⁺/TIGIT⁺ NK cells, while NK CD56^{dim}/CD16⁻/DNAM-1⁻ NK cells were prominent in patients w/oDC. Thus, patients with DC were characterized by lower percentages of ILC1 and the presence of mature activated CD56^{dim} NK cells.

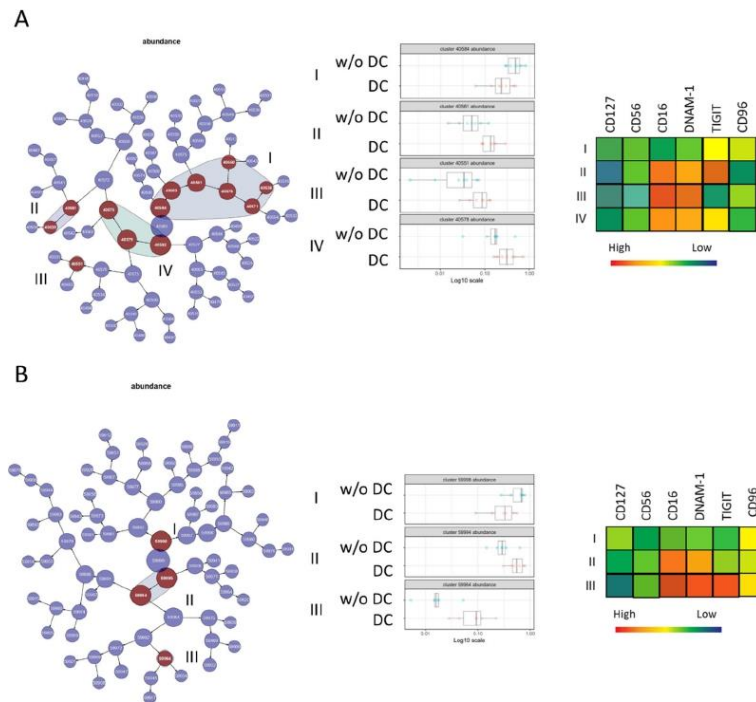


Figure 5. Comparison of natural killer (NK) subpopulations in DC patients and in patients w/oDC with citrus. A hierarchical clustering map of the CITRUS analysis comparing DC patients and w/oDC patients at D0 (A) and W12 (B). Clusters whose cell abundances are different between the two conditions are in dark red. Four groups of clusters are noted at D0 and three at W12. The cell abundance in the different clusters is represented on the left side with a heatmap presenting the phenotypes of these clusters.

4. Discussion

Immunotherapies that use immune checkpoint blockers (ICBs) are efficient treatments for metastatic melanoma patients. However, their efficiency is limited to some patients, and yet numerous patients either resist or relapse. If the reinvigoration of T cell cytotoxicity is the most relevant mechanism underlying antitumor response in responding patients, other immune effectors, which are rarely investigated in immunomonitoring studies, may be involved. The family of ILCs includes helper-type ILCs, which are mainly resident in tissue and control immune homeostasis. Through the secretion of cytokine, they may be involved in the immunosurveillance of tumors. The ILC1 helper type shares the ability to produce $IFN\gamma$ and $TNF\alpha$ with NK cells and is controlled by *tbet*. ILC2 is present in the skin and produces type 2 cytokines, including IL4 and IL13. ILCs rapidly respond to changes in their environment and are able to acquire new secretion capacities. As ILCs, including h-ILCs, are additional players in tissue immunosurveillance, here, we investigated these ILC populations in a series of metastatic melanoma patients that received ipilimumab.

The definition of ILCs implicates that the gating of NK cells is different from the one that is used for specific monitoring of NK cells. Indeed, ILCs are defined as $linCD7^+$ cells, and $CD56/CD16$ or $CD127/CD117/CRTH2$ identify NK cells or h-ILCs, respectively. Among blood ILCs, NK cells largely predominated and h-ILCs represented discrete populations, with a main subset of ILC1 and very low proportions of ILC3. While the proportions of ILCs were similar in donors and patients, distinct correlations between blood-derived NK cells and h-ILCs in HD and in patients were observed. In donors, the NK and h-ILC subsets were independent, while in melanoma patients, there was a strong negative correlation between $CD56^{dim}$ and h-ILCs. The skin contains all ILC subsets, with a predominance of ILC2. In cutaneous melanoma, there is an enrichment of NK, ILC1, and $ILC3NCR^+$ cells. Moreover, NK cells and ILC3 were increased in liver and lung metastases, and ILC2 was increased in lung metastases [33]. In melanoma patients, the dynamic differentiation of ILCs in the tumor environment may allow circulation of other ILC subsets [26].

The most significant phenotypic difference between donors and patients was the reduction of activated NK cells in patients. The comparison of donor and patient blood ILCs using CITRUS outlined a decreased abundance of activated $CD56^+CD16^+NCR^+$ with moderate or high $NKG2D$ expression and no or low $NKG2A$ expression. The downregulation of activation receptors on the $CD56^+CD16^+$ NK subsets from metastatic melanoma patients confirmed previously described numerous studies [34], including ours [35,36] on monitoring NK cells. Immunosuppressive factors and cytokines that are produced by the tumor as soluble molecules or presented on extracellular vesicles (EVs) may contribute to these immune defects of ILCs [33]. Furthermore, NK-derived extracellular vesicles can compromise NK cell function [37]. The higher expression of CD69 and lower CD62L in patients indicate that these ILCs may correspond to recirculating tumor-exposed ILCs [38]. The low CD62L can also indicate their altered cytotoxic function [39,40] and/or capacity to spill out of the tumor and migrate to lymph node.

Our results are in accordance with those of previous studies, showing that ipilimumab affected the ALC, with a sustained increase in T cell counts from W3 to W12 [32]. In contrast, ipilimumab did not affect the absolute ILC counts, but altered the blood proportions and the phenotypes of ILCs. Ipilimumab reduced the proportions of h-ILC subsets at W3, which remained low at W12. The proportions of NK $CD56^{dim}$ were decreased at W3 and W6, but the values at W12 were comparable to values before treatment (D0). As ILCs rapidly adapt to changes in their environment, the impact of ICBs in the tumor lesions may account for these dynamic changes in the blood ILCs.

The proportions $CD56^{bright}$ NK cells were reduced in treated patients at W12 compared to D0. Previous studies reported a change in NK cell subtype in melanoma patients treated with ICBs. Low baseline levels of NK cells and $CD56^{dim}$ NK cells were correlated with a positive response to ipilimumab, and patients with low amounts of $CD56^{dim}$ NK

cells showed a trend of longer survival [41]. While percentages of CD56^{dim} were not reduced in patients with DC, non-supervised CITRUS analyses showed that higher percentages of CD56^{dim}CD16⁺ were associated with patients w/oDC. Furthermore, the relative abundance of CD56^{dim}CD16⁺ NK cells over CD56^{dim}CD16[−] NK cells characterized patients with DC before and after treatment with ipilimumab.

Thus, the most prominent ILC subset that was significantly overrepresented in patients with DC was that of CD56^{dim}CD16[−] NK cells. As CTLA-4 expression by NK cells and h-ILCs was weak, it excluded a direct effect of ipilimumab on NK cells. However, IgG1 antiCTLA-4 mAb can engage FcRIII on NK cells and favor ADCC-mediated lysis. It was reported that ipilimumab engaged CD16 ex vivo on non-classical monocytes by inducing ADCC-mediated lysis of regulatory T cells (Tregs) [42]. Melanoma cells express CTLA-4 [43], and the engagement of CTLA-4 on primary melanoma cell lines induced ADCC and TNF α production by NK cells [44]. Moreover, it was reported that an immunconjugate between ipilimumab and EGFR (epidermal growth factor) targeting breast cancer cells activated NK cells, and inhibited tumor cells more efficiently than each compound alone [45]. The presence of CD56^{dim}CD16[−] NK cells and ADCC may have a prognostic value for ipilimumab treatment outcomes.

In melanoma patients, h-ILC and CD56^{dim} were inversely correlated, and h-ILC1 cells were associated with clinical response to ICBs. Low h-ILC1 percentages were present in patients with DC, while larger proportions of ILC1 characterized melanoma patients that did not benefit from ipilimumab, suggesting that ILC1 exerts pro-tumor immune functions. Accordingly, the conversion from NK to ILC1-like cells that produced VEGF (vascular endothelial growth factor) was recently reported, leading to metastasis development [46]. In addition, TGF β was shown to induce the conversion of NK cells into ILC1, which lacked antitumor capacities [47]. At the baseline, ILC2 frequencies tended to be higher than in donors. Increased numbers of ILC2 were already found in gastric cancers and chronic lymphocytic leukemia [48,49]. In several reports on lung metastasis models, IL-33-activated ILC2s were shown to mediate a potent immunosuppression of NK cells, promoting metastasis [50,51]. On the other hand, ILC2s were reported to amplify the anti-tumor effect of PD-1 blockades by activating tumor-specific T cells in an orthotopic murine model of pancreatic tumors [52], emphasizing the requirement for characterization of intra-tumoral ILC populations [33].

In addition to CTLA-4 and PD-1, additional checkpoint molecules involved in tumor immunosurveillance target ILCs. A comparison of DC and NR patients with CITRUS outlined a high expression of DNAM-1 in DC patients. The involvement of NCR and DNAM-1 in the antitumor function of NK cells against melanoma cells was reported earlier [53]. DNAM-1/CD155 interactions promoted cytokine production and NK-mediated suppression of metastasis of poorly immunogenic murine melanoma [54]. DNAM-1 (CD226) binds several ligands, including CD155 (poliovirus receptor, PVR), which also associate with the inhibitory receptor TIGIT and with CD96; the latter receptor leads to a controversial effect on NK cell function and activity [55]. Soluble ligands (sCD155) also altered DNAM-1 signaling [56,57]. IL15 stimulation with TIGIT blockades reverses altered CD155-mediated NK cell function in melanoma [58]. High CD155 expression by tumor cells was associated with PD-1⁺ CD8⁺T cells and poor response to anti-PD-1, and CD155⁺PD-L1⁺ tumors exhibited a poor response to anti-PD-1/CTLA-4 therapy [59]. Pre-clinical studies suggest that targeting this axis can improve immune-mediated tumor control, particularly when combined with existing anti-PD-1 checkpoint therapies [60]. Combined targeting of CD96 and PD-1 receptors potently reduced lung metastases in murine models. An anti-CD96 antibody that inhibited the CD96/CD155 interaction stimulated the proliferation and local production of IFN γ by NK cells. The targeting host CD96 was shown to complement conventional immune checkpoint blockades [61] and further outlined the relevance of this pathway for NK-cell-based immunotherapy. Another newly developed ICB, anti-NKG2A (Monalizumab), which blocks CD94/NKG2A expressed by

mature NK cells and T cell subsets, exerted antitumor activity as a single agent or in combination with anti-PDL1 or anti-EGFR [62].

Overall, the immune checkpoint inhibitors that alter the tumor microenvironment change the crosstalk between resident ILCs, leading to anti- or pro-tumoral responses [33]. Our studies identify innate immunity parameters in the blood melanoma patients that are correlated with a response to immunotherapy.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6694/13/6/1446/s1, Table S1: List of mAbs used in the studies.

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Data Availability Statement: All data associated with this study are present in the paper or in the Supplementary Materials.

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Conflicts of Interest: Page: 12
The authors declare no conflict of interest.

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Annexe 2: NKG2D/NKG2-Ligand Pathway Offers New Opportunities in Cancer Treatment.



NKG2D/NKG2-Ligand Pathway Offers New Opportunities in Cancer Treatment

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The antitumor functions of NK cells are regulated by the integration of positive and negative signals triggered by numerous membrane receptors present on the NK cells themselves. Among the main activating receptors, NKG2D binds several stress-induced molecules on tumor targets. Engagement of NKG2D by its ligands (NKG2D-Ls) induces NK cell activation leading to production of cytokines and target cell lysis. These effects have therapeutic potential as NKG2D-Ls are widely expressed by solid tumors, whereas their expression in healthy cells is limited. Here, we describe the genetic and environmental factors regulating the NKG2D/NKG2D-L pathway in tumors. NKG2D-L expression is linked to cellular stress and cell proliferation, and has been associated with oncogenic mutations. Tumors have been found to alter their NKG2D-L expression as they progress, which interferes with the antitumor function of the pathway. Nevertheless, this pathway could be advantageously exploited for cancer therapy. Various cancer treatments, including chemotherapy and targeted therapies, indirectly interfere with the cellular and soluble forms of NKG2D-Ls. In addition, NKG2D introduced into chimeric antigen receptors in T- and NK cells is a promising tumor immunotherapy approach.

Keywords: NKG2D, natural killer cells, NKG2D ligands, tumor immunosurveillance, NKG2D CARs

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NKG2D/NKG2D-LS IN TUMOR IMMUNOSURVEILLANCE

The immunosurveillance theory described by Robert Schreiber in 2002 (1) suggested that NK cells are involved in the early control of tumor development, before the successive equilibrium and escape phases when tumor-induced immunosuppression results in the emergence of immune-resistant tumor variants. During the elimination phase, NK cells detect and kill emerging transformed cells. NK cells naturally express receptors detecting stress-induced molecules and altered expression of Major Histocompatibility Complex (MHC) class-I molecules on transformed targets (2). NK cells also potentiate the adaptive immune response through cytokine secretion and by stimulating dendritic cells (DC), notably within lymph nodes (3–8).

Several groups have used samples obtained after curative resection to investigate the role played by NK cells in primary solid tumors. Their results indicated that NK cell infiltrates may correlate with clinical outcome (9–14). In most reports, NK cells were present at low numbers within tumors,

as reviewed in (15). In contrast, in colorectal carcinoma (16), lung cancers (14), and gastrointestinal stromal tumors (GIST) (17) numerous NK cells were present in peritumoral areas. NK cells are generally underrepresented among tumor-infiltrating lymphocytes compared to their circulating proportions, and their effector functions are also altered within the tumor microenvironment, as shown in breast and lung malignancies (18, 19). NK cell dysfunction, based on reduced cytotoxicity and cytokine release, correlates with downregulation of NK-activating receptors (18–21). However, this is a tumor-specific phenomenon, described in renal cell carcinoma (RCC) (22), GIST (17), neuroblastoma (23), melanoma (24, 25), and acute myeloid leukemia (26, 27). Altogether, these data revealed that, more than NK cell numbers, expression of NK cell receptors (including activating NK receptors or inhibitory KIRs) strongly influence prognosis and disease outcome. The only notable exception to this conclusion is chronic myeloid leukemia (CML) patients for whom treatment with imatinib was interrupted; in these patients, NK cell numbers were a significant predictive parameter for relapse (28, 29).

In addition, NK cells are thought to play a role in the emergence of metastases, as high numbers of circulating or tumor-infiltrating NK cells inversely correlated with metastatic disease (30, 31). The mechanisms deployed to limit cancer dissemination could involve activating NK receptors, including NKG2D or NKp46. In murine models of melanoma and prostate cancer, IFN- γ production by NK cells triggered by NKp46 activation was found to induce expression of the extracellular matrix protein fibronectin 1 in tumor cells, altering tumor architecture and controlling metastatic invasion (32).

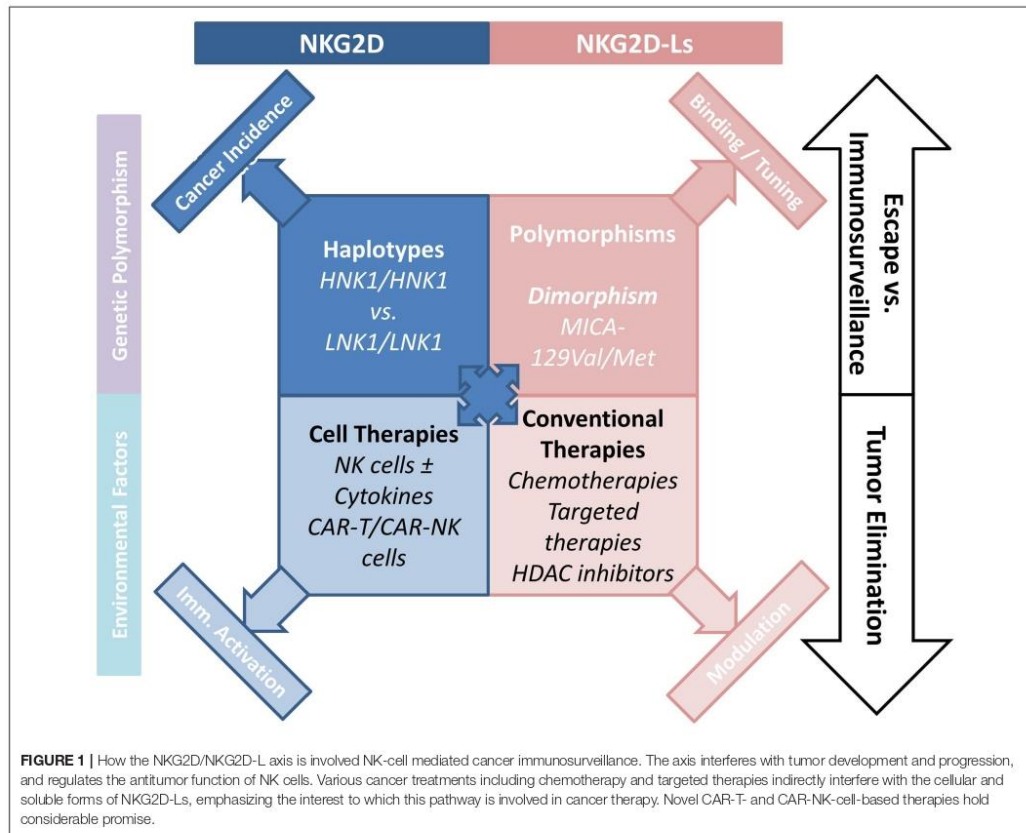
NK cell activation is regulated by signals from activating receptors and inhibitory NK receptors, which bind to HLA-class-I molecules. In addition to natural cytotoxicity receptors, or Ig-type family receptors—the ligands for which have not yet been clearly identified—NKG2D (Natural Killer Group 2, member D)—a C-type lectin receptor—is a major activating receptor for NK cells. In humans, the gene encoding NKG2D (*KLRK1*) lies amid a cluster of genes referred to as the “NK-complex” (NKC) that includes several genes expressed by NK cells [*KLRD1* (CD94), *KLRC4* (NKG2F), *KLRC3* (NKG2E), *KLRC2* (NKG2C), and *KLRC1* (NKG2A)]. NKG2D was first identified on the surface of NK cells as an immunosurveillance receptor. It is also expressed by most CD8⁺ and a small subset of CD4⁺ cytotoxic $\alpha\beta$ T cells, as well as innate-like immune cells, such as some iNKT cells and $\gamma\delta$ T cells (33, 34). NKG2D is a type II transmembrane protein and, in humans, it associates with the transmembrane domain of the adaptor protein DAP-10 (DNAX-Activating Protein 10). Ligand binding causes dimerization of two NKG2D monomers to form an active receptor which phosphorylates DAP10 and triggers NK cell activation signaling pathways which promote Ca²⁺ influx, actin-based cytoskeleton reorganization, and microtubule polarization (35). This signaling cascade leads to the release of the contents of cytolytic granules, and in some cases elicits the production of cytokines by NK cells (36). NKG2D provides co-stimulatory signals in activated T cells (37). NKG2D expression in NK cells and CD8⁺ T cells can be upregulated, in particular in response to cytokines, such

as interleukin (IL)-2, and IL-15, while transforming growth factor (TGF)- β can decrease NKG2D expression.

Our current understanding of the role played by NKG2D in controlling tumor development through NK cell and cytotoxic T-lymphocyte (CTL) activity was aided by the early characterization of its ligands (38). NKG2D binds to eight molecules, members of the following two families: MHC class-I-related chains A or B (MICA/B), and UL16-binding proteins (ULBP1–6). Seven *MIC* genes (*MICA* to *MICG1*) have been identified, located on chromosome 6p21.33. Only *MICA* and *MICB* genes are translated into proteins. As to ULBP, six protein-coding genes (*ULBP1* to *ULBP6I*) have been described on chromosome 6q24.2–25.3. Among all these proteins, MICA, MICB, ULBP4, and ULBP5 are transmembrane-anchored glycoproteins, whereas ULBP1, ULBP2, ULBP3, and ULBP6 are bound to the cell surface by a glycosphatidylinositol (GPI) motif (39). All NKG2D-Ls are composed of one alpha 1 and one alpha 2 extracellular immunoglobulin (Ig)-like domain, which share a strong homology to the corresponding domains in classical HLA-class-I molecules (40). MICA and MICB contain a third, additional extracellular Ig-like domain (alpha 3). NKG2D-Ls are not associated with β 2-microglobulin and bind no antigenic peptide.

ROLE OF NKG2D AND NKG2D-L POLYMORPHISMS IN TUMOR IMMUNOSURVEILLANCE

The pioneering work by Imai et al. attributed a major role to NKG2D polymorphisms in cancer immunosurveillance (41), and in the prevention of cancer formation (42). An 11-years follow-up survey of a cohort including >3,500 members indicated that medium and high natural cytotoxic activity of peripheral-blood lymphocytes was associated with a reduced cancer risk, whereas low natural cytotoxicity correlated with a higher incidence of cancer. These findings suggest a role for NK cell-mediated immunity in controlling cancer. Analysis of 25 SNPs reported with an allele frequency of >10% in the NKC gene cluster identified eight SNPs in the NKG2D locus (*KLRK1*) that form two haplotype blocks (NKG2Dhb1 and hb2). Each of these blocks can generate two major alleles linked to low (LNK) or high (HNK) cytotoxic activity. Patients with the HNK1/HNK1 NK2GDhb1 haplotype had a lower incidence of cancer compared to those with the LNK1/LNK1 haplotype (41). In a Japanese population the HNK1/HNK1 genotype was associated with decreased colorectal (43) and aerodigestive tract cancer (44). A recent report indicated that NKG2D gene polymorphisms also correlated with control of CML by dasatinib (45). Thus, patients with the NKG2D HNK1/HNK1 haplotype achieved deep molecular response (MR4.5) more quickly than those with other haplotypes. Interestingly, phosphorylation of VAV1 on Tyr174, which was proposed as a major mechanism by which dasatinib intensifies NK cell activity (46), could also be enhanced by expression of the NKG2D HNK1 allele (45). These data suggest that the NKG2D HNK1/HNK1 haplotype may influence cancer development and modulate treatment response (Figure 1).



Importantly, MICA and MICB as well as ULBP molecules are highly polymorphic and allelic variation can alter their expression levels or their affinity for NKG2D. As a result, NKG2D-L polymorphisms may strongly influence NKG2D-mediated NK cell triggering by tumor cells or other stressed targets (47). To date, 107 MICA and 47 MICB alleles have been described (updated allele numbers can be found at <http://hla.alleles.org/nomenclature/index.html>). SNPs are located within regions encoding the $\alpha 1$ and $\alpha 2$ extracellular domains. These alleles are transcribed to produce a total of 82 MICA and 30 MICB proteins. The impact of MICA polymorphisms on protein expression and function remains only partly characterized, and MICA-129 is the only SNP described so far that affects NKG2D receptor affinity (48). MICA-129 (rs1051792) dimorphism—the substitution of a methionine (Met) for a valine (Val) at position 129—alters MICA affinity for the NKG2D receptor: MICA-129Met has an affinity 10- to 50-fold higher than MICA-129Val. Significantly, expression levels for MICA-129Met isoforms are reduced compared to the MICA-129Val molecule (49), but

they nevertheless have a higher capacity to trigger the NKG2D pathway, leading to enhanced NK cell activity (50).

Several studies implicated MICA polymorphisms in viral infections and autoimmunity [reviewed in (51)], but few have investigated the impact of MICA polymorphism in cancers. Initial studies focused on cervical cancer and found no association between MICA polymorphism and disease susceptibility (52). In contrast, MICA polymorphism was found to be a significant risk factor for other tumors. For example, MICA-129Val is associated with poor prognosis in nasopharyngeal carcinoma (53) or breast cancer (54) in a Tunisian population. In melanoma, Isernhagen et al. (49) showed that MICA-129Val-homozygous melanoma cell lines expressed higher surface levels of MICA than cells with the Met/Met genotype, which released more soluble MICA. A group of frequent MICA alleles, named MICA-A5.1 (prototype MICA*008), produce a truncated protein that acquires a GPI anchor allowing it to be recruited to exosomes, from where it can downmodulate NKG2D expression

(55). A GWAS study in cervical cancer patients linked one MICA-adjacent region to the disease and identified a SNP (rs2516448) linked to the MICA-A5.1 frame-shift mutation, suggesting that this allele may cause impaired immune activation resulting in cancer development (56). In colorectal liver metastases, in contrast, the MICA-A5.1 polymorphism was associated with better tumor control and response to treatment (57).

Few studies have investigated ULBP polymorphisms, probably because of the limited number of SNP identified within these genes. However, one ULBP6 dimorphism (two SNP at positions 106 and 147) plays a significant role in determining affinity of the protein for the NKG2D receptor. The ULBP0602 molecule (which contains Leu¹⁰⁶ and Thr¹⁴⁷ in contrast to ULBP0601's Arg¹⁰⁶ and Ile¹⁴⁷) binds to NKG2D with a 10- to 1,000-fold higher affinity than other ULBPs. This difference in binding could result in decreased interaction of NKG2D with other ligands; it could thus have a negative effect on NK cell function (58).

REGULATION OF NKG2D AND NKG2D-L EXPRESSION

NKG2D-Ls are rarely expressed by healthy cells, but are induced at the cell surface when the cell is stressed as a result of viral infection or malignant transformation; they are therefore called "induced-self" ligands. NKG2D-L-positive cells are detected and eliminated, mostly by NK cells. Expression of NKG2D-Ls is regulated by several mechanisms, which may be transcriptional, translational or post-translational. Expression of NKG2D-Ls is induced by DNA damage, a characteristic of tumor transformation, which leads to the activation of the ATM-ATR DNA repair pathways (59). In mouse models, hyper-proliferation can also induce NKG2D-L expression through activation of the E2F transcription factor (60). Indeed, following HER2/HER3 or BCR-ABL activation, proliferative signals can induce MICA/B and ULBP expression (61). Cellular stress, such as heat shock, has also been reported to induce heat shock factor 1-mediated MICA/B expression (62, 63).

The NKG2D/NKG2D-L pathway is triggered early in cancer development and participates in the elimination of tumor cells. However, during tumor progression, profound changes occur and the NKG2D and/or NKG2D-Ls are targeted by a range of tumor escape mechanisms. Cancer can sculpt the immune environment by selecting immune-ligand-negative variants (1, 64). Tumor cells expressing high levels of NKG2D-Ls can thus be eliminated as part of the tumor immunoeediting process, which involves NK cells and NKG2D, and progressively results in the emergence of NKG2D-resistant variants (65). Persistent NKG2D-L expression by tumor cells may cause systemic immunosuppression as a result of NK exhaustion and perturbation of the immune synapse (26). Epigenetic and transcriptional regulation mechanisms are often perturbed in tumor cells, and NKG2D-L expression may be altered. In melanoma, endoplasmic reticulum stress can reduce MICA

transcription by modulating activation of the transcription factor E2F1 (66). Indeed, immature isoforms of MICA are retained in the endoplasmic reticulum, resulting in limited membrane-display of MICA (67). Tumors can also inhibit NKG2D-L expression by altering cell surface glycosylation (68), notably in a hypoxic environment (69). In CML, BCR/ABL controls MICA expression through post-transcriptional mechanisms (70), including MICA glycosylation (71). Finally, histone deacetylases (HDAC) may also regulate NKG2D-L expression (72, 73).

Soluble factors secreted by tumor cells and cells from their microenvironment can also alter NKG2D-L expression levels. Thus, TGF- β and IL-10 secreted by regulatory T cells (Tregs) and myeloid-derived suppressor cells downmodulate the expression of NKG2D-Ls (74, 75). Some tumors were demonstrated to secrete TGF- β , resulting in reduced NK cell-mediated lysis (10). ADAM metalloproteinases can catalyze shedding of NKG2D-Ls from the cell's surface, and the released soluble forms can hamper NKG2D signaling (76). Upregulation of ADAM10 or ADAM17 expression in tumors has been linked to the release of solMICA/B, decreased membrane MICA/B expression, and reduced NKG2D expression on NK cells or CD8 T cells (77, 78). Importantly, high serum levels of soluble MIC associated with poor clinical prognosis and the emergence of metastases in RCC (79) and prostate cancer (80). Most leukemia patients present high levels of at least one solNKG2D-L, associated with reduced NKG2D expression by NK cells and impaired anti-leukemic function. In patients who entered complete remission following treatment, solNKG2D-Ls were no longer detected, and NK function was restored (81). Following publication of these data, a meta-analysis of 19 studies, comprising 2,588 patients with 10 different types of tumor, showed that serum concentrations of solMICA/B represent a potential prognostic marker in human cancer (82). In metastatic melanoma, levels of solULBPs are associated with reduced survival in patients treated with immune checkpoint blockers (83); solNKG2D-Ls could thus be a relevant biomarker to select melanoma patients for immunotherapy.

CONVENTIONAL CANCER TREATMENT AND THE NKG2D/NKG2D-L PATHWAY

The frequent and high expression of NKG2D-Ls by tumor cells in various human cancers, and the potent antitumor function of the NKG2D/NKG2D-L pathway are now established (Figure 1). Cancer therapies aiming to improve or restore NK- and T-cell responses through NKG2D activation have attracted considerable interest. Among the options proposed, conventional cancer treatments can be used to increase NKG2D expression and signaling. Indeed, a number of cancer treatments increase NKG2D-L expression on tumor cells. Thus, HDAC inhibitors (Valporic acid) acting on epigenetic regulation of NKG2D-Ls can upregulate their membrane expression (84, 85). Cisplatin, Gemcitabine, Oxiplatin, or 5-fluorouracil chemotherapies have all been shown to increase MICA/B expression on tumor cells through modulation of the ATM-ATR pathway. Other

chemotherapies directly kill tumor cells while also inducing NKG2D-L expression, which stimulates tumor elimination by activating immune processes (86–89). Proteasome inhibitors inducing ULBP2 expression and decreasing HLA-I molecules may promote NK cell immunosurveillance of hematologic malignancies (90). ADAM10 or MMP inhibitors could be used to inhibit NKG2D-L-shedding and promote NK cell activity, this approach has been shown to restore NKG2D-Ls in some cancers (91–93). Similarly, antibodies targeting the proteolytic site can prevent shedding of MICA/B proteins, and was shown to limit tumor growth and reduce the formation of metastases in a humanized murine melanoma model (94).

Inhibitors of oncogene-driven mutations are actively being developed to treat various tumor types. Such inhibitors decrease the constitutive activation of kinases involved in tumor cell proliferation and also affect NKG2D-L expression (61, 95). Treatment with imatinib controls the expression of NKG2D-Ls and membrane ganglioside (GM1), and was shown to interfere with NK cell recognition and cytotoxicity of BCR/ABL cells (70, 71). Treatment of melanoma cells with BRAF and MEK inhibitors modulates the expression of MICA/B and ULBP2, attenuating their recognition by NK cells (96). These effects can be overcome by the simultaneous application of HDAC inhibitors, restoring NKG2D-L expression and stimulating NK cell recognition and function (97). Erk activation was shown to increase NKG2D-L expression (98, 99), but, by acting on MMP, Erk/MEK activation can disrupt the equilibrium between membrane and soluble isoforms of NKG2D-Ls and alter NKG2D function (100, 101). It would therefore be relevant to control membrane and soluble NKG2D-Ls when performing immunotherapy trials.

EXPLOITING THE NKG2D/NKG2D-L PATHWAY FOR CELL THERAPY-BASED CANCER TREATMENT

One of the most promising approaches to cancer immunotherapy relies on revisited immune-cell-based therapies, T cells engineered to express chimeric antigen receptors (CARs) can be highly tumor-specific and have a high killing potential (102). CAR-T cells present a major clinical benefit for patients with malignant hemopathies. For instance, CAR-T cells were shown to be potent against CD19-expressing hematologic tumors in several trials (103, 104). However, the safety of CAR-T cells remains a major obstacle, and CARs must be optimized to increase efficacy and limit treatment-related morbidities due to cytokine-release syndrome (105). Up to now, the use of CAR-T cells for treating solid tumors has been limited by the lack of appropriate tumor antigens (106). Production of modified NK cells expressing CARs could represent an alternative for treatment of solid tumors as they recognize numerous tumor cell types. In addition, infusion of large numbers of NK cells is known not to induce autoreactivity. CAR-NK cells could thus be used in

complement or as an alternative to CAR-T cells (Figure 1). NK cells have recently been engineered with CARs to enhance their killing activity, and trials of these cells for treatment of refractory solid tumors have been initiated (107). NK-92 cells were engineered with tumor antigen-specific CARs (EGFR, EpCAM) and successfully used in xenograft models (108, 109). NK cells have a limited lifespan and do not produce memory cells, as a result the excessive activation observed with CAR-T cells should be avoided. In addition, engineered CAR-NK cell lines could be mass-produced, circumventing the need to generate autologous products for each patient, a process that remains challenging and expensive (110). Another promising option would be to express CARs in human iPSC-derived NK cells. This approach might present several advantages, as it could provide a universal cell therapy product (111).

When considering CAR constructs the NKG2D receptor is worthy of attention. Indeed, as we have seen, NKG2D-Ls are widely expressed by a number of solid human tumors, and their relatively selective expression by transformed cells compared to healthy cells makes them an attractive receptor for CARs constructs in T cells. The extracellular domain of NKG2D is used in different CARs constructs with a view to promoting tumor-reactive T reactions (112–114). However, investigations must be performed with care as NKG2D-ligands can also be expressed by healthy cells (115), potentially leading to significant toxicity (116).

The expression of NKG2D-Ls by myeloid cells, Tregs and endothelial cells in the tumor microenvironment suggests that CAR NKG2D cells could also be used to control *in situ* immunosuppression (117). Thus, CAR-NK cells transduced with NKG2D fused to the TCR CD3 ζ chain could be used to target suppressive myeloid cells and improve infiltration and function of subsequent infusions of tumor-specific CAR-T cells (118).

NKG2D-based CARs with full-length NKG2D or NKG2D-ligand binding domains represent a novel strategy to target several types of solid tumors, and would have the capacity to induce potent antitumor immunity in patients. NKG2D CARs could not only target tumors but also myeloid immunosuppressive cells and Tregs, as well as other cells in the microenvironment that promote tumor progression.

AUTHOR CONTRIBUTIONS

AF, ND, and AC wrote the manuscript. AT, M-FA, MM, and LR read and corrected the manuscript.

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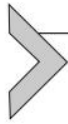
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Annexe 3: Two-dimensional dynamic evaluation of natural killer cell-mediated lysis of adherent target cells



CHAPTER FIFTEEN

Two-dimensional dynamic evaluation of natural killer cell-mediated lysis of adherent target cells

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Contents

1. Introduction	290
2. Assessment of NK cell-mediated lysis using cell impedance dynamic measure	294
2.1 Principles	294
2.2 Protocols	295
3. Concluding remarks	300
4. Notes	301
Acknowledgments	301
References	301

Abstract

Cell-mediated cytotoxicity is a major function of cytotoxic lymphocytes. The cytotoxic function of immune effectors requires accurate and sensitive quantification as tumor immunotherapies are actively developed to treat growing types of tumors. Various methods have been developed to quantify this function. A first approach consists in measuring the externalization of LAMP-1 (lysosomal associated membrane 1), CD107a molecules transitory expressed at the cell surface by degranulating cytotoxic cells and is determined by flow cytometry. The focus of the chapter concerns the second approach that quantifies target cell lysis resulting from the close contact interaction with cytotoxic Natural Killer (NK) lymphocytes. For long time, target cell lysis was evaluated by chromium release assay, requiring use of radioactive labeled salt (⁵¹Cr) and specific devices not compatible with repeated tests performed for immunomonitoring of patients. Other methods include fluorimetric and bioluminescence assays. Monitoring immune cell-mediated lysis of adherent targets by the dynamic measure of cell proliferation using the Real time cell analyzer (RTCA) is a good alternative. The test relies on

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sensitive dynamic measure of cell index values during their interactions with cytotoxic immune effectors. The cell index increasing with cell proliferation rapidly drops in presence of immune cells. The lysis is quantified in reference with targets alone and expressed as percentages of lysis curves. Moreover, the dynamic monitoring of target cell index allows the evaluation of drugs, cytokines, mAbs on target cell lysis. Here we describe a robust and sensitive method for quantification of immune cell-mediated lysis of adherent targets.



1. Introduction

Cytotoxicity is a key function of immune lymphoid cell subsets such as CD8⁺ T lymphocytes and NK cells implicated in tumor immunosurveillance. Cell cytotoxicity is a sensitive read out to monitor the impact of immunotherapies that are now reference treatments for cancer patients. Actual tumor immunotherapies involve on one hand adoptive transfer of engineered chimeric antigen receptor CAR-T cells or CAR-NK cells that show efficiency in hematologic cancers (Sadelain, Brentjens, & Riviere, 2013; Salter, Pont, & Riddell, 2018) and are evaluated in solid tumors (Bollino & Webb, 2017; Watanabe, Kuramitsu, Posey, & June, 2018). On the other hand, patients are treated with immune checkpoint blockers (ICB), mAbs that block immune inhibitory receptors to restore a high lytic function by cytotoxic T lymphocytes (CTL) or NK cells infiltrating the tumor (Andre et al., 2018; Ribas & Wolchok, 2018). To follow the impact of these immunotherapies in patients, it is important to develop and use accurate and standardized methods to measure the cytotoxic capacity of transferred engineered CAR-T cells, CTL or NK cells in ICB treated patients, starting from ex vivo samples immune cells. Lymphoid cell-mediated cytotoxicity is dependent of close contact between effectors and targets and involves the formation of an immune synapse that gather receptor ligands interactions inducing activation of cell signaling in immune effectors and leading to apoptosis of the target (Dieckmann, Frazer, Asano, Stinchcombe, & Griffiths, 2016; Montoya, Sancho, Vicente-Manzanares, & Sanchez-Madrid, 2002). NK cell-mediated cytotoxicity is also governed by the formation of a lytic immune synapse leading to the directed secretion of cytolytic granules and subsequent target cell death (Mace et al., 2014).

Different approaches for evaluating effector cell lytic capacity or target cell lysis were developed (Fig. 1). Lytic potential of effector cells is assessed by flow cytometry by the measure of LAMP-1 externalization (CD107a assay) that comes out as a surrogate marker of cytotoxic potential of immune

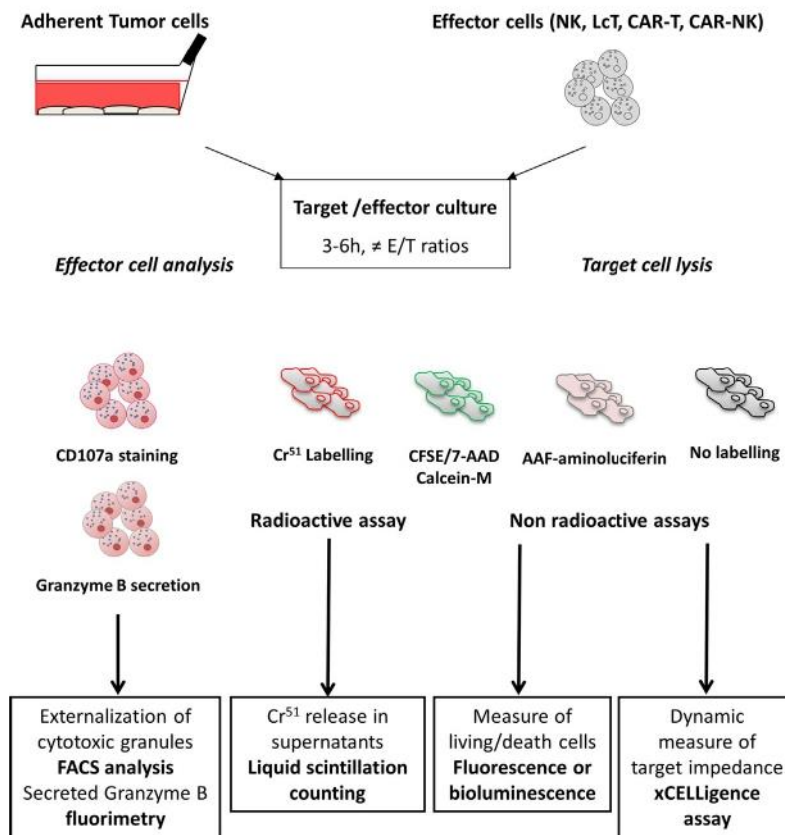


Fig. 1 Measure of cytotoxic effector cell activation and cell-mediated lysis assay of adherent tumor cells. Effector and target cells are mixed together at different E/T ratio for short term cocultures (2–4 h). Activation of effector cell cytotoxic capacity can be evaluated by externalization of CD107a (LAMP-1) detected by flow cytometry in cell suspensions. Membrane staining of immune cells with specific mAbs allows the detection of CD107a in distinct cytotoxic cell subsets in cell suspensions. Concentration of granzyme B in supernatants can be monitored. Tumor target cell lysis can be monitored by different methods. The ⁵¹Cr release assay, the gold standard assay for decades is progressively replaced by nonradioactive assays. These tests allow detection of dead and living cell ratios detected by fluorescence or bioluminescence. RTCA xCELLigence assay allows dynamic measure of cell proliferation of adherent cells and has been adapted to determine immune effector cell-mediated death.

cells (Zaritskaya, Shurin, Sayers, & Malyguine, 2010). The quantification of Granzyme B by ELISPOT assay also refers to lytic potential of NK cells and is used for monitoring cell-mediated cytotoxicity (Shafer-Weaver et al., 2003) and can be used to follow cell-mediated cytotoxicity in cancer vaccine trials (Shafer-Weaver et al., 2006). However, these assays do not inform on the fate of the target cells.

The alternative is to measure target cell lysis by assessing target cell death. Target lysis occurs rapidly after formation of the synapse and leads to apoptosis of the target cell. Immune cell-mediated cytotoxicity focused on the lysis of the target can be determined by different techniques that display various sensitivity and reproducibility (Fig. 1). For decades the gold standard technique has been the use of chromium release assay developed in 1970 (Brunner, Mael, Rudolf, & Chapuis, 1970), based on insertion of chromium salts in the membrane of the target during a short incubation. Then the effector cells at different E/T ratios are added and cocultures are maintained for 2–6h. The measure of the radioactive counts in supernatants of cocultures are determined at given time points of cocultures. Lysis is estimated by the formula:

$$[(\text{experimental release} - \text{minimum release}) / (\text{maximum release} - \text{minimum release}) \times 100],$$

where minimum release is the supernatant from labeled targets incubated with medium, maximum release the supernatant from detergent treated labeled targets. This technique is reliable and sensitive but highly depends on the first step of insertion of chromium salts in the membrane (Fig. 1).

Determination of cytotoxic immune cell-mediated lysis is a useful read out to follow along immunotherapy treatments by adoptive cell transfer of engineered T and NK cells expressing a chimeric antigen receptor (CAR). The precise and standardized evaluation of tumor cell survival after coculture with cytotoxic immune cells is a prerequisite to calculate the percentages of tumor cell lysis.

In that context, the ^{51}Cr release assay is not appropriate as manipulation of radioactive elements is restricted, requiring safe disposal of radioactive waste and cannot be used for routine assay by immunomonitoring laboratories. RTCA with xCELLigence has been used in preclinic trials (Cerignoli et al., 2018; Quereda et al., 2010) and to follow manufacture of CAR-T cells (Gargett, Truong, Ebert, Yu, & Brown, 2019).

Alternative methods to measure target cell death include assays with vital dyes that are incorporated in target before addition of cytotoxic effectors and assessment of dilution of the dye. These nonradioactive assays have been developed in which target cells are loaded with fluorescent dyes. Different Fluorometric Cell Viability Kits have been designed to quantify live cell numbers based on the presence of their cytoplasmic membrane integrity, as end-point assay for cell viability. The fluorescent signal generated from the assay is proportional to the number of living cells in the sample.

Calcein-AM is a nonfluorescent, hydrophobic compound that easily permeates intact, living cells. The hydrolysis of Calcein-AM by intracellular esterases produces Calcein, a hydrophilic strongly fluorescent compound that is retained in the cell cytoplasm. Cells grown in black-walled plates can be quantified in less than 2h using a fluorescence plate reader with excitation at 490 nm and emission at 520 nm. Calcein-AM provides a simple, rapid and accurate method to measure cell viability and or cytotoxicity (Roden, Lee, Panelli, & Marincola, 1999). It has been adapted for measuring immune cell-mediated cytotoxicity (Somanchi, McCulley, Somanchi, Chan, & Lee, 2015).

Fluorescent probes such as 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to label target cells, in combination with red fluorescent probes such as 7-AAD or propidium iodide to label dead effector and dead target cells can be used. CFSE passively diffuses into cells and its acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. 7-AAD and propidium iodide are fluorescent dyes excluded from live cells but penetrating dead or damaged cells to label DNA. The labeling distinguishes living target cells in green, dead target cells in green and red, dead effector cells in red, and live effector cells, which remain unstained. Thus, effector cells cytotoxicity can be evaluated quantitatively at a single cell level (Kim, Donnenberg, Donnenberg, Gooding, & Whiteside, 2007).

Other assays use luminescence for assessing relative dead cell number in cell populations. The CytoTox-Glo™ Assay is a luminescent cytotoxicity assay that measures the extracellular activity of a distinct intracellular protease activity released from membrane-compromised cells. A luminogenic cell-impermeant peptide substrate (AAF-aminoluciferin) is used to measure dead-cell protease activity. The liberated aminoluciferin product is measured as “glow type” luminescence generated by Ultra-Glo™ Recombinant Luciferase provided in the assay reagent. The AAF-aminoluciferin substrate cannot cross the intact membrane of viable cells and does not generate any appreciable signal from the live-cell population. The amount of luminescence directly correlates with the percentage of cells undergoing cytotoxic stress. With the addition of a lysis reagent, the CytoTox-Glo™ Assay also can deliver the luminescent signal associated with the total number of cells in each assay well. Viability is calculated by subtracting the luminescent dead-cell signal from the total luminescent value, thus allowing normalization of the data to cell number. The cytotoxicity protease biomarker is constitutive and conserved across cell lines, and the CytoTox-Glo™ Assay demonstrates excellent correlation with other methods of assessing cell

viability. Bioluminescence based cytotoxicity assay with target cells stably transduced with luciferase was used to measure NK cell activity (Karimi et al., 2014).

However, these tests lack sensitivity, are not easily standardized and are time consuming when numerous conditions are evaluated (E/T ratio, duration of coculture, different target ...).

The RTCA are suitable for addressing cell responses in different applications. It was first used for assessing cell proliferation, cell adhesion and spreading, and cytotoxicity (Atienza et al., 2006; Atienza, Zhu, Wang, Xu, & Abassi, 2005; Solly, Wang, Xu, Strulovici, & Zheng, 2004). Our team has developed for several years a sensitive assay to measure the dynamic lysis of adherent tumor cells by NK cells. We used the RTCA, xCELLigence system (ACEA Biosciences) an impedance based label free cell analysis system that has been adapted to immune cell-mediated cytotoxicity of adherent tumor cells. RTCA is used to follow cell impedance. Our data demonstrate that RTCA can be used to detect tumor cells lysis by immune NK cells in a label free and real time fashion.



2. Assessment of NK cell-mediated lysis using cell impedance dynamic measure

2.1 Principles

The methodology relies on the monitoring by RTCA, xCELLigence System (ACEA Biosciences) of the cell adhesion and proliferation of adherent cells. The RTCA xCELLigence system is a microelectronic biosensor system for dynamic, real-time, label free cellular analysis for a variety of applications such as drug discovery and screening, cell migration and invasion (Atienzar et al., 2011; Ke, Wang, Xu, & Abassi, 2011; Roshan Moniri et al., 2015). This technology allows accurate dynamic measure by capturing data throughout the entire time course of an experiment and obtaining more physiologically time lapse feature of cell-mediated cytotoxicity.

It measures electric impedance across gold plated interdigitated micro-electrodes on specialized 96 well E-Plate (s). Impedance is reported as a cell index (CI) value. The CI value at each time point is calculated as

$$CI = (Z_i - Z_0) / 15 \Omega,$$

where Z_i is the impedance at an individual time point during the experiment and Z_0 is the impedance at the beginning of the experiment. The CI is thus self-calibrated value derived from the ratio of measured impedances. CI is a unitless parameter.

The impedance measurement provides quantitative information about the status of adherent cells, including cell number, viability and morphology. The RTCA station located in the incubator transmits signals from the E-Plate to the RTCA unit. The RTCA unit controls cells, selects wells to automatically measure impedance data to the computer. The control Unit is a laptop computer with a mobile port replicator device connected to it. The software tools are installed and CI derived from impedance measures are continuously displayed on the software user interface. The average measurement rate is from one each 20 s to one each 15 min for a 96-well E-Plate. Proliferation of adherent cells will increase the numbers of adherent cells and thus the cell index.

We have adapted the RTCA for immune cell-mediated lysis of adherent tumor targets. NK cells induce the apoptosis of the target following interactions between the two cells. Target lysis leads to cell detachment, cell index decrease observed from 10 to 20 min after NK cells addition and reaches a plateau at 120–180 min. The inhibition of cell index is proportional to effector/target ratio (Fig. 2). The assay has been used to successfully quantify the cell-mediated lysis of NK cells, TCR γ/δ T cells (Schiller et al., 2016; Seidel et al., 2014) and CD8⁺ T cells (Erskine, Henle, & Knutson, 2012; Guan et al., 2013; Morisada et al., 2017; Peper et al., 2014).

Additional possible applications include the determination of the impact of drug treatment of target on NK cell-mediated lysis, modulation of NK cell-mediated cytotoxicity by blocking mAbs (anti-NKG2D, NKp30, NKp46, anti-HLA-I) (Frazao et al., 2019; Fregni et al., 2013, 2011; Messaoudene et al., 2014). Antibody dependent cell cytotoxicity can also be studied with this method (Glamann & Hansen, 2006).

2.2 Protocols

2.2.1 Cell culture of adherent tumor targets

Various types of human tumor cell lines, renal cell carcinoma RCC5, RCC6, melanoma SK28, M14, A375, MelC, MelS, breast cancer MCF-7, MDA-MB-321, colon cancer HT29, DLD1 are routinely maintained at 37 °C (5% CO₂) in Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 Glutamax supplemented with 10% fetal calf serum (FCS) and 1% penicillin–streptomycin. Cells are passed 24–48 h before the experiment to allow appropriate confluence in the flask and assure exponential growth phase of cells in the xCELLigence assay.

When cultures reach 80% confluence (corresponding to different cell numbers according to the size and shape of cells), exhausted culture medium is discarded and cells are washed with prewarmed PBS. Then

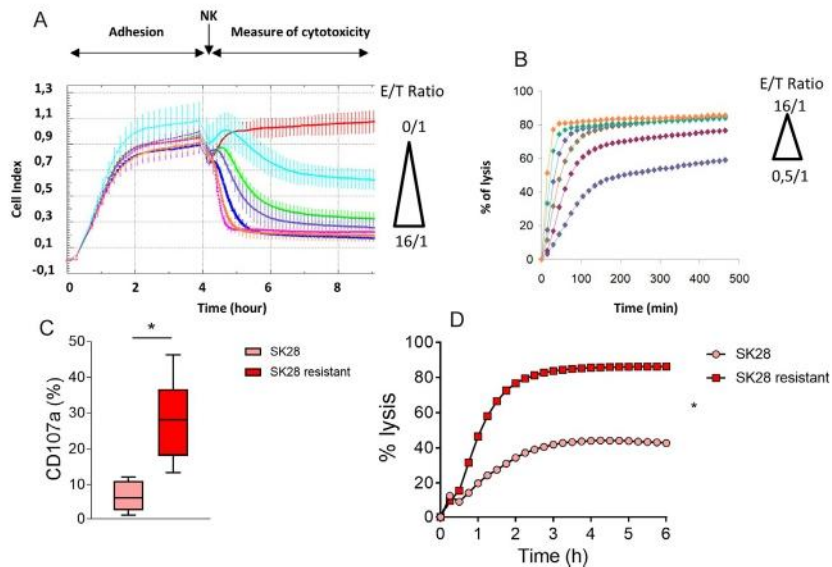


Fig. 2 Evaluation of the dynamic NK cell-mediated lysis of adherent tumor target cells. Tumor targets seeded in the E-Plate adhere, spread and grow for 5–6 h before the addition of NK cells. NK cells induce a rapid drop of CI values in an effector/target ratio dependent manner (A). CI data from the addition of NK cells allow the calculation of percentages of target lysis. Parallel NK cell degranulation (C) and curves of percentages of lysis from RTCA data (D) derived from the same experiment are shown.

the cell monolayer is detached with 0.52% Trypsin/EDTA, washed, diluted and cells are seeded 1 in 5. This procedure is used for maintenance of cell culture and provides cells for experiments. Before performing an assay, cell viability is assessed by Trypan blue exclusion labeling to ensure a viability >90%.

2.2.2 NK cell selection and culture

Blood samples from healthy donors are obtained from Etablissement Français du Sang (EFS) or from patients. Peripheral blood mononuclear cells (PBMC) are isolated by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation. NK cells are purified by negative immunoselection using the NK Cell Isolation Kit and MS Columns (Miltenyi Biotec), leading to >95% of CD3⁻CD56⁺ cells.

Alternatively NK cells are selected from lymph node cell suspensions using the same procedure. When isolated from tumors, NK cells are immunoselected from EpCAM⁻ or CD45⁺ subset of tumor cell suspensions.

Purified NK cells (1×10^6 /mL) are cultured in RPMI medium (Life Technologies) supplemented with 10% human serum AB (Biowest) and IL-2 (10 ng/mL, Miltenyi Biotec) or IL-15 (10 ng/mL, Miltenyi Biotec) for 4–6 days. Half of the medium is changed every 2 days with cytokine renewal.

2.2.3 Monitoring target cell proliferation

The process to monitor cell growth and proliferation is the following. First, for calibration of the E Plate “measure of the background noise,” each wells of the plate are filled with 50 μ L of medium and 150 μ L of PBS are put between the wells. The plate is put in the system and background is measured. PBS between the wells of the E plate allows homogeneous humidity and avoids edge effects altering cell spreading and growth in these E-Plate wells.

Then, 1.3×10^4 (M14, A375) or 1×10^4 (SK28) cells/well are seeded in duplicates in a volume of 100 μ L/well and their adhesion monitored for 5 h. Cell index values depend on the size, shape and spreading of target cells and must be adjusted for each cell line. Preliminary assay of proliferation of each target cell type is required to determine the optimal numbers of cells to be seeded. Indeed a cell index >2 is appropriate at 4–6 h after adhesion of the cells to monitor changes induced by drugs as minimum CI values above background signal is 0.2.

After spreading, targets can be treated with different concentrations of drugs or cytokines to evaluate the impact of treatments on cell growth dynamics. The method sensitivity allows following different phases of the response such as initial growth acceleration before decline of cell index. Results are expressed as Cell index (CI) curves (Fig. 3A).

2.2.4 NK cell-mediated lysis assay

For cell-mediated lysis assays, adherent targets (10,000–13,000) are seeded into 96 E-Plate wells in 100 μ L of medium. Cell adhesion and spreading are monitored for 4–6 h, the optimal time for adhesion and spreading of most tumor cell lines.

After this phase, the E-Plate is taken out of the station and IL-2 or IL-15-activated NK cells are added at different Effector/Target (E/T) ratios (0.5/1, 1/1, 2/1, 5/1...), in a volume of 50 μ L/well. The E-Plate is put back in the station and Cell index (CI) is measured every 15 min for up to 6 h. Addition of NK cells induces a rapid and dramatic decline of target cell index (Fig. 2A).

Lysis of various types of tumor cells (melanoma, breast cancer, colon cancer, renal cell carcinoma) by NK cells was assessed giving sensitive and reproducible results (Frazao et al., 2019; Fregni et al., 2011; Messaoudene et al., 2014; Perier et al., 2011; Rocca et al., 2016).

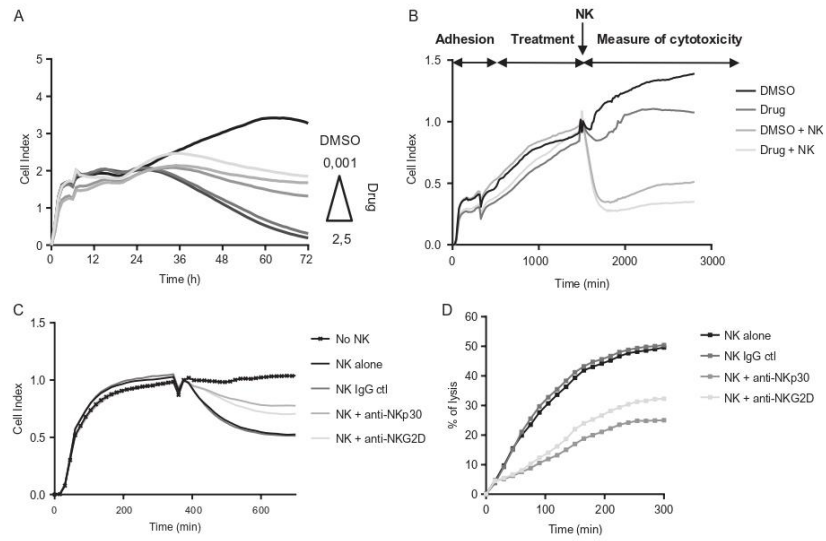


Fig. 3 Additional applications of the RTCA XCELLigence assay for evaluation of NK cell fitness. The treatment of targets before the addition of NK cells allows the evaluation of immunogenicity of this treatment (A and B): cell proliferation of treated tumor targets (A), CI data curves showing the different steps: adhesion, cell growth in response to treatment and NK cell-mediated lysis (B). The modulation of NK cell-mediated lysis by anti-NK receptors mAbs can be monitored: CI data curves (C), percentages of NK mediated lysis (D).

2.2.5 Calculation and statistical analysis

The immune cell-mediated cytotoxic function is expressed as percentages of lysis during the first 6 h after the addition of immune effectors. CI Curves are normalized at the last point before addition of NK cells (Fig. 2A). Results are expressed as percentage of lysis determined from normalized CI curves (normalized CI, nCI) with the following equation:

$$\text{Percentage of lysis} = [\text{nCI (no effector)} - \text{nCI (effector)}] / \text{nCI (no effector)} \times 100.$$

Percentages of lysis are calculated taking as reference CI curves from targets without NK cells. Lysis percentage curves depicted dynamic percentages of target cell lysis for the first 4–6 h of NK/Target cocultures (Fig. 2A and B). Lysis percentage curves allow comparing NK cell-mediated lysis of target cells displaying different CI curves before normalization (Fig. 2A).

Duplicates are used as dynamic CI data generate multiple time points allowing statistical comparison of CI curves.

Nonparametric paired Wilcoxon test is used to compare lysis percentage curves, and to compare CI data curves (treatment of targets with drugs).

2.2.6 Treatment of targets before addition of NK cells

In such assays, target cells are seeded, let to adhere and spread for 4–6 h, and treated with drugs (BRAF, MEK inhibitors, DITC...) or cytokines (IFN α , TGF β ..) or DMSO in control conditions, for various times (6–20 h) (in a volume of 25 μ L/well) before the addition of cytotoxic immune effectors. The treatment duration with the drugs is chosen based on tumor cells impedance curves to avoid overgrowth of nontreated tumors cells in control wells. NK cells are added when control curves are still increasing (Fig. 3B). Normalization at the time of drug addition allows determining the effect of the drug on target cell survival/proliferation in comparison to wells plated with targets incubated with drug diluent (usually DMSO) corresponding to the highest drug concentration condition. Normalization at the time of NK cell addition is done to compare the effect of immune cells on targets pretreated with different concentrations of the drug in reference to targets incubated in presence of diluent.

Percentages of lysis by NK cells are calculated in reference to treated and nontreated targets. Variations in percentages of lysis toward treated cells indicate that the agent used modulates the recognition and lysis of targets by NK cells. Using this setting, we showed that BRAF or MEK inhibitors decreased the lysis of melanoma cells (Frazao et al., 2017).

2.2.7 Modulation of NK cell-mediated lysis by blocking mAbs

To determine the involvement of NK receptors in NK cell-mediated lysis, anti-NKR specific mAbs are used to block the interaction with the cognate ligand on the target. NK cells are incubated for 15–20 min with 40 µg/mL of the following mAbs: NKG2D, NKG2A, NKp30, NKp46, DNAM1 (R&D Systems) or control Igs before their addition to targets in the E-Plate (Fregni et al., 2013, 2011; Perier et al., 2011). The E-Plate is put back in the station and the Cell index (CI) is measured every 15 min for up to 6 h. CI curves are normalized at the last point before NK cell addition. Percentages of lysis are calculated as above and lysis percentage curves in presence of control Igs or anti-NKR mAbs are depicted (Fig. 3C). Blocking with mAbs can be expressed as percentages of inhibition or increase compared to the control condition (Igs, or no mAbs). Comparisons of mean values are performed using Wilcoxon matched paired test and $P < 0.05$ is regarded as significant.

2.2.8 Limits of the method

The dynamic measure of NK cell-mediated lysis is restricted to adherent tumor target cells.

CI values refer to cell detachment and/or cell death. Although CI curves can be monitored for hours, it is important to assess NK-mediated lysis during 2–6 h and to ensure that in control wells (without NK cells) cells are still growing. Immunoselection of effectors of interest (T versus NK cells) is required as there is no possibility to differentiate specific effectors present in heterogeneous cell suspensions.

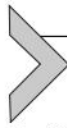


3. Concluding remarks

First developed for accurate, sensitive and dynamic monitoring of adherent cell spreading and cell growth, the xCELLigence system has become a useful and reliable tool to measure the dynamic of adherent cell lysis induced by T and NK immune cells. xCELLigence system presents advantages compared to ^{51}Cr release assay (Erskine et al., 2012; Peper et al., 2014) and correlates with CD107a (Fregni et al., 2013, 2011), (Fasbender & Watzl, 2018), (Friedman et al., 2018).

Impedance based technology is efficient to quantify Antibody dependent cell cytotoxicity (ADCC) against adherent cells (Toth, Szollosi, & Vereb, 2017).

As immunotherapies with Chimeric Antigen receptor (CAR)-T cells (CAR-NKs) display growing interest for solid tumors, the method can be extended to monitor the antitumor activities of engineered T and NK cells.



4. Notes

1. Based on recommendations of the American Type Cell Culture Collection (ATCC, Manassas, VA). Optimal culture conditions for cell lines established in the lab were determined in the lab.
2. Cell lines are maintained in small or larger (25 or 75 cm² flasks). For melanoma cell lines, a 75 cm² flask at 75%–80% confluence contain $0.8 \times 10^6 - 2 \times 10^6$ cells.

Gentle washing of cells with PBS to remove serum (known to inhibit trypsin) facilitates and speeds the detachment of the cells. The time required for Trypsin/EDTA to detach a monolayer varies with the cell type and degree of confluence from 1 to 4 min incubation at 37 °C. Detachment is confirmed by inspection on light microscopy. Clumps are dissolved by repeated pipetting assuring good counting of cell numbers.
3. Note that each cell line has its own pattern of CI curves depending of the cell size and morphology. Preliminary experiments are therefore required to optimize the kinetics of adhesion/spreading time to exponential growth phase.
4. The RTCA station is located in a regular incubator to ensure a controlled temperature, humidity and CO₂ atmosphere required for reproducible cell line growth the E-Plate.

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Annexe 4: Challenges for NK-cell based therapies: what can we learn from lymph nodes?

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Challenges for NK-cell based therapies: what can we learn from lymph nodes?

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Keywords: Natural Killer cells, Innate Lymphoid Cells, lymph nodes, tumor immunosurveillance, metastases, immunotherapy

Abbreviations:

ACT: adoptive cell therapy
ADCC: Antibody Dependent Cellular Cytotoxicity
ALL: Acute lymphocytic leukemia
AML: Acute myeloid leukemia
BM: bone marrow
CAR: chimeric antigen receptor
CTLA-4: cytotoxic T-lymphocyte-associated protein 4
DC: Dendritic cell
HL: Hodgkin lymphoma
NHL : Non Hodgkin lymphoma
ICB: Immune checkpoint blockers
IFN: interferon
IL-: interleukin
ILC: innate lymphoid cells LN: lymph nodes
LTi: lymphoid tissue-inducers
MDS: myelodysplastic
MDSC: myeloid derived suppressor cells
MM: Multiple Myeloma
NCR: Natural Cytotoxicity Receptors
NK: Natural Killer
PD-1: program cell death receptor-1
TDLN: Tumor draining lymph node
TF: transcription factor
TIGIT: T cell immunoreceptor with Ig and ITIM domains
TNF: tumor necrosis factor

No potential conflicts of interest were disclosed

Abstract:

NK cells are innate lymphoid cells described in the 70's for their ability to kill various tumor cells. NK cells originate and differentiate in the bone marrow, migrate to secondary lymphoid organs, namely lymph node, where they terminate their maturation and interact with myeloid immune cells, including dendritic cells and macrophages. Developing programs on NK-cell immunosurveillance of tumor (melanoma, breast cancers), we focus our interest on NK cells from lymph nodes, a crucial relay for the immunosurveillance of several solid tumors. We summarized our original data on lymph node NK cells and their involvement in immune response in tumor draining lymph node, a first site of metastatic invasion. We discuss different options aiming at the manipulation of these immune populations in new immunotherapeutic strategies.

Introduction

Natural Killer (NK) cells are innate lymphoid cells (ILC) playing a major role in host defenses against pathogens and in the immunosurveillance of tumor cells. ILC are divided into five main subsets based on their developmental transcription factor (TF) requirements and cytokine production patterns: NK cells, ILC1, ILC2, ILC3 and lymphoid tissue-inducers (LTi) (1). NK cells and ILC1 produce IFN- γ and depend on the TF T-bet, and Eomes for NK cells. ILC2 produce IL-5 and IL-13 and depend on GATA3. ILC3 produce IL-17 and IL-22 and express the TF ROR γ t. LTi play a critical role in the development of secondary lymphoid organs and the formation of lymph nodes (LN) during fetal life.

The present review is focused on cytotoxic ILC, namely NK cells, important antitumor effectors that may be targeted to improve efficiency of anti-tumor immunotherapies.

NK cells are identified by the absence of CD3 and the expression of the cell adhesion molecule CD56. They can be subdivided into two main subsets: CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺. NK cells are the major ILC population present in blood, constituting 5-15% of PBMC. The majority of NK cells in the blood are CD56^{dim}. In particular, the growing number of studies using single-cell RNA sequencing (scRNA-seq) technologies helped to define comprehensive analysis of the immune system diversity and brought new findings on the heterogeneity of NK cells. In, unstimulated and IL-2-activated NK cells from healthy cytomegalovirus (CMV)-negative donors, three NK-cell subsets resembling the well-described populations; CD56^{bright}CD16⁻, CD56^{dim}CD16⁺CD57⁻, and CD56^{dim}CD16⁺CD57⁺ and additional novel blood NK-cell subset characterized by distinct signature were identified. Analysis of CMV⁺ donors established that

CMV altered the proportion of NK cells in each subset, increasing adaptive NK cells, as well as gene regulation within each subset (2). Another study reports the identification of distinct NK populations in human bone marrow and blood, including one population expressing higher levels of immediate early genes indicative of a homeostatic activation and a transitional population between CD56^{bright} and CD56^{dim} NK cells (3). Two major NK subsets were identified in blood and spleen with similarity between mouse and human (4). These data expanded our understanding of the heterogeneity and development of human NK cells and established an unexpected diversity in blood NK cells. Advances in scRNA-seq can be useful to identify novel distinct immune cell subsets and to build developmental 'trajectories' for immune cells in health and disease. In tumor, it will help to characterize stochastic heterogeneity within a cell population along tumor progression.

NK cells mediate their activity through two main functions. The first one is the ability to recognize and directly lyse cell that have undergone malignant transformation, such as tumor cells, or cells infected by virus or other pathogens. After recognition of the target cells, cytotoxicity is mediated by the release of the intracellular perforin and granzymes. The cytolytic function of NK cells is mainly driven by the CD56^{dim} NK subset and can be either mediated naturally through the triggering of activating receptors expressed on NK-cell surface or by Antibody Dependent Cellular Cytotoxicity (ADCC). The second function of NK cells, mainly driven by the CD56^{bright} subpopulation, is the production of inflammatory cytokines (IFN- γ , TNF- α) and growth factors (GM-CSF) in response to activating receptor stimulation or induced by cytokines within the environment. The cytokines secreted by NK cells play a role in the regulation of the immune response. In particular, in the lymph node, IFN- γ produced by NK cells promotes T_H1 polarization of CD4⁺ T-cells (5). NK-cell functions are regulated by a balance between activating and inhibitory signals received by a broad panel of receptors. Activating receptors including Natural Cytotoxicity Receptors (NCR1-3) or NKG2D recognize stress induced molecules, whereas inhibiting signals are mediated by receptors specific for classical or non-classical HLA-I molecules.

Many studies have demonstrated that NK cells contribute to cancer immunosurveillance, especially in the control of metastasis (6). A first link between cancer development and NK cell activity was reported in 2000 when a Japanese study, that has followed a cohort of 3625 individuals for 11 years, showed a correlation between natural cytotoxic activity of blood NK cells and a reduced incidence of cancer (7). Functional defects of NK cells were associated with particular NKG2D haplotypes (8). Other studies showed correlations between the ratio of NCR isoforms or the NCR transcript levels and development of more aggressive tumors and/or resistance to treatment (9-11). Numerous studies have shown that NK cell infiltration of tumors

areas is associated with better survival or a lower risk of cancer (12-14). A correlation between high numbers of blood or tumor-infiltrating NK cells and the absence of metastasis has been established in gastric (15) and colorectal cancer (12), prostate carcinoma (16) and renal cell carcinoma (17).

The present review is focused on NK cells from LN, a privileged site for NK cells to terminate their maturation but also for tumor cells as a first step of the metastatic spread. The review first recapitulates the NK cell differentiation and maturation in LNs and the role of LN-NK cells in tumor immunosurveillance, then it presents an overview of NK cells in immunotherapies, and finally a revisited role of Tumor draining (TD) LN NK cells in the schedule of immunotherapies for cancer patients.

1- NK differentiation and migration involve lymph nodes

NK cells derive from the Hematopoietic Stem Cells (HSCs) in the bone marrow (BM). Although BM is the major site for the first stages of NK cell development, *in vitro* experiments showed that NK cells can be derived from CD34⁺ hematopoietic progenitor cells (HPCs) coming from thymus, secondary lymphoid tissues or blood (18-20).

In BM, HSCs differentiate into the common lymphoid progenitor (CLP) which can produce either T- and B-cells or the NK precursor (NKP). NKP is characterized by the expression of the IL-2R β , CD38 and IL-15R α transcripts, and do not express CD7, CD16, CD56 nor NKG2A (21). NKP differentiate into immature NK (iNK) cells by the acquisition of CD161. However, these cells do not present yet the capacity to lyse perforin-sensitive targets or to produce IFN- γ (22). Then, iNK cells acquire the expression of CD56, Natural Cytotoxic Receptors (NCR) and other activating NK receptors including NKG2D. The production of functional mature NK cells requires further maturation through a process referred as "education" that results from the capacity to recognize self MHC class I molecules mainly by KIR receptors expressed on NK cells. This mechanism generates educated KIR⁺ NK cells fully functional and, in parallel, hyporesponsive NK cells that have not been armed by HLA-I/KIR interactions (23).

NK cells precursors can be found in secondary lymphoid tissues (SLT), especially in LNs, where NKP coming from the BM, stimulated with IL-15 and IL-2, become CD56^{bright} NK cells (24). The stromal micro-environment of LN and the presence of membrane-bound IL-15 are essential for NK cell differentiation and maturation. In LN, CD56^{bright} NK cells are the most abundant subsets while in blood NK cells are mostly CD56^{dim} (25). The precise developmental relationship between CD56^{bright} and CD56^{dim} is still unclear, however the vast majority of studies

suggests that CD56^{bright} NK cells are the precursors of CD56^{dim} NK cells (26). Resident CD56^{bright} NK may acquire CD16 in the LN and recirculate in the blood (27). In LN, NK cells interact with DC through bidirectional crosstalk leading to NK cell activation and priming of DC (28). NK cell migration to the LN is dependent on the receptor CCR7, through the binding of its ligands CCL21 and CCL19 produced by fibroblastic reticular cells as well as lymphatic endothelial cells (29). CCR7 is mainly expressed on CD56^{bright} NK cells, which co-express the adhesion molecule CD62L (L-selectin). In the mouse, recruitment of NK cells to the LN is CD62L dependent (30).

2. Lymph node relay for antitumor response initiation

LNs, are a major site of innate immunosurveillance and adaptive immune responses, because antigens and antigen-presenting cells (APCs) from distinct organs migrate through the lymph fluid via the afferent lymphatics. In cancer, LN may be invaded by tumor cells that modified the microenvironment of these lymphoid organs and may impact on NK cell development and function (Figure 1). In LN, INF γ and TNF α produced by NK cells will stimulate antigen presenting DC to support the activation of T and B cells. The regulation of NK cell by DC and reciprocally the consequences of NK cell activation on DC functions are important physiological features (31). Prolonged NK/DC contacts in TDLN may turn resident NK cells into antitumor effectors (32) and DC priming by activated NK induce protective cancer specific immune response. Consequently, the DC-NK cell interplay in TDLN will determine the outcome of the ongoing immune response.

Lymphatic fluids from the primary tumor are drained by the co-called "sentinel" lymph node. Tumor factors drained into the sentinel node can modify the microenvironment leading to the development of a pre-metastatic niche (33). Cancer cells can express CCR7 and migrate to the LN (34) through the lymphatic system before systemic dissemination through the blood. Thus, TDLN is the first site of metastatic dissemination in many types of cancers including melanoma and breast cancer, and nodal invasion reflects poor prognosis (35).

While the vast majority of human NK cell studies focused on blood NK cells, NK cells infiltrating secondary lymphoid organs and tissues were also characterized and the studies indicated the presence of tissue specific NK subsets. Our group and others have demonstrated that NK cells can infiltrate TDLN where they can act in the immunosurveillance of metastatic dissemination. However these studies are limited and according to the tumor model, reveal somewhat different data. In melanoma patients, we found that NK cell numbers in sentinel LN correlated with higher rate of 5 year relapse (36). In metastatic LN, percentages of invading melanoma

cells decreased the activation of CD56^{bright}/CD16⁺ TDLN-NK cells. This population of mature NK cells displayed lytic potential towards melanoma cells following *in vitro* activation by IL-15 or IL-2 (37, 38). A different population, CD56^{dim} CD57⁺ NK cells have been found to infiltrate LN from melanoma patients and were cytotoxic against autologous melanoma cells (39), while CD56⁺ NK cells in metastatic regional LN of melanoma patients showed a reduced IFN γ production (40). In colorectal cancer CD57⁺ NK cells were correlated with a diminution of LN metastasis (41). In breast cancer, axillary LN metastases with high amount of CD56⁺ NK cells were associated with pathological complete response (42). We found an increased CD56⁺/CD16⁺ PD-1⁺ NK subset in BC patient LN that may be targeted with Cetuximab and anti-PD-1 therapy (43).

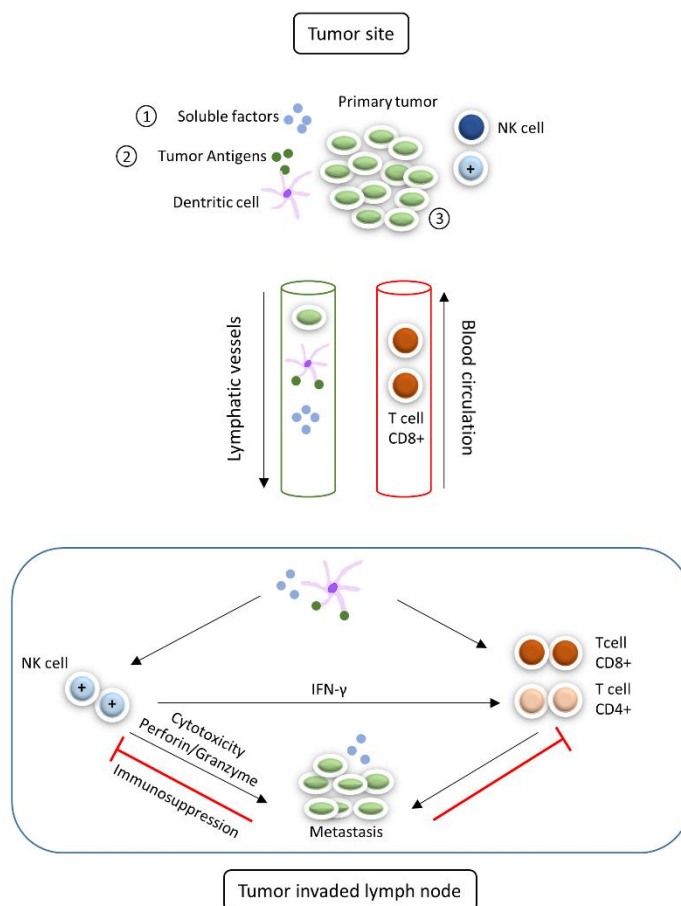


Figure 1: Lymph node in antitumor responses.

3. Tumor immunotherapy, the emerging role of NK cells

In recent years, immunotherapy has become a recognized therapy for a variety of cancer types. The development of immune checkpoint blockers (ICB) therapies, the adoptive transfer

of tumor-specific T cells (adoptive cell therapy (ACT)) or the generation of T cells engineered with chimeric antigen receptors (CAR) have been successfully applied to elicit durable immunological responses and sustained control of the disease in cancer patients. However, not all the patients respond to these therapies and therapeutic improvement is still required. One obvious but yet disregarded option is the NK cells. Beside their direct role on transformed cells, NK cells play a pivotal role in the activation and modulation of the adaptive immune response against the tumor. Recently several efforts were made to develop strategies aimed to harness these cytotoxic effectors in the context of cancer immunotherapy. New therapeutic approaches were implemented to broaden the efficacy of the antitumor functions of NK cells in cancer patients.

3-1 Promoting NK-cell-mediated ADCC

Therapeutic monoclonal antibodies (mAbs) targeting tumor specific antigens are actively developed in the clinic to treat hematologic and solid tumors. One of the mechanisms of action of certain of these mAbs involved ADCC mediated by NK cells, as CD16 (FcRgIII) can bind the Fc portion of the therapeutic mAbs, which have recognized their specific tumor antigen.

Demonstrating the implication of ADCC in the antitumoral response, it has been shown that polymorphisms in the Fc receptor CD16 (V158V (V/V) and V158F (V/F) polymorphism) modulated the clinical response to mAbs (44). Consequently, modified mAbs mediating strong ADCC were developed to overcome these limitations. For instance, Obinutuzumab, a new mAbs targeting CD20, has been glyco-modified to reduce Fc fucosylation (45) and induce higher ADCC than rituximab against B-CLL cells (46).

In metastatic colorectal cancer (CRC), Cetuximab mediated NK cell activity on EGFR+ cells in a CD16 dependent manner (47). In a phase II clinical trial, *ex vivo* ADCC and NK cell response were correlated with clinical response in head and neck carcinomas (48). For HER2 positive metastatic breast cancer patients, Trastuzumab an anti-HER2 mAb was showed to activate ADCC of NK cells *in vitro* (49) and high levels of NK activity and ADCC were correlated with clinical response in metastatic breast cancer (50). To face patient's resistance to Trastuzumab, a second mAb anti-HER2 has been developed, Pertuzumab, binding a different epitope. A recent *in vitro* study demonstrated that the effect of the combination Trastuzumab plus Pertuzumab is increased with IL-2 activated NK cells (51).

Another field of therapeutic mAbs concerns plurispesific killer cells engagers which associate several specificities and therefore interact with two or more surface antigens, building a bridge between the tumor cell and the NK effector cell. BiKEs are bi-specific antibodies targeting the effector cells and the tumor to improve immune response against tumor cells. Yet, BiKEs are

mainly used in liquid tumors (Hodgkin Lymphomas (HL), Non Hodgkin Lymphomas (NHL), mixed lineage leukemia, AML). Usually BiKEs target CD16 on the surface of NK cells and an antigen on the tumor cell, for instance CD33 at the surface of AML cells, or CD30 in HL(52).

A further generation of therapeutic mAbs based on Bike technology, called TriKEs or trispecific Killer Engager is under development. This technology is based on mAbs targeting simultaneously tumor cells and NK cells (through CD16), and also associates to this structure an IL-15 moiety to trigger NK cell activation and proliferation. In a study comparing BiKE and Trike in myelodysplastic (MDS) patients, the authors showed that BiKEs (CD16/CD33) failed to induce the proliferation of NK cells. Moreover, NK cells were still inhibited by MDSCs through CD155-TIGIT engagement. Addition of IL-15 to generate a TriKE CD16/IL15/CD33 allowed the proliferation of NK cells without any increase of TIGIT expression and led to the elimination of MDS cells, avoiding inhibition by MDSCs in the bone-marrow microenvironment (53). Moreover, CD33 expressed by tumor cells as well as MDSCs, this TriKE could eliminate both cell subsets decreasing the immunosuppression in the bone-marrow microenvironment (54). TriKE combining anti-CD16, IL-15 and anti-CD33 were evaluated in AML and high risk MDS. In chronic lymphocytic leukemia (CLL) patients, TriKEs that combined anti-CD16 for NK cells recruitment, IL-15 to provide an activation signal and anti-CD19 to target leukemic cells were developed (55).

To promote more efficient NK cell activation a new family of trifunctional NK-cell engagers (NKCEs) is tested to target NK cell activation with two binding domains: one targeting NKp46 and the second one targeting CD16. The development of such antibodies is in line with studies demonstrating that a co-engagement of NK receptors is needed for a full activation (56). This NKCE contains a third domain, binding to a tumor antigen, here CD20 (57). Preclinical tests in mice showed better results than the anti-CD20 already in clinic (rituximab, obinutuzumab) supporting the development of NKCEs as emerging immunotherapies. Of note, BiKE and TriKE strategies were mainly evaluated in hematological malignancies. However, some molecules were developed against solid tumors to target EpCAM (58) or more recently B7-H3 (59)

3-2 Immune Checkpoint Inhibitors

NK cells and PD-1, CTLA4 inhibitors

Tumor Immunotherapy has been revolutionized by the discovery of immune checkpoint inhibitors. Immune checkpoint (IC) receptors are inhibitory receptors that decrease the anti-tumoral activity of the cytotoxic tumor-induced T cells. Blocking CTLA-4 and PD-1 pathways

with mAbs (Ipilimumab, Nivolumab, ..) reinvigorated the antitumor cytotoxic T cells and induced durable clinical responses and prolonged survival in some patients.

Importantly, NK cell activation can be regulated by PD-1 and CTLA4 following similar signaling pathways than T cells. PD-1 is expressed by KIR⁺CD57⁺CD56^{dim} NK cells by contrast to less mature CD56^{bright} NK cells (60). In multiple myeloma (MM) patient's, NK cells express PD-1 and blockade of PD-1 on NK cells enhanced anti-tumoral NK cell function against autologous MM cells (61). In ovarian carcinoma, PD-1⁺ NK cells are increased and present an impaired anti-tumoral function which can be restored by anti-PD-1 (60). In different mouse models, activated NK cells expressed PD-1, the engagement with its ligand PD-L1 reduced NK cell activity and the use of a PD-1/PD-L1 blocker restored NK cell response (62). Immune cell poor melanomas benefit from PD-1 blockade after targeted activation of type-I IFN that involved coordinated interactions between DC, NK and T cells (63). NK cells numbers was associated with clinical response to anti-PD-1 in metastatic melanoma patients including a subset of patients with downregulated MHC-I expressing tumors (64). A NK stimulatory DC axis involving the cytokine FLT3L enhanced checkpoint therapy response mediated by T cells in the tumor microenvironment (65).

The role of anti-CTLA-4 on NK cells is less clear. A study in a murine model showed that anti-CTLA-4 mAb increase NK cell anti-tumor activity, in a more important way with IL15/IL15Ra (66). They also looked at the CD56 expression in patients with cutaneous melanoma receiving ipilimumab and found that patients who responded expressed more CD56. Similarly, we found a correlation between soluble NKG2D ligands and response to Ipilimumab in melanoma patients (67). Altogether, these observations suggest a close link between NK cells and response to Ipilimumab treatment in melanoma.

KIR and NKG2A inhibitors

Knowing the crucial role of HLA-I specific receptors in NK cell regulation, mAbs targeting these inhibitory HLA-specific receptors, namely KIR and NKG2A receptors were developed. Blocking the KIR/HLA-I interaction switches the balance towards NK-cell activation. These mAbs were evaluated in patients with AML as it was showed that KIR2DL/HLA-C mismatch resulted in improved survival of AML patients transplanted with allogeneic stem cells (68). Preclinical studies with a first generation of anti-KIR2DL1-3 (IPH2101) demonstrated that KIR blockade increased NK-cell mediated cytolytic activity against AML and MM cells (69, 70). First clinical trials in AML and MM demonstrated no severe side effect but no efficacy in phase II (71, 72). The second generation of anti-KIR2DL1-3 (lirilumab/IPH2102/BMS-986015) has been

developed and is currently being tested in combination with other drugs (rituximab, azacitidine) in lymphoma and MDS (73).

CD94/NKG2A heterodimer that binds to HLA-E molecules is a major inhibitory checkpoint of NK cells activation. HLA-E molecules are overexpressed in several solid tumors and hematological malignancies (74-76). NKG2A is expressed by a large fraction of mature NK cells and by antigen experienced CD8⁺ T cells. A mAbs blocking NKG2A (Monalizumab), has recently been described and evaluated for its anti-tumoral activity *in vitro* and *in vivo* as single agent or in combination with anti-PDL1 (Durvalumab) or anti-EGFR (Cetuximab) (77).

Other NK- and T-cell checkpoints

While DNAM-1 is a potent coactivating NK-cell receptor, its ligands are shared with two inhibitory receptors TIGIT and CD96, binding to CD155 (PVR) and CD112 (PVRL2) (78). The expression of CD155 and CD112 was detected on tumors from epithelial origin such as breast cancer, colon cancer, and metastatic neuroblastoma as well as malignant hemopathies (MM and AML). Serum levels of both CD155 (79) and CD112 were associated with the prognostic of colorectal cancer and lymphoma (80). Blockade of CD96 in a mouse model, can increase the anti-metastatic activity of NK cells by an increased production of IFN- γ (81). This increase is more effective with a combination with anti-CTLA4 or anti-PD-1, and in absence of TIGIT expression in mice. TIGIT has been associated with NK cells exhaustion in colorectal cancer and in several mice models, and anti-TIGIT therapy reversed NK cell exhaustion in mice (82).

TIM-3 is an immune checkpoint strongly upregulated, in parallel to PD-1, by exhausted T cells (83) and combined blockade of TIM-3 and PD-1 may restore the anti-tumor activity of exhausted T cells, more effectively than PD-1 blockade alone (84). Similarly, TIM-3 is expressed by resting NK cells and is up-regulated upon activation (85, 86) and in cancer (87, 88). Importantly, NK cells from metastatic melanoma patients expressing TIM-3 are exhausted and can be reactivated by TIM-3 blockade (89).

3-3 Adoptive cell transfer of NK cells

Adoptive NK cell transfer is a cell therapy approach first developed for leukemic patients treated with allogeneic stem cell transplantation to consolidate a graft versus leukemia (GvL) effect (90). Adoptive transfer of autologous NK cells in patients with solid tumors was also evaluated. Autologous transfer of NK cells consist in sorting of NK cells from the peripheral

blood of patients, often exhausted in a tumor microenvironment, stimulate those cells with a cocktail of cytokines (IL-2, IL-15 or IL-21) and reinjected in patients with or without an addition of IL-2 injection to maintain NK cell activation. However, the efficiency of this immunotherapy was very limited when used to treat in renal cancer, metastatic melanoma (91), lymphoma and breast cancer (92). The expansion of Tregs, due to the injection of IL-2, and the expression of self HLA-I molecules by the tumor cells may explain the weak efficiency of this strategy. Such therapy was combined with other immunotherapy and chemotherapy to be effective. A phase I clinical trial studied the safety and efficacy of infusion of NK cells activated *in vitro* in combination with anti-myeloma chemotherapy. Four of five MM patients showed a disease stabilization, and two showed a reduction in BM infiltration and a long-term (>1 y) response (93).

To potentiate an antitumor effect, adoptive transfer of allogeneic NK cells (or the NK cell line NK92/Neukoplast™) was performed. The benefit of using allogeneic NK cells is the KIR/KIR-ligands mismatch setting allowing the presence of donor derived alloreactive NK cells endowed with the cytotoxic potential of tumor cells not engaging inhibitory KIR. Another main advantage is that donor derived allogeneic NK cells have not been altered by the immunosuppressive environment of the patient. Several clinical trials of allogeneic NK cells transfer were attempted or completed. At the time of redaction of this review, more than 160 “allogeneic NK cell” trials were referenced in the ClinicalTrials.gov web site (<https://clinicaltrials.gov/ct2/home>) using NK cells alone or in combination in different indications (AML, non-Hodgkin lymphoma or HER2+breast cancer). A phase I clinical trial (NCT02845999) showed the safety and efficacy of *in situ* delivery of allogeneic NK cells combined with Cetuximab in patients with either colorectal or prostatic cancer bearing liver metastases and a higher efficiency with cell products harboring KIR ligand mismatches. This trial demonstrated that combining allogeneic NK cells transfer via intra-hepatic artery, Cetuximab and IL-2 is feasible, well tolerated and may result in clinical responses (94).

Adoptive transfer in immunotherapy of cytotoxic NK cell lines, especially NK-92 was also evaluated. This cell line is particularly interesting because it is easy to amplify *in vitro* and the cells lack KIR expression avoiding inhibition by HLA recognition (95). Several studies have been done in renal cell carcinoma (96).

CAR-NK cells

The new development in the field of adoptive cell transfer of antitumor immune effectors involved genetically engineered T or NK cells using chimeric antigen receptors (CAR) technologies. The vast majority of the works on this approach concerns CAR-T cells and

hematologic diseases. Promising results have thus been obtained with CD19-CAR-T cells to treat lymphoma patients. However, loss of antigen expression and the cytokine storm induced by high numbers of infused of reacting CAR-T cells are serious side effects of this approach (97)

In that context, CAR-NK cells may present several advantages. NK cell transfer does not induce high cytokine release syndrome or graft versus host effect so that fully functional donor allogeneic NK cells can be engineered. Of note, NK cells derived from induced progenitor stem cells (iPSC) can be engineered providing off-the-shelf resource for cancer therapy (98). New CAR with accurate signaling dedicated to activate NK cells may be designed and known NK ligands expressed by solid tumors may be targeted. First data emerging with CAR-NK cells are indeed promising.

CAR-NK cells have been successfully genetically modified from primary NK cells, and from the NK-92 cell line with a vector to express specific receptor for recognition of tumor cells. CAR-NK cells are being evaluated in several preclinical trials in different hematological tumors such as B-ALL, targeting CD19 (99), MM, targeting CD138 (100) and solid tumor such as breast cancer (101). Some CAR-NK studies are currently in phase I and II clinical trials in AML (NCT02944162) and B-ALL (NCT01974479). A recent study described the encouraging results obtained with CAR-NK cells engineered to recognize CD19 with absence of deleterious cytokine release and a response to treatment to CAR-NK cells of relapsed refractory CD19⁺ cancer patients (102)

4. LN NK cells and immunotherapy

Considering the role of NK cells in the control of metastasis and the frequent involvement of LN in the metastatic spreading of solid tumors, it is of interest to question the impact of immunotherapies on lymph node that drain many tumors (Figure 2). The data presented above raise several questions and suggest some strategies for scheduled NK-based immunointerventions.

As immunotherapies are developed with success for various solid tumors, if surgery remains a recommended act in the treatment of primary tumors, practice is changing. For tumor prone to invade TDLN such as melanoma and breast cancer, the resection of the sentinel LN was recommended for many years. However it was showed that for stage III melanoma patients, there is no improvement of completion of LN dissection for most patients (103, 104). Furthermore, whether TDLN can favor clinical response to immunotherapies is an important issue to determine the utility of the sentinel lymph node procedure.

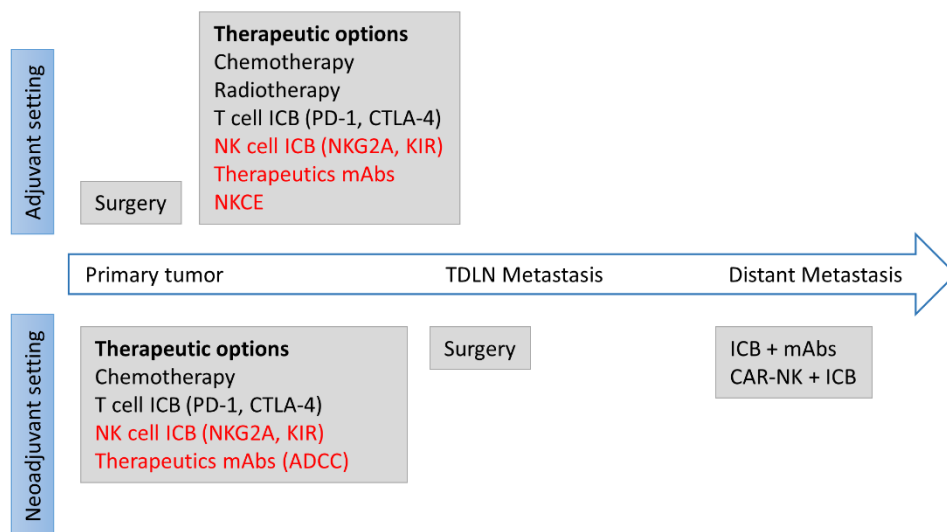


Figure 2: Therapeutic options in adjuvant and neoadjuvant settings.

A better understanding of the involvement of LN in adjuvant neo adjuvant treatment setting is required. After surgical primary tumor resection, patients can receive an adjuvant therapy to kill the remaining metastatic cells as half of the patients can relapse after surgery due to metastatic development. In melanoma patients, clinic trials have evaluated the efficacy of several immunotherapies in adjuvant treatment. It was hypothesized that immunotherapies may impact the TDLN and promotes an antitumor response. Several studies showed that the ICB, specific of PD-1 and CTLA-4 in adjuvant therapies can prevent for relapse (105, 106). For breast cancer patients, in several studies the monoclonal antibody Trastuzumab (anti-HER2) have been evaluated in adjuvant setting after chemotherapy and leads to increased survival (107).

Breast cancer patients may receive neoadjuvant treatment before surgery. This therapy can lead to the decrease of the tumor size, release of tumor antigens and increased immunosurveillance. Chemotherapy was first tested but one clinical study showed a reduced activity of NK cells associated with a high risk of developing metastasis (108).

In metastatic melanoma patients, combined immunotherapies are being evaluated in neoadjuvant setting. The rational is to promote a better activation of the immune system compare to the adjuvant therapies. Numerous studies have been launched, testing ICB alone or in combination. In melanoma, anti-PD-1 or anti-CTLA-4 in neoadjuvant setting increase lymphocytes infiltration in the tumor (109). Thus LN is considered as important relay in the responses to neoadjuvant immunotherapies (110). Neoadjuvant treatment can increase the antigenic presentation by APC to the T cells in the LN and can interfere with NK cell

differentiation and activation, which can be important for the response. Taking in fact that LN are the first site of metastasis and that NK cells play a role in the responses to adjuvant/neoadjuvant therapies, the study of LN NK cells is required to better understand the response and to increase the efficacy of this treatment.

Cancer derived factors may render TDLN permissive to tumor cell invasion and TDLNs have been involved in tumor induced immune escape (111). Thus, direct targeting of TDLN with local delivery of adjuvanted nanoparticles to reshape the antitumor immune response was investigated in an experimental model (112).

Conclusions

NK cells exert antitumor cytotoxic function as well as regulatory function. In TDLN, these effectors require further characterization of their role in immune regulation. Such studies may open new avenues for adjuvant/neoadjuvant treatments for cancer patients.

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Liste des éléments retirés :

Figure 2 : Répartition des ILC dans différents tissus.....	24
Figure 3 : Localisation des sous-populations NK.....	25
Figure 9 : Les trois étapes du concept de l'immunosurveillance.....	44
Figure 10 : Fonctions anti-tumorales des cellules NK.....	46
Figure 13 : Structure et fonction des BiKEs et TriKEs.....	51
Figure 14 : Fonctions pro- et anti-tumorales des ILC auxiliaires.....	61
Figure 15 : Le cancer du sein et ses sous-types.....	88
Figure 16 : Choix thérapeutiques dans le cancer du sein.....	89
Tableau 3 : Classification de l'AJCC.....	67