Natural Killer cell subsets in hematological diseases: learning for immunotherapy
Dang Nghiem Vo

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En Biologie Santé

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Natural Killer cell subsets in hematological diseases : learning for immunotherapy

Présentée par Dang Nghiem VO
Le 03 Juillet 2018

Sous la direction de Dr. Martin VILLALBA-GONZALEZ

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<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ALC</td>
<td>absolute lymphocyte count</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
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<td>BCR</td>
<td>B-cell receptor</td>
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<td>B-NHL</td>
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<td>common lymphoid progenitor</td>
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<td>NF-AT</td>
<td>nuclear factor of activated T cell</td>
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<td>NK</td>
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<td>NKG2-A/C/D</td>
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<td>OBZ</td>
<td>obinutuzumab</td>
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<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
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<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<td>PD-1</td>
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<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<td>PLCγ</td>
<td>phospholipase C gamma</td>
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<td>PRR</td>
<td>pattern recognition receptor</td>
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<td>PTPase</td>
<td>tyrosine-specific protein phosphatase</td>
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<td>poliovirus receptor</td>
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<td>SHIP</td>
<td>SH2 domain-containing inositol 5'-phosphatase</td>
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<td>Src homology region 2 domain-containing phosphatase-1/2</td>
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<td>small interfering RNA</td>
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<td>SLAM</td>
<td>signaling lymphocytic activation molecule</td>
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<td>Full Form</td>
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<tr>
<td>SLT</td>
<td>secondary lymphoid tissue</td>
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<td>Syk</td>
<td>spleen tyrosine kinase</td>
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<tr>
<td>TAM</td>
<td>tumor associated macrophage</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TIGIT</td>
<td>T cel immunoreceptor with Ig and ITIM domains</td>
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<tr>
<td>TIM-3</td>
<td>T cell immunoglobulin mucin 3</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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<tr>
<td>UCB</td>
<td>umbilical cord blood</td>
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<tr>
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<td>ZAP-70</td>
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Abstract

Natural Killer (NK) cells are innate cytotoxic lymphocytes that play an important role in immune control of tumor cell formation and virus infection. In healthy people, NK cells represents heterogeneous populations defined by different phenotypical markers and performing specific functions. NK cells from patients with neoplastic malignancies and viral infection are however typically distinctive from healthy people by the appearance of NK cell subsets, which are differentiated by their CD45 isoform profile. CD45 is a common-leukocyte tyrosine phosphatase abundantly expressed on all nucleated hematopoietic immune cells. Alternative splicing variant resulted in generation of the long-isoform CD45RA and the short-isoform CD45RO, which express differently on naïve and effector/memory T cells. Expression of CD45 isoforms on NK cells is largely unknown. We have previously shown that differential expression of CD45RA and CD45RO isoforms identified specific NK cell subsets in hematological diseases. One question remained unclear: how do these CD45RARO+ NK cells changes when their target cells disappeared? We used NK cells from patients treated with Lenalidomide and the anti-CD20 antibody Obinutuzumab to investigate this and showed a reduction in CD45RARO/CD45RO+ NK cells upon clearance of tumor cells (Chater 4). We observed the same in AML patients after chemotherapy. In this case the CD45RARO+ NK cell subset strongly correlates with trogocytosis of the monocyte/macrophage marker CD14 (Chapter 5). Immunophenotyping of NK cells from HIV-infected patients revealed the presence of CD45RAdim and CD45RO+ cells with reduced CD16 expression and total NKG2D down-modulation. In summary, NK cell from hematological cancers and HIV infection displayed dysfunctional hallmarks and analyzing CD45 isoform profile in these pathological conditions unveils these hallmarks.

Finally, in order to regain the anti-tumor immune response in cancer patients, we present an efficient method for expansion of highly activated NK cells from umbilical cord blood (UCB) in vitro. These NK cells prove substantial antibody-dependent cell cytotoxicity (ADCC) when used in combination with clinical-approved monoclonal antibodies targeting various tumor antigens. This paves their use in allogeneic NK cell-
based immunotherapies.

Keywords: Natural Killer cell, hematological cancer, HIV, CD45, anti-tumor immunotherapy.
Résumé Substantiel

Les cellules Natural Killer (NK) sont des lymphocytes cytotoxiques innés qui jouent un rôle important dans le contrôle immunitaire de la formation de cellules tumorales et de l'infection virale. Chez les personnes en bonne santé, les cellules NK représentent des sous-ensembles hétérogènes définis par différents marqueurs phénotypiques et exécutant plusieurs fonctions spécifiques. Les cellules NK provenant de patients atteints de tumeurs malignes néoplasiques et d’infection virale sont cependant distinctes de celles issues de personnes en bonne santé par la composition de leurs sous-ensembles, l’un d’entre eux est le profil composé de l’isoforme CD45. CD45 est la tyrosine phosphatase à leucocytes communément exprimée parmi toutes les populations de cellules immunitaires hématoïdétiques nucléées. Un variant d’épissage alternatif a entraîné la génération de l’isoforme CD45RA longue et de l’isoforme courte CD45RO qui s’expriment différemment dans les cellules T naïves et effetrices/mémoires. Cependant, l’expression des isoformes de NK CD45 est largement inconnue. Nous avons précédemment montré que la co-expression des isoformes CD45RA et CD45RO identifiait des sous-ensembles de cellules NK spécifiques dans les maladies hématoïdétiques (Chapitre 3). Une question reste floue: comment ces cellules CD45RARO+ NK changent-elles lorsque leurs cellules cibles disparaissent? La première partie de cette thèse a tenté d'aborder cette question par des approches immuno-phénotypiques détaillées sur les populations de cellules NK concernant les isoformes CD45 dans différents types de tumeurs malignes hématoïdétiques (Chapitre 3-5) et l’infection virale (VIH - Chapitre 6). La deuxième partie de cette thèse a fourni une méthode efficace de génération de cellules NK allogéniques avec un potentiel hautement activé pour l’immunothérapie anticancéreuse. Ces cellules NK expansées peuvent être utilisées en tant qu’agent unique en monothérapie ou en combinaison avec des anticorps monoclonaux cliniquement approuvés ou à des médicaments modulateurs métaboliques qui modifient l’immunogénicité des cellules tumorales pour renforcer l’effet anti-tumoral NK-dépendant. La discussion pour chaque section des résultats est présentée selon l’ordre suivant.
CHAPITRE 4: Activation des cellules NK et récupération des sous-populations de cellules NK chez les patients atteints de lymphome après traitement par obinutuzumab et lénalidomide

Le chapitre 4 a examiné les résultats de l’analyse des sous-ensembles de cellules NK dérivés de lymphomes non hodgkiniens à cellules B (B-NHL) provenant de l’étude clinique GALEN (NCT01582776). L’essai clinique GALEN est une étude de phase Ib/II sur les effets de l’obinutuzumab (OBZ) associé au lénalidomide (LEN) pour le traitement des lymphomes B récidivants/réfractaires et agressifs (DLBCL et MCL). L’OBZ montre une cytotoxicité cellulaire dépendante des anticorps plus forte (ADCC) par rapport au rituximab et une activité clinique améliorée pour le traitement de certaines néoplasies CD20+. Cependant, l’efficacité de l’anticorps monoclonal (mAb) en monothérapie est limitée. Les cellules Natural Killer (NK) sont des médiateurs de l’ADCC. Les patients atteints d’un cancer hématologique possèdent des cellules NK antitumorales qui sont incapables de contrôler la maladie, peut-être parce qu’elles sont dysfonctionnelles. Le médicamente immunomodulateur lénalidomide (LEN) pourrait être un traitement pour restaurer les fonctions cytotoxiques épuisées des cellules NK. Pendant le traitement, nous avons analysé des aspects spécifiques de la biologie des cellules NK. Le traitement a inversé le phénotype NK immature des patients et augmenté l’expression des récepteurs d’activation des NK. Les récepteurs inhibiteurs étaient soit inchangés, soit diminués. Il y avait une forte réponse des NK à la fin du 1er cycle: le nombre de NK et l’expression de la granzyme B intracellulaire (GrzB) diminuaient, la dégranulation augmentait et les NK répondaient mieux à la provocation allogénique. De plus, l’interaction des cellules NK avec les cellules B cibles, mesurée par trogocytose, a diminué pendant le traitement. A la fin du traitement, lorsque les cellules cibles ont été éliminées, la proportion de cellules NK réactives (CD69+, CD45RARO+, CD107a+, CD19+) a diminué fortement. Cependant, parce que tous les patients avaient reçu du LEN et d’OBZ, il était incertain quel médicament était responsable de nos observations, ou même si une combinaison des deux produits était nécessaire pour les effets décrits sur cette lignée lymphocytaire.
CHAPITRE 5: Étude des populations de cellules NK chez des patients atteints de leucémie myéloïde aiguë (LAM) au cours d'une chimiothérapie

Les patients atteints de leucémie myéloïde aiguë (LAM) sont typiquement caractérisés par une accumulation et une prolifération rapide de précurseurs myéloïdes hématopoïétiques immatures appelés myéloblastes. Le traitement standard pour ces malignités est généralement une chimiothérapie avec des anthracyclines et de la cytarabine en traitement de première intention, souvent suivie d'une greffe de cellules souches hématopoïétiques (TCSH) chez des patients en rechute. Cependant, la TCSH n’est pas toujours réalisable. Les cellules NK des patients atteints de LAM présentent généralement des dysfonctionnements sévères (Costello et al. 2004; Farag and Caligiuri 2006). Pour mieux comprendre le phénotype des cellules NK chez les patients atteints de LAM après un traitement de chimiothérapie (CT), nous avons analysé les phénotypes de cellules NK chez 7 patients à 2 temps: au diagnostic et 30 jours après la CT. 30 jours après CT, le schéma leucocytaire est partiellement restauré. Ceci est corrélé à une forte diminution des myéloblastes CD33+CD14- immatures. Fait intéressant, la cellule NK du patient de notre étude a montré une proportion accrue de la population CD62bright NK CD62L tout en réduisant les cellules exprimant CD57. Ceci indique un phénotype immature après CT. De plus, nous avons également signalé une diminution de la dégranulation ex vivo et une baisse du niveau de PD-1 dans l’ensemble du compartiment des cellules NK, suggérant un phénotype moins activé après la diminution de la charge des cellules tumorales. Enfin, nous avons confirmé que les cellules NK CD45RARO+ sont en corrélation avec la charge tumorale comme dans notre étude précédente (Chapitre 4). Curieusement, CD14, mais pas CD33, s’associe fortement à la quantité de cellules NK CD45RARO+. Étant donné que CD14 est un marqueur spécifique des populations de monocytes/macrophages, comment les cellules NK CD45RARO+ interagissent préférentiellement avec ces populations mais pas les précurseurs myéloïdes exprimant CD33 reste à explorer.

CHAPITRE 6: Empreintes phénotypiques distinctes dans les cellules NK de patients infectés par le virus immunodéficitaire humain (VIH)
Le rôle des cellules NK dans l’infection par le VIH a été bien établi. Des études récentes suggèrent que l’infection par le VIH cause une homéostasie des cellules NK atténuée et un répertoire aberrant de leurs récepteurs (Mikulak et al. 2017; Scully and Alter 2016). Les cellules NK de patients exposés à long terme à la virémie VIH présentent un phénotype distinct avec une diminution du taux de CD56 tandis que le CD16 intact (c.-à-d. CD56negCD16+). Ces cellules NK CD56neg dysfonctionnelles voient leur cytotoxicité compromise par rapport à leurs homologues CD56dimCD16+. De plus, ils sont également déficients dans la production de cytokines, tels que l’IFNγ et le TNF-α, lors de leur stimulation (Alter et al. 2005; Mavilio et al. 2005). Pour étudier l’impact de l’infection par le VIH sur la population de cellules NK en général et sur l’expression des isoformes de CD45 en particulier, nous avons analysé des échantillons de sang de patients atteints de VIH à haute virémie et virémie absente (avirémie). Chez ces patients, nous avons observé une baisse du pourcentage des compartiments NK et NKT par rapport aux HD (donneurs sains), ce qui suggère que l’infection par le VIH peut avoir un impact sur l’homéostasie des cellules NK. De façon intéressante, la population de cellules NK de notre cohorte de patients a montré des marques de réduction de NKG2D. Étant donné que la diminution de NKG2D est vraisemblablement liée au ligand, nous avons supposé que l’infection par le VIH entraînait des taux plus élevés de NKG2DL chez les patients comparativement aux HD. Le profil d’isoformes CD45 des cellules NK dérivées de nos patients est distinct de celui des HD: avec des proportions plus élevées de sous-populations NK CD45RAdim et CD45RO+ NK chez les patients. La plupart des cellules NK des HD présentaient un phénotype CD45RA+. Il est important de noter que ces cellules NK CD45RAdim et CD45RO+ spécifiques au VIH exprimaient progressivement un taux inférieur de CD16 dans des cellules NK CD45RO+ provenant à la fois des patients avirémiques et virémiques dont la plupart sont CD56dimCD16−. La perte de CD16 sur la population de NK CD56dimCD16+ lors de l’activation via la métalloprotéinase ADAM17 a été démontrée comme caractéristique de la stimulation prolongée des NK (Romee et al. 2013). Par conséquent, nous avons émis l’hypothèse que l’augmentation des populations de NK CD45RAdim et CD45RO+ ayant un faible niveau de CD16 chez les patients infectés par le VIH, résultait d’interactions cellulaires entre les NK et les cellules infectées par le virus in vivo.
De plus, nous avons démontré in vitro que les cellules NK primaires allogéniques sont capables d’effectuer une trogocytose sur des lignées de lymphocytes T CD4+. Résultant de la trogocytose, les cellules NK ont acquis des récepteurs d’entrée du VIH, comme CD4 et CCR5, pouvant sensibiliser à une infection ultérieure par le VIH. Le VIH peut infecter non seulement les lymphocytes T CD4+ mais aussi les macrophages (Honeycutt et al. 2016). Nos résultats suggèrent que les cellules NK pourraient devenir une cible du VIH après la trogocytose des antigènes des cellules T provenant de cellules infectées par le VIH, cependant des preuves directes pour répondre à cette question sont encore en cours d’investigation.

**CHAPITRE 7: Expansion des cellules NK allogéniques avec une cytotoxicité cellulaire efficace dépendant des anticorps contre des tumeurs multiples**

Les anticorps monoclonaux (mAbs) ont significativement amélioré le traitement de certains cancers. Cependant, en général, les mAb seuls ont une activité thérapeutique limitée. L’un de leurs principaux mécanismes d’action est d’induire une ADCC, qui est médiée par des cellules NK. Malheureusement, la plupart des patients atteints de cancer ont des dysfonctionnements immunitaires sévères affectant l’activité NK. Cela peut être contourné par l’injection de cellules NK allogéniques et expansées, inoffensif. Néanmoins, malgré leur fort potentiel cytolytique contre différentes tumeurs, les résultats cliniques ont été médiocres.

**Méthodes:** Nous avons combiné des cellules NK allogéniques et des mAbs pour améliorer le traitement du cancer. Nous avons généré des cellules NK expansées (e-NK) avec de fortes réponses ADCC *in vitro* et *in vivo* contre différentes tumeurs et en utilisant différents mAb thérapeutiques, à savoir le rituximab, l’obinutuzumab, le daratumumab, le cetuximab et le trastuzumab.

**Résultats:** Remarquablement, les cellules e-NK peuvent être conservées congelées et, après décongélation, armées de mAbs. Ils médient l’ADCC par des mécanismes dépendants et indépendants de la dégranulation. En outre, ils surmontent certains mécanismes anti-apoptotiques présents dans les cellules leucémiques.

**Conclusion:** Nous avons établi un nouveau protocole d’activation/expansion des cellules NK avec une activité ADCC élevée. L’utilisation de mAbs en combinaison avec des cellules e-NK pourrait potentiellement améliorer le traitement du cancer.
CONCLUSIONS GÉNÉRALES

En résumé, le travail sur l’analyse des isoformes CD45 dans la sous-population de cellules NK issues de divers cancers hématologiques tels que B-NHL (chapitre 4) et LAM (chapitre 5) a révélé des preuves de dysfonctionnement de ces cellules au moment du diagnostic. De plus, les profils d’isoformes CD45 sur les cellules NK de ces pathologies les différencient des personnes en bonne santé. Ainsi, les cellules NK CD45RO+ sont pertinentes car associées à l’élimination des cellules tumorales (c’est-à-dire LEN plus OBZ, Chapitre 4).

Les cellules NK provenant de patients infectés par le VIH contiennent des sous populations caractérisées par CD45RAdim et CD45RO+ dont le niveau de CD16 diminue progressivement, suggérant une activation récente in vivo. Ces cellules NK provenant de patients VIH + présentent également un récepteur activateur NKG2D diminué, ce qui pourrait être une marque d’altération fonctionnelle induite par un virus.

Enfin, l’expansion des cellules NK d’UCB s’est avérée efficace en combinaison avec plusieurs anticorps monoclonaux contre des antigènes tumoraux. Cela résulte en une ADCC forte in vitro et in vivo. Ce travail soutient l’hypothèse de l’utilisation de cellules eNK en immunothérapie.

Mots-clés: Cellule tueuse naturelle, cancer hématologique, VIH, CD45, immunothérapie anti-tumorale.
CHAPTER 1.

GENERAL INTRODUCTION
1.1 General overview about the immune system and its component

Immunity is one important aspect of life. Indeed, during the course of lifetime individual living need to constantly protect itself from the threats of foreign invader pathogens. In order to do so, living organisms need to develop multiple means to ward off against different invading threats. In general, the mammalian immune system can be divided into 2 parts: the innate immune system and adaptive immune system. These two components of the host immune system cooperate in function to provide protection from variety of pathogenic microorganisms, viruses and cancer.

The innate immune components are the first line of defense that response to presence of pathogens in a broad and non-specific manner. The cells of innate immunity generally include granulocytes, macrophages, neutrophils, monocyte, dendritic cells (DC) and natural killer cells (NK). Due to the non-specific nature of the innate immunity that allows it to quickly scan for pathogenic invading signals collectively known as pathogen-associated molecular patterns (PAMPs) and rapidly mount an immune response towards these pathogenic factors that latter shapes the development of an antigen-specific adaptive immune response giving by T and B cell of the adaptive immunity. The subsequent adaptive immunity requires longer time to develop in term of protection by immunoglobulin (i.e. antibodies) and cytotoxic T cells that are not only eliminate antigen more efficiently but also provide long term protection by mean of immune cell memory. It was generally appreciated that immune memory cells only occur in adaptive T and B cell responses but recent emerging evidences about adaptive features in murine and human NK cell is challenging this long-standing belief and offer a new view to the innate-adaptive discrimination concept (Lanier and Sun 2009). Needlessly to say, there is much crosstalk between cells of the two parts of the immune system in a coordinated manner that determine
Figure 1.1 Schematic model of hematopoiesis and lineage commitment.

the outcomes whether leading to clearance of the invading pathogen or immune tolerance and chronic infections.

Although to some extent cells of non-hematopoietic origin have their role in immune response to pathogens, cells of the immune system from both the innate and adaptive components are those derived from hematopoietic origin. Hematopoietic stem cells (HSCs) are self-renewable CD34+ stem cells that are located in the bone marrow, which give rises to the common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) respectively. These lineages committed progenitors will, in turn, give rise to the subsequent cell types of the myeloid lineage from the CMP origin or lymphoid lineage from the CLP origin respectively. The schematic diagram describes the origin and differentiation cascades of the HSCs is demonstrated in Figure 1.1. In the scope of this thesis work, various aspects of NK cell biology and physiology in cancers and virus infection will be closely examined and discussed, therefore it is essentially important to review what we have known about NK cell from the literature.

1.2 NK cell ontogeny

In the mid 1970s, the identity of NK was started to be recognized by its potential to autonomous lysis of cancer cells without prior antigen immunization hence the term
“Natural Killer” arose (Herberman et al. 1975; Kiessling et al. 1975). Soon after it was realized the need for a surface marker to essentially identify this “Natural Killer” population of cells from T and B cells of the lymphoid lineage. Contemporary development of hybridoma for making monoclonal antibodies and flow cytometry allowing answer to this problem became possible. The first monoclonal antibody successfully identify NK cell from the rest of lymphoid populations was Leu-11 (now known as CD16) (Lanier et al. 1983). Subsequently another surface marker, CD56 (Leu-19) has been identified to successfully describe human NK cell (Lanier et al. 1989). CD56 is a 140kDa isoform of the neural cell adhesion molecule 1 (NCAM-1) that abundantly expressed in circulating NK cells that previously proposed to play a role in mediating NK cell to target cells interactions. Recent evidence further suggested that CD56 also participates in NK cell development via facilitating interaction with stroma cells and serves as pathogen recognition receptor (PRR) in anti-fungal innate immune response (Mace et al. 2016; Ziegler et al. 2017). Interestingly mouse NK cell does not express an ortholog of CD56 hence NKp46, a highly conserved natural cytotoxicity receptor (NCR) is a marker for identify NK cell across species (Walzer et al. 2007). In circulating peripheral blood, NK cell generally takes up 5-15% of total lymphocyte

**Figure 1.2** Two main populations of NK cells in circulating peripheral blood with their difference in CD56 and CD16 expression (Cooper, Fehniger, and Caligiuri 2001)
populations. Based on the surface expression of CD56 and CD16, total NK cell population can be further divided into two sub-populations: CD56brightCD16- (designated as CD56br) and CD56dimCD16+ (designated as CD56dim) with characteristic different in many surface markers expression as well as cytokine secretion and cytotoxicity potential (Figure 1.2).

In peripheral blood, CD56dim NK population is predominantly enriched whereas the opposite situation occurs in secondary lymphoid tissues (STL) where CD56br population presents in prevalent. This due to the fact that CD56br expresses high level of the chemokine receptors CCR7 and CXCR3 as well as the adhesion molecule CD62L that giving homing and entry signal to the STL environment where the reciprocal ligands for these receptors are abundantly expressed (Freud et al. 2017). In contrast CD56dim does not express these STL-associated receptors therefore they are more predominant in peripheral blood. In term of cytokine receptors, it has been demonstrated that CD56br NK cell constitutively expresses c-kit (CD117), IL7Rα (CD127) and IL2Rα (CD25) that enable them to response to low dose of c-kit ligand (KL), IL7 and IL2 respectively. Both CD56br and CD56dim NK cells constantly express the low affinity IL-2R subunits β (CD122), and γ (CD132) but lacking the α sub-unit of the IL-2R complex renders CD56dim NK cell only response to intermediate/high dose of IL-2. In turn, CD56dim NK cell produce little cytokine IFNγ upon stimulation with IL-1β, IL-2, IL-12, IL-15 or IL-18 produced by macrophages, DC or T cells whereas CD56br NK cell produce abundant cytokines including IFNγ, TNFα or MIP-1 in response to monokines stimulation. Both CD56br and CD56im NK cells express NKp46 but lacking CD16 expression in CD56br cell making them unable to perform antibody-dependent cytotoxicity (ADCC), a key function for eliminating antibody coated microbes and target cells. In this regard, CD56dim population with high level of CD16 and cytotoxic granules are more capable of performing ADCC or natural cytotoxicity activity towards harmful cells. Taken together, inherent difference between CD56br and CD56dim NK population making them more powerful in immunomodulatory tasks (for CD56br) or direct cytotoxic tasks (for CD56dim) that sum up their wide roles in the function of the immune response. Figure 1.3. summarized phenotypic and functional differences between the two populations of NK cells.
Figure 1.3 Phenotypic and functional differences between CD56br and CD56dim NK cell populations. (Cooper et al. 2001)

Besides the two well characterized conventional NK (cNK) populations, growing evidence also suggest the presence of tissue-specific resident NK (trNK) cell populations that are localized in liver, uterus, lung, kidney and gut that are considered distinct functionally and immunophenotypically compared to the cNK populations (Björkström, Ljunggren, and Michaëlsson 2016; Melsen et al. 2016).

Where does NK cell develop? Early studies proposed a model in which NK cell develops strictly in bone marrow (BM) since NK differentiation can be induced by in vitro culture of HSC with IL-15 or BM-derived stroma cells (Colucci, Caligiuri, and Di Santo 2003; Miller, Alley, and McGlave 1994; Mrózek, Anderson, and Caligiuri 1996).
However progressing studies on NK cell precursors revealed their predominant presence in the STL including lymph nodes (LN), tonsils and spleen suggesting NK cell can be developed from various sources (Eissens et al. 2012; Freud et al. 2005, 2006; Scoville et al. 2016). According to the linear model of development, mature NK cells are generated from HSC going through 5 stages of developmental intermediates (NKDI) starting with multipotent HSC give rises to NKDI precursor 1 CD34+CD45RA+ that are able to leave BM environment to localize in STL in which will further committed into NK cell lineage (Scoville, Freud, and Caligiuri 2017). NKDI stage 2 marks an important point where NK cell precursor gains IL-15 receptor (CD122) that render them responsible to soluble or trans presented IL-15 upon FLT3 ligand and c-kit ligand stimulation (Boos, Ramirez, and Kee 2008). Stage 3 NKDI further committed into NK cell lineage differentiation by the lack of ability to give rise to T/DC precursors although stage 3 NKDI still lack Tbet and EOMES expression that specific for mature

Figure 1.4 Summarized stages of NK cell developments. (Yu et al. 2013)
NK cell. Finally stage 4 and stage 5 NKDIIs are phenotypically identical to CD56br and CD56dim NK population as discussed previously. It worth to mention, according to this model CD56br therefore regarded as the direct progenitor of the more mature CD56dim. Although this direct maturation relationship between CD56br vs CD56dim is widely accepted, in vitro studies has shown certain plasticity between the two NK populations and not necessarily exclude the possibility that each of the individual cells in these stages are terminally differentiated (Luetke-Eversloh, Killig, and Romagnani 2013; Yu, Freud, and Caligiuri 2013). Therefore until further evident emerges to validate or disregard the model in which CD56br NK are direct progenitors to CD56dim NK cells, this model (Figure 1.4) supports CD56dim NK cells as a more mature population.

1.3 NK cell education and tolerance

In 1971, mouse studies of hybrid resistance in bone marrow transplantation leading to the concept of ‘missing-self” recognition (Ljunggren and Karre 1990). This phenomenon was later on proven thymic-independent hence not involved in T cell but NK cell-mediated (Cudkowicz 1971; Kiessling et al. 1977). In the “missing-self” hypothesis, it was proposed that NK cells constantly sense the presence of MHC class I

![Figure 1.5](image-url) The missing-self hypothesis in NK cell (Ljunggren and Malmberg 2007)
molecules express on healthy cells and remain tolerant. In contrast when viral infection or tumor transformation down modulate MHC-I molecules, cells are sensitized to NK cell mediated-cytotoxicity (Figure 1.5).

In human, inhibitory receptors that recognize classical MHC class I molecules (HLA-ABC) are called killer-cell immunoglobulin-like receptor (KIR). There are 17 KIR-encoding genes have been identified hence KIR locus are undergoing extensive polymorphism (Parham 2005). The similar situation occurs in mice where KIR-homologous in mouse is Ly49 receptor family that recognizes H-2 genes encoding MHC-I molecules in mouse. Besides the interaction between KIR and classical MHC-I, NK cells in human and mouse also express the inhibitory receptor NKG2A for the non-classical HLA-E ligand in human and Qa-1b in mice. According to the “missing-self” hypothesis therefore it requires a condition whereby every individual NK has at least one KIR that recognize self-MHC class I to maintain tolerance, this notion is supported by early works using NK cell clones (Valiante et al. 1997). Nevertheless, recent evidence suggested the presence of NK cell subsets that do not express any inhibitory KIR that is corresponding to self-MHC class I and these NK cells are hyporesponsive upon stimulation (Anfossi et al. 2006; Fernandez et al. 2005; Kim et al. 2005). This requires an expansion of the ‘missing-self” concept into NK cell education/licensing model. The educated/licensed NK cell remains fully functional by expressing at least one KIR or the inhibitory NKG2A recognizing the classical MHC class I (HLA-A/B/C) and non-classical HLA-E. The un-educated NK cells fail to express any self-ligand KIR and remained hyporesponsive, hence tolerant to normal healthy cells. Several models have been proposed to explain the process of NK cell education (Figure 1.6) (Höglund and Brodin 2010):

- **Arming model.** NK cell expressing self-MHC ligand receptors promote functional maturation leading to NK cell cytotoxicity competence by interacting with educating HLA molecules, hence “arming”.

- **Disarming model.** This model suggest NK cell are fully functional by default and “disarmed” if fail to express one of the KIR for self-MHC class I
Figure 1.6 Different models of NK cell education (Höglund and Brodin 2010)

- to retain tolerance, NK cells that acquire inhibitory KIR are therefore avoid this effect hence remained functional. Comparing to the “arming” model, this model does not require a secondary role for KIR except inhibiting activation signal.
• **Cis-interaction model.** This model emerged by the observation that self MHC class I molecules can interact with their corresponding KIR in the NK cell in *cis*, therefore retain the inhibitory KIR from recognizing self-MHC class I molecules presented on target cells. However the question whether all KIRs can interact with MHC-I molecules in *cis* makes it unclear whether this model fully explains the NK education process.

• **Rheostat model.** By far the “arming” and “disarming” models imply a definite state of functional/hyporesponsive status but NK cell education can be a quantitative process suggested by the finding that different MHC class I alleles possess non-equal ‘education effect’ on their corresponding receptors (Brodin et al. 2009; Johansson et al. 2005). This model therefore suggests a diversified spectrum of NK cell populations with different functional potential depending on which inhibitory receptors they possess.

Notably, ongoing studies on activating members of the KIR (aKIR) receptor family revealed additional mechanism of NK cell education. Recent evidence reported the presence of KIR2DS1 NK cell subsets in HLA-C2+ donors lacking a self-inhibitory KIR or NKG2A expression suggesting these KIR2DS1 NK cells would be highly autoreactive towards self MHC-I expressing cells (Cognet et al. 2010). However, subsequent characterization studies revealed that KIR2DS1-carrying NK cells are hyporesponsive in HLA-C2 (which serves as ligands for the respective aKIR) homozygous donors but not in HLA-C1 donors suggesting self-reacting aKIR-expressing NK cells lacking any inhibitory receptors were tuned down their responsiveness in an opposite direction to iKIR-acquisition in NK cell education (Fauriat et al. 2010; Ivarsson, Michaëlsson, and Fauriat 2014).

Finally, it is worth to mention that MHC-I-independent mechanisms mediate NK cell education. The SLAM family receptors 2B4 (CD244), SLAMF6 and other inhibitory receptors such as TIGIT, CD161 (NKR-P1A) can educate NK cells via their respective
ligands (He and Tian 2017; Orr and Lanier 2010) although the exact mechanisms of these non-conventional education pathways are largely non elucidated.

Role of educated vs un-educated NK cell in immunity? The presence of both NK populations in normal individual hosts seems like an un-necessary expense in efficiency perspective. Therefore there must be a different role of both populations: educated and non-educated NK cells in term of protection in the immune system. Recent studies in mouse model of murine cytomegalovirus (MCMV) infections suggesting that un-educated mouse NK populations play a more important role in viral clearance compare to educated NK cells (Orr, Murphy, and Lanier 2010; Tu, Mahmoud, and Makrigiannis 2016). In contrast, the educated NK cell population, by possessing inhibitory self-receptors, is more potentially active against cells that down modulated MHC class I, e.g. newly transformed tumor cells and or virus that used these immune-evasion strategies.

1.4 NK cell receptor repertoire and signaling pathways

In spite the lack of rearranged antigen-specific receptor like other lymphocyte counterparts such as T cell and B cell, NK cells express a wide spectrum of germline-encoded both inhibitory and activating receptors that works in synergy to exert variety of functions in host defense mechanism and homeostasis (Lanier 2003). It is now widely accepted that the integration of both inhibitory and activating signaling input governs the outcome of NK cells and their target interaction leading to target cell elimination or not. Therefore it is necessary to briefly summarize the diversity of NK cell receptors and their downstream mediated signaling pathways that influent NK cell activity.

Inhibitory receptors. Perhaps the most well characterized class of NK cell inhibiting receptors are members of the KIR family as discussed in detail previously. Human KIRs can appear in two forms: KIR-L receptors with long cytoplasmic tail containing immunoreceptor tyrosine-based inhibition motif (ITIM) are inhibitory whereas short cytoplasmic tail counterparts KIR-S lacking ITIM but can coupled with
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<td>CD96</td>
<td>Nectin-1, PVR (CD155)</td>
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<td>TIGIT</td>
<td>Nectin-2 (CD112), Nectin-3 (CD13), PVR (CD155)</td>
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<td>HLA-A*11, some HLA-C alleles</td>
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<td>HLA-E</td>
<td>Expansion of NKG2C+CD57+ in various virus infections</td>
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**Table 1.** Summary of NK cell major activating and inhibitory receptors and the ligands that they recognize.

DAP12, an activating-associated receptor therefore promote NK cell activating signaling. KIR2DL4 is the only exception among KIR family in which this receptor contains an ITIM domain but also capable of coupling to ITAM-adapter molecules, KIR2DL4 mainly involved during pregnancy upon recognition of its selectively soluble ligand HLA-G in the uterus. In addition to KIR receptor category, the NKG2A-CD94 heterodimer also belong to MHC class I inhibitory receptor that recognize the non-classical HLA-E (Braud et al. 1998). Finally ITIM-containing inhibitory receptors for non-MHC class I ligands such as KLRG1 and NKR-PiA (CD161) and Siglec family receptors also present on NK cells but their roles in regulating NK cell inhibiting signals remained largely unexplored (**Table 1**).

**How ITIM-receptors mediate NK cell inhibition?** It is speculated that upon engaging with the ligands, ITIM domain of the inhibitory receptors is phosphorylated
by possibly an Src family kinase resulting recruitment of several phosphatases including SH2-domain-containing protein tyrosine phosphatase 1 (SHP1), its homolog SHP2 and the SH2-domain-containing inositol-5-phosphatase (SHIP) (Lanier 2008). It has been reported that KIR family members and NKG2A-CD94 preferentially recruit tyrosine phosphatase SHP1/SHP2 whereas KLRG1 is more associated with the lipid phosphatase SHIP (Tessmer et al. 2007). In turn, these phosphatases predominantly inhibit downstream activating signaling mediated by Vav-1 (Stebbins et al. 2003). More recently two additional alternative inhibiting mechanism have also been suggested in which the tyrosine protein kinase c-Abl mediated disruption of the Crk, c-Cbl and C3G complex. In basal condition c-Cbl continuously inhibits phosphorylated Vav-1 by which prevents spontaneous activating signal in resting NK cells (Yenan T Bryceson et al. 2006; Kim et al. 2010; Peterson and Long 2008). This, at least, partly explains why NK cell in steady condition requires synergistic co-activating receptors to overcome the basal inhibiting effect of Vav-1 dephosphorylation by c-Cbl (Figure 1.7).

**Figure 1.7** ITIM-mediated inhibitory signaling pathway. (Höglund and Brodin 2010)
NK cell activating receptors: ITAM-associated receptor members. ITAM-mediated signaling represents a major activating signaling pathway in NK cell. Members of ITAM-associated activating receptors include the low affinity FcγRIIIa (CD16), the heterodimer NKG2C-CD94, activating receptor members of the KIR family (KIR-S) and the family of natural cytotoxicity receptors (NCRs). In NK cell, CD16 confers major function in ADCC to eliminated IgG1-coated target cells. The importance of CD16 function in NK cell perhaps demonstrated by the fact that it is the only activating receptor capable of mediate degranulation and cytotoxicity in resting freshly isolated NK cell (Y. T. Bryceson et al. 2006). CD16 can pair with both CD3ζ and FcεRIγ homodimer or CD3ζ- FcεRIγ heterodimer to trigger downstream signaling. There is another adapter molecule an ITAM sequence motif that can associate with NK cell activating receptors, DAP12, which was identified in 1997 (Lanier et al. 1998). DAP12 was shown to pair with MHC-I-binding receptors like NKG2C-CD94 and activating-KIR. The self-ligands for these MHC-I-specific activating receptors are still poorly identified however on-going researches pointed out the roles of these receptors in NK cell responses in HIV and CMV viral infections and also impact on NK cell education (Fauriat et al. 2010; Gumá et al. 2004; Lopez-Verges et al. 2011; Martin et al. 2002). Final class of ITAM-associated receptors are the 3 members of the NCR family includes NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3) with NKp46 was most well known as a pan-NK cell marker across species. Although NKp46 and NKp30 constitutively expressed on all NK cells, NKp44 only upregulated after NK cell activation (Arnon, Markel, and Mandelboim 2006; Vitale et al. 1998). Cytoplasmic domains of NKp46 and NKp30 can pair with both CD3ζ and FcεRIγ chains but NKp44 can only pair with DAP12. NKp46 and NKp44 are reported to recognize viral protein ligands however whether other types of ligands (if present) can bind to these receptors will require further studies (Arnon et al. 2006). One member of the B7 superfamily, B7-H6 presents on several tumor cell lines has been shown to serve as a ligand for NKp30 (Brandt et al. 2009).

ITAM-mediated downstream signaling cascade. Although greatly diverged in receptor-ligands possibilities, downstream signaling by ITAM-containing adapter
molecules CD3ζ, FcεRIγ and DAP12 in NK cell is greatly resemble of BCR and TCR signaling that are well understood in B and T cell. Simply speaking, engagement of the corresponding ligand to ITAM-associated receptors leading to phosphorylation by one of the member of Src kinase, this will in turn results in recruitment of the tyrosine kinase Syk or ZAP-70 to the intracytoplasmic receptor complex. In response to this, several further downstream signaling complexes including the phosphatidylinositol-3-OH kinase (PI3K), phospholipase C (PLC-γ1, PLC-γ2) and Vav-1,2,3 activation. Results of these signaling complexes activation finally leads to nuclear translocation of key transcription factors of the family NF-AT, NF-κB and AP-1 responsible for cytokine secretion but also prepare for cytotoxic granule exocytosis by rearrangement of actin-cytoskeleton. ITAM-mediated signaling cascade was summarized in Figure 1.8.

**Figure 1.8** NK cell activating signaling pathway via ITAM-coupled receptors (Lanier 2008)

*NK cell activating receptor: NKG2D-DAP10 complex and the “induced-self” recognition.* The C-type lectin activating receptor NKG2D, which belong to the NKG2
gene family, can be considered one of (if not) the most important receptors in NK cell function in eliminating danger target cells. Although its function most studied on NK cell but NKG2D expression is not restricted to NK cell but also found in γδT cells and CD8+ T cells (Bauer 1999; Jamieson et al. 2002). It has long been speculated that NK cells are able of recognize and kill cells that are undergoing stress such as virus infected cells or transformed tumor cells, this hypothesis was regarded as the “induced-self” hypothesis. The finding of NKG2D and its respective ligands widely upregulated in “stressed” cells confirmed this theory (Gasser et al. 2005). In human, NKG2D recognizes 8 ligands including MHC-I related molecules MIC-A, MIC-B, UL16-binding proteins (ULBP1-6) with extensive polymorphism (Bauer 1999; Chalupny et al. 2003; Cosman et al. 2001). Interestingly in human there is only one version of NKG2D but in mouse alternative splicing resulted in 2 versions of the molecule denoted as NKG2D-L (for long isoform) expressed mainly on resting NK cells and NKG2D-S (for short) which induced after NK cell activation (Diefenbach et al. 2002; Gilfillan et al. 2002). NKG2D was not found to be associated with ITAM-containing molecules but

![Figure 1.9](image)/

**Figure 1.9** NK cell activating signaling pathway via NKG2D-DAP10 complex (Lanier 2008)
instead binds to an YINM-motif-containing DAP10 with an exception of NKG2D-S isoforms that binds both to DAP10 and DAP12 molecules (Wu et al. 1999). Consequently, human NK cell can only trigger NKG2D-mediated signaling via DAP10 signaling. Upon phosphorylated by NKG2D crosslinked, DAP10-YINM motif recruits the binding of either p85 of PI3K or Grb2 and finally leading to activation of several downstream signaling including PLC-γ, MEK1/2-Erk pathway and Akt pathway (Figure 1.9).

**NK cell activating receptor: SLAM family receptors.** SLAM family receptors expressed on NK cells include 2B4 (CD244), NTB-A and CRACC. Different from ITAM-associated receptors and NKG2D, cytoplasmic tail of these receptors contains an immunoreceptor tyrosine-based switch motif (ITSM) which can be both inhibitory or activating receptors. Perhaps the most well studied member of this group is CD244 (also known as 2B4), since 2B4 receptor can serve a dual function on NK cells, exact mechanism on how 2B4 transfer signaling to downstream molecules in certain physiological contexts is still under investigation. Current model suggesting 2B4 can

![Diagram of CD244 receptor with signaling pathways](image)

**Figure 1.10** NK cell can be activated or inhibited via the CD244 receptor depending on coupled-adapter molecules (Lanier 2008)
bind to both SAP and EAT-2 (only in human) in which SAP-dependent activation requires Fyn recruitment and involved different activating complexes such as Grb2 and LAT- PLC-γ and Vav-1, whereas inhibiting signal required the phosphatases SHP-1/2, SHIP, Csk and this process involved EAT-2 association (Figure 1.10) (Bottino et al. 2000; Chen et al. 2006; Veillette 2006; Watzl, Stebbins, and Long 2000).

NK cell activating receptor: DNAM-1. DNAX accessory molecule-1 (known as DNAM-1, CD226) is an immunoglobulin (Ig)-like molecule in NK cell and other cell types which recognize specifically CD112 (nectin-2) and CD155 (poliovirus receptor, PVR) (Bottino et al. 2003; Shibuya et al. 1996). Recently published result indicated that DNAM-1 mediated signaling can be activated by Fyn member of Src kinase family and recruitment of Grb2 and other downstream signaling complexes (Zhang et al. 2015). Interestingly, independent of its signaling function, DNAM-1 also promotes cell adhesion by participating with another adhesion molecule LFA-1 therefore promote immune synapse formation between NK cell to target cells (Shibuya et al. 2003). Expression of DNAM-1 on NK cell underlined an alternative maturation pathways governs human NK cell functions (Martinet et al. 2015).

LFA-1 and other coactivating receptors. As mentioned above, NK cells require formation of the immunological synapse with their targets for efficiently perform cytotoxicity and avoiding bystander cells co-lateral damage, this synapse formation promote by LFA-1 (also known as CD18, integrin β-2) (Hsu et al. 2016). NK cell is unique in its capability to promote adhesion by LFA-1 binding to its target ligand ICAM-1 (CD54) in autonomous manner which usually only followed after other co-activating receptors as seen in other cell types such as T cell (Watzl and Long 2010). Furthermore, binding of LFA-1 to ICAM-1 alone is sufficient to induce granule depolarization on NK cells (Barber, Faure, and Long 2004; Bryceson et al. 2005). There are many other activating and co-activating receptors expressed on NK cell such as NKp80, NKp65, CD2, CD69, CD137, CD27 and CD160 to name a few, although the list goes on but the mechanism of these receptors on NK cell functions are still largely unexplored and therefore rely on the need for future works.
1.5 How does a NK cell kill its target?

Being developed from the same progenitor, despite the vast difference on the target cell receptor recognition, cytotoxic T lymphocyte (CTL) and NK cell are strikingly similar on how they trigger target cell apoptosis. Mainly, NK cells and CTL mediate cell death via two different mechanisms: cytotoxic granule exocytosis, and death ligand/death receptor interaction from the TNF superfamily members. Although one would not exclude the intrinsic pro-apoptotic nature of secreted cytokines such as IFNγ and TNFα by NK cell and CTL, perhaps in the real physiological context, all these mentioned mechanisms contributed into target cell death.

*Cytotoxic granule inducing cell death.* Mature NK cells are featured with preformed secretory granules containing the pore-forming protein (perforin, PRF1) serine proteases known as granzymes (Grz). There are 5 types of Grz in human but only Grz-A and B are mostly studied in NK cells and CTLs, in which GrzB is mostly crucial for inducing cell death while other member roles are still in subject of further investigation (Bovenschen and Kummer 2010; Pardo et al. 2009; Voskoboinik, Whisstock, and Trapani 2015). Upon activating receptor recognition, cytotoxic granules are polarized into the NK-target cell interface (i.e, immunological synapse) by actin rearrangement and release effector molecules. In turn, perforin forming holes in the target cell membranes which enable entering of GrzB into the cytosol where it can cleave pro-apoptotic caspases leading to the activation of the caspase-mediated apoptosis machinery. Concurrent to this process, the protein LAMP-1 (lysosomal associated membrane protein 1, CD107a) which resides in the endosomal side of the granules are exposed to the plasma membrane which can be served as a marker of recent degranulation.

*Death-ligand mediated cell death.* Besides degranulation mechanism, NK cells also express death ligands of the TNF family notably Fas-ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) (Warren and Smyth 1999). It was speculated that immature NK precursors employ TRAIL as a main way for inducing cell death while
both FasL and degranulation are more important for mature NK cell-mediated cytotoxicity (Zamai et al. 1998). The observation that NK cell and CTL can trigger target cell death independent of Ca2+ influx (which is essential for granules mobilization) underlined the role of death receptors pathway as an alternative mechanism of NK cell cytotoxicity. Downstream signaling by death receptor leading to pro-apoptotic signal has been extensively studied and discussed (Cullen and Martin 2015; Schulze-Osthoff et al. 1998). In summary, upon target cell recognition by appropriate activating receptor, NK cell can mediate apoptosis on target cell both
degranulation and death ligand/death receptor pathways (detailed mechanism summarized in Figure 1.11).

1.6 Human NK cell and the immune control of viral infection

Although NK cell was originally identified due to its spontaneous anti-tumor activity, very soon later it was appreciated that NK function is a crucial part in antiviral immune response. This was clearly demonstrated in case studies where individuals with NK cell deficiency affected severely by virus infections, particularly those of herpes family (Biron, Byron, and Sullivan 1989; Mace and Orange 2016). NK cell activity during viral infection closely linked to DC in reciprocal manner, where type 1 IFN and pro-inflammatory cytokines (IL-12, IL-15, IL-18) produced by DC activates NK cell and trigger IFNγ secretion which in turn, required for DC maturation and functions. IL-15 in soluble form or trans-presented by DC-derived IL-15Rα chains greatly enhances NK cell survival and proliferation.

Perhaps the most well studied model of NK cell-mediated antiviral response is from mouse cytomegalovirus (MCMV) of the herpesvirus family. In this model it was firstly demonstrated that mouse NK cells express a viral specific receptor (named Ly49H) that recognize the MCMV encoded protein m157 (Arase et al. 2002; Smith et al. 2002). Why MCMV affords to carry a gene that recognized by NK cell hence playing against its advantage remained a myth, although this underlined an intimated co-evolution relationship between virus and NK cell. Also in this mouse MCMV infection model it was later presented first evident for NK cell adaptive feature that widely accepted is a characteristic attributed only to cells of the adaptive immune response such as T and B cell (Sun, Beilke, and Lanier 2009). Therefore, NK cell remembers. This raised the next question whether there is an equivalent behavior of human NK cell against HCMV, the answer is: possible. Studies with HCMV+ patients shown a specific population of human NK cells with CD94-NKG2C+ phenotype is preferentially expanded and persist (Gumá et al. 2004, 2006; Lopez-Verges et al. 2011). UL16-binding proteins (ULBPs) which were found to be a ligand for NKG2D is one of the target of HCMV encoded protein (UL16), this protein was used by the virus as an evasion
strategy for avoiding NKG2D-mediated recognition. UL16 was also found to target to MIC-A, another NKG2D ligand. Besides UL16, HCMV also has different alternative NK-dependent immune evasion strategies for successful host infection (Chalupny et al. 2006; Stern-Ginossar et al. 2007). As mentioned above, the antiviral role of NK cell is not only restricted to HCMV but also expand to other viruses of the herpes family like HSV and EBV, etc.

One of the reported ligand for the NK cell receptor NKp46 and NKp44 is influenza HA therefore it is rational to argue NK cell has a role in influenza infection control. According, other study shown that NK cell became activated in the presence of influenza-infected DC and this activation further prevented by NKG2D and NKp46 blocking (Draghi et al. 2007). Furthermore, NK cells are found to be recruited to influenza-infected mice’s lung and NKp46/- NK cells cannot rescue infected mice (Gazit et al. 2006).

Immunogenetic studies reported several interesting correlation between patient pathological progress with certain KIR- HLA alleles combinations in HCV and HIV infected (Flores-Villanueva et al. 2001; Khakoo et al. 2004). Notably, KIR3DS1 and HLA-Bw4 alleles-carrying patients are better at delaying AIDS symptoms, KIR3DS1+ NK is also shown to control HIV replication in HLA-Bw4 infected target cells in vitro (Alter et al. 2007; Cohen et al. 1999). Finally, HIV-encoded protein Nef was found to block NKG2D ligands expression such as MIC-A and ULBPs indicated the role of NK cell as a selective pressure for HIV immune evasion (Cerboni et al. 2007).

1.7 Tumor immunosurveillance from the NK cell perspective: tumor immune evasion strategies

The concept of tumor immunosurveillance was first proposed by Paul Erlich, suggesting a novel role of the immune system not only in control of infection but also in control of spontaneous tumor formation. This idea was later formalized into Sir Macfarlane Burnet’s hypothesis of “cancer immunosurveillance” by his own words (Burnet 1970):
“In large, long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessary that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character.”

This extraordinary idea by Sir Burnett is nowadays widely accepted and supported by various studies in cancer immunology, most notably from the results of IFNγ-/− and RAG-/− knockout mice models and the discovery of tumor-associated antigens (TAA). Since NK cell are known much later than T cells, the main effector type of cells that were regarded as anti-tumor cells were T cells. However, it is soon realized that NK cell and CD8+ T cells are the 2 arms of the immune system in anti-tumor effect. In both human and mouse, NK cell deficiencies are largely at the potential expose of uncontrolled virus infection but also are a higher risk of tumor development (Orange 2013). Transgenic mice with NKG2D depletion show higher chance of primary tumor development (Guerra et al. 2008). In these mice, primary tumor cells were found to express NKG2D ligands, which is mostly absent in wide-type mice. This underlined the role of NKG2D-expressing cells in controlling tumor growth (majority of NK cells and small subsets of T cells). Tumor mediates immune escape from NKG2D/NKG2D-ligand (NKG2DL) by multiple mechanisms in which notably by shedding of NKG2DL from their membrane surface by matrix metalloproteases (MMP) or through exosomes (Salih, Rammensee, and Steinle 2002; Wu et al. 2004). In lines with these, soluble NKG2DLs were detected on sera of patients with various cancers (Fernández-Messina et al. 2010). Encountering with soluble NKG2DL causes NKG2D down-modulation in NK cells and T cells therefore could further suppress NKG2D-mediated cytotoxicity. Interestingly recent evident suggested that this is not always the case since a mouse NKG2D ligand namely MULT1 in fact promote NK cell-mediated tumor rejection (Deng et al. 2015). How this result can be interpreted together with abundant immunosuppressive effect of other shedding NKG2DL in previous studies need to be resolved.
Tumor microenvironment is widely accepted to suppress the effector functions of both NK cell and CTL through various mechanisms. Tumor microenvironment is typically characterized by having strong immunosuppressive effect by cytokines and metabolites secreted by the tumor itself and tumor-associated populations such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and M2 macrophages. Tumor cells are characterized with distinctive metabolism than healthy cells by higher glucose consumption and lactate production as a by-product of the Warburg effect, this in turn suppress tumor-infiltrating NK cells and CTL effector functions by hypoxia and lactate-mediated effect (Villalba et al. 2013). Other tumor-derived metabolite products such as Indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2) and adenosine are also known to inhibit NK cell effector functions (Böttcher et al. 2018; Villalba et al. 2013).

Tumor cells preferentially recruited immune cell populations with suppressive phenotypes, in which Tregs are commonly associated with tumor progression. Notably Tregs are not affected by low glucose concentration in the tumor microenvironment since they mainly utilize fatty acids oxidation as the source of energy (Michalek et al. 2011). Tregs are phenotypically characterized by CD4+CD25hiFoxP3+ in which CD25, the high affinity IL-2 receptor alpha chain is constitutively expressed therefore can interfere the CD4+ effector cells and NK cells crosstalk via IL-2 sequestration. Accumulation and expansion of Tregs are found to be associated with different types of cancer and correlate with poorer prognostic (Fu et al. 2007; Ormandy et al. 2005; Sinicrope et al. 2009). Tregs can also dampen NK cell antitumor effect via the TGF-β pathway although TGF-β is also widely secreted by other immunosuppressive populations such as MDSC and tumor-associated macrophages (TAM). Collectively, these tumor-associated populations are commonly recruited within the tumor microenvironment that via their various yet overlapping immunosuppressive mechanisms contribute to the immune escape from effector cytotoxic lymphocytes including NK cells (Figure 1.12). Hence, several approaches to enhance NK cell activity and overcoming these suppressive effects showed to be promising in cancer immunotherapy, one of which is the ex vivo expansion protocols of activated NK cells.
Figure 1.12  Tumor cells can develop various immune evasion strategies to avoid NK cell mediated attack (Morvan and Lanier 2016).

1.8 Ex vivo expansion of allogeneic NK cell for cancer immunotherapy

Although NK cell possesses intrinsic anti-tumor potential, yet they fail to control tumor growth in cases of cancer patients. Indeed, as discussed above, circulating and tumor-infiltrating NK cells from patients with cancer progression displayed impaired functional characteristics and abnormal phenotypes as a consequent of various tumor-induced suppression mechanisms. Furthermore, freshly isolated naïve NK cells exhibit low natural cytotoxicity and only represent a minor
**Figure 1.13** Autologous or allogeneic NK cell adoptive transfer can be achieved by different approaches of ex vivo expansion/activation to regain the anti-tumor potential of NK cell–based immunotherapy, adapted from (Guillerey, Huntington, and Smyth 2016).

subset within total population of peripheral blood mononuclear cells (PBMC). Therefore, ex vivo expansion strategies are required in order to re-gain anti-tumor activity of NK cells in NK cell based immunotherapy approaches (Figure 1.13).

*Sources of allogeneic NK cells.* Circulating NK cells can be effectively isolated from Ficoll buffer preparation of healthy donor PBMC, alternatively umbilical cord blood (UCB) is another potential source of NK cell for ex vivo expansion. Compared to NK cells derived from PBMC, UCB mononuclear populations contains more NK cell with immature phenotypes hence greater proliferation potential (Condiotti et al. 2001; Mehta, Shpall, and Rezvani 2016; Sarvaria et al. 2017). Moreover UCB-derived mononuclear populations also contain CD34+ hematopoietic precursors that can further be differentiated into fully mature NK cells in vitro. Finally, various stem cells isolation and generation methods which can produce human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) can be served as a source to generate in vitro expanded NK cells, although the cost efficiency and multiple in vitro
manipulation steps involved in generating these cells could make them less approachable than PBMC and UCB-derived NK cells.

Cytokines for NK cell in vitro expansion. The first use of cytokine to generate NK cells for clinical purpose was applied very early since 1980 by using of high dose IL-2 to generate Lymphokine-activated Killer (LAK) cells from autologous PBMC (Rosenberg et al. 1985). It was soon later realized that the tumor rejection activity by this IL-2 activation method was mainly attributed to NK cell (Phillips et al. 1987). However the method to generate LAK cells are pretty much hindered by T cell populations which also require IL-2 for their expansion and survival, especially for Tregs due to high level expression of CD25. Alternatively, T cell population can be depleted from the PBMC mix to acquire higher purity of expanded NK cells without cytokine competition. IL-15 is another important promoter of NK cell differentiation, survival and homeostasis (Cooper et al. 2002). Notably, IL-15 promote NK cell survival via the anti-apoptotic MCL-1 and targeting IL-15-mediated inhibitory checkpoint CIS in the NK cell enhanced the anti-tumor function of NK cell in vivo (Carson et al. 1997; Delconte et al. 2016; Huntington et al. 2007). Combination treatment of IL-12, IL-15 and IL-18 has been shown to generate a long-lived population of NK cells with memory-like properties (Leong et al. 2014). Recent studies also highlighted on the role of newly identified member of common-gamma chain receptor cytokines, IL-21 in NK cell activation and the potential use of IL-21 in synergy with IL-2 and IL-15 (Ozaki et al. 2000; de Rham et al. 2007). However, generally NK cell generated by cytokine stimulation alone gives lower yield of expansion in comparison with expansion method using both cytokine and feeder cell.

Role of feeder cells in NK cell expansion protocols. Besides cytokines, NK cell also requires co-stimulatory signals from surrounding cells for survival and proliferation. Combination approaches using both stimulatory cytokines such as IL-2/IL-15 together with irradiated feeder cells seem to enhance NK cell expansion rate compares to each of the single factor alone. There are different ways of using feeder cells in NK expansion protocols, one would be irradiated autologous PBMC. Alternatively, the HLA-null target cell prototype K562 also commonly used in different NK expansion
approaches. There are multiple advantages of using irradiated K562 as feeder cells: unlike autologous PBMC, these cell lines are very readily accessible and can be preserved in batch, moreover K562 can also be genetically engineered to express ectopic membrane-bound IL-15, IL-21 and 41BBL which are NK-stimulatory factors (Childs and Carlsten 2015; Denman et al. 2012; Zhang et al. 2011). Lastly, Epstein-barr virus (EBV)-transformed lymphoblastoid cell lines (EBV-LCL) can also preferentially promote NK cell expansion and survival in vitro (Perussia et al. 1987). EBV-LCL based expansion protocols has been developed to produce large scale of NK cells and compatible with GMP regulations (Berg et al. 2009; Vasu et al. 2015).

1.9 CD45 expression and alternative splicing isoforms on NK cell

CD45 is a transmembrane glycoprotein expressed on all hematopoietic-origin populations except erythrocytes and platelets. In these types of cells, CD45 served as a phosphotyrosine phosphatase (PTPase) that directly regulates ITAM-mediated downstream signaling of TCR and BCR in T and B lymphocytes respectively. In NK cells, CD45 is also shown to control ITAM signaling probably through ITAM-coupled activating receptors such as CD16, NCRs or NKG2C/KIR-S members of MHC-class I binding receptors. Studies with CD45-deficient mice revealed distinct role of this molecule in regulating NK cell ITAM-signaling: indispensable for ITAM-mediated cytokine production but not cytotoxicity (Hesslein et al. 2006). Furthermore, CD45-deficient NK cells are unable to control MCMV infection, probably via ITAM initiated signaling (Hesslein et al. 2011). Primary signaling regulatory function of CD45 cytoplasmic phosphatase domain D1 is enabling Src kinases activity: CD45 D1 phosphatase domain can dephosphorylate the constitutive inhibited p56lck and p59fyn members of the Src family, thereby activating Syk and ZAP70 signaling downstream of ITAM-coupled receptors.

CD45 is a multifunctional protein, since besides its positive role in regulating ITAM signaling in lymphocyte populations, CD45 is also reported to be a negative regulator of the Jak-STAT pathways (Sasaki, Sasaki-Irie, and Penninger 2001) (Figure 1.14). The 'speudo'-phosphatase domain D2 of the molecule was found to associated
Figure 1.14  Dichotomy of CD45 phosphatase role in controlling Src family kinase (SFK) activation and Jak-STAT inhibition (Sasaki et al. 2001)

with Jak, this resulted in dephosphorylation of Jak and inactivate downstream STAT-dependent signal. Consequently, loss of CD45 leading to increase in signaling of cytokines such as IL-3 and IFN-α (Penninger et al. 2001).

One of the remarkable features of CD45 is the ability to generate multiple isoforms through alternative splicing of CD45 pre-mRNA. In particular, exons of
location 4, 5 and 6 (traditionally denoted A, B and C, respectively) are selectively spliced to form multiple versions of the CD45 molecules with different combination in the A, B and C components. As a result of this effect, potentially there are total 8 possible combinations between the A, B and C regions however in reality there are only 5 isoforms that are detected at the cellular surface level of lymphocytes, those are: CD45RABC, CD45RAB, CD45RBC, CD45RB and CD45RO (which is lacking of all the A, B and C regions) (Figure 1.15). What could be the implication in term of function between these different isoforms? In ectopically expression of chimeric molecules of the defined isoforms of CD45 with the cytoplasmic domain of CD45, T cell activation and development are not affected suggesting that the PTPase domain of CD45 is most essential for T cell function function (Kozieradzki et al. 1997). This begs the question:

**Figure 1.15** Alternative splicing of CD45 and different isoform expression on lymphocytes. a) gene structure of PTPRC (CD45) and exon-splicing of CD45 pre-mRNA
at location 4,5 and 6 generate 8 possible isoforms. b) schematic structure of CD45 protein with the longest and shortest isoforms and their difference in O-linked glycosylation pattern. c) Different expression in CD45 isoforms in naïve/effector T cell. Adapted from (Tchilian and Beverley 2006).

what is the difference in various extracellular domains of the different isoforms? One hypothesis proposed the dimerization model of CD45 is differently regulated due to the size of extracellular isoforms, however the implication of this hypothesis still not yet comply with contemporary results in T cell studies (Majeti et al. 2000). Alternative splicing of CD45 is mostly studied with T cells where it is generally observed that naïve T cell in the peripheral blood preferentially express the long isoform CD45RA, upon antigenic stimulation via TCR T cell rapidly “switch” to CD45RO expression profile. Based on this phenomenon, CD45RA vs CD45RO are generally used to differentiate T cell “naïve” or “effector/memory” phenotypes in the T cell pool. However, in NK cells, the differential expression of CD45 isoforms and its correlation with NK cell in vivo activities in different pathological conditions are largely unknown.

1.10 Aims and structure of the thesis

NK cells are considered frontline in the anti-tumor function of the immune system yet in patients with hematological malignancies and solid tumors they are unable to keep these transformed tumor cells in check, partly because of tumor-induced impaired functions. Within the scope of my thesis work, I set to investigate the hallmarks in NK cell phenotypes and functional attributes in different pathological conditions. This might be linked with disease progression or remission during treatment. I made a special focus, but not exclusively, on the expression of CD45 isoforms on the NK cell population.

In chapter 3, I report, together with some co-workers, that NK cell populations with different CD45 isoform profile, notably the increased frequency of CD45RA+CD45RO+ (hereafter CD45RARO NK) is linked with several hematological
cancers (Krzywinska et al. 2015, 2016). Results from this study will be presented in form of published article in the journal *Ebiomedicine*.

Chapter 4 presented the GALEN study: this is a longitudinal pilot study in NK cell phenotypes and functions in relapsed patients with non-Hodgkin lymphoma (NHL) in a phase Ib/II clinical trial of combination treatment of GA101 (anti-CD20 monoclonal antibody) and the immunostimulatory agent Lenalidomide (Revlimid; Celgene). The results of this study are presented in form of the published article in journal of *Oncoimmunology*.

Chapter 5 discussed results in NK cell phenotyping study in acute myeloid leukemia (AML) patients during chemotherapy intervention.

In Chapter 6, and based on the similar argument, we present that NK cells are probably showing clues of functional impairment in patients with various pathological conditions, I presented my analysis of NK cell population in a small cohort of HIV infected individuals as a collaborative project with Immuno-Pathology team based in CHU-Montpellier and IGH and directed by Prof. Pierre Courbeau.

In chapter 7, we presented a method of generating activated NK cells *in vitro* from healthy donor UCB. These highly activated NK cells were shown to perform efficient ADCC *in vitro* and *in vivo* when used in combinaton with several clinical-approved mAbs.

Chapter 8-10 presented seminal research articles on cancer cell metabolism that I have shared co-authorship in these studies. In particular, Chapter 8 described the novel role of the PDK1 inhibitor, dichloroacetate (DCA) in regulating cholesterol homeostasis via ERK5-MEF2-dependent upregulation of LDLR (CD36). Furthermore, in Chapter 9 we showed the ERK5-MEF2 signaling pathway controls ABC transporters in mutant p53 cancer cells but not wt p53 cells, indicating a different role of this stress-induced pathway in developing multiple drug resistance between cancer cells depending on their p53 status. Finally, Chapter 10 described the role of ERK5-MEF2
pathways in the mitochondrial-complex-I dependent antioxidant response via regulation of NRF2 expression. Due to space constrains, results from these chapters will not be focused in discussion together with NK cell related studies in the previous chapters.

Chapter 11. General discussions and perspectives of the result sections.
CHAPTER 2.

MATERIALS AND METHODS
2.1 GALEN study:

Patients
All patients belong to the BioGALEN study and signed specific informed consent form before biological samples collection of BioGALEN. This study is recorded in website ClinicalTrials.gov with number NCT01582776. Phase IB was for follicular lymphoma (FL) patients and Phase II for follicular and aggressive (DLBCL and MCL) B-cell lymphoma patients. 3 x 3 ml of heparinized blood or 4 ml of bone marrow aspirate were collected at day 0. At the end of first cycle or at the end of treatment (supplemental Fig. 1) we collect 3 x 3 ml of heparinized blood.

Cell culture
The B cell lymphoblastoid Daudi cell line was maintained in logarithmic growth in RPMI 1640 medium (Gibco® GlutaMAX™ media) with 10% fetal bovine serum (FBS) (Gibco®). Cells were cultured at 37°C in a humidified chamber with 5% CO₂ in air, and passaged 1:10 twice a week.

Peripheral blood mononuclear cell (PBMC) purification
Bone marrow and peripheral blood samples were obtained from patients and total PBMC were isolated using Ficoll. Briefly, 3-6 ml of 1:2 diluted blood or 1:3 diluted bone marrow samples in RPMI were added on top of 5 ml of Histopaque (Sigma). Cells were centrifuged at 1600 rpm and at 20°C without break for 30 minutes. Mononuclear cells were collected from the interlayer white ring. After washing in RPMI, cells were cryopreserved in liquid nitrogen in medium comprise of FBS plus 10% culture-grade DMSO (CliniMACS) until analyzing.

Flow Cytometry analysis
Isolated PBMCs were stained with 7AAD (Beckman) to identify viable cells and with the following -CD45RO-FITC, -CD161-FITC, -CD3-PE, -CD19-PE, -CD62L-PE, -CD69-PE, -CD314(NKG2D)-PE, -CD3-ECD, -CD19-ECD, -CD56-PECy7, CD3-APC, -CD56-APC, -GzB-AlexaFluor700, -CD19-AlexaFluor700, -CD20-APC-AlexaFluor750, -CD45RA-APC-AlexaFluor750, -CD5-PacificBlue, -CD16-PacificBlue, -CD57-PacificBlue, -CD16-KromeOrange (Beckman), -CD158a-V450, -CD158b-FITC, -CD158a-PE, -CD107a-HV500, -
In vitro CD107a Degranulation Assay

*In vitro* degranulation assay was performed to evaluate NK reactivity to the B cell target Daudi by measuring CD107a expression on the surface after cytotoxic granule release. In summary, isolated PBMC were pre-stained with CD3/CD56 to determine NK frequency in the sample. Next, PBMC were incubated with Daudi cells at a 1:10 ratio NK:Daudi in the presence of 1.5 ul of anti-CD107a (BD Biosciences, Franklin Lakes, NJ) and 1 ul Golgi-stop (BD Biosciences) (containing monensin) to inhibit vesicle trafficking. Cell mixture was then resuspended in RPMI Glutamax 10% supplemented with 10 IU/ml Interleukin 2 (eBiosciences) and incubated overnight. After stimulation, cell mixture was collected and stained for FACS using an antibody cocktail containing 7AAD, the anti-CD45RO-FITC, -CD69-PE, -CD19-ECD, -CD56-PECy7, -CD3-APC, -CD45RA-APCAlexaFluor750, -CD107a-HV500 and -CD16-KO antibodies (BD Biosciences, Beckman). A bivariate plot of CD56 versus CD3 was used to acquire at least 10,000 NK cells.

Multicolor Staining for Intracellular Markers

Cell permeabilization and intracellular staining was performed as previous described (Krzywinska et al. 2016). Briefly, 1-10 million cells were incubated with 10% normal human serum at RT for 15 min and then stained with an antibody mix for cell surface markers (anti-CD45RO-FITC, -CD19-ECD, -CD56-PC7, -CD3-APC, -CD45RA-APCAlexaFluor750 and -CD16-KO antibodies) (BD Biosciences, Beckman). After surface staining, cells were washed twice and permeabilize with CytoFix/CytoPerm (BD Biosciences) reagent according to the manufacturer protocol. After fixation and permeabilization, cells were washed twice in BD Perm/Wash solution and follow FACS staining for intracellular markers Granzyme B-PE (Miltenyi Biotec) and Ki-67-V450 (BD Biosciences) at 4°C for 30 minutes in the dark. Finally,
cells were washed twice in BD Perm/Wash solution and resuspended in PBS 2% FBS prior to acquisition on flow cytometer Gallios (Beckman). A bivariate plot of CD56 versus CD3 was used to acquire at least 10,000 NK cells.

**Statistical analysis**

Experimental figures and statistical analysis were performed using GraphPad Prism (v6.0). Statistical significance between day 0 and the following time-points was determined using paired Student t-test on the sample patients for each sampling point. For determine statistical significance between healthy donors and patients, one-way ANOVA test was used to compare between healthy donors versus patients at every time-points. All statistical values presented as *: p<0.05; **: p<0.01; ***: p<0.001. Average values were expressed as mean plus or minus the standard deviation (SD).

2.2 AML study:

**Patients**

AML patient blood samples were collected from the Clinic Hematology Department of CHU Montpellier under patient’s consent. Patient blood samples were collected in EDTA or Heparin tubes and subsequently processed for PBMC isolation step.

**PBMC isolation**

Patients’s PBMC was processed by Ficoll gradient centrifugation as described above. After centrifugation and washing step, patient’s PBMC was counted and cryopreserved using FBS plus 10% culture-grade DMSO (CliniMACS) and stored at minus 80 celsius (°C) until analysis.

**Multiparameter flow cytometry analysis**

Cryopreserved patient PBMCs were thawed in pre-warmed RPMI-Glutamax (Gibco) serum-free medium and wash once in this medium. If cell clumps appeared, they were treated with a DNase digestion step (StemCell) for 15m at RT and followed by another washing step. Finally, 0.2 - 1 x 10⁶ PBMC per sample are stained with a multicolor FACS antibody panel with PD-1 – FITC, CD57 – PerCP-Cy5.5, CD62L – PE-CF594, CD16 – AF700,
CD14 – BV650, CD33 – BV711, CD3 – BV786, CD107a – BUV395, CD19 – BUV737 (BD Biosciences), CD56 – PE-Vio770, NKG2C – APC, CD45RA – APC-Vio770, CD45RO – VioGreen, CD7 – VioBlue (Miltenyi Biotec) and CD69 – PE (Beckman Coulter) as described in table 2. Cells were stained for 30m at 4°C, washed with PBS and resuspended in FACS staining buffer before acquiring on cytometer. Stained samples were acquired by Fortessa cytometer (BD) for at least 10'000 events on NK cell gate defined by CD56+CD3-. Results were analysed by Kaluza software 5.1 (Beckman Coulter).

**Statistical analysis**

Experimental figures and statistical analysis were performed using GraphPad Prism (v6.0). Statistical significance between samples at “diagnostic” (i.e. “before treatment”) and “after treatment” of the same patient was determined using paired Student t-test. All p values presented as *: p<0.05; **: p<0.01; ***: p<0.001. Average values were expressed as mean plus or minus the standard deviation (SD).

**2.3 HIV study**

**Patients**

Blood samples from HIV+ patients and healthy donors (HD) were kindly provided by Pr. Pierre Corbeau (Département d’Immunologie, CHU de Montpellier). During the initial phase study, blood samples from HIV+ patients (both aviremic and viremic) and HD were lysed to eliminate red blood cells and directly used for subsequent FACS analysis.

**Multiparameter flow cytometry analysis**

After red blood cell lysing step, whole leukocytes were stained with the FACS antibody panel (as described in Table 3) with CD45RO – FITC, NKG2D – PE, CD19 – PE-Cy5.5, CD14 – PE-Cy5.5, CD16 – KromeOrange (Beckman Coulter), CD56 – PE-Vio770, CD3 – APC, CD45RA – APC-Vio770 and CD107a – VioBlue (Miltenyi Biotec). Cells were stained for 30m at 4°C as in the standard protocol described before and subsequently analysed using Beckman Coulter Gallios flow cytometry to obtain at least 5’000 events on NK cells (defined as CD56+CD3-).
<table>
<thead>
<tr>
<th>Laser</th>
<th>Fluorescence Channel</th>
<th>Antibody-conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Laser 488nm</td>
<td>FITC, Alexa Fluor 488</td>
<td>PD1-FITC</td>
</tr>
<tr>
<td></td>
<td>PerCP, PerCP-Cy5.5, PE-Cy5</td>
<td>CD57-PerCP-Cy5.5</td>
</tr>
<tr>
<td>Yellow-Green 561nm</td>
<td>PE</td>
<td>CD69-PE</td>
</tr>
<tr>
<td></td>
<td>PE-CF594</td>
<td>CD62L-PE-CF594</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7</td>
<td>CD56-PE-Vio770</td>
</tr>
<tr>
<td>Red Laser 640nm</td>
<td>APC, Alexa Fluor 647</td>
<td>NKG2C-APC</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 700</td>
<td>CD16-AF700</td>
</tr>
<tr>
<td></td>
<td>APC-H7, APC-Cy7</td>
<td>CD45RA-APC-Vio770</td>
</tr>
<tr>
<td>Violet Laser 405nm</td>
<td>BV421, V450, Pacific Blue</td>
<td>CD7-VioBlue</td>
</tr>
<tr>
<td></td>
<td>BV510, Vio Green</td>
<td>CD45RO-VioGreen</td>
</tr>
<tr>
<td></td>
<td>BV605</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BV650</td>
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</tr>
<tr>
<td></td>
<td>BV711</td>
<td>CD33-BV711</td>
</tr>
<tr>
<td></td>
<td>BV786</td>
<td>CD3-BV786</td>
</tr>
<tr>
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<td>BUV395</td>
<td>CD107a-UV395</td>
</tr>
<tr>
<td></td>
<td>DAPI</td>
<td>live/dead marker</td>
</tr>
<tr>
<td></td>
<td>BUV737</td>
<td>CD19-UV737</td>
</tr>
</tbody>
</table>

**Table 2.** Multiparameter flow cytometry panel for AML samples on BD Fortessa instrument.
For the second phase of HIV study, whole PBMC from patients and HD were stained according to the panel described in **Table 4-5** and fixed with 2% PFA in PBS before analysis. Stained samples were acquired in parallel using BD Fortessa and Beckman Gallios flow cytometers.

<table>
<thead>
<tr>
<th>Beckman Gallios™</th>
<th>FL</th>
<th>conjugation</th>
<th>Panel 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue laser 488nm</td>
<td>1</td>
<td>FITC/AF488</td>
<td>CD45RO-FITC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>PE</td>
<td>NKG2D-PE</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>DsRed, PI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>PerCP-Cy5.5</td>
<td>CD19/CD14-PE-Cy5.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>PE-Cy7</td>
<td>CD56-PE-Vio770</td>
</tr>
<tr>
<td>Red laser 638nm</td>
<td>4</td>
<td>Alexa647/APC</td>
<td>CD3-APC</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Alexa-700</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>APC-H7</td>
<td>CD45RA-APC-Vio770</td>
</tr>
<tr>
<td>Violet laser 405nm</td>
<td>9</td>
<td>V450</td>
<td>CD107a-VioBlue</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>BV510/KO</td>
<td>CD16-KromeOrange</td>
</tr>
</tbody>
</table>

**Table 3.** Multiparameter flow cytometry panel for HIV samples on Beckman Gallios instrument.
<table>
<thead>
<tr>
<th>Laser</th>
<th>Fluorescence Channel</th>
<th>Antibody-conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Laser 488nm</td>
<td>FITC, Alexa Fluor 488</td>
<td>CCR5 - FITC</td>
</tr>
<tr>
<td></td>
<td>PerCP, PerCP-Cy5.5, PE-Cy5</td>
<td>NKG2A - PerCP</td>
</tr>
<tr>
<td>Yellow-Green 561nm</td>
<td>PE</td>
<td>CD158a/b/e - PE</td>
</tr>
<tr>
<td></td>
<td>PE-CF594</td>
<td>CD57 – PE-CF594</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7</td>
<td>NKp46 – PE-Vio770</td>
</tr>
<tr>
<td>Red Laser 640nm</td>
<td>APC, Alexa Fluor 647</td>
<td>NKG2C - APC</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 700</td>
<td>CD14/CD19 – AF700</td>
</tr>
<tr>
<td></td>
<td>APC-H7, APC-Cy7</td>
<td>CD45RA-APC-Vio770</td>
</tr>
<tr>
<td>Violet Laser 405nm</td>
<td>BV421, V450, Pacific Blue</td>
<td>CD7-VioBlue</td>
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<td></td>
<td>BV510, Vio Green</td>
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<tr>
<td></td>
<td>BV605</td>
<td>CD62L – BV605</td>
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<tr>
<td></td>
<td>BV650</td>
<td>NKG2D – BV650</td>
</tr>
<tr>
<td></td>
<td>BV711</td>
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<td>BUV395</td>
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</tr>
<tr>
<td></td>
<td>BUV737</td>
<td>CD4 – BUV737</td>
</tr>
</tbody>
</table>

**Table 4.** Multiparameter flow cytometry panel (extended) for HIV samples on BD Fortessa instrument.
Table 5. Multiparameter flow cytometry panel (extended) for HIV samples on Beckman Gallios instrument.

**In vitro trogocytosis assay**

For studying NK cell trogocytosis activity in vitro, patient PBMCs were co-cultured with CD4 and CCR5-expressing target cells (MT4-R5 and CEM). In summary, target cells using in the assay were labeled with cell-trace violet (CTV) (Invitrogen) according to the manufacturer protocol for 20m at 37°C then further co-incubated with patient whole PBMCs for 4h at 37°C in 10% FBS supplemented RPMI-Glutamax (Gibco). After co-incubation, the target cells and PBMC mix was collected and stained for FACS analysis using Beckman Gallios cytometer to detect membrane transfer from target cells to NK cells by gating on CD4 and/or CCR5 expression in patient NK cell as defined by CD56+CD3- and excluding duplets.
**Statistical analysis**

Experimental figures and statistical analysis were performed using GraphPad Prism (v6.0). Statistical significance between HD samples and HIV+ patients (both aviremic and viremic) was determined using one-way ANOVA. All $p$ values presented as *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Average values were expressed as mean plus or minus the standard deviation (SD).

**2.4 In vitro NK cell expansion from healthy donor UCB**

To study NK cell characteristics and functions *in vitro*, NK cells were activated and expanded from healthy donor UCB according to our protocol described previously (Sánchez-Martínez et al. 2015, 2016). In short, UCB mononuclear cells (UCBMC) from healthy donors were obtained from CHU Montpellier Hospital. UCBMC were collected by Ficoll gradient centrifugation and frozen in medium containing FBS supplemented with 10% clinical-grade DMSO (Miltenyi Biotec). NK cell expansion start with either freshly isolated or cryopreserved UCBMC. In case of cryopreservation, a preliminary DNase digestion step (DNase I Solution, StemCell) can be done if frozen cells were aggregated due to mechanical stress. Before expansion, T cells were eliminated using CD3+ positive selection kit (StemCell) according to the manufacturer protocol. T cell depletion were carried by a total of 3 consecutive purification step by magnet separation to avoid T cell competitive expansion. Finally CD3- cells were cocultured with the gamma-irradiated (100Gy) accessory PLH cell line (EBV+ lymphoblastoid cell line) at the ratio NK:iPLH [1:4], at the initial concentration $1.5 \times 10^6$ cell/ml in NK cell medium: 10% FBS RPMI-Glutamax containing 100 IU/ml interleukin-2 (Peprotech), 5 ng/ml interleukin-15 (Miltenyi Biotec). Expanded NK cell culture is refreshed every cycle of 3-4 days by replacing NK culture with fresh medium (NK medium completed supplement) and maintaining NK:iPLH ratio at [1:1] until NK cell percentage in the culture is higher than 90% determined by CD3-CD56+ population. Typically, *in vitro* expanded NK cells were used for further cytotoxicity assays, functional/phenotypical analysis from day 14 until day 21 post expansion. Remaining cells can be kept for future use by cryopreservation as previously described in liquid nitrogen.
CHAPTER 3.

Identification of anti-tumor cells carrying Natural Killer (NK) cell antigens in patients with hematological cancers

(published article - eBiomedicine)

[In this study that I shared co-authorship, I was involved in planning and execution of the experiments, collecting and isolating patient’s sample PBMC, performing FACS analysis and DEEParray imaging experiment]
1. Introduction

The immune system recognizes and eliminates tumor cells (Dunn et al., 2002), which defend themselves by different mechanisms (Villalba et al., 2013). The lymphocyte lineage natural killer (NK) cell belongs to the innate immune system (Lanier, 2008; Vivier et al., 2008) and shows strong anti-leukemia activity when engrafted in allogeneic settings in hematological cancer patients (Velardi, 2008; Ruggeri et al., 2007; Anel et al., 2012). However, the presence of an anti-leukemia NK cell population in patients with hematological malignancies has not been proven.

NK cells are not a homogenous population and different subsets have different physiological activities. Moreover, different stimuli (e.g., cytokines vs targets cells) give rise to different immunophenotypes (Fujisaki et al., 2009; Sanchez-Martinez et al., 2014). In peripheral blood, human NK cells are mostly CD3−CD56dim cells with high cytotoxic activity, while CD3+CD56bright cells excel in cytokine production (Bryceson et al., 2011). In vitro evidence indicates that CD56bright NK cells are precursors of CD56dim NK cells and this might also be the case in vivo (Domaica et al., 2012). In addition, combined analysis of CD56 and CD16 expression during NK cell development indicates that their profiles change as follows: CD56brightCD16− → CD56brightCD16dim → CD56dimCD16− → CD56dimCD16dim. Additional markers can be used to identify specific subsets within these NK cell populations (Moretta, 2010; Freud et al., 2014).

Identification of antileukemic NK cells in vivo is complex. CD69 expression increases after NK cell activation but it is not exclusive of NK cells encountering tumor cells (Fogel et al., 2013; Elpek et al., 2010). Due to the clinical interest of NK cells, it is therefore highly relevant to identify more precisely the NK cell population(s) with antitumor functions.

CD45 is a protein tyrosine phosphatase that is specifically expressed in leucocytes (Kaplan et al., 1990). CD45 regulates receptor signaling by...
direct interaction with components of the receptor complexes or by dephosphorylating and activating various Src family kinases (SFK) (Rhee and Veillette, 2012). However, it can also hinder cytokine receptor signaling by inhibiting Janus kinases (JAK) (Irie-Sasaki et al., 2001) or by dephosphorylating Src activating residues (Rhee and Veillette, 2012). CD45 activity is critical for efficient immune response, because its deficiency results in severe combined immunodeficiency (SCID) in mice (Kishihara et al., 1993; Byth et al., 1996; Mee et al., 1999) and humans (Kung et al., 2000; Tchilian et al., 2001).

Total CD45 expression increases with T cell maturation (Hermiston et al., 2009), but the CD45 family comprises several isoforms derived from a single complex gene (Hermiston et al., 2009). Naive T lymphocytes usually express the long CD45RA isoform. Activated and memory T cells express CD45RO, the shortest CD45 isoform, generated by activation-induced alternative splicing of CD45 pre-mRNA (Warren and Skipsey, 1991; Roth, 1994; Lynch and Weiss, 2000; Hermiston et al., 2009). It has been proposed that CD45RO expression also identifies memory NK cells (Fu et al., 2011). Little is known about CD45 expression and function in NK cells, although it is commonly accepted that CD45 positively regulates their activation by dephosphorylating the inhibitory site of SFKs, thus leading to cytokine and chemokine production. However, in vitro cytotoxicity is only slightly impaired in NK cells derived from CD45-deficient mice (Huntington et al., 2005; Hesselin et al., 2006; Mason et al., 2006). As most of what is known is obtained in mouse models and cannot be transposed to humans, the role of CD45 in human NK cells is an open issue and could depend on the type and strength of the activation.

Here we show that, in contrast to T cells, NK cells can express both CD45 isoforms: CD45RA and CD45RO. Moreover, these CD45R0 NK cells recognize tumor cells in patients with hematological cancers and, subsequently, degranulate.

1.1. Experimental Procedures

1.1.1. Cell Culture

The K562 cell line (ATCC CCL 243) and the lymphoblastoid EBV cell line PHL (IHW Number: 9047) were maintained in logarithmic growth in RPMI 1640 medium (Gibco® GlutaMAX™ media) with 10% fetal bovine serum (FBS) (Gibco®). Cells were cultured at 37 °C in a humidified chamber with 5% CO2 in air, and passed 1:10 twice a week.

1.1.2. Peripheral Blood Mononuclear Cell (PBMC) Purification

Bone marrow and peripheral blood samples were obtained from patients with different hematological diseases and from healthy donors after informed consent. Cells were purified by Ficoll-Hypaque (Sigma) density-gradient centrifugation as described earlier (Allende-Vega et al., 2015). Briefly, 3–6 ml of 1:2 diluted blood or 1:3 diluted bone marrow samples in RPMI were added on top of 5 ml of Histopaque. Cells were centrifuged at 1600 rpm and at 20 °C without break for 30 min. Mononuclear cells were collected from the interlayer white ring. After washing in RPMI, cells were suspended in complete RPMI medium supplemented with 10% FBS (Invitrogen).

1.1.3. In Vitro NK Cell Stimulation Protocol

PBMCs, 1.10⁶ cells/ml, were stimulated during 10 or 20 days with a high dose of IL-2 (1000 U/ml, eBiosciences) or with the lymphoblastoid EBV cell line PHL together with IL-2 (100 U/ml) and IL-15 (5 ng/ml, Miltenyi).

1.1.4. Selection of Patients and Healthy Donors

Data and samples from patients with different hematological cancers were collected at the Oncology and Clinical Hematology Department of the CHU Montpellier, France, after patient’s informed consent (Allende-Vega et al., 2015). Patients were enrolled in two independent clinical programs approved by the “Comités de Protection des Personnes Sud Méditerranée I”: ref 1324 and ID-RCB: 2011-A00924-37. All samples from cancer patients were collected at diagnosis and included HD, Healthy donor (n₉= 10); MM, multiple myeloma (n₉= 19, n₁₀= 20); B-CLL, B-cell chronic lymphocytic leukemia (n₉= 15); BCL, B-cell lymphoma (n₉= 14); AML, acute myeloid leukemia (n₉= 14); bs, blood samples; bms, bone marrow samples.

1.1.5. Multicolor Staining of Cell Surface Markers

PBMCs were stained with 7AAD (Beckman) to identify viable cells and with the following anti-CD25-FITC, − CD45RO-FTTC, − CD161-FITC, − CD3-PE, − CD19-PE, − CD62L-PE, − CD69-PE, − CD138-PE, − CD314 (NK2GD)-PE, − CD2-EC, − CD19-EC, − CD38-EC, − CD56-PECy7, − CD3-APC, − CD56-APC, − G2B-AlexaFluor700, − CD19-AlexaFluor700, − CD20-APC-AlexaFluor750, − CD45RA-APC-AlexaFluor750, − CD5-PacificBlue, − CD16-PacificBlue, − CD57-PacificBlue, − CD45-KromeOrange, − CD16-KromeOrange (Beckman), − CD158b-FTTC, − CD158a-PE, − CD107a-HV500, − Ki-67-V450 (BD Biosciences), − CD45RA-HTC, − CD45RO-PE, − CD159a(NK2A)-PE, − CD335 (NKp46)-PE, − CD94-Vio770, − CD235 (NKp46)-Vio770, − CD45RO-APC, − CD14-VioBlue, − CD19-VioBlue, − CD15158e-VioBlue (Miltenyi Biotec) and − CD71-APC (ImmunoTools) antibodies against surface markers for cell phenotyping. Briefly, 1x10⁶ cells were incubated with the different antibodies in PBS with 2% FBS at 37 °C for 30 min. Cells were then washed and resuspended in 200–250 μl PBS 2% FBS and staining was analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

Viable lymphocytes were gated using FSC-SSC and 7AAD staining. B lymphocytes (CD19⁺), T lymphocytes (CD3⁺ CD56⁻) and NK cells (CD56⁺ CD3⁻) were differentiated based on CD19, CD3 or CD56 expression. NK cells were then separated in four distinct populations based on CD45RA and CD45RO expression: CD45RA-RO⁺ (CD45RA⁺ RO⁻ CD45RAR0), CD45RA⁺RO⁺ (CD45RA⁺ RO⁺ CD45RAR0), CD45RA⁺RO⁻ (CD45RA⁺ RO⁺ CD45RAR0), CD45RA⁺RO⁺ (CD45RA⁺ RO⁻ CD45RAR0). These different populations were then analyzed for CD16, CD57, CD62L, CD69, CD71, CD94, CD107a, CD158, CD158b, CD158e, CD159a (NKG2A), CD161, CD314 (NK2GD), CD335 (NKp46), Ki-67, GzB expression and cell size and granularity (FSC and SSC).

1.1.6. In Vitro CD107a Degranulation Assay

After PBMC purification and NK cell quantification, 3 million cells were incubated at 37 °C for 4 h or overnight with K562 target cells at an Effector (NK cell): Target ratio of 1:10 in a final volume of 500 μl (RPMI Glutamax with 10% FBS and 100/ul IL2). The medium also contained 1.5 μl anti-CD107a antibody (BD Biosciences, Franklin Lakes, NJ) and 1 μl monensin to prevent CD107a degradation (BD Golgi-Stop BD Biosciences). Then, cells were resuspended in 50 μl of an antibody cocktail containing the anti-CD45RO-FTTC, − CD69-PE, − CD19-EC, − 7AAD, − CD56-PECy7, − CD3-APC, − CD45RA-APC-AlexaFluor750, − CD107a-HV500 and − CD16-KO antibodies (BD Biosciences, Beckman). Samples were analyzed on a Beckman Coulter FACs Gallios flow cytometer using the Kaluza software. Events were initially gated on forward and side scatter (SSC) to identify lymphocytes. A bivariate plot of CD56 versus CD3 was used to acquire at least 10,000 NK cells.

1.1.7. Multicolor Staining for Cell Surface and Intracellular Markers

After PBMC purification, 1 million cells were pre-blocked by incubation with 10% normal human serum at RT for 15 min and then stained with 50 μl of the PANEL Ki-67 antibody cocktail against cell surface markers (anti-CD45RO-PE, − CD19-EC, − CD56-PC7, − CD3-APC, − CD45RA-APC-AlexaFluor750 and − CD16-KO antibodies) (BD Biosciences, Beckman). Cells were washed twice with Staining Buffer and re-suspended in 250 μl BD Cytofix-Cytoperm solution at 4 °C for 20 min. Cells were washed twice in BD Perm-Wash solution. Next, cells were fixed-permeabilized in 50 μl BD Perm-Wash solution containing an antibody cocktail against intracellular markers (anti-GzB-AlexaFluor700, − Ki-67-V450) as described in the figures at 4 °C for 30 min in the dark. Cells were washed twice in BD Perm-Wash solution and re-suspended in Staining Buffer prior to flow cytometric analysis on a
Beckman Coulter FACS Gallios flow cytometer using the Kaluza software. Events were initially gated on forward and side scatter (SSC) to identify lymphocytes. A bivariate plot of CD56 versus CD3 was used to acquire at least 10,000 NK cells.

1.1.8. Identification of Pure Single NK Cells
Primary CD56⁺ NK cells were enriched and purified from PBMCs of B-cell lymphoma patients with the CD56⁺ NK cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). The purity (%) of CD56⁺ CD3⁻ of CD56⁺ NK cells, measured by flow cytometry, was >90%. Purified CD56⁺ NK cells have been stained with anti-CD135/NKp46-PE to formally identify NK cells together with anti-CD45RA- FITC, —CD45RO-APC and —CD19-VioBlue (detection of trogocytosis-capable NK cells). Purified and stained NK cells have been analyzed with the DEPArray™ System (Silicon Biosystems, Menarini). This technology allowed us to detect, enumerate and take pictures of single cells.

1.1.9. DEPArray™ Procedure
Cell sorting experiments were performed as described in the manufacturer’s instructions and in (Lianidou et al., 2013). Briefly, DEPArray cartridges were manually loaded with 14 μl of sample and 800 μl of the buffer solution in which purified and stained NK cells had to be recovered. After loading the cartridge into the DEPArray system, ∼9.26 μl of sample was automatically injected by the system into a microchamber of the cartridge where the cells were spontaneously organized into a preprogrammed electric field consisting of 16,000 electrical cages in which individual cells are trapped. Image frames covering the entire surface area of the microchamber for each of four fluorescent filter cubes (FITC, PE, APC and DAPI-Hoechst-VioBlue-PacificBlue) and bright field images were captured. Captured images were digitally processed and presented in a software module that enables selection of cells of interest by the operator.

2. Results

2.1. Expression of Different CD45 Isoforms in Patients With Hematological Malignancies
In healthy donors, NK cells were mainly CD45RA cells with few CD45RA/B cells, found particularly in immature NK cell subsets. CD45RARO cells represented between 0 and 0.75% of all NK cells and belonged exclusively to the fully mature CD56⁺ CD16⁺ subset (Fig. 1A and supplemental Table 1). NK cells derived from healthy donor bone marrows showed equal distribution (Fig. 1B). Blood samples from patients with multiple myeloma (MM) contained four times more CD45RA/B cells and between 1 and 20% of CD45RARO cells (Fig. 1A and supplemental Table 2). As MM is characterized by accumulation of tumor cells in the bone marrow, we also investigated whether bone marrow NK cells, which should be in closer contact with tumor cells, were more activated than circulating NK cells. This was not the case as the percentage of CD45RA/B and CD45RARO cells was similar in blood and bone marrow samples (Fig. 1A and supplemental Table 2).

Similar increases in the CD45RA/B and CD45RO populations were also observed in bone marrow samples from patients with acute myeloid leukemia (AML) or in blood samples of patients with B-cell chronic lymphocyte leukemia (B-CLL) and B-cell lymphoma (BCL) (Fig. 1A and supplemental Table 3). In summary, the CD45RARO cell population was statistically increased in all analyzed samples from patients with blood malignancies compared to healthy controls (Fig. 1B and supplemental Fig. 1). The gating strategy to identify CD45RARO cells is described in supplemental Fig. 1B.

2.2. Phenotypic Characterization of CD45RARO Population
As indicated in Fig. 1, CD45RARO cells belonged to the CD56⁺ CD16⁺ subset (Fig. 2A) and mostly express the maturation marker CD57 (Fig. 2B) although CD62L was coexpressed by half of them. The CD45RARO population contained higher percentage of cells that expressed KIRs, although it was statistically significant only for CD158e (Fig. 2C and supplemental Fig. 2). CD45RARO cells expressed similar levels of CD94 glycoprotein and, probably, the inhibitory NK receptor NK2G2R (Fig. 2D and supplemental Fig. 3). In summary, CD45RARO cells are fully mature NK cells that mainly express NK receptors of mature cells.

2.3. Expression of Different CD45 Isoforms in Vivo: Patients With Cytomegalovirus (CMV)-Reactivation
We next asked whether other conditions that lead to NK cell activation, such as viral infections, could give rise to a similar phenotype. We thus analyzed peripheral blood mononuclear cell (PBMC) samples from patients with reactivation (CMV⁺) or not (CMVneg) of CMV infection following kidney transplantation. CMV reactivation induced an increase in the total number of NK cells (Fig. 3A). In addition, CMV⁺ patients showed an increase in CD56dimCD16dim cells associated with a reduction of the CD56bright subsets compared to CMVneg patients (Fig. 3B). The reason of these changes is not clear to us, but could be due to different factors, such as CMV-induced NK cell maturation (Della Chiesa et al., 2013), or an effect on the expression of the different NK cell markers in CMV-infected cells, as previously described for decidual NK cells (Siewiera et al., 2013). These changes were accompanied by minor variations in the expression pattern of CD45 isoforms (Fig. 3C). These results indicate that the expression pattern of CD45 isoforms in NK cells activated by viral infection or hematological cancers is different. Specifically, CD45RARO cells are mainly present in samples from patients with hematological cancers, whereas they represent a minor fraction in virus-infected patients.

2.4. Metabolic Characterization of CD45RARO Population
Activated lymphocytes generally increase their size (Zarcone et al., 1987; Skak et al., 2008) and become highly metabolically active cells
That loss of CD45RA and gain of CD69 expression identify two different ways associated with gain of CD69 expression. In fact, patients' samples including ex vivo (Fig. 4A) showed an increase in size (FCS) and granularity (SSC) that was more relevant at day 20 (supplemental Fig. 4A).

After 3 days of in vitro cultivation, NK cells started losing CD45RA (supplemental Fig. 4B). However, it was questionable if a real CD45RARO population appeared or cells were losing CD45RA whereas gaining CD45RO. 10 days after initial activation, most cells were CD45RA−CD45RO+. However, at day 20 a CD45RARO population appeared in the culture. This was not exclusive of the presence of accessory cells because long-term activation with cytokines produced a similar pattern (supplemental Fig. 4B). In summary, CD45RARO NK cells also exist in vitro after long activation. Next, we evaluated in vitro activation of patient CD45RARO population. Three days of cytokine-induced activation induced a strong rearrangement on the expression of CD45 isoforms and it was impossible to evaluate the fate of individual populations (Supplemental Fig. 4C). These results additionally suggested that CD45RARO cells could change their CD45 phenotype, at least in vitro.

We then assessed the expression of transferrin receptor protein 1 (TfR1 or CD71), which is required for iron delivery from transferrin to the cells. CD71 expression increases in active metabolic cells because iron is a cofactor for fundamental biochemical activities, such as oxygen transport, energy metabolism and DNA synthesis (Wang and Pantopoulos, 2011). In agreement with the superior metabolic activity suggested by high FS and SS of CD45RARO cells, CD71 expression was higher in CD45RO− cells in both healthy controls and patients with hematological malignancies (Fig. 4B and supplemental Fig. 5A). Moreover, most of these cells also expressed the proliferation marker Ki-67 (Fig. 4C and supplemental Fig. 5B). In summary, CD45RARO cells represent a NK subset of highly metabolically active cells in proliferation.

CD69 expression increases after NK cell stimulation and is considered a bona fide marker of NK cell activation (Elpek et al., 2010), including ex vivo (Vey et al., 2012). Analysis of CD69 expression in the different CD45 populations in patients showed that CD45RO− cells were mainly CD69− (Fig. 4D); but not vice versa, as most CD69− cells were not CD45RO. The very low amount of CD45RO− cells in healthy donors precluded any meaningful analysis of this population.

In healthy donors, CD45RA dim cells were mainly CD69− (Fig. 4E). CD45RA dim cells were significantly increased in patients and many were also CD69−. However, reduction of CD45RA expression was not always associated with gain of CD69 expression. In fact, patients' samples were enriched particularly in CD45RA dim CD69− and CD45RA CD69+. and, to a lower extent, in CD45RA dim CD69+ cells. This finding suggests that loss of CD45RA and gain of CD69 expression identify two different physiological processes and that these two populations might have different functions.

The phenotypic characterization of CD45RARO shows that they are fully mature cells. PBMCs from a representative BCL patient were stained as in Fig. 1 to identify the CD45RARO population and the maturation development was revealed by expression of CD56 CD16 (A) or CD57 CD62L (B). Numbers in the quadrant indicate the percentage of cells. C (D) PBMCs from 5 BCL patients were stained as in Fig. 1 to identify the CD45RARO population and the expression of different molecules on the different NK cell subsets was revealed by using antibodies against KIRs 158a, b and e, GzmB, the Lectin Like Transcript-1 (LLT1) receptor CD161, the NCR NKp46, the activating receptor NKG2D, the inhibitory receptor NKG2A (D) and the molecule CD54.

2.5. Functional Characterization of CD45RARO Population

To identify the function of the different NK cell subsets, first we assessed cell degranulation by ex vivo staining of PBMCs with anti-CD107a antibodies (Fig. 5A). In healthy donors, around 1% NK cells were CD107a+. Most of these cells were CD45RA, with a small number of CD45RO− cells. Remarkably, most CD45RARO and half of CD45RA−RO cells were CD107a+ (Fig. 5B).

NK cells from patients with hematological cancers showed a large increase in CD107a+ cells (Fig. 5A and supplemental Fig. 6A), particularly among the CD45RO− subsets, which are specifically increased in these patients (Fig. 1). Reduction of CD45RA expression was not associated with increased degranulation (Fig. 5B). Like in healthy donors, the CD45RARO and, to a lower extent, CD45RA dim RO fractions contained mostly cells that had degranulated (Fig. 5B and supplemental Fig. 6B). The median CD107a− mean fluorescence intensity (MFI) of these two populations was largely increased compared to CD45RO− populations (supplemental Fig. 6A). This was not exclusive of circulating NK cells, because similar results were obtained also for NK cells derived from bone marrow samples of patients with MM and AML (Fig. 5B and C upper panels). In contrast, CD45RARO cells showed low GzmB content (Fig. 2C). Our explanation is that CD45RARO cells had recently degranulated in vivo.

CD45RARO cells continued to show the higher degranulation rate after an in vitro analysis using K562 as target cells (Fig. 5C bottom panels), although other populations significantly increased degranulation. Interestingly, the different CD45 NK cell subsets did not change after the 4-hour in vitro cytotoxic assay (supplemental Fig. 7). This and the in vitro activation results (supplemental Fig. 4) showed that expression of CD45RA and CD45RO is stable at short times but can change after long lasting activation.

CD45RARO Have Performed Trogocytosis in Vivo

To investigate if CD45RARO cells were performing antitumor activity in vivo, we investigated if these cells have performed trogocytosis on tumor targets. Trogocytosis is a process whereby lymphocytes, i.e. NK cells (Suzuki et al., 2015; Nakamura et al., 2013), gain surface molecules from interacting cells and express them on their own surface and has been observed in B lymphoblastic leukemia (B ALL) ex vivo (Soma et al., 2015). We observed that long-time activated NK cells (see supplemental Fig. 4) performed trogocytosis in two AML cell lines (supplemental Fig. 8). In fact, NK cells extracted at least two proteins expressed in AML cells, CD14 and CD33, with considerable efficiency. This showed that human NK cell efficiently performed trogocytosis and we investigated if this was the case in vivo. Because in this experiment we studied markers of other cell types, we used a double labeling to identify NK cells and gated on CD56− NKp46− cells. In a BCL patient, we observed that 14% of the NK cells expressed the BCL marker CD19 in their membrane (Fig. 6A). This value increased to 52% in the CD45RARO population and it was much lower in the other populations. NK cells also gained at lower level expression of the myeloid marker CD14, although the population was predominantly CD45RARO+. However, the NK cells that stained positive for both CD19 and CD14 were very rare. This suggested that two different NK cell populations were performing trogocytosis. The CD45RARO cells were doing it on tumor cells. We observed the very similar results in another CD19+ disease: B-CLL (supplemental Fig. 9). Next, we used the purified NK cells (CD56 selection) from whole blood of a B-CLL patient and analyzed them with the
A. NK cells

CD56

CD45RA

CD45RARO

CD45RA_{dim}

CD45RA_{dim} RO

CD16


B. NK cells

CD57

CD45RA

CD45RARO

CD45RA_{dim}

CD45RA_{dim} RO

CD62L


C. C. 


D. D.

Total NK cells

CD45RA

CD45RA_{dim}

CD45RA_{dim} RO

CD45RARO

n = 8

% of CD158b NK cells

% of CD158a NK cells

% of CD158c NK cells

% of CD158d NK cells

% of GzmB NK cells

GzmB (MFI)
DEPArray™ System, which allowed identifying, visualizing and taking pictures of single cells. We labeled cells with NKp46 to formally identify NK cells together with CD45RA, CD45RO and CD19. Fig. 6B showed that single cells expressed all markers. Thus, we showed a picture of a human NK cell that has just performed in vivo trogocytosis in a tumor (see also the graphical abstract).

To exclude that NK cells were not gaining CD19 in all tumors, we investigated NK cells from an AML patient (Fig. 6B). Around 10% of NK cells expressed the AML marker CD14 and only 3% expressed CD19, which was expressed in all NK cell populations. In contrast, 90% of CD45RARExpressed CD14 showing that they have massively performed trogocytosis in a CD14+ population in AML patients.

Next, we investigated if CD45RARExpressed cells derived from an AML patient that had performed trogocytosis and gained CD14 expression were able to take CD19 from tumor cells of a BCL patient. Tumor CD19+ cells and NK cells from the AML patient did not express the same membrane markers (Fig. 7A). We distinguished BCL cells by CD19 and CD10 staining and after 16-h cytotoxic assay, we observed that 4% of NK cells form the AML patient gained expression of both CD10 and CD19 (Fig. 7A). The CD45RARExpressed cell population was mainly stable all through the assay (Fig. 7B). The population that performed trogocytosis mainly was the CD45RARExpressed (Fig. 7C). This showed that the CD45RARExpressed population was prompted to recognize and interact with allogeneic tumor cells. In summary, our data showed that NK cells performed trogocytosis on tumor cells and that the CD45RARExpressed cell population is mainly responsible of this.

3. Discussion

Identification of human NK cell populations is important for understanding their physiology and for improving their therapeutic use in the clinic. Altogether our results indicate that CD45RARExpressed cells are fully mature NK cells (CD56dimCD16+CD57+KIR+CD161+), which are activated (high size and granularity, CD69+CD71+Kl67+NKG2D+) and that have degranulated (CD107a+ and low GzmB content) and performed trogocytosis (CD19+ in BCL and B-CLL and CD14+ in AML). Moreover, they are prompted to perform trogocytosis on different target cells. These findings suggest that they are effector cells with maximal cytotoxic activity against cancer cells. It seems that a population of highly mature NK cells encounters its targets and respond by becoming effector cells. In addition, we observed that a population of NK cells has performed trogocytosis in non-tumor, myeloid, cells at least in BCL and B-CLL patients. It is well known that NK cells kill dendritic cells and macrophages in several contexts, but the role here is unknown. Moreover, the population that has performed it is mainly CD45RARExpressed, a generally minor population.

The large size and granularity of CD45RARExpressed cells could preclude their observation when standard FCS-SSC parameters for the classical lymphocyte populations are used. It is essential to understand that activated lymphocytes increase in size and granularity, which distinguish them for naive lymphocytes. This is important for future studies of CD45RARExpressed NK cells in solid cancers, which could also induce a similar phenotype because NK cell infiltration is associated with a good prognosis in several cancers (Senovilla et al., 2012; Mamessier et al., 2013; Mamessier et al., 2012). However, our work does not show the irrefutable proof that CD45RARExpressed cells are bona-fide NK cells, although all results point in this direction. Alternative analyses are needed to definitively state the nature of these cells.

Target cell availability is probably maximal for NK cells in blood borne cancers, hence, we believe that these diseases will show the highest CD45RARExpressed NK cell numbers; although these cells are unable to control the disease. Leukemogenesis in mouse is enhanced when the host immune system is impaired (Garaude et al., 2008; Kaminski et al., 2012) and more hematological cancer patients present severe NK cell dysfunctions (Baier et al., 2013). Others and we have shown
the requirement of fully functional NK cells to eradicate blood-borne tumors in several mouse models (Karre et al., 1986; van den Broek et al., 1995; Pardo et al., 2002; Aguilo et al., 2009; Charni et al., 2009; Charni et al., 2010; Ramírez-Comet et al., 2014). The use of alloreactive NK cells may represent a new cancer treatment, specifically for tumors of hematopoietic origin. Indeed, KIR–KIR ligand incompatibility in the graft-versus-host (GvH) direction, which is mainly based on NK cell alloreactivity, improves the outcome after unrelated cord blood stem cell transplantation (UCBT) in the clinic (Willemze et al., 2009; Stern et al., 2008). Moreover, NK cells: i) are not responsible of GvH disease (GvHD); ii) can be injected as “differentiated” cells and thus do not need to survive within the patient’s body for a long time; iii) protect from opportunistic infections (Willemze et al., 2009), probably through their immunoregulatory effects on B and T cells, macrophages and, more importantly, polymorphonuclear cells (Bhatnagar et al., 2010). However, evaluation of NK cell activation in vivo is difficult because

Fig. 4. Functional characterization of CD45RAO NK cells. A) FS and SS values of the different NK cell subsets (based on the expression of CD45 isoforms) derived from a blood sample of a patient with MM. B–C) Expression of CD71 and Ki67 in the different NK cell subsets in a representative BCL patient. D–E) Representative graphs showing the expression of CD45RO or CD45RA versus CD69 in NK cells from blood (bs) or bone marrow samples (bms) of patients with different blood-borne cancers. Numbers in the quadrant indicate the percentage of cells.
Fig. 5. CD45RA^RO identifies degranulating NK cells. PBMCs from healthy donors (HD) and patients with different hematological malignancies were purified as in Fig. 1. A) Number of CD107a^+ cells in each NK cell subset (CD45RA^RO expression described in Fig. 1A) per million of NK cells. Bars represent the mean ± SD for each medical condition; Student t-test compare to healthy donor samples. B) Percentage of CD107a^+ NK cells in the four different subsets. C) Upper panels, Percentage of CD107a^+ cells in different NK cell subsets isolated from bone marrow samples (bms) of patients with MM (shown also the percentage in the corresponding blood sample, bs, for comparison) or AML. Bottom panels, Percentage of CD107a^+ cells in different NK cell subsets after exposure to target K562 tumor cells (in vitro cytotoxicity assay). PBMCs were incubated for 4 h with target K562 tumor cells at the effector:target ratio of 10:1.
Fig. 6. CD45RARO cells have performed trogocytosis on tumor cells. PBMCs from patients with BCL (A) or AML (C) were purified as in Fig. 1 and were stained with different antibodies. Numbers in the quadrant indicate the percentage of cells. In this experiment, the NK cell population corresponded to CD56^+ NKP46^+ cells. B) Purified NK cells (CD56^- selection) from a B-CLL patient have been stained with NKP46, to formally identify NK cells, together with CD45RA, CD45RO and CD19. They were analyzed with the DEPArray™ System.
we lack effective methods for their analysis. CD69 expression has routinely been used (Elpek et al., 2010; Vey et al., 2012); though, our results show that CD69 expression does not imply degranulation, which is believed to be the most essential component of the NK cell anti-tumor activity (Bryceson et al., 2011), or trogocytosis. Conversely, our work indicates that CD45RO expression identifies degranulating NK cell subsets in patients with hematological malignancies. We believe that efficient antitumor treatments that involve also NK cell activity, such as monoclonal antibodies against tumor antigens, should also increase these NK cell populations. Other options for treatment include new chemicals that can be associated with immunotherapy to boost the immune response (Villalba et al., 2014) and that could improve the NK cell-mediated response (Catalán et al., 2015).

CD45 activity is regulated by dimerization and spontaneous CD45 homodimerization at the plasma membrane inhibits its activity (Xu and Weiss, 2002). The size of CD45 extracellular domain is inversely proportional to the extent of CD45 dimerization and thus self-inhibition (Xu and Weiss, 2002). Larger CD45 isoforms, such as CD45RA, dimerize less efficiently and, accordingly, they should better promote TCR signaling than smaller isoforms, such as CD45RO.
(Rhee and Veillette, 2012). However, CD45 activity also depends on its plasma membrane localization and thus on its extracellular domain (Mustelin et al., 2005; Rhee and Veillette, 2012). At least in T cells, too high CD45 activity leads to dephosphorylation of the activating residues in Src kinases, whereas too low CD45 activity might leave phosphorylated the inhibitory residues. Therefore, it is important for efficient NK cell activation that CD45 activity remains within a specific window (Hermiston et al., 2009) and the amount of specific CD45 isoforms will regulate the final activity. We found that CD45R0 NK cells show maximal degranulation and cytotoxicity, suggesting that expression of both CD45RA and CD45RO isoforms might give to NK cells the appropriate level of CD45 activity for efficient signaling to boost cytotoxicity. CD45 is required for full NK cell cytotoxicity in vivo in mice (Hesslein et al., 2011); however, it is not required in vitro (Mason et al., 2006; Hesslein et al., 2006; Huntington et al., 2005). In agreement, we observed that other NK cell subsets, which express different CD45 isoforms, improved degranulation in vitro. This suggests that NK cells depend less of CD45 expression in vitro than in vivo.

Expression of different CD45 isoforms changes the recognition of CD45 ligands. For example, the abundance and types of O-glycans on the different CD45 isoforms regulate the cell sensitivity to galectin-1 (Earl et al., 2010). Galectin-1, which is abundantly produced by tumor cells, blocks T cell-mediated cytotoxic responses (Ito et al., 2012) and induces apoptosis of thymocytes and T cells (Earl et al., 2010). It is possible that anti-tumor NK cells are selected based on their resistance to galectin-1 or to other ligands through expression of CD45RO. Indeed, as O-glycans bind mainly to CD45 extracellular domain, cells that express short CD45 isoforms, like CD45RO, will have relatively fewer O-glycans and thus will be more resistant to galectin-1.

Ex vivo we found very few NK cells that express CD45RO in peripheral blood samples from healthy donors. This is surprising, especially if CD45RO expression identifies memory NK cells, as it has been proposed (Fu et al., 2011). This finding suggests that the amount of memory NK cells might be extremely low in blood or bone marrow samples, or that CD45RO may not be a marker of memory NK cells. Alternatively, CD45RO expression in NK cells could have been specifically lost during ex vivo sample handling. We think that this is unlikely because NK cells express slightly higher levels of total CD45 than other lymphocyte types (data not shown). In fact, we found that CD45RO is mostly associated with effector NK cells; however, differently from what observed in most T cell populations, CD45RA down-regulation is not required for CD45RO cell activation. This suggests that in NK cells the expression of different CD45 isoforms plays a different role than in T cells.

In summary, we show here that NK cells that recognize tumor cells are present in all examined patients of hematological cancers. Hence, NK cells are actively recognizing tumor cells in leukemia patients; but CD45 isoforms plays a different role than in T cells.

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### References


### Conflicts of Interest

The authors and MV have presented a patent application for the use of CD45RO cells as a biomarker of hematological cancers (Martin Villalba and Ewelina Krzywinska. Methods for Diagnosing Hematological Cancers. EP14306134.9).

### Appendix A: Supplementary Data

Supplementary text can be found online at [http://dx.doi.org/10.1016/j.ebiom.2015.08.021](http://dx.doi.org/10.1016/j.ebiom.2015.08.021).
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CHAPTER 4.

NK cell activation and recovery of NK cell subsets in lymphoma patients after obinutuzumab and lenalidomide treatment

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**ORIGINAL RESEARCH**

**NK cell activation and recovery of NK cell subsets in lymphoma patients after obinutuzumab and lenalidomide treatment**

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**ABSTRACT**

Obinutuzumab (OBZ) shows stronger antibody-dependent cell cytotoxicity (ADCC) compared to rituximab and improved clinical activity for treating certain CD20\(^+\) neoplasia. However, the efficacy of monoclonal antibody (mAb) as a monotherapy is limited. Natural Killer (NK) cells are mediators of ADCC. Hematological cancer patients possess antitumor NK cells that are unable to control disease, possibly because they are dysfunctional. The immunomodulatory drug lenalidomide (LEN) could be a treatment to restore exhausted NK cell cytotoxic functions. The clinical trial GALEN is a Phase Ib/II study of OBZ combined with LEN for the treatment of relapsed/refractory follicular and aggressive (DLBCL and MCL) B-cell Lymphoma. During treatment, we analyzed specific aspects of NK cell biology. Treatment reversed the immature NK phenotype of patients and increased expression of NK activating receptors. Inhibitory receptors were either unchanged or decreased. There was a strong NK response at the end of the 1st cycle: NK number and intracellular granzyme B (GrzB) expression decreased, degranulation increased and NK responded better to allogeneic target challenge. Moreover, the interaction of NK cells with B cell targets, measured by trogocytosis, decreased during treatment. At the end of treatment, when target cells had been wiped out, the proportion of reactive NK cells (CD69\(^+\), CD45RA\(^+\), CD107a\(^+\), CD19\(^+\)) strongly decreased. Because all patients received LEN and OBZ, it was uncertain which drug was responsible of our observations, or even if a combination of both products was necessary for the described effects on this lymphocyte lineage.

**Introduction**

The anti-CD20 IgG1 monoclonal antibody (mAb) rituximab (RTX) has improved the treatment of B-cells lymphocytic leukemia (B-CLL) and B-cells non-Hodgkin lymphomas (B-NHL). Its success is related to its capacity to induce Fc-(antibody-dependent cell-mediated cytotoxicity (ADCC). One receptor for human IgG1 is FcRIIIa (CD16 a), which is expressed on natural killer (NK) cells and macrophages. The influence of FcRIIIa-158VF polymorphism on RTX clinical response strongly suggests that ADCC is critical.\(^1\) Based on these results, there has been an attempt to produce new anti-CD20 mAbs that exhibit higher affinity for FcRIIIa either by Fc mutations or by glycoengineering.\(^2,3\) This later strategy, leading to low fucose content of the N-glycan, is currently under clinical investigations in B-cell malignancies with the mAb obinutuzumab (OBZ; previously GA101, Roche, Genentech), which shows stronger ADCC in vitro and in a lymphoma xenograft mouse model compared to RTX\(^4\) and improved clinical activity for treating chronic lymphocytic leukemia (CLL).\(^5\) This clinical benefit has been observed in other B-cell malignancies.\(^4,6,7\) OBZ is approved for first-line CLL in association with chlorambucil and in combination with bendamustine for the treatment of patients with follicular lymphoma (FL) who relapse or are refractory to RTX-containing regimen.\(^8\)

However, it is remarkable to note that the mAbs themselves have modest clinical activity. For example, RTX or OBZ when used as monotherapy in patients with relapsed follicular lymphoma have demonstrated short progression-free survival (PFS).\(^8\) These data indicate that there is a need to optimize their use in co-therapy. In this sense, hematological cancer patients possess antitumor NK cells that are unable to control disease.\(^9,10\) Blood-borne cancer cells use different mechanisms for immune escape.\(^11,12\) e.g. inducing NK cell dysfunction.\(^13,14\) In addition, NK cell differentiation may be inhibited by the presence of tumor cells e.g. acute myeloid leukemia (AML) cells infiltrating bone-marrow.\(^15,16\) Therefore, the failure of mAb as monotherapy could be related to impaired NK cell function.

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and hence, there is a clinical interest to reactivate patient NK cells.\textsuperscript{17}

Lenalidomide (LEN; Revlimid; Celgene) is an immune-modulatory drug that can activate NK cells.\textsuperscript{3,18–21} LEN treatment during and after stem cell transplantation (SCT) increases NK cell proliferation, enhances NKP44 expression on NK cells,\textsuperscript{14} and increases circulating NK-cell numbers in leukemia patients.\textsuperscript{22,23} LEN increases co-stimulatory receptor expression on NK cells, such as CD16 and Lympocytes Function-associated Antigen (LFA)\textsuperscript{14} and stabilizes NK cell:target cell immunological synapse.\textsuperscript{20,23,24} These effects lead to increased cytotoxic activity and increased proliferation of LEN-stimulated NK cells.\textsuperscript{14,19,20} LEN has similar effects in B-NHL patients restoring synapse formation, ADCC, and cytotoxic functions in NK cells.\textsuperscript{25,26} Of particular clinical importance, LEN allows NK cells to be activated by lower doses of RTX.\textsuperscript{20} Finally, it also favors target recognition by inducing expression of NKG2D and DNAM-1 ligands on malignant cells.\textsuperscript{27} LEN mechanism of action is thus predominantly immune-mediated, making LEN a suitable treatment to restore exhausted NK cell cytotoxic functions.

With this view, the clinical trial GALEN is a Phase Ib/II study of OBZ combined with LEN for the treatment of relapsed/refractory follicular and aggressive B-cell lymphoma (diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL)) by the LYSAL Lymphoma Study Association. The primary objective of the Phase II part of the study was to determine the recommended dose (RD) of LEN when administered in association with OBZ. The primary objective of the Phase II part of the study was to assess the efficacy of the association of the recommended dose of LEN in combination with OBZ, as measured by the overall response rate (ORR) at the end of 6 cycles in these 2 different populations of lymphoma patients. We developed a pilot exploration of some specific aspects of NK cell biology. In this respect, we monitored the following time points: i) C1D1 predose; ii) C1D28 and iii) C6D28 (supplemental Fig. 1).

Results

Effect of treatment on lymphocyte populations

Patients were treated with a combination of LEN (orally administered) and 3 doses of OBZ in the first cycle and a single dose on the first day of the following for total of six consecutive treatment cycles (see supplemental Fig. 1 for treatment and sampling protocol). We did not observe differences in the NK cell parameters tested between the different lymphoma types in our pilot study, hence we analyzed them together (both FL and DLBCL patients). We observed a transient decrease in hemoglobin levels, a significant decrease in leucocytes and a trend towards a decrease in lymphocytes (Fig. 1A). T cell numbers were unchanged and there was a transient decrease in NK cells at the end of the first cycle (Fig. 1B). B cells (CD19\textsuperscript{+}) decreased in numbers (Fig. 1C). The CD20\textsuperscript{+} population, which is the main target of OBZ, showed a tendency to decrease (Fig. 1C), similar to CD5\textsuperscript{+} cells (Fig. 1C). The remaining CD19\textsuperscript{+} cells showed increased expression of the major histocompatibility complex-I (MHC-I), as has been observed in other hematological neoplasias\textsuperscript{14}; but also of the stress ligands MHC class I polyomavirus-related sequence A (MICA) and MICB (Fig. 1D). The increased expression of MHC-I and MICA/B could have countervailing effects on NK cells because they are recognized by KIRs, inhibitory receptors, and NKG2D, activating receptor, respectively. Hence the final effect on NK cell recognition in remaining target cells is unclear.

Treatment induces maturation of the immature NK cell population

We next directly investigated the physiological status of NK cells during treatment. In the peripheral blood, human NK cells are mostly CD3\textsuperscript{−}CD56\textsuperscript{dim} cells with high cytotoxic activity, while CD3\textsuperscript{−}CD56\textsuperscript{bright} cells excel in cytokine production.\textsuperscript{28} In vitro evidence indicates that CD56\textsuperscript{bright} NK cells are precursors of CD56\textsuperscript{dim} NK cells and this might also be the case in vivo.\textsuperscript{29} In addition, combined analysis of CD56 and CD16 expression during NK cell development indicates that their profiles changes as follows: CD56\textsuperscript{+}\textsuperscript{CD16\textsuperscript{−}} → CD56\textsuperscript{+}\textsuperscript{CD16\textsuperscript{dim}} → CD56\textsuperscript{+}\textsuperscript{CD16\textsuperscript{bright}} → CD56\textsuperscript{+}\textsuperscript{CD16\textsuperscript{bright}} → CD56\textsuperscript{+}\textsuperscript{CD16\textsuperscript{−}}. Additional markers can be used to identify specific subsets within these NK cell populations.\textsuperscript{20,31} As previously described,\textsuperscript{10} we observed a tendency to a higher proportion of immature NK cells in patients compared to HD, which correlated with a decrease in the full mature CD56\textsuperscript{+}\textsuperscript{CD16\textsuperscript{−}} (Fig. 2A). At the end of treatment most patients lost the immature subsets and gained a NK distribution similar to healthy donors (Fig. 2A), i.e. with less immature cells.

We next analyzed the maturation marker CD161-killer cell lectin-like receptor subfamily B, member 1 (KLRB1) that is expressed early in NK cell development and before CD56\textsuperscript{+}. The expression of this marker did not change during treatment in the CD56\textsuperscript{+} NK compartment (Fig. 2B).

During in vivo maturation CD56\textsuperscript{bright} cells become CD56\textsuperscript{dim}\textsuperscript{CD26\textsuperscript{−}CD57\textsuperscript{−}} cells that produce perforin, while maintaining high IFN-γ production in response to cytokines.\textsuperscript{29,33} On the other hand, CD56\textsuperscript{dim}\textsuperscript{CD26\textsuperscript{+}CD57\textsuperscript{−}} cells show low response to cytokines and higher cytotoxic capacity.\textsuperscript{28,34} CD62L was slightly increased in patients and the treatment decreased the expression (Fig. 2B). In contrast CD57 was lower in patients and remained unchanged by the treatment (Fig. 2B). This suggests that at the end of treatment the NK cells show decreased expression of an immature marker, i.e. CD62L. When NK cells reach fully mature CD56\textsuperscript{dim}\textsuperscript{CD16\textsuperscript{−}} status, they gain full expression of killer inhibitory receptors (KIRs) receptors. KIR expression in patients before and after treatment was variable and expression of the 3 KIRs taken together was similar in patients and healthy donors (Fig. 2C).

The CD94 glycoprotein heterodimerizes with the natural-killer group 2 (NKG2) receptors, which are type II transmembrane proteins. CD94/NKG2A is an inhibitory receptor that recognizes HLA-E and it is the first inhibitory receptor expressed during NK cell maturation.\textsuperscript{32,33} CD94 can also associate with the activating receptors NKG2C and E\textsuperscript{32,33}. The activating receptor NKG2D represents an

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exception: it is a homodimer.\textsuperscript{32,35} CD94 was lower in patients and increased on treatment (Fig. 2D). In contrast, NKG2A was higher in patients and was not modified by treatment (Fig. 2D). NKG2D, which was lower in patients, significantly increased after treatment (Fig. 2D). In summary, NK activating receptors tend to increase while inhibitory receptors are either unchanged or decreased.

**Treatment decreases the activated NK cell population**

The proliferation marker Ki-67 is increased in NK cells from hematological cancer patients.\textsuperscript{9,10} Fig. 3A showed that the elevated values remained unchanged during treatment. In contrast, levels of the activation marker CD69, which were similar to healthy donors, decreased at the end of treatment (Fig. 3A).

The antitumor NK cell population is easily recognized by expression of CD45RO (CD45RO cells) in general together with CD45RA (CD45RARO cells). Patients show high levels of these cells, leading to a decrease in the CD45RA\textsuperscript{+}RO\textsuperscript{−}population (CD45RA cells).\textsuperscript{9,10} Patients in our cohort clearly showed this phenotype (Fig. 3B). At the end of treatment this phenotype tended to converge versus a healthy donor phenotype with increase in CD45RA cells and a decrease in CD45RO\textsuperscript{+} cells. Taken together, these data...
suggest that elimination of target cells decreases NK cell activation status.

**Treatment modulates NK cell cytotoxic activity**

If NK cell target cells were disappearing, we also expect to find a decrease in NK degranulation because cytotoxicity is probably the main antitumor function of these lymphocytes in hematological neoplasias.\(^9,10\) The proportion of granzyme\(^+\) cells was similar to healthy donors and slightly decreased after treatment (Fig. 4A). The amount of granzyme, as measured by the median fluorescence intensity (MFI) values, was higher in patients, underwent a significant reduction at the end of cycle 1, and recovered to normal values at the end of treatment (Fig. 4A). In agreement with our previous results,\(^9,10\) we observed that more NK cells were degranulating in patients, measured by CD107a expression in the plasma membrane (Fig. 4B). At the end of treatment the proportion of degranulating cells had significantly decreased (Fig. 4B). NK cells degranulated at similar levels at the beginning and at the end of the treatment against the
allogeneic non-Hodgkin B lymphoma cell line Daudi (Fig. 4C and D). Interestingly, after the first cycle NK cells were more active against the allogeneic targets (Fig. 4C and D). This suggests that the presence of targets cells and the treatment activate NK cells in vivo. CD56<sup>dim</sup> cells were responsible for CD107a expression ex vivo, whereas CD56<sup>bright</sup> cells lacked expression of this marker (Fig. 4E). CD56<sup>dim</sup>CD16<sup>+</sup> cells are considered immature cells and precursors of CD56<sup>dim</sup>CD16<sup>−</sup> cells Unexpectedly, CD56<sup>dim</sup>CD16<sup>−</sup> cells expressed higher CD107a levels than CD56<sup>dim</sup>CD16<sup>+</sup> cells. This is probably related to CD16 downregulation after NK cell activation and also explains the relative high CD56<sup>dim</sup>CD16<sup>−</sup> cell numbers in patients (Fig. 2A).

**Treatment decreases the trogocytosis of tumor-associated markers by NK cells**

These results suggested that NK cells were actively killing their targets during treatment and the absence of such targets at the end of treatment generated resting NK cells. To test this hypothesis, we investigated the proportion of NK cells that have killed CD19<sup>+</sup> targets at the different time-points. We took advantage of the fact that NK cells gained target cell antigens, e.g. CD19, by trogocytosis. As expected, the percentage of CD19<sup>+</sup> NK cells was higher in patients than in healthy donors and decreased with treatment (Fig. 5A). The CD45RARO NK subset expressed the highest level of CD19 and treatment successfully decreased CD19 expression (Fig. 5A). Other subsets also decreased CD19 expression (Fig. 5A). These results suggest that efficient treatments leading to elimination of NK cell target cells reduce NK: target cell interaction and lead to lack of target antigens on NK cell surface.

We next investigated which populations were responsible for other variations in NK cell markers and focused on three of them that changed after treatment. CD69 expression was higher in patients and decreased after treatment (Fig. 3A). CD45RA<sup>+</sup> cells showed higher CD69 levels, and treatment did not decrease it. CD45RO<sup>−</sup> cells showed lower levels that decreased with treatment (Fig. 5B). Therefore the decrease in CD69 expression is linked to both the decrease in the number of CD45RO<sup>+</sup> cells, which expressed higher CD69 levels and the decrease of CD69 in CD45RO<sup>−</sup> cells.

NKG2D expression was lower in patients and increased after treatment (Fig. 2D). NKG2D was lower in CD45RO<sup>−</sup> cells and treatment increased expression in both CD45RO<sup>−</sup> and CD45RO<sup>+</sup> cells (Fig. 5B). Hence the increase in NKG2D expression is linked to both the increase in the number of CD45RO<sup>+</sup> cells, which expressed higher CD69 levels and the decrease of CD69 in CD45RO<sup>−</sup> cells.

CD94 expression was not modified in patients but increased during treatment (Fig. 2D). CD45RO<sup>−</sup> cells expressed less CD94 and both CD45RO<sup>+</sup> and RO<sup>−</sup> non-significantly increased CD94 expression during treatment (Fig. 5B).
increase of CD94 on the whole NK cell population is thus related to both, the decrease in CD45RO\textsuperscript{+} cells and the general increase of CD94.

**Treatment does not exhaust NK cells**

Finally, we investigated the effect of treatment on two receptors regulated on NK cells by CD16-mediated activation: the stimulatory CD137 receptor and the inhibitory PD-1 receptor\textsuperscript{17}. We used samples from a different cohort of patients in the GALEN clinical study and investigated the effect of OBZ treatment at the following time points: 1) during LEN course, just before OBZ injection (D7 before OBZ); 2) 1 hour after the end of OBZ infusion (D7 after OBZ); 3) at D0 of cycle 2 before LEN (cycle 2); and 4) at assessment of clinical response after 6 cycles (end of induction). OBZ increased CD137 in both FL and the stimulatory CD137 receptor and the inhibitory PD-1 receptor.

![Figure 4. Treatment modulates NK cell cytotoxic activity.](image)
and DLBCL patients (Fig. 6). The effect was found within hours after OBZ treatment and increased until the end of treatment. In contrast, PD-1 expression was unchanged (Fig. 6). This suggests that OBZ induces NK CD137 receptor expression in the presence of LEN and that continued infusion of the mAb keeps levels of this activating receptor high. In contrast, under these conditions, the inhibitory PD-1 receptor was not expressed.

**Discussion**

Although RTX-based therapy is efficient in a large number of patients, there is still a need for improvement. The development of new mAbs such as OBZ aims to fulfill this demand. However, even the best-designed mAb could be inefficient in some patients if they lack proper effector cells. The use of LEN to activate NK cells could address this problem. Here we observe that treatment with LEN reverses the immature phenotype of patient NK cells (Figs. 2 and 3) and induces expression of activating ligands, i.e. NKG2D (Fig. 2) and CD137 (Fig. 6). During treatment and in the presence of target cells (at end of first cycle), NK cells from patients degranulated more than those from healthy donors (Fig. 4). Once target cells disappear (Fig. 1), the activated markers CD69 and CD45RO (Fig. 3), the degranulation marker CD107a (Fig. 4) and the marker of trogocytosis CD19 (Fig. 5) decrease on NK cell membrane. This suggests that is possible to follow disease development by studying NK cell markers; at least, when NK cells are the effectors of the therapy, e.g. some clinical mAb. At the end of first cycle, when target cells are still present in relative numbers, NK cells show increased cytotoxicity _in vitro_ and _ex vivo_ and low GrzB levels (Fig. 4). However, the NK cell number decreases (Fig. 1). We propose the following scenario. NK cells are constitutively killing target cells (Fig. 5 and 9). Some NK cells die during this immune response generating an increase in immature cells. OBZ and LEN induce improved target cell recognition and NK cell activation. NK cells degranulate in larger numbers but also die in larger numbers. At the end of treatment, most targets cells have disappeared and NK cells are no longer dying, so there is less _de novo_ formation of NK cells and they are becoming more mature. However, NK cells continue to show high Ki-
67 levels. Perhaps this can be explained by adaptive differentiation of NK cells and subsequent growth of a larger population of mature “memory” NK cells, as has been suggested after CMV infection. Almost all phenotypic changes observed in NK cells disappear with the lack of target cells suggesting that treatment keeps NK activated only in the presence of target cells. Several populations are responsible for the changes in NK cell phenotype (Fig. 5B) although cytotoxicity ex vivo is almost exclusively associated to CD56\textsuperscript{dim} cells (Fig. 4E). Unexpectedly, we observed that CD45RO\textsuperscript{+} NK cells showed low NKG2D expression (Fig. 5B). We speculate that once NK cells are engaged on killing, NKG2D expression is not anymore required. Our results show an increase in immature, CD56\textsuperscript{bright}, NK cells in lymphoma patients (Fig. 2A). CD56\textsuperscript{bright} cells produce high cytokine levels.\textsuperscript{28} In the context of anti-CD20-induced ADCC, we believed that NK cell cytotoxic function would be more relevant than cytokine production. In hematological cancer patients, cytotoxicity ex vivo is mainly mediated by CD56\textsuperscript{dim} cells (Fig. 4E). Unexpectedly, we observed that CD45RO\textsuperscript{+} NK cells showed low NKG2D expression (Fig. 5B). We speculate that once NK cells are engaged on killing, NKG2D expression is not anymore required.

Our results show an increase in immature, CD56\textsuperscript{bright}, NK cells in lymphoma patients (Fig. 2A). CD56\textsuperscript{bright} cells produce high cytokine levels.\textsuperscript{28} In the context of anti-CD20-induced ADCC, we believed that NK cell cytotoxic function would be more relevant than cytokine production. In hematological cancer patients, cytotoxicity ex vivo is mainly mediated by CD56\textsuperscript{dim} cells (Fig. 4E). When we planned our analysis, we decided to maximize the study of cytotoxic, CD56\textsuperscript{dim}, NK cells and did not investigate cytokine production because it is believed that CD56\textsuperscript{dim} cells produce low cytokine levels.\textsuperscript{28} However, in view of our current results it would be interesting to investigate the cytokine profile of the immature NK cell populations that accumulate in lymphoma patients.

LEN targets the E3 ligase cereblon that degrades the Ikaros transcription factors IKZF1 and IKZF3.\textsuperscript{38} In vivo, LEN induces tumor cell apoptosis and blocks bone marrow stromal support,\textsuperscript{39} but also activates immune cells, e.g. NK cells.\textsuperscript{14,18–21} Our results support that NK cells are important mediators of the clinical benefits of LEN+OBZ.

It is noteworthy that PD-1 is absent on NK cells isolated from healthy donors but it is expressed on those from MM patients.\textsuperscript{40} We observe that there is a large heterogeneity of PD-1 expression in our patient cohort, and only a few of them constitutively express PD-1 (Fig. 6). As discussed above, there is probably continual production of mature NK cells to replace those dying during the immune response. These new cells are probably not exhausted and lack PD-1 expression. Hence, the continual renewal of NK cells might preclude PD-1 expression on NK cells in some patients. Treatment did not significantly affect PD-1 expression. This suggests that the role of PD-1 on treatment is minor. Perhaps LEN partially reversed the exhaustion of effector cells as previously suggested.\textsuperscript{41} LEN is probably the most active treatment (alone or combined with anti-PD-1/ PD-L1 antibodies or other drugs) able to restore cytotoxic function to exhausted NK cells.\textsuperscript{14} Our results show the hypothesis that LEN in combination with OBZ increases several NK cell biological parameters associated with maturation and activation. However, because we did not obtain samples in mono-therapy, i.e. LEN or OBZ alone, we cannot identify the relative contribution of these two drugs. Cancer patients show NK cell subsets that are significantly different of those found in healthy donors.\textsuperscript{9,10} However, one question was unresolved: what is the fate of these NK cell subsets when their target cells disappear? Here we show for the first
time that in our situation the NK cell subsets come back to a normal situation, i.e. similar to healthy donors, for the vast majority of markers. This is probably related to the disappearance of target cells because both LEN and OBZ are NK cell activating molecules that should not promote NK cell resting markers. This suggests that NK cells strongly react to effective treatment and that NK cell monitoring could be interesting to follow-up anti tumor treatments; mainly those involving mAb therapy.

Disclosure information

The authors declare the following conflict of interests: Roch Houot: Honoraria from Celgene and Roche; Guillaume Cartron: Honoraria and consultancy from Roche and Celgene; Franck Morschhauser: honoraria Celgene, Roche advisory boards and scientific lectures; Karin Tarte: Celgene, Roche for advisory boards and scientific lectures; Cedric Menard: Celgene for scientific lectures.

Patients and methods

 Patients

All patients belong to the BioGALEN study and signed specific informed consent form before biological samples collection of BioGALEN. This study is recorded in website ClinicalTrials.gov with number NCT01582776. Phase Ib was for follicular lymphoma (FL) patients and Phase II for follicular and aggressive (DLBCL and MCL) B-cell lymphoma patients. 3 x 3 ml of heparinized blood or 4 ml of bone marrow aspirate were collected at day 0. At the end of first cycle or at the end of treatment (supplemental Fig. 1) 3 x 3 ml of heparinized blood was collected.

Cell culture

The B cell lymphoblastoid Daudi cell line was maintained in logarithmic growth in RPMI 1640 medium (Gibco® Glutamax® media) with 10% fetal bovine serum (FBS) (Gibco®). Cells were cultured at 37°C in a humidified chamber with 5% CO2 in air, and passaged 1:10 twice a week.

Peripheral blood mononuclear cell (PBMC) purification

Bone marrow and peripheral blood samples were obtained from patients and total PBMC were isolated using Ficoll. Briefly, 3–6 ml of 1:2 diluted blood or 1:3 diluted bone marrow samples in RPMI were added on top of 5 ml of Histopaque (Sigma). Cells were centrifuged at 1600 rpm and at 20°C without break for 30 minutes. Mononuclear cells were collected from the white ring at the interface. After washing in RPMI, cells were cryopreserved in liquid nitrogen in medium comprised of FBS plus 10% culture-grade DMSO (CliniMACS) until analyzing.

Flow cytometry analysis

Isolated PBMCs were stained with 7AAD (Beckman) to identify viable cells and with the following -CD45RO-FITC, -CD161-FITC, -CD3-PE, -CD19-PE, -CD62 L-PE, -CD69-PE, -CD314 (NKGD2)-PE, -CD3-ECD, -CD19-ECD, -CD56-PECy7, CD3-APC, -CD56-APC, -GzB-AlexaFluor700, -CD19-AlexaFluor 700, -CD20-APC-AlexaFluor750, -CD45RA-APC-AlexaFluor 750, -CD5-PacificBlue, -CD16-PacificBlue, -CD57-PacificBlue, -CD16-KromeOrange (Beckman), -CD158 a-V450, -CD158 b-FITC, -CD158 a-PE, -CD107 a-HV500, -Ki-67-V450, HLA-ABC-BV711 (BD Biosciences), MIG-A/B-PE, -CD45RA-FITC, -CD45RO-PE, -CD159 a(NKG2 A)-PE, -CD94-PE-Vio770, -CD45RO-APC, -CD19-VioBlue, -CD158e-VioBlue (Miltenyi Biotec) antibodies against surface markers. Briefly, 1 to 10 x 10⁶ cells were incubated with the different antibodies in PBS containing 2% FBS at 4°C for 30 minutes. Cells were then washed with PBS and suspended in 200–250 µl PBS 2% FBS. Finally, sample acquisition was performed using Gallios flow cytometer (Beckman) or Fortessa (BD Biosciences). Acquired samples were later analyzed using Kaluza software v5.1 (Beckman).

In vitro CD107a degranulation assay

In vitro degranulation assay was performed to evaluate NK reactivity to the B cell target Daudi by measuring CD107a expression on the surface after cytotoxic granule release. In summary, isolated PBMC were pre-stained with CD3/CD56 to determine NK frequency in the sample. Next, PBMC were incubated with Daudi cells at a 1:10 ratio NK:Daudi in the presence of 1.5 ul of anti-CD107a (BD Biosciences, Franklin Lakes, NJ) and 1 ul Golgi-stop (BD Biosciences) (containing monensin) to inhibit vesicle trafficking. Cell mixture was then resuspended in RPMI Glutamax 10% supplemented with 10 IU/ml Interleukin 2 (eBiosciences) and incubated overnight. After stimulation, cell mixture was collected and stained for FACs using an antibody cocktail containing 7AAD, the anti-CD45RO-FITC, -CD69-PE, -CD19-ECD, -CD56-PECy7, -CD3-APC, -CD45RA-APCAlexaFluor750, -CD107a-HV500 and -CD16-KO antibodies (BD Biosciences, Beckman). A bivariate plot of CD56 versus CD3 was used to acquire at least 10,000 NK cells.

Multicolor staining for intracellular markers

Cell permeabilization and intracellular staining was performed as previously described.9,10 Briefly, 1–10 million cells were incubated with 10% normal human serum at RT for 15 min and then stained with an antibody mix for cell surface markers (anti-CD45RO-FITC, -CD69-PE, -CD19-ECD, -CD56-PC7, -CD3-APC, -CD45RA-APCAlexaFluor750 and -CD16-KO antibodies) (BD Biosciences, Beckman). After surface staining, cells were washed twice and permeabilized with CytoFix/CytoPerm (BD Biosciences) reagent according to the manufacturer protocol. After fixation and permeabilization, cells were washed twice in BD Perm/Wash solution and follow FACs staining for intracellular markers Granzyme B-PE (Miltenyi Biotec) and Ki-67-V450 (BD Biosciences) at 4°C for 30 minutes in the dark. Finally, cells were washed twice in BD Perm/Wash.
solution and resuspended in PBS 2% FBS prior to acquisition on flow cytometer Gallios (Beckman). A bivariate plot of CD56 versus CD3 was used to acquire at least 10,000 NK cells.

**Statistics**

Experimental figures and statistical analysis were performed using GraphPad Prism (v6.0). Statistical significance between day 0 and the following time-points was determined using paired Student t-test on the sample patients for each sampling point. To determine statistical significance between healthy donors and patients, one-way ANOVA test was used to compare between healthy donors versus patients at every time-points. All statistical values presented as "": p<0.05; "\*": p<0.01; "\*": p<0.001. Average values were expressed as mean plus or minus the standard error (SD).

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FACS analysis was performed at the platform Montpellier Rio Imaging (MRI).

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**References**


CHAPTER 5.

Study of NK cell populations in Acute Myeloid Leukemia (AML) patients during chemotherapy treatment

(unpublished results)
**Total PBMC analysis on AML patients during chemotherapy intervention**

To study NK cell phenotype in AML disease, we collected and analysed blood samples from 7 patients (in which 6/7 patient data were analysed both before and after treatment and 1 patient with “after treatment” was un-analysed due to cell lost during the freeze/thaw steps). PBMC samples from patients with leukemia, in particular AML are typically overpopulated by immature myeloid blasts therefore created a technical obstacle for conventional gating strategy for NK cell (i.e. CD3-CD56+) due to overlapping regions in a bi-directional dot-plot by the abundant leukemic cells. To overcome this issue, we utilized a secondary NK-specific marker, CD7 into the analyzing pipeline. In this study, NK cell population was defined as CD3-CD7+CD56+ (Figure 5.1). General composition of patients PBMC was changed after 30 days (or more) post-induction with higher percentage of T cell and NK cell (not significant) and decrease in CD33+CD14-myeloid population (Figure 5.2). This change in total PBMC proportion in patients are most likely due to leukemic cells cytotoxic effect by chemotherapy agents.

**NK cell displayed more immature phenotype after treatment**

With the notion that NK cell maturation status can be examined via several well-studied markers such as CD62L and CD57 that previously reported to described NK cell at different stages of development, the current model in human NK cel suggests that CD56br gaining maturation phenotype to become CD56dim via following steps: CD56brCD16− -> CD56dimCD62L+ ->CD56dimCD62L−CD57+ implying that gaining CD57 marks the terminal differentiation/maturation step in the NK cell lineage (Juelke et al. 2010; Krzywinska et al. 2016). Bearing this model, NK cell in our AML cohort showed an increase in CD62L level and loosing CD57 expression simultaneously after the treatment (Figure 5.3 C-D). Together with this, CD56br populations (both CD56brCD16- and CD56brCD16+) increased in frequency after treatment indicating whole NK population skewed toward a less mature phenotype (Figure 5.5 B). This perhaps suggests a turnover of general NK cell population after introduction of chemotherapy agent, either directly or indirectly via tumor-associated killing activity.

**NK cell showed less in vivo cytotoxicity-associated markers after treatment**
We next investigate NK cell in vivo activity through cytotoxicity-related markers to see how treatment intervention may affect on these makers. As expected, the ex vivo degranulation marker CD107a on NK cells was significantly lower after treatment as observed before in another cohort of B-NHL (Chapter 4) (Figure 5.4 B). This decrease is also in agreement with less NK cells carrying myeloid-specific marker (e.g. CD14 and CD33), an effect mediated by trogocytosis on the NK cell (Figure 5.4 C-D). After treatment, NK cells gained more NKG2C expression, an activating receptor that recognize HLA-E which may highly expressed on transformed tumor cells (Figure 5.4 A). Unfortunately we did not know whether this correlates with changing in HLA-E level on AML leukemic cells as HLA-E antibody was not used in the analysing FACS panel (Tabel 2). Interestingly, the activation/exhaustion marker PD-1 on NK cells also decreased after treatment which suggests NK cells are in less activation state after decrease in leukemic tumor load, therefore this observation intergrated well with other phenotypic changes (Figure 5.3 A). Surprisingly a reduction in CD69 level, a marker associated with activation after treatment was not observed in whole NK cell population (Figure 5.3 B), this led us to further analyse on NK subpopulation based on CD45 isoforms to better understand how each of these subsets changed in CD69 level during treatment.

**CD45RARO+ NK cell correlates with CD14 myeloid marker and CD69 activation marker**

We have previously the distinctive phenotype of the CD45RARO+ subpopulation which shown strong trogocytosis activity in vivo therefore it is of interest to re-validate our findings in this new study (Chapter 3). In general, the NK cell CD45 isoform profiles did not changed dramatically after patient’s treatment although a slight increase trend in CD45RA+ population was observed (Figure 5.5 A). To further examine the trogocytosis of CD45RARO+ NK to potential tumor-associated markers of AML (i.e. CD14 and CD33, which are myeloid-specific), we made correlation analysis between % of myeloid populations (including CD33+CD14- and CD33+CD14+) and % of CD45RARO+ NK cell in each sample as observed before and after treatment. As shown in Figure 5.6 A, the presence of CD33+CD14+ myeloid population is strongly correlated with CD45RARO+ NK population but not CD33+CD14- myeloid population. With a similar correlation
analysis between CD33/CD14 trogocytosed NK cells with proportions of CD45RARO+ NK subset, CD14 but not CD33 is strongly related with higher CD45RARO+ NK percentage (Figure 5.6 B). Given that CD14 molecules only expressed on hematopoetic-origin cells committed to the monocyte lineage, this seems to indicate CD45RARO+ NK preferentially interacts (hence, trogocytosis) with monocyte/macrophage populations.

To better understand how CD45RARO+ NK cells can be used to predict NK cell general phenotypic change, we took advantage of the fact that only half number of patients (i.e. 3 of total 6 patients) in our AML cohort showed a decrease in % CD45RARO+ NK cells to divide total patients into 2 groups: “RARO-down” (i.e. %CD45RARO+ NK decrease after treatment) and “RARO-up” (i.e %CD45RARO+ NK unchanged/increased) and further investigate these 2 groups in dynamic change of previous markers (Figure 5.7 A). In contrast to the unchanged level of CD69 in the total NK population, CD69 level was decreased in “RARO-down” group (not significant) and increase in “RARO-up” group (Figure 5.7 C). Since the number of CD45RARO+ NK that changed during treatmet was much smaller in scale to total NK population, the decrease observed in CD69 (approximately 20% in average) cannot be accounted for the number of CD45RARO+ NK cells alone. Furthermore, the decrease in PD-1 level observed on the “RARO-down” group was more dramatic than in the “RARO-up” (Figure 5.7 B). However, the phenotypic difference in NK cells in “RARO-down” versus “RARO-up” groups are not distinguishable for the majority of other markers (Figure 5.7 D-E, supplementary Figure 5.1, 5.2). Taken together, the dynamic changes in CD45RARO+ NK cell can be a factor to predict CD69 activation marker in total NK cell population in AML patients.
**Figure 5.1** Diagram of gating strategy for NK cell and other lymphocytes populations in AML. Representative plots of a patient’s PBMC (after treatment) showing the gating strategy applied in the study. In this study, NK cell population defined as CD3-CD7+CD56+. 
**Figure 5.2** Proportion of lymphocyte and monocyte populations. Proportions of each lymphocyte and monocyte populations presented in percentage in T, B and NK cell compartments (upper panels) and monocytes (lower panels) of CD33+ and CD33+CD14+ phenotypes. Statistical significant was determined by paired t-test between diagnostic versus after treatment. N=7 with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
**Figure 5.3** NK cell activation and maturation markers. A) Percentage of PD-1 expressing NK cell. B) CD69 activation marker on NK cell population during treatment. C-D) Expression of maturation markers of CD62L and CD57 on NK cell population during treatment. Statistical significant was determined by paired t-test between diagnostic versus after treatment. N=7 with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
**Figure 5.4** NK cell ex vivo degranulation and trogocytosis. A) Level of NKG2C activating receptor on NK cell during treatment. B) NK degranulation ex vivo identified by CD107a. C-D) Measure of NK cell trogocytosis activity by myeloid makers before and after treatment. Statistical significance was determined by paired t-test between diagnostic versus after treatment. N=7 with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
**Figure 5.5** NK subpopulation analysis. Analysis of NK cell subpopulations based on CD45 isoform subsets (CD45RA+, CD45RA0+, CDRAdim and CD45RO+) (A) and level of CD56 and CD16 expression that divides NK cells in 4 subpopulations: CD56brCD16-, CD56brCD16+, CD56dimCD16- and CD56dimCD16+ (B). Statistical significant was determined by paired t-test between diagnostic versus after treatmet. N=7 with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
**Figure 5.6** A) proportion of CD45RA+ NK cell correlates with CD33+CD14+ monocyte population but not CD33+CD14- monocyte population. B) proportion of CD45RA+ NK cell correlates with CD33+CD14+ trogocytosed NK cell. Correlation between populations analyzed by Pearson method with r= correlatin coefficient and p = statistical significance of correlation.
**Figure 5.7** CD45RARO+ stratification: analysis of various surface markers based on changes in CD45RARO+ NK cells percentage after treatment. A) Total patients divided into 2 groups based on CD45RARO+ response after treatment. B-E) Comparison for various markers PD-1 (B), CD69 (C), CD62L (D) and CD57 (E) between 2 groups based on CD45RARO+ stratification described in (A). Statistical significant was determined by paired t-test between diagnostic versus after treatment. N=3 with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
**Supplementary Figure 5.1** CD45RARO+ stratification (continued). Analysis of surface markers based on CD45RARO+ stratification as described in Fig. 5.7.

**Supplementary Figure 5.2** CD45RARO+ stratification (continued). Analysis of surface markers based on CD45RARO+ stratification as described in Fig. 5.7.
CHAPTER 6.

Distinct phenotypic imprints in NK cell from patients with Human Immunodeficient Virus (HIV) infection

(unpublished results)
Examine the general landscape in lymphocyte populations in HIV+ patients

To obtain understanding about HIV infection effect on NK cell, we analysed blood samples from HIV+ patients (in which 4 patients are aviremic i.e. no serum virus particles, and 3 viremic patients i.e. virus presence in blood serum) and 3 healthy donors as control group. Figure 6.1 summarized the gating strategy used in this study. Due to limitation in fluorescent channel options compared to our desirable immunophenotypic panel, CD14 and CD19 were pooled analysed (Table 3). Hence in subsequent PBMC analysis, lymphocyte populations are defined as CD14-CD19- (i.e. without B cells). Proportional analysis on the CD14-CD19- lymphocytes indicated a decrease in both frequencies of NKT and NK cell populations, simultaneously CD3+ T cell percentage was increase (Figure 6.2) compared to HD control. This suggests 2 possibilities: NK and NKT cell decreased in cell number in HIV+ patients leading to higher proportion of CD3+ T cells, or NK and NKT cell number remained steady while T cells proliferate more in infected patients. We postulated that the former scenario is more physiological relevant however absolute lymphocyte count (ALC) values are needed to validate this hypothesis. In general, the distribution landscape in cytotoxic lymphocyte populations in HIV-infected patients is distinctively different from healthy individuals.

Impairement in the activating receptor NKG2D in HIV+ patient NK cells

In the context of HIV viral infection, at least one viral protein, namely Nef has been reported to interfere with NKG2D-NKG2DL signaling axis between infected cell and NK cell, emphasizing the importance of this well known “stress induced” mechanism in NK-mediated viral clearance (Cerboni et al. 2007). To address the role of NKG2D in our study, we analyzed the level of NKG2D expression on the NK cells from HIV+ patients and HD control. As expected, we have observed both reduction in % of NKG2D+ NK cells and reduction in NKG2D level in NKG2D-expressing cells on HIV+ patients compared to healthy controls, implying a difference in NKG2D profile in patient NK cell (Figure 6.3). This could either mean NK cells down-modulated NKG2D molecules in a ligand-dependent endocytosis manner possibly due to NKG2DL-expressing infected cells or perhaps NKG2D level decreased indirectly by virus mediated immune escape mechanisms.
**Different subpopulation profiles in NK cells among HIV-infected patients**

To gain better understanding on NK cell dynamics in HIV+ patients, we divided total NK population into each of individual small subsets based on their profile of CD56 versus CD16 or CD45RA versus CD45RO (Figure 6.4). In normal people, majority of peripheral NK cells show CD56dimCD16+ phenotype (~90%) and remaining cells have CD56brCD16- which related well with our data. However, in HIV+ patients (both aviremic and viremic) the highly cytotoxic population CD56dimCD16- does indicate a tendency of lower proportion, especially in viremic patients the CD56dim vs CD56br composition seems to skew toward higher immature phenotype (i.e. increase CD56br percentage) (Figure 6.4 A). Similarly, CD45 profile on NK cells from infected patient are distinctively different from that of HD, with an increase in NK cell having CD45RAdim and CD45RO+ phenotype where most of NK cells from HD are CD45RA+ corresponding to a “resting” state (Figure 6.4 B-C).

**Down modulation of CD16 in CD45RAdim and CD45RO NK cell from patients**

In the highly cytotoxic CD56dimCD16+ NK cell subset, down-modulation of CD16 after engagement to target cells has been previously reported (Grzywacz, Kataria, and Verneris 2007; Lajoie et al. 2014). Moreover, CD16 down-modulation also occurred in associated with in vitro cytotoxic activity and in proportion with CD107a, marker for degranulation (unpublished observation). With this notion, we attempted to interpret the change in CD45 profiles in patient NK cells to link between different CD45 subsets with CD16 level. Interestingly, in both groups of patients we observed a link between CD45RAdim plus CD45RO+ NK (which does not exist in HD) and a gradual decrease in CD16 level (Figure 6.5 A-B). Noticeably, the “grade” of CD16 down-modulation is progressive as NK cell subsets gaining CD45RO expression while loosing CD45RA (Figure 6.5 B). This led us to propose a consecutive sequence of NK cell activation scenario in vivo as follow: CD45RA+ -> CD45RAdim -> CD45RO+ in which CD45RA+ NK cells with high level of CD16 expression should be regarded as “naive” cells, as they encounter the infected cells in vivo these events promoted activation with molecular signature of CD45RA-CD45RO+ and CD16 loss. Hence in this situation CD45RAdim NK
cell can be regarded as an “intermediate” state between “naive” and “activated” cells, which has been proposed before in patients with hematological cancers (Krzywinska et al. 2016).

**Transferring of HIV-entry receptors from T cell lines and primary NK cells via mechanism of trogocytosis in vitro**

Previous results providing evidents that NK cells from HIV infected patients associated more with activated state *in vivo*, probably due to infected cells engagement. This led us to investigate the outcome of this cell-cell interaction between NK cell and virus infected cell. NK cells have been shown to perform trogocytosis activity *in vitro* and *in vivo*, as a consequent of this NK cell can carry patches of target cell membrane including membrane-anchored receptor molecules (Miner et al. 2015; Nakamura et al. 2013). To examine if NK cell can perform trogocytosis on T cell (which in HIV infection context means target cell), we co-cultured primary isolated NK cells (from a hematological cancer patient) with T cell lines CEM and MT4 that are previously labeled with a violet dye (CTV). As shown in Figure 6.6 A, after co-culturing window of 4h with T cell targets, violet dye associated with target cells can be detected from NK cells with some proportion of NK cells gained as high amount of dye as primary stained target cells. We next asked if this membrane-transferring phenomena between NK/T cells could imply a physiological-relevant consequent. It was known that HIV virus particle exploits the T cell membrane receptor CD4 together with chemokine receptor CXCR4/CCR5 for virus entry. This led us to hypothesize if these HIV-receptors can also be transferred from virus infected cell into NK cell via trogocytosis. To answer this question, we repeated the *in vitro* trogocytosis assay between primary isolated NK cells to dye-labeled CEM/MT4 and simultaneously using anti-CD4 and anti-CCR5 antibodies to detect (if possible) transferring of these molecules from these target cells to NK cells. As shown in Figure 6.6 B-C, CEM and MT4 both expressed CD4 (partially for CEM) however only MT4 but not CEM express the chemokine receptor CCR5. In contrast, primary NK cells isolated from 2 different patients with hematological diseases co-cultured with labeled CEM/MT4 gained substantial level of CTV dye as well as CD4 (both with CEM and MT4) and CCR5 (only with MT4). Therefore, it was indicated that, via trogocytosis, NK cells can gain transient expression of virus-receptors by interacting
with CD4/CCR5-expressing target cells. This begs the next interesting question: does NK cell become HIV susceptible host by gaining CD4/CCR5 via trogocytosis from previous infected cells? To our knowledge it has not yet been described before.
**Figure 6.1** Gating strategy for NK cell and other lymphocytes populations in healthy donors and HIV+ patients. Representative plots of an aviremic patient's PBMC showing the gating strategy applied in the study.
Figure 6.2  General proportion of lymphocyte populations in HD and HIV+ patients. Percentage of T, NKT and NK cell populations respectively on infected patients (aviremia and viremia) and on healthy donors. HD: n=3, Aviremic: n=4, Viremic n=3. Statistical significant was determined by one-way ANOVA between HD versus patients with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.

Figure 6.3  Reduced NKG2D expression in NK cells in infected patients measured by percentage of NKG2D+ NK cells (left panel) and expression level of NKG2D+ NK cells (right panel). HD: n=3, Aviremic: n=4, Viremic n=3. Statistical significant was determined by one-way ANOVA between HD versus patients with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
Figure 6.4 NK subpopulation analysis on HD and HIV+ patients. A) Analysis of NK cell subpopulations based on level of CD56 and CD16 expression that divides NK cells in 3 subpopulations: CD56br, CD56dimCD16- and CD56dimCD16+. B) Representative dot-plots showing CD45RA versus CD45RO expression profiles between HD and HIV+ patients.
patients. C) Summarized bar-graph showing percentage of NK cell subsets divided into 4 populations based on CD45RA versus CD45RO expression. HD: n=3, Aviremic: n=4, Viremic n=3. Statistical significant was determined by one-way ANOVA between HD versus patients with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
Figure 6.5  Reduced CD16 expression in CD45RAdim and CD45RO+ NK subpopulations in HIV+ patients. A) representative dot-plots on HD and infected patients showing
CD56/CD16 subsets compositions between total NK cells, CD45RA+, CD45RAdim and CD45RO+ NK cells. B) Quantitative bar-graph summarized CD56/CD16 composition within the different CD45 NK cell subsets as demonstrated in A. Aviremic: n=4, Viremic n=3. Statistical significant was determined by one-way ANOVA between CD45RA+ versus CD45RAdim and CD45RO+, with * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
Figure 6.6 Transfer of HIV receptor CD4 and CCR5 from target cell to NK cell by trogocytosis. A) In vitro trogocytosis assay performed by co-incubation of CTV-labeled CD4+ target cells (CEM and MT4) togethe with patient’s PBMC, NK cell trogocytosis on target cells are measured by transfering of CTV by FACS analysis after 4h of co-incubation.
B-C) In vitro trogocytosis assay performed as described above with CD4 (B panel) and CCR5 (C panel) HIV co-receptor staining showing transfering of these receptors onto NK cell surface upon co-incubatin period.
CHAPTER 7.

Expansion of allogeneic NK cells with efficient antibody-dependent cell cytotoxicity against multiple tumors

(published article - Theranostics)

[In this study that I shared co-authorship, I was involved in planning and execution of the experiments, collecting and isolating patient’s sample PBMC and UCB donors, and performing analysis of results included in supplementary materials]
Expansion of allogeneic NK cells with efficient antibody-dependent cell cytotoxicity against multiple tumors

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Abstract

Monoclonal antibodies (mAbs) have significantly improved the treatment of certain cancers. However, in general mAbs alone have limited therapeutic activity. One of their main mechanisms of action is to induce antibody-dependent cell-mediated cytotoxicity (ADCC), which is mediated by natural killer (NK) cells. Unfortunately, most cancer patients have severe immune dysfunctions affecting NK activity. This can be circumvented by the injection of allogeneic, expanded NK cells, which is safe. Nevertheless, despite their strong cytolytic potential against different tumors, clinical results have been poor.

Methods: We combined allogeneic NK cells and mAbs to improve cancer treatment. We generated expanded NK cells (e-NK) with strong in vitro and in vivo ADCC responses against different tumors and using different therapeutic mAbs, namely rituximab, obinutuzumab, daratumumab, cetuximab and trastuzumab.

Results: Remarkably, e-NK cells can be stored frozen and, after thawing, armed with mAbs. They mediate ADCC through degranulation-dependent and -independent mechanisms. Furthermore, they overcome certain anti-apoptotic mechanisms found in leukemic cells.

Conclusion: We have established a new protocol for activation/expansion of NK cells with high ADCC activity. The use of mAbs in combination with e-NK cells could potentially improve cancer treatment.

Key words: NK cells, monoclonal antibodies (mAbs), antibody-dependent cell cytotoxicity (ADCC), cancer

Introduction

Recent progress in cancer treatment is primarily related to the development of novel targeted therapies [1]. These require the identification of suitable targets that are mainly expressed by the tumor cell population and/or playing a critical role in neoplastic cell growth. Therapeutic monoclonal antibodies
(mAbs) particularly illustrate this concept. Indeed, rituximab (RTX), an IgG1 mAb directed against CD20 antigen, has now become the treatment of choice for most B-cell chronic lymphocytic leukemias (B-CLL) and B-cell non-Hodgkin’s lymphomas (B-NHL). The combination of RTX with conventional chemotherapy has shown better efficacy in randomized clinical trials. Similar success has been found with other cytotoxic mAbs, such as trastuzumab in breast cancer or cetuximab in colorectal carcinoma and squamous cell carcinoma of the head and neck [2, 3]. Nevertheless, mAbs alone generally have modest clinical activity. For example, the anti-CD20 mAbs RTX and obinutuzumab (OBZ; previously GA101, Roche, Genentech), when used as monotherapy in patients with relapsed follicular lymphoma (FL), have only led to short progression-free survival (PFS) [4]. Thus, there is a need to optimize their use in combination therapy.

RTX success is related to its capacity to induce Fc-antibody-dependent cell-mediated cytotoxicity (ADCC). One receptor for human IgG1 is FcγRIIIa (CD16a), which is expressed on natural killer (NK) cells and macrophages. The link between FcγRIIIa-158VF polymorphism and RTX clinical responses strongly suggests that ADCC is critical [5]. This polymorphism is located on the extra-cellular domain of FcγRIIIa, and amino-acid 158 is involved in the interaction with CH2 of human IgG1 [4]. Human IgG1 has a higher affinity for VV-NK cells compared to FF-NK cells [5]. Based on these observations, there has been an attempt to produce new anti-CD20 mAbs by either Fc mutations or by glycoengineering that exhibit higher affinity for FcγRIIIa [4, 6]. Lowering the fucose content of the N-glycan is currently under clinical investigation in B-cell malignancies with the mAb OBZ, which shows stronger ADCC in vitro and in a lymphoma xenograft mouse model relative to RTX. It also demonstrated improved clinical activity for treating B-CLL and other B-cell malignancies [4]. OBZ is approved for first-line B-CLL in association with chlorambucil, and in combination with bendamustine for the treatment of patients with FL who relapse or are refractory to a RTX-containing regimen [4]. Initial results show that lenalidomide, which stimulates NK cell activity [7], activates NK cells in OBZ-treated patients [8].

NK cells mediate ADCC but also possess natural cytotoxicity, which is mediated by engagement of their natural cytotoxicity receptors (NCRs). These play a central role in triggering NK activation. In humans, NKp30, NKp46, and NKp80 are constitutively expressed on resting and activated NK cells [9]. The NK cell-activating receptor CD16 mediates ADCC. Hematological cancer patients possess antitumor NK cells that are unable to control disease [10, 11]. Notably, blood-borne tumor cells use different mechanisms for immune escape [12, 13], e.g., by inducing NK cell dysfunction [7, 14]. This mechanism has also been observed in a variety of patients of solid tumors [3]. In addition, NK cell differentiation may be inhibited by the presence of tumor cells, e.g., acute myeloid leukemia (AML) cells infiltrating bone marrow [15, 16]. Therefore, the failure of mAbs in monotherapy could be related to impaired NK cell function. Hence, there is a clinical interest to reactivate or replace patient NK cells [17]. Clinical-grade production of allogeneic NK cells is efficient and NK cell-mediated therapy after hematopoietic stem cell transplantation (HSCT) seems safe [16, 18, 19]. Despite the strong cytolytic potential of expanded NK cells against different tumors, clinical results have been very limited [16, 18, 19].

The combination of allogeneic NK cells with mAb could improve cancer treatment by replacing the defective effector immune cells. In addition, mAbs would effectively guide these effectors to their tumor targets. Several groups have tried this combination with varying results that could be due to deficient CD16 expression or lack of proper activation of expanded NK [20-23]. In addition, these studies did not include a systematic evaluation of the effect of these cells in combination with several mAbs on different tumors, nor did they include primary tumor cells.

The aim of this work was to generate allogeneic NK cells with strong ADCC response against different tumors and mediated by different therapeutic mAbs. In addition, NK cell production should be easily scaled up and developed with good manufacturing practices (GMP). We have produced umbilical cord blood (UCB)-derived NK cells because UCB are rapidly available, present low risk of viral transmission and have less strict requirements for HLA matching and lower risk of graft-versus-host disease (GvHD) [18]. For NK cell expansion we used Epstein-Barr virus (EBV)-transformed lymphoblastoid B cell lines as accessory cells, which induce a unique genetic reprogramming of NK cells [24]. This generates effectors that overcome the anti-apoptotic mechanism of leukemic cells [25] and that are able to eliminate tumor cells from patients with poor prognosis [26]. We show that NK cells obtained with our protocol are able to perform ADCC in vitro and in vivo. The ADCC response was induced by using different therapeutic antibodies and against multiple target cells.
Methods

Ethics statement

Experimental procedures were conducted according to the European guidelines for animal welfare (2010/63/EU). Protocols were approved by the Animal Care and Use Committee “Languedoc-Roussillon” (approval number: CEEA-LR-12163). The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient prior to surgery.

Chemicals

The D1D2 peptide has been previously described [27]. IL-2 and IL-15 were obtained from Miltenyi Biotec. To produce deglycosylated cetuximab, a commercial cetuximab solution was treated overnight with PNGaseF (Promega) at 37 °C in 50 mM sodium bicarbonate buffer (pH 7.8) at 125 U/mg of cetuximab. Deglycosilated cetuximab was purified by gel filtration using a Sephadex 75 column in PBS, and sterilized by filtration. The AF 647 Goat F(AB’)2 anti human IgG (H+L) min x (BOV, HRS, MS) was from Interchim.

B-CLL patients

Data and samples from patients were collected at the Clinical Hematology Department of the CHU Montpellier, France, after patients’ written consent and following French regulations. Patients were enrolled in two independent clinical programs approved by the “Comités de Protection des Personnes Sud Méditerranée I”: ref 1324 and HEMODIAG_2020 (ID-RCB: 2011-A00924-37). Samples were collected at diagnosis and kept by the CHU Montpellier [11, 28]. For analysis, peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors from the CHU Montpellier. Prof. John de Vos is the responsible of the “Collection du Centre de Resources Biologiques du CHU de Montpellier” – http://www.chu-montpellier.fr/fr/plateformes (Identifiant BIOBANQUES - BB-0033-00031). PBMC and UCB mononuclear cells (UCBMC) were respectively collected from peripheral blood samples and UCB units using Histopaque -1077 (Sigma). Briefly, 13 mL Histopaque was added to 50 mL centrifugation tubes and 30 mL of 1/2 diluted blood in RPMI (Invitrogen) was slowly added at the top. Tubes were centrifuged at 400 rcf for 30 min at 20 °C without brake. Mononuclear cells were collected from the interlayer white ring, washed in RPMI and suspended in RPMI medium supplemented with 10% FBS (Invitrogen).

Isolation and activation of human NK cells

Frozen UCBMCs were depleted of T cells by using EasySep™ CD3 Positive Selection Kit (STEMCELL technologies). Cells were cultured for 10 to 20 days with γ-irradiated PLH cells at 1:1 NK cell:accessory cell ratio in the presence of IL-2 (100 U/ml) and IL-15 (5 ng/mL), or with IL-2 alone (1000 U/ml). PLH cells were added every four days and fresh cytokines every two days. At the end of the process, NK cell purity (CD56+/CD3-) was always higher than 90%.

FACS analysis

For phenotype analysis, cells were stained with 7AAD (Beckman) to identify viable cells and antibodies against the surface markers CD25-FITC, CD45RO-FITC, CD69-PE, CD62L-PE, CD19-PE, CD3-PE, CD19-PECy7, CD56-APC, CD3-APC, CD45-APCAlexaFluor750, CD45RA-APC-AlexaFluor750, CD16-PacificBlue, CD57-PacificBlue, CD45-KromeOrange, CD16-KromeOrange (Beckman), CD158b-FITC, CD158a-PE, CD107a-HV500 (BD Biosciences), CD158e-Vioblue (Miltenyi). 1×10^5–3×10^5 cells were incubated for 20-30 min at 4 °C with different antibodies in PBS containing 2.5% FBS. Cells were then washed and suspended in 200-250 µL of the same media. Staining was analyzed on a Gallios flow cytometer (Beckman) using the Kaluza software. Alive lymphocytes were gated using FSC/SSC and 7AAD staining. B lymphocytes (CD19+), T lymphocytes (CD3+CD56-) and NK cells (CD56+CD3-) were distinguished using, respectively, CD19, CD3 and CD56 antibodies.
NK cell-mediated cytotoxicity

Fresh or frozen (stored in liquid nitrogen) NK cells were labeled with 3 µM of CellTracker™ Violet BMQC Dye (Life Technologies) and incubated overnight with target cells at different E:T ratios. Subsequently, phosphatidylserine (PS) translocation and membrane damage were analyzed in the violet fluorescence-negative target cell population by flow cytometry using Annexin V-FITC (Immunostep) and 7AAD (BD Biosciences) or propidium iodide (PI) as previously described [25, 29]. We consider all cells positive for annexin-V and/or PI (or 7-ADD) as dead (or dying).

In ADCC experiments, we incubated target cells with the relevant antibodies (RTX and OBZ at 10 µg/mL; daratumumab, cetuximab and trastuzumab at 5 µg/mL) for 30 min at 37 °C. To arm NK cells, we incubated them at the same concentration of antibodies before washing and incubation with target cells. EGTA was used at 1 mM to block the granular exocytosis pathway and MgCl₂ at 1.5 mM to maintain the osmotic pressure.

We used the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to determine cellular viability. We dissolved the crystals and quantified absorption at 550 nm using a spectrophotometer.

In all experiments, we calculated the basal cell death in the absence and presence of the different mAbs. These values were subtracted from those obtained after NK cell or NK cell+mAb treatments to generate the specific natural cytotoxicity or specific ADCC, respectively. All mAbs gave very low levels (<3%) of cytotoxicity in presence of heat-inactivated serum media.

Evaluation of RTX-armed e-NK

e-NK cells (2×10⁶) were incubated for 1 h with 10 µg/mL RTX, washed and incubated with 1:800 solution of a goat F(ab')2 anti-human IgG (H+L) for 30 min at 4 °C. After incubation, NK cells were washed with PBS and RTX binding was analyzed by FACs. As a control, cells were only stained with the goat F(ab')2 anti-human IgG.

NK degranulation assay

Briefly, 50×10³ target cells per well were placed in RPMI, 10% FBS, IL-2 100 U/mL with monensin (BD Biosciences) in a 96-well V-bottom plate. NK and target cells were incubated overnight at 37°C in 5% CO₂ and living cells were counted using a Muse cytometer (Millipore) with the count and viability kit (Millipore). As a control, NK cells were incubated without targets. CD107a⁺ NK cells were analyzed on a Gallios flow cytometer (Beckman Coulter) using 7AAD, CD45RO-FITC, CD19-PE, CD56-PECy7, CD3-APC, CD45RA-AlexaFluor750, CD16-KromeOrange and CD107a-HV500 (BD Biosciences). Results were analyzed using Kaluza software.

In vivo experiments

In vivo experiments were carried out using 6–8-week-old male NOD scid gamma (NSG) mice. Mice were bred and housed in pathogen-free conditions in the animal facility of the European Institute of Oncology–Italian Foundation for Cancer Research (FIRC), Institute of Molecular Oncology (Milan, Italy). For engraftment of human cells, mice were subcutaneously engrafted with 5×10⁶ BCL-P2 or 10×10⁶ LNH1 primary tumor cells derived from a B-cell lymphoma (BCL) patient (BCL P2) or a diffuse large B-cell lymphoma (DLBCL) patient (LNH1). At day 4, we engrafted 15 (BCL-P2) or 10 (LNH1) million e-NK cells and at day 6, mice were treated i.p. with RTX (in saline medium) 3 mg/kg once a week for 3 weeks; or with a combination of both treatments e-NK and RTX. Tumor growth was monitored at least once a week using a digital caliper, and tumor volume was calculated according to the formula: L × W²/2 (mm³), where W represents the width and L the length of the tumor mass.

Statistical analysis

Experimental figures and statistical analysis were performed using GraphPad Prism (v6.0). All statistical values are presented as * p<0.05; ** p<0.01; *** p<0.001 and **** p<0.0001. Mean values are expressed as mean plus or minus the standard error of the mean (SEM).

Results

Costimulation with the EBV lymphoblastoid PLH cell line more efficiently expands UCB NK cells for clinical use than IL-2 stimulation

Cytokines and encounter with target cells induce dissimilar gene expression on NK cells [24]. We used umbilical cord blood (UCB) cells and compared two NK cell activation/expansion protocols: one using a high dose of the cytokine IL-2 (1000 U/mL) and the other using costimulation. The costimulation protocol was performed with the EBV cell line PLH together with low concentrations of IL-2 (100 U/mL) and IL-15 (5 ng/mL) [9]. NK cell expansion is jeopardized by T cells, therefore we depleted them from UCB before expansion. NK cell cultures underwent massive cell death at day 12 in IL-2-driven
expansion (data not shown). So, we compared different parameters that reflect NK activity at day 10:

Proliferation. Costimulation-driven expansion was considerably more efficient (Figure 1A).

Cytotoxicity. IL-2-driven expansion led to NK cells with superior natural cytotoxicity against K562 (Figure 1B), Daudi (Figure S1A) and primary CD20+ B cell lymphoma cells (BCL P2; Figure 1C). Moreover, these NK cells also showed higher ADCC activity with RTX (Figure 1C and Figure S1A). However, natural cytotoxicity gradually increased in the costimulation protocol when cells were activated for longer periods of time (Figure 1D). This correlated with a notable large-scale expansion of cells (median ± SD, IL-2 10 d (16.8 ± 22.2), costimulation 10 d (140.5 ± 235.8) and costimulation 20 d (260.9 ± 141.2), n=10).

Activation markers. Both protocols increased the expression of the activation marker CD69 (Figure S2B) and decreased CD45RA expression to that of CD45RA^{dim} cells (Figure 2B). This was associated with an increase in the activation marker CD45RO, as previously published [10]. Costimulation maintained higher CD16 expression (Figure S2B).

Exhaustion markers. We investigated the expression of two markers that could suppress NK cell-mediated cytotoxicity: TIM-3 [30] and PD-1 [31]. While both protocols did not affect their expression, the mean fluorescence intensity (MFI) of positive populations tended to increase (Figure S2B). This probably reflects that, after long activation, some NK cells become exhausted.

Maturation and homing markers. UCB NK cells showed a low percentage of CD62L^{+} cells (18.1% ± 6.7%, n=3) that increased 10 d after IL-2 treatment (68.2% ± 13.5%) and costimulation (56.7% ± 11.9%). However, at day 20 post-costimulation, CD62L expression was lost (1.6% ± 0.4). IL-2-stimulated cells did not survive this long; so, we could not measure CD62L levels. CD62L is a "homing receptor" facilitating naive lymphocytes to enter secondary lymphoid tissues. Mature NK cells express low CD62L, which favors their peripheral trafficking [32].

In agreement with others [33], few naive UCB NK cells expressed CD57 (1.2% ± 1.3%, n=3). IL-2 stimulation barely increased expression (7.3% ± 3.9%) and costimulation did not change it (0.6% ± 0.6). Longer costimulation, i.e., 20 days, also had no effect (1.6% ± 0.5%). The lack of CD57 expression did not impair NK cell cytolytic activity (see below).

In summary, costimulation led to higher numbers of activated and functional NK cells with higher CD16 expression. This prompted us to only use costimulation for the next experiments. On the other hand, IL-2 induced higher and faster cytotoxicity and could be the best option for autologous NK cell grafts.

Frozen/thawed NK cells keep their cytolytic activity

For clinical purposes, it would be advantageous to have a bank of cryopreserved expanded NK cells ready to use [34]. Compared with fresh expanded NK cells, frozen/thawed NK cells lost roughly 35% of CD16 expression and 50% of their cytolytic activity (Figure 1E). As shown in Figure 1D, 20 day-activation showed higher cytolytic activity than shorter expansions. For the next experiments, we used 20-21 days costimulation-induced expansion of UCB-derived NK cells containing more than 90% of NK cells that were kept frozen until use. Hereafter, we call them e-NK.

e-NK cells mediate ADCC against target cells with diverse CD20 levels

We observed that e-NK performed ADCC similarly on Raji and Daudi cells, which express high CD20 levels (Figure S2A), as on primary tumor cells, which express low levels (Figure S2B). Even though P2 cells probably express more CD20 than P148, they were slightly less sensitive to RTX-mediated ADCC. In fact, e-NK performed ADCC even if their natural cytotoxicity against some patient cells was low or absent (Figure 2A and Figure S2B-E). Hence, e-NK show strong ADCC with RTX independently of their natural cytotoxicity and with lower variability (Figure 2A-B). The glycoengineered mAb OBZ [4, 35] induced higher ADCC than RTX (Figure 2C and Figure S2B-C).

e-NK cells can be “armed” with mAbs to facilitate treatment

We next used e-NK cells coated with anti-CD20 mAb (“armed” e-NK cells) as an alternative to antibody-coated target cells. “CD20-armed” e-NK showed similar results to opsonizing tumor cells with anti-CD20 (Figure S3A). The presence of RTX after e-NK “arming” was visualized by using a fluorescent anti-IgG (Figure S3B). “OBZ-armed” e-NK also efficiently generated ADCC (Figure S4).

Statistical analysis of several e-NK productions on cells from 5 CLL patients did not show any differences between opsonizing targets or “arming” e-NK (Figure 3A). Moreover, the analysis of these 4 e-NK expansions on the CLL targets showed that all productions could be armed (Figure 3B). Combining all these results statistically showed that significant ADCC was mediated by e-NK (Figure 3C).

Cytotoxicity requires degranulation and cell interaction by ICAM

Primary human NK cell cytotoxicity is largely independent of degranulation [36] and resides in
death receptor binding on tumor cells by ligands expressed by NK cells. In agreement with this, e-NK natural cytotoxicity was only partially diminished by the degranulation inhibitor EGTA (Figure 4A), which in contrast, largely blocked ADCC.

Figure 1. Optimization of NK cell expansion protocol. (A) Comparison of costimulation (PLH accessory cells + IL-2 100 U/mL + IL-15 5 ng/mL) and IL-2 (1000 U/mL) expansion protocols using three UCB donors (2903, 3464, 2928). (B) K562 cells were incubated overnight with costimulation- (circles) or IL-2-activated (triangles) NK cells from two different donors at different effector:target (E:T) ratios. Cell death was analyzed by 7-AAD staining. (C) BCL Patient 2 cells were incubated overnight with costimulation- or IL-2-activated NK cells from three different donors, in the presence (black symbols) or absence (white symbols) of RTX (10 µg/mL). (D) NK cells from 2 donors were expanded by costimulation for different days. At these days, NK cells were frozen. They were thawed at the same time and tested for cytotoxicity against the cell lines. (E) NK cells from 2 donors were expanded for 20 days and frozen or kept fresh before testing their cytotoxicity.
The interaction of NK cell-expressed LFA-1 with its target cell ligand ICAM modulates NK cell cytotoxicity [37]. Blocking this interaction with the D1D2 peptide [27] partly reduced natural cytotoxicity and almost completely abolished ADCC (Figure 4B). Therefore, our e-NK use similar mechanisms for eliminating target cells as primary human cells.

**e-NK mediate ADCC with daratumumab**

Next we tested if e-NK produced ADCC with the anti-CD38 daratumumab [38]. We used 3 target cells that express CD38 (MM.1S, MV4-11 and BCL-P2; Figure S5A) and observed that three different e-NK preparations showed ADCC with daratumumab (Figure S5B-D). In contrast, daratumumab failed to induce ADCC against U266 that are negative for CD38 (Figure S5A, E). Several e-NK productions efficiently performed ADCC with daratumumab on MM.1S and P2 that was statistically significant (Figure 4C).

**e-NK mediate ADCC with cetuximab**

Next, we analyzed if e-NK cells mediate ADCC with other mAbs used to treat solid tumors. We used the cell lines Calu-1 and A549. Calu-1 cells express more epidermal growth factor receptor (EGFR) than A549 (data not shown). Both lines are targets of the anti-EGFR cetuximab, which has been proposed for use with adoptively-transferred expanded allogeneic NK cells in clinical trials for cervical cancer [39]. In fact, in vitro and clinical data suggest that cetuximab mediates ADCC through NK cells [23, 34]. We observed a relatively large variation in the natural cytotoxicity of the different e-NK donors versus these target cells. The decrease in cell viability, as measured by MTT formation, was low (Figure S6A). However, the increase in cell death, measured by annexin-V / 7-ADD staining, was higher. This showed that e-NK had induced the initial steps of apoptosis (annexin-V staining), but longer times were required to evaluate cell viability with MTT. Cetuximab increased early apoptosis and accelerated the process of cell death, decreasing viability. Several e-NK productions efficiently performed ADCC with cetuximab on CALU-1 and A549 that was statistically significant (Figure 5A).

EGTA diminished ADCC but insignificantly (Figure S6B-C). This suggested that the mechanism of action only partly involved degranulation, indicating a possible participation of death ligand-induced apoptosis.
Figure 3. Anti-CD20-armed e-NK show ADCC activity. PBMCs from CLL samples were incubated for 1 h with 10 µg/mL of RTX and overnight with donor e-NK cells at a 3:1 E:T ratio (antibody-coated target cells condition). Alternatively, e-NK cells were incubated for 1 h with 10 µg/mL of RTX before incubating them overnight with target cells (antibody-armed NK cell condition). (A) The bars represent cell death of each individual CLL sample to 4/5 e-NK preparations. (B) The bars represent cell death of each individual e-NK preparation to 4/5 CLL samples. B-CLL cell death was analyzed by 7-AAD. Graphics represent mean ± SEM. Significance was determined by paired t-test; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 and **** p ≤ 0.0001.
Figure 4. ADCC requires degranulation and LFA-1/ICAM interaction. (A-B) Daudi cells were incubated overnight with e-NK cells from two different donors and/or RTX (10 µg/mL) as described in Figure 2. Cytotoxic assays were performed also in the presence of 1 mM EGTA (A) or 15 µg/mL D1D2 protein (B). Cell death was analyzed by 7-AAD staining. (C) e-NK produced ADCC with daratumumab. Three different e-NK cell productions were tested against the CD38+ cell line MM.1S and BCL-P2 cells that express CD38. Target cells were pre-incubated for 1 h with 5 µg/mL daratumumab before overnight incubation at different E:T ratios with e-NK. Cell death was analyzed by 7-AAD staining. Graphics represent mean ± SEM. Significance was determined by paired t-test; * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
e-NK mediate ADCC with trastuzumab

We next tested the anti-HER2 mAb trastuzumab on SK-BR-3 cells, which express high HER2, and A549 cells, which express low levels. Under this condition, e-NK performed ADCC in both cell lines by decreasing viability or increasing apoptosis (Figure S7A). Natural cytotoxicity, as well as ADCC, heavily depended on degranulation because EGTA largely decreased both (Figure S7A-B). Statistical analysis of several e-NK productions on SK-BR-3 and A549
showed that cell viability was significantly reduced and apoptosis tended to increase, although this was not statistically significant. This suggested that e-NK efficiently performed ADCC with trastuzumab (Figure 5B). However, the increase in apoptosis was not statistically significant. Finally, we extended this study to the ovarian cell line SK-OV-3 that was resistant to both natural cytotoxicity and ADCC during short treatment (data not shown). A 6-day treatment revealed that NK cells, mainly with trastuzumab, efficiently killed these cells (Figure 5C).

e-NK mediated ADCC in vivo

We next evaluated e-NK activity in vivo by engrafting primary tumor cells from a B-cell lymphoma (BCL) patient (P2) or from a diffuse large B-cell lymphoma (DLBCL) patient (LNH1) into NSG mice. NSG mice have a complete null mutation by knockout of the γ chain of the interleukin 2 receptor (Il2γ), which is a common component of the cell surface receptors for several cytokines, including IL-2 and IL-15. Therefore, the signaling pathways for these cytokines are blocked in Il2γ knockout mice. They should lack functional NK cells, which require IL15 signaling to develop. Four days later, mice were engrafted with e-NK and, 2 days later, treated with RTX; the latter decreased tumor growth (Figure 6A), showing that RTX possesses direct, non-effector functions independently of NK cell-mediated effects. While e-NK also decreased tumor growth (Figure 6A), co-treatment was more effective, protecting all mice from BCL cells and 4 out of 5 mice from DLBCL cells.

e-NK cells showed ADCC against chemoresistant cells

EBV-activated NK cells overcome anti-apoptotic mechanisms active in chemoresistant cells [25, 26]. Overexpression of BCL-XL and MCL1 are common features of several hematological cancers [40]. Jurkat

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**Figure 6.** e-NK show ADCC in vivo and overcome mechanisms of drug resistance. (A) 5 NSG mice/group were subcutaneously engrafted with 5×10⁶ BCLP2 (left) or 10×10⁶ LNH1 (right) cells and treated with e-NK and/or RTX. (B) e-NK cell-induced ADCC overcome anti-apoptotic mechanisms of drug resistance. CD20⁺ MEC-1 cells overexpressing BCL-XL and MCL1 were incubated with RTX (10 µg/mL). After 1 h, e-NK cells from 3 different donors were added overnight. Cell death was analyzed by 7AAD/annexin-V labeling. Graphics represent mean ± SEM. Significance was determined by paired t-test: * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
cells over-expressing BCL-X<sub>L</sub> are resistant to doxorubicin and to soluble TRAIL [41]. MCL1 over-expression protects them from ibrutinib cytotoxicity [42]. e-NK killed the CD20<sup>+</sup> B-CLL cell line MEC-1 overexpressing BCL-X<sub>L</sub> or MCL1, neither of which conferred protection against ADCC compared to wild type cells (Figure S8). Several e-NK productions kill chemoresistant cell lines to statistically significant levels (Figure 6B). However, BCL-X<sub>L</sub> overexpression significantly decreases ADCC at high E:T ratios. This suggests that chemoresistance could partially protect tumor cells from e-NK-mediated ADCC, but not from e-NK natural cytotoxicity.

**Discussion**

Clinical mAbs fail to improve prognosis in a large number of patients. This could be due to the impairment of NK cells observed in most cancer patients [3, 16, 18]. Therefore, restoring this immune function should improve mAb clinical benefits. We focused on developing a protocol to obtain NK cells in sufficient number and with high ADCC activity together with different therapeutic mAbs. From UCB-derived NK, we produced e-NK that only partially lost ADCC function after cryopreservation (Figure 1) and preserved ADCC in vivo (Figure 6). e-NK do not require relatively high Ag levels to perform ADCC, since they were effective with different cell lines expressing variable Ag levels.

The coupling of mAbs and e-NK should synergize in several clinical contexts. First, e-NKs should bypass NK dysfunction by increasing mAb-induced ADCC in patients with immune defects. Second, the clinical activity of NK cells is uncertain in solid cancers [16, 18]. Probably these effectors scarcely recognize solid tumor targets in vivo and/or fail to infiltrate these tumors—e.g., NK have been detected in the tumor stroma but not within the tumor lesion in some cases [43-45]. Moreover, the adoptive transfer of autologous NK cells in patients as single therapy maintained high levels of circulating NK cells but did not mediate tumor regression [3, 18, 46]. mAbs should recruit e-NK to the selected targets and can also facilitate target elimination by favoring the recognition of opsonized cells. In fact, haploidentical NK cells combined with anti-GD2 mAb therapy has shown promising antitumor activity in pediatric recurrent/refractory neuroblastoma [47, 48]. Third, e-NKs overcome anti-apoptotic mechanisms active in leukemic cells (Figure 6B and [25]), allowing elimination of tumor cells from patients with poor prognosis [26].

e-NK could also have anti-tumor activity per se. First, high numbers of tumor-infiltrating NK correlates with a better prognosis in some tumors [16]. Second, NK are the first lymphocytes to recover after HSCT including after umbilical cord blood transplantation (UCBT). The speed of recovery correlates with the prognosis [18]. In spite of these findings, NK cell adoptive immunotherapy has provided clinical benefit. Perhaps current expansion protocols fail to produce enough NK cells to support clinical success or generate cells with impaired activity [16]. An inconvenience of engraftment of allogeneic expanded NK cell is their low survival in vivo [49]. The persistence of ex vivo haploidentical IL-2-activated and expanded NK-DLIs reaches a maximum of 7 days in lymphoma patients [50]. This leaves grafted NK cells little time to eliminate their targets. The advantage is that NK will be less likely to generate the clinical problems found with CAR-T cells, which produce some chronic effects related to their long-term persistence ([51]; http://www.medscape.com/viewarticle/876591). One of the main concerns in using allogeneic immune cells is the incidence of GVHD. Allogeneic NK cells infusion is well tolerated in cancer patients [3, 18] and the severity of aGVHD correlates with impaired reconstitution of the NK cell compartment [52]. To our knowledge, engraftment of NK cells has been linked to GVHD only when combined to HLA-matched, T-cell-depleted nonmyeloablative peripheral blood stem cell transplantation [53]. NK cells likely contributed to GVHD in this setting by augmenting underlying T-cell alloreactivity [53].

An interesting alternative to allogeneic NK is KIR/KIRL blocking antibodies that activate endogenous NK cells [54]. This approach has the inconvenience that cancer patient NK cells are hyporeactive [3, 16, 18], suggesting that they are too inefficient to totally eliminate tumors. Moreover, recent clinical data suggest that such antibodies modify the endogenous NK repertoire. This would make KIR-expressing NK cells, which are those with higher cytolytic activity, hyporeactive [55]. This raises concerns about the clinical use of these antibodies. There are other ways to activate endogenous NK cells, such as the use of lenalidomide (LEN) [7, 8]. Preliminary results from the Phase Ib/II clinical trial GALEN suggest that LEN could facilitate OBZ-mediated NK cell activation [8], as was observed with RTX [56].

In collaboration with the University Hospital of Montpellier, we wish to test the clinical efficiency of e-NK in lymphoma patients resistant to standard treatments, including RTX.

**Abbreviations**

ADCC: antibody-dependent cell-mediated cytotoxicity; AML: acute myeloid leukemia; B-CLL:
B-cell chronic lymphocytic leukemia; B-NHL: B-cell non-Hodgkin’s lymphoma; BCL: B-cell lymphoma; DLBCL: Diffuse large B-cell lymphoma; EBV: Epstein-Barr virus; EGFR: epidermal growth factor receptor; FL: follicular lymphoma; GMP: good manufacturing practices; GvHD: graft-versus-host disease; HSC: hematopoietic stem cell transplantation; LEN: lenalidomide; NCRs: natural cytotoxicity receptors; NK cells: natural killer cells; OBZ: obinutuzumab; PFS: progression-free survival; RTX: rituximab; UCBL: umbilical cord blood; UCBT: umbilical cord blood transplantation; e-NK: expanded NK cells; mAbs: monoclonal antibodies.

Supplementary Material

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Competing Interests
The authors have declared that no competing interest exists.

References
CHAPTER 8.

The PDK1 Inhibitor Dichloroacetate Controls Cholesterol Homeostasis Through the ERK5/MEF2 Pathway

(published article – Scientific Reports)

[In this study that I shared co-authorship, I was involved in planning and execution of the experiments, including prepare and maintain cell culture and primary cells used in the study]
Elevated level of low-density lipoprotein (LDL) in blood is a predominant risk factor for atherosclerosis, a large cause of mortality. Control of plasma cholesterol levels largely depends on low-density lipoprotein receptor (LDLR), which mediates the endocytosis of cholesterol-rich LDL. This process takes place mainly in the liver. LDL is degraded in lysosomes and cholesterol made available for repression of microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting step in cholesterol synthesis. Hence, LDLR regulates intracellular and extracellular cholesterol homeostasis and is involved in atherosclerosis due to accumulation of LDL-cholesterol in blood.

Lipid and carbohydrate metabolic pathways are interconnected and targeting the latter may result in altered cholesterol levels. The pyruvate dehydrogenase (PDH) kinase (PDK) inhibitor dichloroacetate (DCA) activates PDH, the rate-limiting enzyme of aerobic glucose oxidation. PDH converts glycolysis-produced pyruvate in acetyl-CoA that enters the mitochondria and is consumed in the process of oxidative phosphorylation (OXPHOS). Thus, DCA inhibits glycolysis and lactate production and induces OXPHOS. DCA concentration in DCA-treated patients is unclear because its half-life is less than 1 hour and it is not detectable in patients during the initial phase of treatment that can last 2 to 3 months. However, DCA inhibits its own metabolism.
and serum concentrations increased, eventually reaching a plateau, with plasma concentrations around 0.3 mM\(^{15}\). Michelakis et al. gave 50 mg/Kg/day of DCA to patients and obtained similar values: 0.44 ± 0.16 mM\(^{16}\). DCA treatment decreases plasma triglyceride and cholesterol in animal models and humans\(^{17,18}\). This effect is likely indirect due to the decrease in plasma of very-low-density lipoproteins (VLDL) by stimulation of triglyceride oxidation\(^{17,19}\), although the mechanism remains unknown. After these findings there was an attempt to use DCA to treat two cases of familial hypercholesterolemia (FH)\(^{15}\). DCA reduced circulating cholesterol levels in both patients by decreasing LDL cholesterol\(^{15}\). Unfortunately, DCA had to be discontinued following neuropathological effects and this precluded its use to treat high cholesterol levels\(^{11,12}\). The biological mechanism underlying DCA effects on cholesterol was unknown and, to the best of our knowledge, no further studies were conducted to investigate it. Nowadays, the most accepted explanation would be that the change in carbohydrate metabolism alters lipid metabolism. In this sense, DCA, by inhibiting glycolysis, activates AMPK\(^{20}\), which inhibits cholesterol synthesis\(^{21,22}\) leading to LDLR expression\(^{16,19}\). Enhanced LDLR expression is mediated by a MAPKK because the MAPKK inhibitors PD98059\(^{23}\) and U0126\(^{20,21}\) restrain it. This would indicate that DCA has a similar mechanism of action than the alkaloid berberine and its analogs\(^{20,24}\). An alternative explanation could be that DCA induces ROS production\(^{5,10}\) and the cellular oxidative state regulates LDLR expression\(^{22}\). Hence, changes in ROS could mediate DCA-induced LDLR expression.

In preliminary experiments, we found by transcriptome analysis that LDLR was one of the downregulated genes in hematopoietic cells expressing a small hairpin RNA for ERK5 (shERK5) and one of the most upregulated genes in hematopoietic cells (Fig. 1A). In contrast, in all cell lines but not in others\(^{32–24}\), we first observed that DCA increased LDLR mRNA in hematopoietic cells (Fig. 1A, left panel). Since liver is the main organ that takes up LDL-cholesterol from blood, we tested the effect of DCA in two hepatic cell lines, finding that LDLR mRNA levels were also increased (Fig. 1A, right panel). Augmented LDLR mRNA levels correlated with an increase of LDLR protein in the plasma membrane (Fig. 1B and Supplemental Fig. 1A). We then tested the functional consequence of this enhanced expression by incubating control or DCA-treated cells with fluorescently labeled LDL. DCA increased LDL transport into these cell lines (Fig. 1C and Supplemental Fig. 1B).

**Results**

**DCA enhances LDLR expression.** We first observed that DCA increased LDLR mRNA in hematopoietic cells (Fig. 1A, left panel). Since liver is the main organ that takes up LDL-cholesterol from blood, we tested the effect of DCA in two hepatic cell lines, finding that LDLR mRNA levels were also increased (Fig. 1A, right panel). Augmented LDLR mRNA levels correlated with an increase of LDLR protein in the plasma membrane (Fig. 1B and Supplemental Fig. 1A). We then tested the functional consequence of this enhanced expression by incubating control or DCA-treated cells with fluorescently labeled LDL. DCA increased LDL transport into these cell lines (Fig. 1C and Supplemental Fig. 1B).

**Cells performing OXPHOS increase LDLR activity.** DCA induces OXPHOS in leukemic cells\(^{8–10,24,25,31}\). To investigate whether the metabolic switch from aerobic glycolysis to OXPHOS mediated by DCA affect LDLR expression, we used a glucose-free culture medium containing a final glutamine concentration of 4 mM and 10 mM galactose. Glutamine is used to drive mitochondria to utilize OXPHOS and galactose allows cells to synthesize nucleic acids through the pentose phosphate pathway\(^{24,32–34}\). We called it ‘OXPHOS medium’ because it forced leukemic cells to use OXPHOS as primary ATP source\(^{8,21}\). We observed that acute myeloid leukemia (AML) OCI-AML3 cells growing in OXPHOS medium for 2 weeks, such as those treated with DCA, presented an increase of ERK5 and LDLR mRNA (Supplemental Fig. 2A), LDLR protein and LDL intake (Fig. 1D). These results indicated that, as expected, the effect of DCA on LDLR expression was due to a metabolic switch. DCA and OXPHOS also increased LDLR mRNA and protein as well as LDL intake in primary lymphoma cells derived from a B cell lymphoma patient (BCL-P2; Fig. 1A, Supplemental Fig. 1B and Fig. 1E). We found similar results in the hepatic cell lines HepG2-C3A and HuH7, with OXPHOS media increasing LDLR protein and uptake (Supplemental Fig. 3).

In primary human hepatocytes, DCA also induced LDLR expression at 6 and 24 h post treatment (Fig. 2A). However, effects disappeared at 48 and 72 h with a net decrease (Fig. 2A). This is likely due to the short DCA half-life, since LDLR mRNA levels were kept high when fresh DCA was added to the medium every 24 h (Fig. 2B).

**Reactive Oxygen Species (ROS) do not mediate DCA-induced LDLR expression.** The cellular oxidative state can regulate LDLR expression\(^{23}\) and DCA induces ROS production in certain hematopoietic cell lines but not in others\(^{6,10}\). We observed a similar phenomenon in two different hepatic cell lines. In HuH7 cells there was an increase of ROS, but not in HepG2-C3A cells, after DCA treatment (Fig. 3A). In contrast, in all cell lines utilized in this study, DCA increased LDLR expression, suggesting that ROS production was not essential (Figs 1A and 3B). Next, we incubated both hepatic cell lines with the antioxidant N-acetyl-cysteine (NAC). NAC efficiently blocked DCA-induced ROS production in HuH7 cells (Fig. 3A); however, it did not affect DCA-induced LDLR mRNA (Fig. 3B) or plasma membrane protein (Fig. 3C). To definitively exclude that ROS played any role in LDLR induction, we incubated primary hepatocytes with DCA in presence or absence of NAC. We found that NAC did not inhibit and in fact increased LDLR mRNA expression (Fig. 3D). These results exclude a major role for de novo ROS production in LDLR expression after DCA treatment.

**DCA induces LDLR in vivo.** We next assessed whether DCA enhances LDLR expression in vivo. We engrafted human AML primary cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID)-interleukin-2 receptor-γ chain null (NSG) mice, as previously described\(^{8,9}\). Mice with established tumors (day 80 post-graft) were treated daily with DCA (Fig. 4A). The treatment was not toxic and did not show any
Figure 1. OXPHOS induced LDLR expression and LDL uptake. (A) The hematopoietic cell lines Jurkat and OCI-AML3 and primary cells from a BCL patient (BCL-P2) as well as HepG2-C3A and Huh7 hepatic cell lines were treated with 10 mM DCA for 24 h and LDLR mRNA was analyzed by RT-qPCR. (B) Cell lines were treated for 72 h with 5 mM DCA and LDLR protein in plasma membrane was analyzed by FACs. (C) Cells were treated as in (B) and fluorescent LDL intake analyzed by FACs. (D) OCI-AML3 cells were grown in OXPHOS medium for 2 weeks and LDLR expression (left) and LDL intake (right) were analyzed by FACs. (E) BCL-P2 cells were treated with 5 mM DCA for 1 week (left) or were grown in OXPHOS medium for 2 weeks (center) and LDLR protein in plasma membrane analyzed by FACs. LDL intake (right) was analyzed in cells growing in OXPHOS. The bar graphs represent means ± SD of 3 independent experiments performed in triplicate; *p < 0.05, **p < 0.01, ***p < 0.005 student t-test compared to control cells.
notable effect on mice survival\(^\text{1}\). Human tumor AML cells gather in murine spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers and observed that DCA significantly increased expression of LDLR mRNA (Fig. 4A).

We also found augmented mouse LDLR mRNA levels in normal liver and spleen from wt mice that were treated daily, for 1 and up to 3 days, with DCA (Fig. 4B). The effect was first observed in hematopoietic cells

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**Figure 2.** DCA induced LDLR expression in primary human hepatocytes. (A) Primary hepatocytes were treated with the indicated concentrations of DCA for the indicated times. (B) Cells were treated at time 0 with DCA and some were treated every 24 h before harvesting as indicated. LDLR mRNA was analyzed by RT-qPCR. The bar graphs represent means ± SD of 3 independent donors performed in triplicate; *p < 0.05, **p < 0.01, ***p < 0.005 one-way ANOVA with post-hoc Tukey test.
Figure 3. Increase in ROS levels was not required for LDLR expression. (A) Both hepatic cell lines were treated with 2 mM NAC 1 h before adding DCA (10 mM) for 24 h. Cells were labeled with CH-H2DCFDA and analyzed by FACS for ROS production. LDLR mRNA (B) or protein (C) from these cells were analyzed as described in Fig. 1. Results represent the means ± SD of these donors with experiments performed in triplicate. The data represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.005 student t-test compared to cells non treated with DCA. D) Primary hepatocytes from 2 independent donors were treated for 6 and 24 h as in (A) but with two different DCA concentrations before analyzing LDLR mRNA expression. The data represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.005 one-way ANOVA with post-hoc Tukey test.

gathering in spleen and, later, in liver. Thus, DCA induced LDLR expression in multiple cell populations in vivo. This could, at least partially, explain the reduction in plasma cholesterol levels after DCA treatment in several species including humans.5-15,34.
We further investigated the underlying mechanism of DCA-induced LDLR expression and the role of the ERK5/MEF2 pathway, which is activated by DCA\(^8\),\(^10\),\(^23\),\(^24\). To this end, we targeted ERK5 utilizing a small hairpin RNA (shERK5). Reducing ERK5 expression resulted in decreased LDLR mRNA levels in non-treated hematopoietic cells (Fig. 5A). We could not investigate DCA effects on cells expressing shERK5 because ERK5 is essential to perform OXPHOS and hence DCA is highly toxic in cells with reduced ERK5 levels\(^8\),\(^10\),\(^23\),\(^24\). Conversely, overexpression of ERK5 increased LDLR mRNA levels (Fig. 5A). As shown in Supplemental Fig. 4A, transfection with shERK5 or ERK5 expressing vectors efficiently decreased and increased ERK5 protein levels respectively. Decreasing ERK5 levels with small interference RNA for ERK5 (siERK5) also impaired LDLR expression in primary hepatocytes (Fig. 5B) or in HuH7 hepatic cells (Supplemental Fig. 5A), in which we observed a reduction of 40% on ERK5 protein levels (Supplemental Fig. 4B). Overexpression of ERK5 in Jurkat cells augmented LDLR protein and enhanced LDL uptake (Fig. 5C). The MAPKK MEK5 activates ERK5 in different physiological contexts\(^8\). Thus, we next used the MEK5 inhibitor BIX02189, which inhibits its catalytic function, and showed that it decreased LDLR protein and LDL uptake in Jurkat and OCI-AML3 cells (Fig. 5D). We validated these findings in primary tumor cells derived from a BCL patient (Supplemental Fig. 6). Treatment of these cells with an extremely selective ERK5 inhibitor, XMD8–92, resulted in the reduction of LDLR protein (Supplemental Fig. 6). Taken together, these results indicate that ERK5 is essential for LDLR expression and function in multiple cell lines.

**Figure 4.** DCA induced LDLR expression *in vivo.* (A) NSG mice were engrafted with primary human AML cells. At day 80 post-graft, they were treated with DCA (n = 4) or leave untreated (n = 4). At day 140, mRNA from bone marrow or spleen was isolated and human LDLR mRNA expression was quantified by qPCR. The data represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.005 student t-test compared to non treated mice. (B) B6 wt mice (n = 4/5 per group) were treated with a dose of DCA (50 mg/kg) everyday intraperitoneally and mouse LDLR mRNA was analyzed in spleen and liver at indicated time points. The data represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.005 one-way ANOVA with post-hoc Tukey test.
**Figure 5.** ERK5 controlled LDLR expression and LDL uptake. (A) $10^7$ Jurkat-TAg cells were transfected with 5 µg of the empty pSUPER Neo vector (control) or with this vector containing a small hairpin RNA for ERK5 (shERK5) or with a pcDNA vector expressing ERK5 (ERK5). Forty-eight hours later mRNA expression of the whole population was analyzed by qPCR and represented as the % of mRNA compared to cells transfected with the empty vector. (B) Primary hepatocytes were transfected with control siRNA (control) or with siRNA against ERK5 (siERK5). 96 h later mRNA was collected and ERK5 and LDLR mRNA expression was analyzed by qPCR. (C) Jurkat cells were transfected with ERK5 as described in (A) and LDLR plasma membrane protein (left) and LDL intake (right) were analyzed by FACs. D) Jurkat (left and center) and OCI-AML3 (right) cells were treated with 5 µM of the MEK5 inhibitor BIX02189 for 24 h and LDLR protein (left) or LDL intake (center and right) were analyzed by FACs. Bar graphs represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.005 student t-test compared to empty vector transfected cells (control).
**AMPK does not mediate DCA-induced LDLR expression.** DCA induces AMPK activation, the main cellular metabolic sensor\[^6\]. AMPK, by blocking de novo cholesterol production\[^4\], \[^5\], could mediate LDLR expression as it has been observed with berberine and its analogs\[^8\], \[^9\]. The effect of berberine on LDLR expression was inhibited by PD98059\[^4\] and U0126\[^6\], \[^7\], In fact, a PD98059\[^4\] and U0126\[^6\], \[^7\], sensible pathway mediated berberine effect. Although these MAPK inhibitors were initially described as specific MEK1 inhibitors, they also inhibit the ERK5 upstream kinase MEK5\[^4\]. This indicates that DCA may have a similar mechanism of action than berberine. To test this hypothesis, we used metformin, which stimulates AMPK in Jurkat and OCI-AML cells\[^6\]. Surprisingly, metformin did not increase, but rather decreased, LDLR mRNA levels (Fig. 6A), protein levels (Fig. 6B) and LDL intake (Fig. 6C) in two hematopoietic cell lines. Metformin also decreased LDL uptake in HEPG2-C3A cells (Fig. 6D). Moreover, blocking expression of the catalytic subunit of AMPK, AMPK\[^\alpha\], with two different siRNA that effectively decrease AMPK\[^\alpha\] levels\[^6\], did not statistically decrease LDLR mRNA. In summary, these data indicated that the AMPK pathway did not modulate DCA-induced LDLR expression and suggested that we uncovered a totally new pathway that controlled LDLR expression.

**LDLR expression requires MEF2.** ERK5 mediates part of its metabolic functions through the MEF2 family of transcription factors\[^8\], \[^10\], \[^21\], \[^24\]. Interestingly, LDLR promoter contains predicted binding sites for MEF2A and C that have been validated in several cell lines (http://genome.ucsc.edu/). Therefore, we performed a knockdown of MEF2A and C in OCI-AML3 cells by siRNA. These siRNA halved mRNA expression of both transcription factors (Fig. 7A) and reduced between 25 and 50% protein levels (Supplemental Fig. 4C). The level of knockdown was sufficient to significantly decrease LDLR mRNA levels (Fig. 7A). Finally, to further investigate whether DCA activated LDLR promoter, we checked the Histone H3 lysine 27 acetylation (H3K27Ac), a modification associated with active gene expression\[^9\]. We observed that H3K27Ac increased after DCA treatment indicating an increase of LDLR gene transcription (Fig. 7B).

**The ERK5/MEF2 pathway also controls expression of the LDL receptor-adapter protein 1 (LDLRAP1).** The LDL receptor-adapter protein 1 (LDLRAP1) is a cytosolic protein that interacts with the cytoplasmic tail of LDLR\[^7\]. LDLRAP1 promoter contains MEF2 binding sites (http://genome.ucsc.edu/), suggesting that the same ERK5/MEF2 pathway could regulate this gene. Consequently with this hypothesis, DCA enhanced LDLRAP1 expression in hepatic cell lines and primary hepatocytes (Fig. 8A). OCI-AML3 and primary tumor B cells also increased LDLRAP1 mRNA after DCA treatment or after incubation in OXPHOS medium (Fig. 8B). In non-stimulated cells, siERK5 reduced LDLRAP1 mRNA in primary hepatocytes, HuH7 cells and primary tumor cells (Fig. 8C left), Supplementary Fig. 5C. Similarly, OCI-AML3 cells transfected with siMEF2 repressed the expression of LDLRAP1 mRNA (Fig. 8C right). In summary, cells performing OXPHOS increased the expression of an additional protein involved in LDLR activity.

**Discussion**

Carbohydrate and lipid metabolism are intrinsically bound and their dysfunction play a major role in cardiovascular disease. Diabetes is typically associated to dyslipidemia, but vice versa, lipid changes also disturb glucose metabolism\[^41\]. DCA, by stimulating PDH activity, decreases glucose catabolism and stimulates OXPHOS\[^2\]. To fuel it, cells could rely on fatty acid oxidation (FAO), suggesting that DCA could increase lipid catabolism. LDL particles transport cholesterol and triglycerides; hence an increase in LDLR should allow cells to increase fat availability. We propose that the avidity for fatty acids induces DCA-treated cells to increase LDLR expression and, subsequently, cholesterol uptake. This is supported by our observations showing that OXPHOS, which mediates FAO, reproduces DCA effects on LDLR expression in vitro (Fig. 1 and Supplemental Figs 2 and 3). The mechanism requires ERK5, which directs the choice of catabolic substrates\[^10\], \[^28\] and, hence, is a good candidate to control increase on fat avidity. Inhibiting ERK5 function by pharmacological or genetic means decrease LDLR intake (Fig. 5). This reduces the consumption of exogenous cholesterol but should also affect the consumption of fatty acids. In contrast, in vivo ERK5 stimulation, e.g.; by DCA treatment, induces LDLR mRNA (Fig. 4) and would induce triglyceride oxidation as previously observed\[^5\], \[^14\].

The ERK5 target MEF2 also regulates this pathway, likely because LDLR promoter contains MEF2 binding sites (see Introduction). ERK5 induces activation of several members of the MEF2 family of transcription factors by several mechanisms. It induces direct phosphorylation at different serines and threonines on MEF2A, C and D\[^27\], \[^32\]. It can also activate MEF2A and D by direct interaction because ERK5 serves as a MEF2 coactivator through its signal-dependent direct association with the MEF2 MADS domain. Although, at least MEF2A-dependent transcription requires ERK5 kinase activity\[^29\], \[^30\]. MEF2 mediates several ERK5 effects on metabolism\[^33\]. However, the exact mechanism via which DCA stimulates LDLR expression could be multifactorial, although it is a general phenomenon that we have confirmed in several hematopoietic, colon and hepatic cells (Fig. 1), including primary human hepatocytes (Fig. 2), which are the main regulators of cholesterol levels\[^6\], \[^7\].

We cannot exclude that other mechanisms play a role in DCA-induced LDLR expression. For example, DCA inhibits HMG CoA reductase activity in liver and leukocytes\[^6\]. This could lead to an even higher demand on exogenous cholesterol and subsequently to an increase in LDLR levels. In addition, we have also recently shown that the MAPK ERK5 induces Sirt1 expression\[^4\] that also stabilizes LDLR protein\[^2\]. Therefore, ERK5 could target LDLR function in multiple ways and some of them independently of MEF2 family. In contrast, we have mainly excluded that ROS levels or AMPK activation play a major role in this process (Figs 3 and 6).

Other drugs could modulate the ERK5/MEF2 pathway to regulate the expression of LDLR. Berberine induces LDLR expression by a MAPK pathway sensitive to PD98059\[^4\] and U0126\[^6\], \[^7\], two inhibitors that block the ERK5 pathway\[^5\]. Then, ERK5 could also partly mediate berberine effects, although independently of AMPK (Fig. 6).

DCA decreases cholesterol plasma levels in several animal models and humans\[^3\], \[^13\], \[^14\]. In this study, we have mainly used high (10 mM) DCA concentrations for acute responses and “physiological” concentrations (1 to
5 mM) for chronic treatments. These latter values are in the range of those used in DCA-treated patients\textsuperscript{11,12}. Michelakis et al. gave 50 mg/Kg/day of DCA to patients\textsuperscript{12}. On average, this amount of DCA should give a blood concentration of 4.6 mM, i.e. by considering 70 Kg/patient and a total of 5 L of blood. However, DCA plasma concentration was around 0.4 mM\textsuperscript{11,12} and the ultimate destination of the DCA that was not in blood was unknown.

Figure 6. AMPK did not regulate DCA-induced LDLR expression and LDL uptake. Two different hematopoietic cell lines were treated with 5 mM metformin for 24 h and LDLR mRNA (A), protein (B) and LDL uptake (C) were analyzed. (D) HepG2-C3A cells were treated as in (A) and LDL uptake was measured. (E) HCT116 cells were transfected with 2 small interference RNA (siRNA) for AMPK\textalpha or with control siRNA and treated with 20 mM DCA for 6 h before mRNA analysis.
Perhaps the liver processes DCA very fast as suggested by our results using primary hepatocytes (Fig. 2). There was an attempt to use DCA for treating hypercholesterolemia using similar DCA doses to ours in vivo. DCA reduced circulating cholesterol levels in two patients through a mechanism involving a reduction in LDL cholesterol, although both patients initially showed low LDLR surface activity. However, DCA was halted due to its neuropathological effects and this precluded its use to treat high cholesterol. These pathological effects have been observed in other clinical contexts, e.g. lactic acidosis and stroke-like episodes (MELAS). At the beginning of their DCA treatment, patients quickly eliminate DCA, and during this phase neuropathy appears. From a pharmacological point of view, it would be desirable to use drugs with the LDLR-stimulating effect of DCA, but without its neuropathological effects. The uncovering of ERK5/MEF2 pathway as a regulator of LDLR expression opens an interesting pharmacological possibility.

**Figure 7.** DCA required the transcription factor MEF2 to target LDLR promoter. (A) OCI-AML3 cells were transfected with 40 nM siRNA control or with 20 nM siRNA for each MEF2A and MEF2C (siMEF2). Twenty-four hours later cells were incubated for 24 h with 10 mM DCA. mRNA expression was analyzed by qPCR and represented as the % of mRNA compared to cells transfected with the empty vector. (B) OCI-AML3 cells were incubated for 72 h with 10 mM DCA. Cells were prepared for ChIP analysis using an antibody against H3 acetylation on lysine 27. Acetylation was revealed at different points of the LDLR promoter by using specific oligonucleotides. Bar graphs represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.005 student t-test compared to empty vector transfected cells (control).
Figure 8. DCA induced LDLRAP1 expression. (A) Two hepatic cell lines or primary hepatocytes were treated with DCA as in Figs 1 and 2 and LDLRAP1 mRNA was analyzed. (B) OCI-AML3 cells (left) and primary cells from a BCL patient (BCL-P2; right) were treated with 5 mM DCA or grown in OXPHOS medium for 2 weeks and LDLRAP1 mRNA was measured. (C) Primary hepatocytes were transfected as in Fig. 5 and expression of LDLRAP1 was analyzed by q-PCR. The bar graphs represent means ± SD of 3 independent experiments performed in triplicate; *p < 0.05, **p < 0.01, ***p < 0.005 student t-test or one-way ANOVA with post-hoc Tukey test.
Methods

Ethical statement. Experimental procedures were conducted according to the European guidelines for animal welfare (2010/63/EU). Protocols were approved by the Animal Care and Use Committee “Languedoc-Roussillon” (approval number: CEEA-LR-12163). The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient prior to surgery.

In vivo mouse experiments. In vivo experiments were carried out using 6 to 8 weeks/old male NSG mice. Mice were bred and housed in pathogen-free conditions in the animal facility of the European Institute of Oncology–Italian Foundation for Cancer Research (IFRC), Institute of Molecular Oncology (Milan, Italy). For engraftment of human cells, 1 million AML cells were injected intravenously (i.v.) through the lateral tail vein in non-irradiated mice. NSG mice with established human AML tumors (day 80 post-graft) were treated with DCA (50 mg/kg, 1 dose/day by gavage, starting at day 1 for 16 consecutive days). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to visualize expression of human LDLR mRNA. In a different experiment B6 wt mice were treated with a daily single dose of DCA (50 mg/kg/day) intraperitoneally and mouse LDLR mRNA was analyzed in spleen and liver at different time points.

Cell lines and culture conditions. The leukemic human cell lines T Jurkat TAg and OCI-AML3 were grown in RPMI 1640–Glutamax ( Gibco) supplemented with 5% (Jurkat) or 10% (OCI-AML3) FBS †44. Primary cells from a lymphoma B cell patient (BCL-P2) were grown in the same medium with 10% FBS. In certain experiments cells were grown in RPMI 1640 without glucose (Gibco 11879) with the addition of 2 mM glutamine and 10 mM galactose (OXPHOS medium). The Jurkat TAg cells carry the SV40 large T Ag to facilitate cell transfection. HepG2-C3A and HuH7 cells were grown in MEM and DMEM respectively supplemented with FBS, sodium pyruvate, glutamine, penicillin and streptomycin. The HCT116 human colon cancer cells were cultured in low glucose (5 mM) DMEM medium supplemented with 10% FBS. Cellular confluence during experiments was between 80–85%.

Human liver samples and preparation of PHHs cultures. Liver samples were obtained from liver resections performed in adult patients for medical reasons. Human hepatocytes isolation and culture were performed as described previously †44. Briefly, after liver perfusion, hepatocytes were counted and cell viability was assessed by trypan blue exclusion test. A suspension of 1 × 10⁶ cells/mL per well was added in 12-well plates pre-coated with type I collagen (Beckton Dickinson) and cells were allowed to attach for 12 h. Then the supernatant containing dead cells and debris was carefully removed and replaced with 1 mL of serum-free long-term culture medium (Lanford medium, LNF). The number of confluent attached cells was estimated at ~1.5 × 10⁵ cells/cm².

Reagents and antibodies. DCA was from Santa Cruz Technologies. Galactose and glutamine were from Gibco. Human anti-LDLR-PE and IgG were from BD Biosciences and 7AAD from Beckman. The MEK5 inhibitor BIX02189 and the ERK5 inhibitor XMD8–92 were from Selleck. RIPA buffer to prepare protein extracts was from GIBCO. Human anti-LDLR-PE and IgG were from BD Biosciences and 7AAD from Beckman. The MEK5 inhibitor BIX02189 and the ERK5 inhibitor XMD8–92 were from Selleck. RIPA buffer to prepare protein extracts was from GIBCO. Human anti-LDLR-PE and IgG were from BD Biosciences and 7AAD from Beckman. The MEK5 inhibitor BIX02189 and the ERK5 inhibitor XMD8–92 were from Selleck. RIPA buffer to prepare protein extracts was from GIBCO.

Transient transfection. Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation †44. In each experiment, cells were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at RT with the DNA mix and electroporated using the Gene Pulser Xcell™ Electroporation system (Bio-Rad) at 260 mV, 960 mF in 400 µl of RPMI 1640. Expression of the different proteins was confirmed by western blot. The transfection efficiency in Jurkat TAg cells is between 60 and 80%. OC-AML-3 cells were transfected using Amaxa D-Nucleofector™ Lonza Kit according to manufactured protocol. In HuH7 and HCT116 cells, transfection of 30–50 nM siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen), according to the manufacturer’s instructions. Primary hepatocytes were transfected twice at days 1 and 3 post-seeding. Cells were harvested 48 to 96 h post-transfection.

Plasmids. The expression vectors for ERK5, the pSUPER expression vector for GFP alone or GFP plus shERK5 and the pSiren-retroQ-puro (BD Biosciences) retroviral vectors for shERK5 and control have been previously described †45. Control, MEF2A and C and ERK5 siRNA were ON-TARGETplus SMARTpools (mixture of 4 siRNA) were from Dharmacon.

Counting and determination of cell viability. Cell number, viability and cell death was analyzed with the Muse Cell Analyzer (Millipore) by incubating cells with Muse Count & Viability and Annexin V and Dead Cell kits respectively, following manufacturer’s instructions.

ROS measurement. Cells lines were plated at 300,000 cells/mL and treated with DCA for the indicated times, harvested and counted to perform further analysis. To evaluate ROS levels, we labeled cells with CellROX® Deep Red Reagent or with CH-H2DCFDA (Life Technologies) for 30 minutes and analyzed them by FACs following manufacturer’s instructions.

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RT-PCR and DNA sequencing. Total RNA was extracted using NucleoSpin RNA isolation columns (Macherey-Nagel), reverse transcription was carried out using iScript™cDNA Synthesis Kit (Biorad). Quantitative PCR was performed with KAPA SYBR Green qPCR SuperMix (Clinscience) and a CFX Connect™ Real-Time qPCR machine (Biorad) with LDLR, LDLRAP1, ERK5 and actin primers (Supplemented Fig. 7). All samples were normalized to β-actin mRNA levels. Results are expressed relative to control values set at 100.

Immunoblotting. Protein analysis by immunoblotting was performed essentially as previously described 45. Briefly, samples were collected, washed out with PBS and lysed with RIPA buffer. Protein concentration was determined by BCA assay (Pierce) before electrophoresis in 4–15% TGX gels (BioRad) and equal amount of protein was loaded in each well. Protein transfer was performed in TransTurbo system (BioRad) in PVDF membranes. After blocking for 1 h with 5% non-fat milk, membranes were incubated overnight at 4 °C in agitation with primary antibodies, washed three times with PBS-Tween 0.1% and incubated with the appropriate HRP-labeled secondary antibody for 1 h. Membranes were washed out three times with PBS-Tween 0.1% and developed with Substrat HRP Immobilon Western (Millipore). Band quantification was performed using the “ImageLab” software from BioRad and represented as the ratio between the protein of interest and a control protein i.e. actin. The value of 1 is arbitrarily given to control cells. One blot representative of several experiments is shown.

LDL Intake. After treatment cells were incubated with BODIPY FL LDL (Invitrogen) in PBS with 2% FBS and incubated at 37 °C for 30 min. Cells were then washed and suspended in 200–250 µL PBS 2% FBS and analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

Flow Cytometry. Briefly, 1 × 10^6 cells were stained with antibody in PBS with 2% FBS and incubated at 37 °C for 30 min. Cells were then washed and suspended in 200–250 µL PBS 2% FBS and staining was analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

ChIP analysis. OCI-AML3 cells were treated with 10 mM DCA for 72 h. Ten million cells were centrifuged (5 min; 1200 rpm) and the pellet was washed twice in 1X phosphate-buffered saline (PBS) at room temperature and suspended in 10 mL of 1X PBS. Cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences) at room temperature for 5 min. Fixation was lysed in 1 ml of cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP40, Na Butyrate 10 mM + 2X protease inhibitor cocktail (Halt™ Protease Inhibitor Cocktail, EDTA-Free (100X), Thermofischer)) at 0 °C for 10 min. Nuclei were recovered by centrifugation (10 min, 1200 rpm) and the pellet was washed two times in 1X phosphate-buffered saline (PBS) at room temperature for 5 min. Fixation was lysed in 1 ml of cell lysis buffer (50 mM Tris-HCl pH 7.5, 1% SDS, 10 mM EDTA, Na Butyrate 10 mM + Halt™ Protease Inhibitor Cocktail (3X)) at 4 °C for at least 2 hours. 250 µl of each sample were then sonicated 2 times for 5 min (30 s on/off) at 4°C using a Bioruptor (Diagenode). After sonication, absorbances at 280 nm (A280) of 1/100 diluted samples were measured and A280nm and was adjusted to 0.133 with nuclei lysis buffer. One hundred microliter were used for ChIP experiments in a final volume of 1 mL. Samples were incubated under gentle agitation at 4 °C overnight in the presence of 1 µg of either a specific antibody or a negative control. Antibodies (anti-K27Ac Ab4729 (Abcam) and negative control IgG (Diagenode)) were previously bound to DYNA Beads Protein G Novex (Life Technology) according to the supplier’s recommendations. Dynabeads-bound immunoprecipitates were sequentially washed once with a low salt buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% triton, 0.1% SDS, 1 mM EDTA, 1 mM Na Butyrate + Halt™ Protease Inhibitor Cocktail (1X)), a high-salt buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1% triton, 0.1% SDS, 1 mM EDTA, 1 mM Na Butyrate) and a LiCl-containing buffer (20 mM Tris-HCl pH 7.5, 250 mM LiCl, 1% NP40, 1% Na deoxycholate, 1 mM EDTA, 1 mM Na Butyrate) and, then, with a TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, Tween 20 0.02%). Samples were then eluted in 250 µL of elution buffer (100 mM NaHCO3, 1% SDS), and DNA-protein complexes were incubated at 65 °C for 5 hours to reverse crosslinks. Samples were then treated with 100 mg/ml proteinase K and 100 mg/ml RNase A at 45 °C for 2 hours to digest proteins and contaminating RNA. DNA was purified with an extraction kit (NucleoSpin Gel and PCR clean-up, Macherey-Nagel) according to the manufacturer’s recommendations and qPCR analysis was performed using the Roche LightCycler 480 real-time PCR system. The data were normalized with inputs taken from samples before the immunoprecipitation and treated under the same conditions. The primers used to amplify various regions of LDLR gene promoter.

Statistical analysis. The statistical analysis of the difference between means of paired samples was performed using the paired t test. Analysis of multiple comparisons with single control was performed using one-way ANOVA with post-hoc tukey test. The results are given as the confidence interval (*p < 0.05, **p < 0.01, ***p < 0.005). All the experiments described in the figures with a quantitative analysis have been performed at least three times in duplicate. Other experiments were performed three times with similar results.

Data availability. All data are available upon request.

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Author Contributions

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CHAPTER 9.

Changes in metabolism affect expression of ABC transporters through ERK5 and depending on p53 status

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[In this study that I shared co-authorship, I was involved in planning and execution of the experiments, including prepae and maintain cell culture and primary cells used in the study, prepare cells performing OXPHOS]
Changes in metabolism affect expression of ABC transporters through ERK5 and depending on p53 status

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ABSTRACT

Changes in metabolism require the efflux and influx of a diverse variety of metabolites. The ABC superfamily of transporters regulates the exchange of hundreds of substrates through the impermeable cell membrane. We show here that a metabolic switch to oxidative phosphorylation (OXPHOS), either by treating cells with dichloroacetate (DCA) or by changing the available substrates, reduced expression of ABCB1, ABCC1, ABCC5 and ABCG2 in wild-type p53-expressing cells. This metabolic change reduced histone changes associated to active promoters. Notably, DCA also inhibited expression of these genes in two animal models in vivo. In contrast, OXPHOS increased the expression of the same transporters in mutated (mut) or null p53-expressing cells. ABC transporters control the export of drugs from cancer cells and render tumors resistant to chemotherapy, playing an important role in multiple drug resistance (MDR). Wtp53 cells forced to perform OXPHOS showed impaired drug clearance. In contrast mutp53 cells increased drug clearance when performing OXPHOS. ABC transporter promoters contain binding sites for the transcription factors MEF2, NRF1 and NRF2 that are targets of the MAPK ERK5. OXPHOS induced expression of the MAPK ERK5. Decreasing ERK5 levels in wtp53 cells increased ABC expression whereas it inhibited expression in mutp53 cells. Our results showed that the ERK5/MEF2 pathway controlled ABC expression depending on p53 status.

INTRODUCTION

The main purpose of cell metabolism is the conversion of metabolic substrates to energy (catabolism) or to build blocks for generating new molecules (anabolism). Metabolism requires the intake of substrates and the outtake of metabolites through the highly impermeable cell plasma membrane and multiple transporters with differences in specificity have been described. One of the biggest and better-conserved
families of transporters is the ATP-binding cassette (ABC transporters [1, 2]). The ABC superfamily has 48 members that have been classified into 7 groups (subfamilies A to G). One of their main biological functions is the transport of lipids [3], essential molecules for cell metabolism. Although it is conceivable that changes in metabolism regulate ABC expression, this remains uninvestigated.

ABC transporters are well known for their role in multiple drug resistance (MDR) because many of them export anticancer drugs and render tumors resistant to chemotherapy [4]. The tumor suppressor gene p53 is an important regulator of metabolic homeostasis by promoting OXPHOS and inhibiting glycolysis [5]. It induces the expression of different metabolic genes, such as cytochrome c oxidase 2 (SCO2), glutaminase 2 (GLS2), p53 up-regulated modulator of apoptosis (PUMA), glucose transporter 1 and 4 (GLUT1 and GLUT4) and TP53-induced glycolysis and apoptosis regulator (TIGAR) [5-7]. More than half of all human tumors harbor mutations in the p53 gene. Most of these mutations abrogate its DNA binding and transactivation activity [8], but others can provide mutant p53 (mutp53) with gain-of-function (GOF) activity that is dependent on its de novo ability to regulate directly or indirectly gene expression [9, 10]. Furthermore, certain p53 mutants enhance drug resistance in liver cancer cells and B-CELL cells [11, 12].

Several ABC promoters contain consensus p53 binding sequences [4]. The direct regulation of ABC promoters by p53 is controversial and depends on the architecture of the promoter, the nature of p53 (mutant or wild type), the presence of other p53 family members and variations cell- and tissue-specific [4, 13]. We have observed that inducing OXPHOS in leukemic cells sensitizes wt p53-expressing NB4 cells to genotoxic drugs such as doxorubicin and vincristine [7]. In contrast, cells carrying mutp53 were more resistant to these anticancer drugs.

OXPHOS promotes the expression of the MAPK extracellular signal-regulated kinase-5 (ERK5), which regulates the choice of metabolic substrates in hematopoietic cells [14-19]. ERK5 induces activation of several members of the MEF2 family of transcription factors by different mechanisms. It mediates direct serine and threonine phosphorylation of MEF2A, C and D [20, 21]. It activates MEF2A and D by direct interaction because ERK5 serves as a MEF2 coactivator through its signal-dependent direct association with the MEF2 MADS domain [22, 23]. The effect of activated ERK5 on MEF2A-dependent transcription requires ERK5 kinase activity. MEF2 mediates several ERK5 effects on metabolism, including NRF2 activation [15, 16]. This transcription factor and the related NRF1 are master regulators of metabolism that are coordinated with MEF2 family to induce expression of metabolic genes [24]. The promoters, or very proximal regions, of several ABC genes, i.e. ABCB1, ABCB4, ABCB8, ABCB9, ABCB10, ABCB11, ABCC1, ABCC4, ABCC12 and ABCG1, have MEF2A binding sites that have been validated by ChIP (http://genome.ucsc.edu/). Most of these transporters also have NRF1 or NRF2 binding sites, i.e. ABCB8, ABCB9, ABCB10, ABCB11, ABCC1, ABCC3, ABCC5, ABCC4, ABCC10, ABCC12, ABCC13, ABCG1, which have also been validated by ChIP (http://genome.ucsc.edu/). This information is based on ChIP-seq experiments performed by the ENCODE consortium[25]. Hence, we investigated here if metabolism, through ERK5, controls ABC expression and the role of p53 status.

RESULTS

OXPHOS regulated ABC transporters in AML cell lines

We induced OXPHOS either by inhibiting pyruvate dehydrogenase kinase (PDK) with dichloroacetate (DCA; [7, 14, 16, 26-28]) or by incubating cells in the absence of glucose. When glucose is no longer available, cells use alternative energy substrates, such as glutamine (Gln). Gln oxidation, or glutaminolysis, generates ATP through OXPHOS [29, 30] and this pathway is functional in leukemic cells [15, 31]. We called OXPHOS medium a glucose-free medium supplemented with 10 mM galactose and 4 mM Gln [14, 15, 31]. OXPHOS induces expression of both wild type (wt) and mutp53 depending on the cellular context [7]. We used DCA concentrations on the range of that found in plasma of DCA-treated patients [32, 33] and tested the effect on the expression of 4 ABC transporters, ABCB1, ABCC1, ABCC5 and ABCG2, involved in MDR, in 3 AML cell lines with different p53 status: OCI-AML3 cells express wt p53, HL60 are p53 null and NB4 are mutp53 [7]. DCA decreased by half the mRNA of the different ABC transporters on OCI-AML3 cells and this correlated with protein reduction in the 2 examined transporters (Figure 1A). In contrast, in HL-60 and NB4 cells, DCA increased mRNA and protein expression (Figure 1B and 1C).

Consequently with our observations in cell lines, we observed that DCA decreased ABC mRNA and protein expression in primary cells derived from a B-cell lymphoma (BCL) patient with wt p53 (Figure 1D). On the contrary DCA increased expression of ABCC5 and ABCG2 in two mutp53 patients and ABCCI in one patient. In contrast, ABCB1 expression was unchanged (Figure 1E). Due to lack of enough patient samples, we could not analyze protein expression.

Moreover, OCI-AML3 cells growing in OXPHOS medium displayed reduced mRNA expression of the above-mentioned ABC transporters (Figure 2A). Conversely, mutant p53-expressing NB4 cells increased ABC expression in the same medium (Figure 2A). In contrast to DCA, OXPHOS medium did not increase ABC expression in null p53 HL60 cells (Figure 2A).
Figure 1: DCA-induced ABC transporters expression depended on p53 status in leukemic cells. Different hematopoietic cell lines OCI-AML3 wtp53 (A), HL-60 nullp53 (B), NB4 mutp53 (C) and primary cells from BCL-P2 wtp53 patient (D) or B-CLL mutp53 patients were treated with 1 and 5 mM DCA for 1 week and RNA and plasma protein levels were analyzed by qPCR or FACS respectively. Data represent the % of mRNA compared to control cells. The bar graphs represent means ± SD of 3 independent experiments performed in triplicate; *p<0.05, **p<0.01, ***p<0.005 student t-test compare to control cells.
This showed that although DCA and OXPHOS medium could have similar effects, they could also induce different outcomes depending on p53 status. This could be related to the different stimulation periods needed to optimize changes in cell metabolism.

To verify that OXPHOS was affecting transcription of these genes, we assessed histone modifications that have been linked to ABCC1 promoter activation [11, 12] in OCI-AML3 cells. We found that DCA inhibited acetylation on H3 K27 on the ABCC1 promoter (Figure 2: OXPHOS inhibited or stimulated ABC expression in wtp53 or mutp53 cells, respectively. (A) OCI-AML3, NB4 and HL60 cells were grown in OXPHOS medium for 2 weeks and expression of ABC transporters was analyzed as in Figure 1. The bar graphs represent means ± SD of 3 independent experiments performed in triplicate; *p<0.05, **p<0.01, ***p<0.005 student t-test compare to control cells. (B) Cells were treated with 5 mM DCA for 3 days and processed for ChIP analysis. The enrichment of K27 acetylation on Histone 3 in the ABCC1 promoter was analyzed as described in material and methods by using different primers around the transcription start site (TSS).

Figure 2: OXPHOS inhibited or stimulated ABC expression in wtp53 or mutp53 cells, respectively. (A) OCI-AML3, NB4 and HL60 cells were grown in OXPHOS medium for 2 weeks and expression of ABC transporters was analyzed as in Figure 1. The bar graphs represent means ± SD of 3 independent experiments performed in triplicate; *p<0.05, **p<0.01, ***p<0.005 student t-test compare to control cells. (B) Cells were treated with 5 mM DCA for 3 days and processed for ChIP analysis. The enrichment of K27 acetylation on Histone 3 in the ABCC1 promoter was analyzed as described in material and methods by using different primers around the transcription start site (TSS).
We conclude that DCA inhibited transcription of \(ABC\) transporters on wtp53 cells.

**OXPHOS regulated ABC transporters in hepatic cells**

The main detoxifying organ is liver. We tested DCA effect in 2 hepatic cell lines expressing wtp53 (HepG2-C3A) or mutp53 (HuH7). When wtp53 was present, an acute dose of DCA decreased \(ABC\) transporters mRNA and protein (Figure 3A and 3C). In contrary, and similar to mutp53 leukemia cells, DCA increased \(ABC\) mRNA expression in HuH7 cells and also ABCC1 protein (Figure 3B and 3C). Surprisingly, ABCB1 protein decreased after treatment suggesting alternative posttranscriptional regulation. Finally, we examined the effect of OXPHOS on ABC transporters expression in non-transformed cells. Primary hepatocytes from non-cancer patients, which were obviously wtp53, showed the same pattern than cell lines expressing wtp53 (Figure 3D).

**DCA decreased mRNA of ABC transporters in vivo**

We had observed that DCA increased doxorubicin efficacy in vivo on tumor cells expressing wtp53 [7]. We engrafted human wtp53 AML primary cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID)-interleukin-2 receptor \(\gamma\) chain null (NSG) mice, as previously described [7, 16]. Mice with established tumors (day 80 post-graft) were treated daily with DCA. The treatment was not toxic and did not show any notable effect on mice survival [7]. Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs and used human-specific primers. We observed that DCA significantly reduced expression of \(ABC\) transporters mRNA (Figure 4A).

Additionally, we assessed the effect of DCA in normal tissues of non-tumor bearing wt mice. A daily injection of DCA, for 1 and up to 3 days, also reduced mouse \(ABC\) transporters mRNA in liver and spleen (Figure 4B). The effect largely increased with the number of doses received. Hence, DCA reduced \(ABC\) expression in multiple wtp53 cell populations in vivo. This could, at least partially, explain its effect on genotoxic drug treatment in wtp53 tumor cells in vivo [7].

**OXPHOS modulated drug clearance from tumor cells depending on its p53 status**

A direct functional consequence of modulating ABC transporters expression in tumor cells could be changes in their responsiveness to chemotherapeutic drugs. To investigate if the changes in the expression of ABC transporters affect drug clearance, we incubated tumor cells with the genotoxic drug daunorubicin, which is a fluorescent compound. This allows monitoring its intracellular accumulation by FACs. In addition, it is used in the clinic to treat leukemia and lymphoma, which are at the origin of some cell lines that we have used in our study. Finally, like other anthracyclines, it is exported from cells by a broad range of transporters [34]. We used OCI-AML3 cells treated for one week with 1 mM and 5mM DCA or growing in OXPHOS medium. Then, cells were further incubated with daunorubicin and drug clearance was followed by FACs. We found that OXPHOS OCI-AML3 cells were impaired on decreasing drug levels (Figure 5A and 5C). The effect was mainly observed at short time points, but it was still noticeable as much as 24 h after (Figure 5A). Primary wtp53 tumor cells from a BCL patient also showed decreased daunorubicin export (BCL-P2; Figure 5D). As expected, the outcome was opposite in cells with alterations in p53. In tumor cells not expressing p53 (HL-60) or expressing mutp53 (NB4) DCA induced a faster drug clearance (Figure 5E) in agreement with an increase on ABC transporters. Furthermore, blocking expression of wtp53 in OCI-AML3 cells by treating them with a small interference RNA against p53 (sip53), which effectively decreased p53 expression (Supplementary Figure 1), prevented DCA-induced decreased daunorubicin clearing (Figure 5B), whereas a control siRNA did not show any effect (data not shown). Therefore, changes in mRNA and/or protein expression of ABC transporters due to metabolic changes lead to alterations in drug clearance, which depended in p53 status.

To further investigate the role of p53 in ABC transporter expression after metabolic changes, we used two isogenic colon cancer cell lines (p53+/- and p53-/- HCT116 cells) that differently respond to DCA treatment [7]. DCA decreased the \(ABCB1\) and \(ABCC1\) mRNAs without changing those of \(ABCC5\) and \(ABCG2\) (Figure 6A). p53-/- HCT116 cells did not downregulate any \(ABC\) mRNA after DCA treatment (Figure 6B). These results from HCT116 +/- cells suggest that the role of p53 in ABC expression is cell type dependent and depends in multiple factors [4, 13]. The absence of DCA effect on HCT116-/- and of OXPHOS medium in null p53 HL60 cells, suggest that p53 is essential to mediate the effect of metabolism on ABC expression.

**ERK5 was essential for DCA-induced effects**

We further investigated the underlying mechanism of OXPHOS-induced ABC transporters expression. We focused on the role of the ERK5/MEF2 pathway, which is activated in cells performing OXPHOS [14–16, 31]. However, we could not reduce ERK5 levels in cells performing OXPHOS because this kinase is essential for cell survival in this metabolic status [14–16, 31]. We speculated that decreasing ERK5 levels in non-treated
Figure 3: DCA-induced ABC transporters expression depended on p53 status in hepatic cells. (A-B) Hepatic cell lines HepG2-C3A wtp53 and HuH7 mutp53 were treated with 20mM DCA for 24h before analyzing mRNA level as described in Figure 1. (C) Cells were treated with the indicated concentration of DCA for 1 week and protein level was analyzed by FACS. (D) Primary hepatocytes were treated with indicated amount of DCA for 72 hours. The data represent means ± SD (n=3); *p < 0.05, **p < 0.01, ***p < 0.001 Student's t-test compared to control cells or as depicted in the graphic.
cells would have the opposite effect than DCA treatment. Reducing expression of ERK5 with a small interference RNA for ERK5 (siERK5), which reduced ERK5 levels (Supplementary Figure 2A and 2B), decreased and increased ABC transporters mRNA in HuH7 and primary hepatocytes, respectively (Figure 7A and 7B). This suggested that ERK5 effects, like those of DCA, depended on p53 status. To test this hypothesis, we used Jurkat cells that express very low levels of mutp53 [35]. DCA or OXPHOS medium induced a small increase in ABCB1 protein (Supplementary Figure 3). Consequently a shERK5, which encodes for a different sequence that the previous described siRNA and decreased ERK5 expression (Supplementary Figure 2C), decreased mRNA of multiple ABC transporters (Figure 7C). In agreement, the ERK5 inhibitor XMD8-92 or the MEK5 inhibitor BIX02189 also decreased basal ABCB1 protein expression (Figure 7D). Finally, overexpression of ERK5 (Supplementary Figure 2C) increased ABC mRNA expression (Figure 7C).

DISCUSSION

ABC transporters have attracted great attention in recent years due to their role in MDR [4]. But, it should be kept in mind that their main physiological role in eukaryotes is possibly the transport of other substances

![Figure 4: DCA induced ABC transcription in vivo. (A) NSG mice were engrafted with primary human AML cells. At day 80 post-graft, they were treated with DCA (n=4) or leave untreated (n=4). At day 140, mRNA from spleen or bone marrow was isolated and human ABCB1, ABCC1, ABCC5 and ABCG2 mRNA expression was quantified by qPCR. (B) B6 wt mice (n=4/5 per group) were treated with a dose of DCA (50 mg/kg) everyday intraperitoneally and mice mRNA in spleen and liver at different times was obtained to analyze ABC transporters. The data represent means ± SD; *p<0.05, **p<0.01, ***p<0.001 student t-test compare to non treated cells or mice.](https://www.impactjournals.com/oncotarget)
Figure 5: Metabolism-controlled drug outtake depended on p53 status. (A, B, D and E) Different cell lines were treated with 5 mM DCA for 7 days or incubated in OXPHOS medium for 14 days (C). In (B), OCI-AML3 cells were transfected with a siRNA for p53 (sip53) 48 h before treatment. Cells were then incubated with 5 uM daunorubicin and clearance was measured at various time points (10, 30, 60, 120 minutes). Some cells were incubated with 1uM daunorubicin for 24 h. Experiments were repeated at least three times.
out of the cell, e.g. lipid transport [3]. It is somehow intuitive that changes in metabolism regulate the transport of a variety of molecules. We show here that wt-p53 cells, which comprise the vast majority of non-transformed cells, while performing OXPHOS decrease expression of ABC transporters. During OXPHOS, cells use most of the carbon of metabolic substrates to produce energy and CO2. Then, they should avoid releasing metabolites to external media. In contrast, during glycolysis, cells generate a number of intermediate metabolites that can not accumulate and would need to be exported. We hypothesize that this is the physiological interest to increase expression of the exporter ABC transporters during high glycolysis. However, tumor cells take advantage of this fact to acquire MDR. Transformed cells present a metabolism more orientated to anaerobic glycolysis than their non-transformed counterparts, which mainly use respiration/OXPHOS. This should favor tumor MDR by increasing ABC transporters activity in wt-p53 expressing cells. In fact, MDR1-dependent chemoresistant cells show an even more glycolytic metabolism than the sensitive counterparts [36, 37]. Interestingly, hypoxia or changes in glucose levels increase MDR1 expression by hypoxia-inducible factor-1 (HIF-1; [38, 39]). This could partly explain the observation that hypoxic tumor cells are more chemoresistant [13]. These observations could be exploited in the clinic to prevent MDR. Since more glycolytic tumors show higher MDR, inducing a metabolic switch should decrease MDR, although as we prove here the p53 status should be taken into account.

High expression of ABC transporters is often observed in drug-naive tumors compared with the tissue of origin and, as previously described, this correlates with a more glycolytic metabolism. Hence, constitutive MDR1 or MRP over expression is likely regulated in some cells by pathways involved in malignant transformation, e.g. metabolic changes [13]. Metabolic drugs such as DCA could revert tumor metabolism and decrease ABC transporters expression. However, DCA accumulates both wt and mut-p53 protein [7]. If the last induces ABC expression as suggested in several cell lines by our results and in previous works [4, 13], DCA would finally increase MDR. HSP90 inhibitors, e.g. 17-AAG, degrade mut-p53 by favoring its MDM2-mediated ubiquitination [40], and we have shown that DCA cooperates with 17-AAG to kill mut-p53 tumor cells [7]. This could be a possibility to treat mut-p53 tumors. In summary, it is essential to know p53 status before treatment with metabolic drugs as we have previously shown [7].

ERK5 could use different ways to transcriptionally activate ABC promoters, e.g. p53, NF-κB, NRF2, HIF1 and MEF2 [13]. ERK5 inhibition induces p53 activation.

Figure 6: DCA-induced ABC transporters expression required wt-p53. (A and B) Two isogenic colon cancer cell lines (p53+/+ and p53-/- HCT116 cells) were treated with 1 and 5 mM DCA for 1 week and RNA was analyzed by qPCR. Data represent the % of mRNA compared to control cells. The bar graphs represent means ± SD of 3 independent experiments performed in triplicate; *p<0.05, **p<0.01, ***p<0.005 student t-test compare to control cells.
and increases sensitivity to 5-fluorouracil in colon cancer cells but the role of ABC was not investigated [41]. We propose that ERK5 activity by regulating metabolism affects p53 effect in ABC promoters.

Oxidative stress induces expression of ABC transporters, which is likely mediated by NRF2 [42, 43]. ERK5, through MEF2, generates an antioxidant response independently of de novo ROS generation through NRF2 activation [16]. Hence, by activating NRF2, ERK5 could modulate ABC expression.

HIF regulates ABC expression [38, 39] and ERK5 regulates in normoxia an array of genes regulated by HIF1 in hypoxia [44]. In fact, ERK5 by modulating metabolism regulates genes that are regulated by HIF. This could be

Figure 7: The ERK5 pathway regulated ABC transcription. (A) HuH7 cells were transfected with control siRNA or siERK5. Thirty-six hours later mRNA expression was analyzed by qPCR. (B) Primary human hepatocytes were transfected with control siRNA or siERK5 at day 1 and 3 post seeding. 96 h later mRNA levels were assessed. (C) Jurkat cells were transfected with a small hairpin RNA for ERK5 (shERK5) or ERK5 expression vector. 36h later mRNA expression was analyzed by qPCR. (D) Jurkat cells were treated with 10 μM XMD8-92 or 10 μM BIX01298 for 24 h before analyzing expression of ABCB1 by FACS. The data represent means ± SD n=3; *p < 0.05, **p < 0.01, ***p < 0.001 Student's t-test compared to empty vector transfected cells (control).
the case of *ABC* genes. Indeed, NF-κB mediates ABC expression by direct binding to *ABC* promoters [13, 45] or by regulating intermediate mediators. In particular during changes in glucose levels, NF-κB activates HIF1 that targets ABC promoters [39]. Given the fact that ERK5 activates NF-κB [46], this could mediate the effect of metabolism on ABC expression.

As previously described, the promoters of several *ABC* genes studied here, i.e. ABCB1 and ABCC1, have MEF2A binding sites. MEF2 mediates a large part of the observed ERK5 effects on metabolism [15, 16, 47]. Hence, this could also be the case of ABC transporters. The existence of multiple ERK5 targets mediating ABC expression makes it difficult to elucidate their individual relevance. The contribution of each factor could depend, as discussed for p53 [4, 13], on the architecture of the promoter, the level of expression of each target, the presence of other targets and variations cell- and tissue-specific.

ERK5 inhibition in mutp53 cells decreased ABC expression (our results) and should decrease MDR and favors chemotherapy as it has been shown [41], although the mechanism was not described. Our results show that metabolism targets multiple *ABC* promoters, depending on p53 status, which will have a clear effect on drug export. In this context, DCA overcomes resistance to paclitaxel in A549/taxol cells [48]; however, p53 status is unknown in this subline although the parental cell line, i.e. A549, is wtp53. Then DCA could decrease expression of ABC transporters in this study as we have observed here. However, Zhou et al proposed that ABCB1 expression did not change and expression of other ABC members was not analyzed [48]. Authors propose that citric acid accumulation induces apoptosis, but DCA and paclitaxel can also activate alternative pathways leading to cell death. In our case, we have shown that inducing OXPHOS in wtp53 cells cooperates with genotoxic drugs to eliminate tumor cells *in vitro* and *in vivo* [7].

An interesting clinical approach would be to treat patients whose tumors express wtp53 with DCA before standard chemotherapy. We have used here DCA concentrations similar to that used in patients [7, 32, 33]. These concentrations were effective to change ABC expression and then should sensitize tumor cells to chemotherapy as we have observed in mice [7]. In fact a combinatorial therapy involving DCA in preclinical settings have been proposed with several compounds such as metformin [49], Nutlin-3 [50] or paclitaxel [51]. But until our knowledge there is a current lack of clinical results.

The control of ABC transporters by cell metabolism is a physiologic process required to optimize substrate use. Therefore wtp53 cells in proliferative states, which enter aerobic glycolysis, would also increase ABC expression and become more resistant to drug treatment. This could have implications for the treatment of non-cancerous proliferative conditions. For example, in coronary artery stent restenosis and pulmonary artery hypertension (PAH) there is an unwanted proliferation of endothelial cells. Notably, inhibition of ABCC4 prevents and reverses pulmonary hypertension in mice [52] and ABCA3 deficiency is related to PAH of the newborn [53]. Moreover, ABCG2 clears hypoxia-induced intracellular metabolites and protects the heart from pressure overload-induced ventricular dysfunction [54]. Hypoxia-induced aerobic glycolysis could obviously affect the effectiveness of drug treatment in these situations. This could be also the case in other situations requesting high proliferation such as inflammation or wound healing.

In summary, the effects of metabolism on the transcription of ABC transporters clearly depend on p53 status. However, sensitization to chemotherapy by metabolic drugs does not depend only on ABC transcription because pro-apoptotic pathways are also activated that could lead to synergism between metabolic drugs and standard chemotherapy.

**MATERIALS AND METHODS**

**Ethical statement**

Experimental procedures were conducted according to the European guidelines for animal welfare (2010/63/EU). Protocols were approved by the Animal Care and Use Committee “Languedoc-Roussillon” (approval number: CEEA-LR-12163).

**Reagents and antibodies**

DCA was from Santa Cruz Technologies. Galactose and glucose were from Gibco. Human anti-ABCB1, ABCC1 and control IgG1 were from Miltenyi Biotec and 7AAD from Beckman. The ERK5 inhibitor XMD8-92 and the MEK5 inhibitor BIX02189 were from Selleck.

**Plasmids**

The expression vectors for ERK5, the pSUPER expression vector for GFP alone or GFP plus shERK5 have been previously described [46]. Control, ERK5 and p53 siRNA were ON-TARGETplus SMARTpools (mixture of 4 siRNA) from Dharmacon and has been previously used [7, 47]. ERK5 shRNA (AGCTGCCCTGCTCAAGTCT) was transfected in Jurkat cells as previously described [46].

**In vivo mouse experiments**

*In vivo* experiments were carried out using 6 to 8 weeks/old male NSG mice. Mice were bred and housed in pathogen-free conditions in the animal facility of the European Institute of Oncology—Italian Foundation for Cancer Research (FIRC), Institute of Molecular Oncology (Milan, Italy). For engraftment of human cells, 1 million
AML cells were injected intravenously (i.v.) through the lateral tail vein in non-irradiated mice. NSG mice with established human AML tumors (day 80 post-graft) were treated with DCA (50 mg/kg, 1 dose/day by gavage, starting at day 1 for 16 consecutive days). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to visualize expression of human ABC mRNA. In a different experiment B6 wt mice were treated in pathogen-free conditions at the INM animal facility at Montpellier, France, with a daily single dose of DCA (50 mg/kg/day) intraperitoneally and mouse LDLR mRNA was analyzed in spleen and liver after different times.

Cell lines and culture conditions

The leukemic human cell lines, HL-60, NB4, Jurkat TAg and OCI-AML3 were grown in RPMI 1640–Glutamax (GIBCO) supplemented with 5% (Jurkat) or 10% (OCI) FBS [15, 16]. Primary cells from a lymphoma B cell patient (BCL-P2) were grown in the same medium with 10% FBS. In certain experiments cells were grown in RPMI 1640 without glucose (GIBCO 11879) with the addition of 2 mM glutamine and 10 mM galactose (OXPHOS medium). The Jurkat TAg cells carry the SV40 large T Ag to facilitate cell transfection. The hepatic cell lines HepG2-C3A and HuH7 were grown in MEM and DMEM respectively supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine, penicillin and streptomycin. The HCT116 human colon cancer cells were cultured in low glucose (5 mM) DMEM medium supplemented with 10% FBS. Cellular confluence during experiments was between 80-85% for adherent cells.

Human liver samples and preparation of PHHs cultures

Liver samples were obtained from liver resections performed in adult patients for medical reasons (CRB-CHUM - Biological Resource Center of the Montpellier University Hospital, Dr. Jeanne Ramos and Pr. Sylvain Lehmann). The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient prior to surgery. Human hepatocytes isolation and culture were performed as described previously [55]. Briefly, after liver perfusion, hepatocytes were counted and cell viability was assessed by trypan blue exclusion test. A suspension of 1x10⁶ cells/mL per well was added in 12-well plates pre-coated with type I collagen (Beckton Dickinson) and cells were allowed to attach for 12h. Then, the supernatant containing dead cells and debris was carefully removed and replaced with 1 mL of serum-free long-term culture medium (Lanford medium, LNF). The number of confluent attached cells was estimated at ~1.5x10⁶ cells/cm².

Transient transfection

Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation [46, 56]. In each experiment, cells were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at RT with the DNA mix and electroporated using the Gene Pulser Xcell™ Electroporation system (Bio-Rad) at 260 mV, 960 mF in 400 µl of RPMI 1640. Expression of the different proteins was confirmed by western blot. The transfection efficiency in Jurkat TAg cells is between 60 and 80%. In HuH7 cells, transfection of 30–50 nM siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen), according to the manufacturer’s instructions. Primary hepatocytes were transfected twice, at day first and third post-seeding. Cells were harvested 48 to 96 h post-transfection. OCI-AML3 cells (2 x 10⁶ in 100 µl) were transfected with 300nM p53 siRNA, or control siRNA by electroporation using Nucleofactor Electroporation system (Lonzza). Cells were harvested 24 to 72 h post-transfection.

Counting and determination of cell viability

Cell viability and cell numbers were determined using the Muse® Cell Analyzer (Millipore). Number of cells means number of live cells and viability is the number of live cells counted divided by total cell counted (alive and dead cells).

RT-PCR

Total RNA was extracted using NucleoSpin RNA isolation columns (Macherey-Nagel). Reverse transcription was carried out using iScript™ cDNA Synthesis Kit (Biorad). Quantitative PCR was performed with KAPA SYBR Green qPCR SuperMix (Cliniscience) and a CFX Connect™ Real-Time qPCR machine (Biorad) with ABCB1, ABCC1, ABCC5, ABCG2 and actin primers. All samples were normalized to β-actin mRNA levels. Results are expressed relative to control values arbitrarily set at 100. The primers used were:

Mouse primers

ABCB1: Forward: (5'-TTCTCTTTTGTCGCAGGG AGTC-3') Reverse: (5'- GAATGTCTCCAGGCATAA GGC-3'), ABCC5: Forward: (5'-CTAGCTGTCTTTTCA CGGT -3') Reverse: (5'-CCTCTTCAGAGCCACT ATAC-3'), ABCC1: Forward: (5'-CAAAACGGG TGAAAATGGG-3') Reverse: (5'-GTGGGA AGACGAGTTGCTGA-3'), ABCG2: Forward: (5'- CTCTTGCCCAGATAAGAGGG-3') Reverse: (5'-
CCTCAGTTAATTTCCAGGACGACAG-3'), Actin: Forward: (5'-GGGACTGTTAGTGAAGTCGTCG-3') Reverse: (5'-TGTGTTGCTCAACCAAACGCTGTC-3')

Human primers

ABCB1: Forward: (5'-GGAGGCGACACACA-TGCGT-3') Reverse: (5'-AGGCTGTCAACAGACGAC-3'), ABCC5: Forward: (5'-CTTGT-TTTGTGCGAGGCCTC-3'), ABCC1: Forward: (5'-CCGGCTGTGGAGCCTGGAA-3'), GATA2: Forward: (5'-TGGTCTATGATATGCTGTGTC-3') Reverse: (5'-TGGTCTATGATATGCTGTGTC-3'), Actin: Forward: (5'-GGGAGGAAATCCGTCGGGACA-3') Reverse: (5'-AATAGTGATGACCTGGCCCTG-3')

Flow cytometry

Briefly, 1x10^6 cells were stained with antibody in PBS with 2% FBS and incubated at 37°C for 30 min. Cells were then washed and suspended in 200–250 μl PBS 2% FBS and staining was analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

ChIP experiment

OCI-AML3 cells were treated with 10mM DCA for 72 h. Ten million cells were centrifuged (5min; 1200rpm) and the pellet was washed twice in 1X phosphate-buffered saline (PBS) at room temperature and suspended in 10mL of 1X PBS. Cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences) at room temperature for 5 min and lysed in 1 ml of cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP40, Na Butyrate 10mM + 2X protease inhibitor cocktail (Halt™ Protease Inhibitor Cocktail, EDTA-Free (100X), Thermofischer)) at 0°C for 10 min. Nuclei were recovered by centrifugation (10min, 5000rpm) at 4°C and lysed in 250μl nuclei lysis buffer (50 mM Tris- HCl pH 7.5, 1% SDS, 10 mM EDTA, Na Butyrate 10mM + Halt™ Protease Inhibitor Cocktail (3X)) at 4°C for at least 2 hours. 250μl of each sample were then sonicated 2 times for 5 min (30 s on/off) at 4°C using a Bioruptor (Diagenode). After sonication, absorbances at 280 nm (A280) of 1/100 diluted samples were measured and A280nm and was adjusted to 0.133 with nuclei lysis buffer. One hundred microliter were used for ChIP experiments in a final volume of 1 ml. Samples were incubated under gentle agitation at 4°C overnight in the presence of 3μg of either a specific antibody or a negative control. Antibodies (anti-K27Ac Ab4729 (Abcam) and negative control IgG (Diagenode)) were previously bound to DYNA Beads Protein G Novex (Life Technology) according to the supplier’s recommendations. Dynabeads-bound immunoprecipitates were sequentially washed once with a low salt buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% triton, 0.1% SDS, 1 mM EDTA, 1mM Na Butyrate + Halt™ Protease Inhibitor Cocktail (1X)), a high-salt buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1% triton, 0.1% SDS, 1 mM EDTA, 1mM Na Butyrate) and a LiCl-containing buffer (20 mM Tris-HCl pH 7.5, 250 mM LiCl, 1% NP40, 1% Na deoxycholate, 1 mM EDTA, 1mM Na Butyrate) and, then, twice with a TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, TWEEN 20 0.02%). Samples were then eluted in 250μL of elution buffer (100 mM NaHCO3, 1% SDS), and DNA-protein complexes were incubated at 65°C for 5 hours to reverse crosslinks. Samples were then treated with 100mg/ml proteinase K and 100 mg/ml RNase A at 45°C for 2 hours to digest proteins and contaminating RNA. DNA was purified with an extraction kit (NucleoSpin Gel and PCR clean-up, Macherey-Nagel) according to the manufacturer’s recommendations and qPCR analysis was performed using the Roche LightCycler 480 real-time PCR system. The data were normalized with inputs taken from samples before the immunoprecipitation and treated under the same conditions. The primers used to amplify various regions of LDLR gene promoter. Supplementary Table 1 shows the primers used in this experiment.

Daunorubicin accumulation / drug uptake Studies

The intracellular daunorubicin accumulation in cells was examined by flow cytometer [57]. The logarithmically growing cells were cultured in 48-well plates and incubated with or without 5 μM daunorubicin for different times (10’, 30’, 60’, 120’ minutes) and 1 μM for 24 h). After incubation, cells were placed on ice, washed twice with PBS and analyzed by flow cytometry (Beckman-coulter, Elite), excitation 488 nm (argon laser) for the mean fluorescence intensity (MFI) of intracellular daunorubicin. A minimum of 10000 events was analyzed for each histogram.

Statistical analysis

The statistical analysis of the difference between means of paired samples was performed using the paired t test. The results are given as the confidence interval (*: p<0.05, **: p<0.01, ***: p<0.005). All the experiments described in the figures with a quantitative analysis have been performed at least three times in duplicate. Other experiments were performed three times with similar results.

Author contributions

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CONFLICTS OF INTEREST

Authors declare no competing financial interests.

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CHAPTER 10.

Mitochondrial Complex I activity signals antioxidant response through ERK5

(published article – Scientific Reports)

[In this study that I shared co-authorship, I was involved in planning and execution of the experiments, including prepare and maintain cell culture and primary cells used in the study, prepare cells performing OXPHOS]
Mitochondrial Complex I activity signals antioxidant response through ERK5

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Oxidative phosphorylation (OXPHOS) generates ROS as a byproduct of mitochondrial complex I activity. ROS-detoxifying enzymes are made available through the activation of their antioxidant response elements (ARE) in their gene promoters. NRF2 binds to AREs and induces this anti-oxidant response. We show that cells from multiple origins performing OXPHOS induced NRF2 expression and its transcriptional activity. The NRF2 promoter contains MEF2 binding sites and the MAPK ERK5 induced MEF2-dependent NRF2 expression. Blocking OXPHOS in a mouse model decreased Erk5 and Nrf2 expression. Furthermore, fibroblasts derived from patients with mitochondrial disorders also showed low expression of ERK5 and NRF2 mRNAs. Notably, in cells lacking functional mitochondrial complex I activity OXPHOS did not induce ERK5 expression and failed to generate this anti-oxidant response. Complex I activity induces ERK5 expression through fumarate accumulation. Eukaryotic cells have evolved a genetic program to prevent oxidative stress directly linked to OXPHOS and not requiring ROS.

Energy consumption in organisms should be finely regulated to spare resources. The vast majority of eukaryotic cells perform oxidative phosphorylation (OXPHOS), which uses the energy generated by mitochondrial oxidation to produce adenosine triphosphate (ATP). This metabolic pathway is highly efficient in releasing energy but it produces reactive oxygen species (ROS) as a byproduct. ROS are involved in normal cell signaling and homeostasis. However, under stress conditions levels may rapidly increase resulting in cell damage, a process known as oxidative stress. Hence, cells using mitochondria as first energy source must regulate ROS levels. Logically, ROS and mitochondria are functionally linked in several ways. First, ROS in the short-term regulate mitochondrial morphology and function via non-transcriptional pathways. Second, ROS lead to Kelch-like ECH-associated protein 1 (KEAP-1) degradation, thereby activating nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2)3-4, which regulates expression of mitochondrial genes5. In addition, NRF2 controls ROS production by mitochondria1 and mitochondrial function5-6. NRF2 arguably mediates the strongest anti-oxidant cellular response by binding to anti-oxidant response elements (ARE) in gene promoters and, consequently, regulates oxidative stress2-3. On the other hand, mitochondrial activity induced by acute exercise promotes Refl/Nrf2 signaling and increases mitochondrial antioxidant activity and capacity in myocardial and skeletal muscle4-5. Remarkably, restraining OXPHOS in vivo in the liver strongly decreases Nrf2 levels8. Moreover, tumor cells forced to perform

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OXPHOS generate a NRF2-mediated anti-ROS response\(^1\). However, how mitochondria transcriptionally signal the genetic program to block the ROS they produce remains unknown.

NRF2 activation depends on its dissociation from the repressor protein KEAP1 and its subsequent translocation into the nucleus\(^2\). In hematopoietic cells, the MAPK extracellular signal-regulated kinase-5 (ERK5), through the transcription factor MEF2, induces expression of miR-23 that inhibits KEAP-1 mRNA leading to NRF2 activation\(^3\). Several types of oxidative stress activate ERK5\(^4\), notably in leukemic cells\(^5\). In fact, ERK5 is considered a redox MAPK\(^6\). In endothelial cells, steady laminar blood flow (s-flow) activates ERK5 that induces up-regulation of NRF2-dependent gene expression, although the mechanism is not fully elucidated\(^6,7\).

Growing evidence indicates that there are alternative pathways leading to de novo production of NRF2\(^8\). In this context, KEAP-1 inhibition only partially accounts for OXPHOS-induced antioxidative response\(^9\). Chip-seq experiments performed by the ENCODE consortium have shown that the NRF2 promoter contains MEF2 binding sites\(^10\). Moreover, predicted networks of transcription factor interactions in skeletal muscle unveil direct regulation of NRF2 by MEF2\(^11\) and MEF2D binds and activates the Nrf2 promoter\(^12\). Hence, ERK5 could transcriptionally induce NRF2 expression through MEF2, a transcription factor that mediates some of the metabolic effects of ERK5\(^11,13,14,21–23\). In fact, ERK5 regulates the choice of catabolic substrates in hematopoietic cells\(^11,13,14,21–23\), suggesting that is a good candidate to mediate the link between OXPHOS and the antioxidative response. We hypothesize that mitochondrial activity triggers the ERK5 pathway that, through MEF2, induces NRF2 expression and NRF2-mediated antioxidative response. We validate this by showing that mitochondrial complex I activity and fumarate accumulation induce the transcriptional expression of ERK5. ERK5 through MEF2 induces NRF2 de novo expression. Therefore, mitochondrial activity is directly linked to the most important antioxidative response in the absence of de novo increase in ROS levels. This implies that eukaryotic cells have evolved a genetic program to prevent oxidative stress directly linked to OXPHOS and not requiring ROS.

**Results**

**OXPHOS-induced de novo expression of NRF2.** We have previously described that leukemic cells performing OXPHOS generated an anti-oxidant response independently of ROS\(^1\). This response was partially mediated by an ERK5-induced increase in miR-23 that impairs expression of KEAP-1\(^2\). In parallel experiments, we found that NRF2 mRNA was also increased in three hematopoietic cell lines and in primary cells obtained from a B-cell lymphoma (BCL) patient growing in OXPHOS medium (Fig. 1A). This glucose-free culture medium has final concentrations of 4 mM glutamine and 10 mM galactose. Glutamine is used to drive mitochondria to utilize OXPHOS and galactose allows cells to synthesize nucleic acids through the pentose phosphate pathway\(^1,11,14,25,26\). We called it ‘OXPHOS medium’, because it forced leukemic cells to use OXPHOS as primary ATP producer\(^11,24,27\). The PDK1 inhibitor dichloroacetate (DCA), which stimulates OXPHOS in all tested leukemic cells\(^11,13,14,22,27,28\), also increased NRF2 mRNA (Fig. 1A). Both ways to stimulate OXPHOS also induced NRF2 protein (Fig. 1B). The effect of DCA on NRF2 mRNA and protein is reproduced in two hepatic cell lines (Supplemental Fig. 1A) and in a group of primary leukemic cells from 4 patients (Supplemental Fig. 1B). Of relevance, we observed that in primary human hepatocytes DCA also increased ERK5 and NRF2 mRNA as well as that of the NRF2 targets HO-1 and NQO-1 (Fig. 1C). In summary OXPHOS induced expression of NRF2 in multiple cell contexts.

**OXPHOS induced NRF2 translocation to the nucleus.** NRF2 must translocate to the nucleus to activate its target genes and generate the antioxidative response. HuH7 hepatic cells treated with DCA showed NRF2 accumulation in the nucleus (Fig. 2A). These results were reproduced in non-adherent Jurkat cells by western blotting (Fig. 2B) and in the hepatic cell line HepG2C3A (Supplemental Fig. 2A). We observed a total increase in NRF2 that was more predominant in the nuclear fraction. OXPHOS medium also induced NRF2 translocation to the nucleus in Jurkat cells (Supplemental Fig. 2B).

**OXPHOS induced de novo expression of NRF2 in vivo.** To test if enhanced OXPHOS could exert a similar effect on NRF2 expression in vivo, we engrafted AML primary cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID)-interleukin-2 receptor γ null (NSG) mice, as previously described\(^27\). Mice with established tumors (day 80 post-graft) were treated with DCA. The treatment was not toxic and did not show any notable effect on mouse survival\(^27\). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to analyze the expression of the selected mRNAs and found an increase in NRF2 mRNA (Fig. 3A). This increase paralleled that of ERK5 and NQO-1 under similar conditions\(^1\).

DCA also induced mouse Erk5, Nrf2 and Nqo1 mRNA in liver and spleen in a separate experiment in which C57BL/6 wild type mice were treated for different periods of time, 1 to 3 days, with DCA (Fig. 3B). The effect was first observed in spleen and later in liver tissue. Nrf2 was likely active because we observed an increase in its target gene Nqo-1 (Fig. 3B). Hence DCA induced NRF2 expression in multiple cell populations in vitro and in vivo.

**Increase in ROS levels is not essential for NRF2 expression.** The cellular oxidative state can regulate NRF2 expression\(^1\). Therefore, we investigated whether NRF2 expression is regulated by ROS in our setting. DCA induces ROS production in some hematopoietic cell lines, e.g. OCI-AML3, but not all, e.g. Jurkat\(^11,17,27,29\). In contrast, both cell lines increased NRF2 expression suggesting that ROS production was not essential for this induction (Fig. 4). Next, we incubated both cell lines with the antioxidant N-acetyl-cysteine (NAC), which failed to consistently reduce DCA-induced ERK5, NRF2 or NQO-1 mRNA (Fig. 4), although efficiently blocked DCA-induced ROS increase\(^11,29\). We observed similar results in primary leukemic cells from a BCL patient (BCL-P2). DCA does not increase ROS in the hepatic cell line HepG2C3A, but it did in Huh7\(^27\). However, DCA significantly increased ERK5, NRF2 or NQO-1 mRNA in both cell lines and in the presence of NAC (Supplemental Fig. 3). These results excluded a major role of ROS in NRF2 expression after DCA treatment. Normally ROS...
activate NRF2. Unexpectedly in AML cells, there is no relationship between high ROS levels and high nuclear NRF2. Furthermore, the use of NAC, which successfully sequesters endogenous ROS in AML, has no effect on nuclear NRF2 levels. Taken together, this excludes ROS as causing nuclear accumulation of NRF2 in resting human AML cells.

**ERK5/MEF2 controls NRF2 expression.** Next, we investigated the mechanism responsible for mitochondria activity-induced NRF2 expression. Reducing expression of ERK5 with a small hairpin RNA (shERK5)
diminished NRF2 mRNA expression in hematopoietic cells under resting conditions (Fig. 5A). NRF2 protein levels were also reduced after shERK5 transfection (Fig. 5B). We could not treat shERK5-expressing cells with DCA because they die due to lack of appropriate mitochondrial functions and antioxidant response.\textsuperscript{11,13,21-23} Conversely, overexpression of ERK5 increased NRF2 mRNA (Fig. 5A). Reducing expression of ERK5 in primary human hepatocytes (Fig. 5C) and hepatic cell lines, HuH7 and HepG2C3A (Supplemental Fig. 1A,B), with small interference RNA for ERK5 (siERK5) also impaired expression of NRF2 and its target genes NQO-1 and HO-1.

To further study the role of the ERK5/MEF2 pathway in NRF2 expression, we overexpressed several proteins of this pathway. Strong activation of the ERK5 pathway by co-overexpression of a constitutively active mutant of MEK5 (MEK5D), the upstream kinase of ERK5, and ERK5 induced a greater increase in NRF2 mRNA (Supplemental Fig. 4C). In those experiments, only 30–60% of the cells are effectively transfected. To overcome this issue, we use a luciferase reporter plasmid driven by a DNA fragment of 1.5 kb of the human NRF2 promoter\textsuperscript{30}. In this context, cells expressing the reporter plasmid also contain the overexpressed proteins. ERK5

Figure 2. OXPHOS induced NRF2 translocation into the nucleus. (A) Huh7 cells were treated with 10 mM DCA for 48 h and nuclear translocation was revealed by immunofluorescence. (B) Jurkat cells were treated with 10 mM DCA for 48 h and NRF2 nuclear translocation was revealed by subcellular fractionation and western blotting.
significantly activated the reporter and MEK5D increased this effect (Fig. 5D). Expression of a dominant negative form of MEF2C (MEF2C-DN) decreased the effect of ERK5 and MEK5D. This DN construct also diminished basal or DCA-stimulated reporter expression. In contrast, MEF2C overexpression increased both basal

Figure 3. Cells performing OXPHOS induce NRF2 expression in vivo. (A) NSG mice were engrafted with primary human AML cells. At day 80 post-graft, they were treated with DCA (n = 4) or leave untreated (n = 4). At day 140, mRNA from bone marrow or spleen was isolated and the expression of different human mRNA was quantified by qPCR. (B) B6 wt mice (n = 4/5 per group) were treated with a dose of DCA (50 mg/kg) everyday intraperitoneally and mouse Erk5, Nrf2 and Nqo-1 mRNA was analyzed in spleen and liver at different times. The data represent means ± SD; statistics were performed using student t-test (A) or One-way ANOVA with post-hoc Tukey test (B). *p < 0.05, **p < 0.01, ***p < 0.001. Different times posttreatment were compared to non-treated mice (control) if not specified in the graph.
and DCA-induced activity (Fig. 5D). DCA, which induced strong activation, did not show a synergistic, but rather an additive, effect with the activating proteins. These results suggested that ERK5 controls NRF2 expression through MEF2. To test this, we transfected a small interference RNA for MEF2 (siMEF2) in the hepatic cell line HepG2C3A (Supplemental Fig. 4D) and the AML cell line OCI-AML3 (Fig. 5E). This efficiently decreased MEF2 mRNA and also decreased both basal and DCA-induced NRF2 mRNA (Fig. 5E).

**Mitochondrial complex I activity signals ERK5 expression.** The previous experiments had shown that OXPHOS generates a signal that induces ERK5 expression, which contributes to the NRF2-mediated antioxidant response. We confirmed this in vivo by using a transgenic Tet-Off mouse that express a mutant active form of the ATPase Inhibitory Factor 1 (IF1) in hepatocytes to restrain OXPHOS in the liver. Interestingly Santacaterina et al. describe in the Fig. 8C of their MS that liver Nrf2 levels are lower in mice expressing IF1. We confirmed it by analyzing expression of Nrf2 mRNA (Fig. 6A). This correlated with lower expression of Erk5 mRNA as compared to wild type mice (Fig. 6A). This shows that OXPHOS also induces Erk5 mRNA expression in vivo.

We confirmed the essential role of mitochondrial function on ERK5 expression by using fibroblasts derived from patients with strong mitochondrial disorders (Supplemental Table 1). ERK5 and NRF2 mRNA were significantly reduced in these patients (Fig. 6B).

We next focused on the molecular mechanisms underlying our observations. When we inhibited the mitochondrial complex I with metformin, we observed a decrease on ERK5, NRF2 and NQO-1 mRNA and protein expression (Fig. 6C). Both metformin and DCA induce AMPK activation, however, they blocked and induced ERK5 expression, respectively. This suggested that AMPK and its associated metabolic changes were not involved in ERK5 expression. We confirmed this by reducing the expression of the catalytic subunit of AMPK, AMPK$\alpha$, with 2 different siRNA that effectively blocked several AMPK-mediated metabolic changes. This did not affect expression of ERK5 or NRF2 mRNA (Supplemental Fig. 5). Therefore, AMPK activation was not responsible for generating the antioxidant response in cells performing OXPHOS.

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**Figure 4.** Increase in ROS levels is not required for NRF2 expression. OCI-AML and HuH7 cell lines and primary leukemic cells from a BCL patient were treated with 2 mM NAC 1 h before adding DCA (10 mM) for 24 h. mRNA was analyzed as described in Fig. 1. Experiments were done in triplicate and data represent means ± SD; statistics were performed using One-way ANOVA with post-hoc Tukey test; *p < 0.05, **p < 0.01, ***p < 0.001. Treatments were compared to non-treated cells (control) if not specified in the graph.
Figure 5. ERK5 controls NRF2 expression. (A) $10^7$ Jurkat-TAg cells were transfected with 5µg of the empty pSUPER Neo vector or with this vector containing a small hairpin RNA for ERK5 (shERK5) or with a pcDNA vector expressing ERK5. Forty-eight hours later mRNA expression was analyzed by qPCR and represented as the % of mRNA compared to cells transfected with the control vector. (B) Cell transfected with control (Neo) or shERK5 were analyzed for protein expression by western blotting at 24 and 48 h post-transfection. Graphic bars show the NRF2/actin ratio of the depicted experiment. (C) Primary human hepatocytes were double transfected with control siRNA or with siRNA against ERK5 (siERK5). 96 h later mRNA was collected and mRNA expression was analyzed by qPCR. (D) $10^7$ Jurkat-TAg cells were co-transfected with 5µg of the following vectors ERK5 wild type, a constitutively active MEK5 mutant (MEK5D, M5), MEF2C and MEF2C with dominant negative function (MEF2DN) together with 2µg of a luciferase reporter plasmid driven by the NRF2 promoter along with 1µg of β-galactosidase expression vector. Cells were incubated in regular glucose media (gray bars) or containing 10 mM DCA (black bars) 24 h after transfection and analyzed 2 days later for luciferase activity. (E) OCI-AML3 cells were transfected with 1µg of a luciferase reporter plasmid driven by the NRF2 promoter along with 1µg of β-galactosidase expression vector. Cells were incubated in regular media (gray bars) or containing 10 mM DCA (black bars) 24 h after transfection and analyzed 2 days later for luciferase activity.
and β-galactosidase activities. The graph represents the relative luciferase units (RLU). (E) OCI-AML3 cells were transfected with siRNA for MEF2A and C and 24 h later treated with 10 μM DCA for 36 h. NRF2 mRNA and NRF2 protein were analyzed as in (A) and (B) respectively. Experiments were done in triplicate. The data represent means ± SD; statistics were performed using student t-test (C) or One-way ANOVA with post-hoc Tukey test (A, D and E); *p < 0.05, **p < 0.01. ***p < 0.001. Treatments were compared to empty vector transfected cells (control) if not specified in the graph.

In Fig. 6C we showed that complex I inhibition decreased ERK5 mRNA. The electron transport chain complex III removes electrons from ubiquinol (QH$_2$) and sequentially transfer them to cytochrome c. The reduction of ubiquinone (Q) to QH$_2$ could either be due to mitochondrial complex I, which removed electrons from NADH, or mitochondrial complex II, which removed them from succinate and transferred through FAD. Then, we investigated the effect of the complex II inhibitor thenoyltrifluoroacetone (TTFA). This drug strongly induced ERK5 mRNA expression (Fig. 6D). DCA did not increase TTFA effects suggesting that both shared the same target.

TTFA was slightly toxic (Supplemental Fig. 6A) and, like metformin, could have off-target effects. Therefore, we used an array of cell lines with impaired activity of the different mitochondrial complexes (Supplemental table 2), p0 cells that lack mitochondrial DNA and thus a functional ETC, did not induce ERK5 expression after DCA treatment (Fig. 6E, right lower panel), in agreement with our previous results showing that mitochondrial activity induced ERK5 expression (Fig. 6A,B). We next used 3 different cell lines in 2 different mitochondrial backgrounds with defects in mitochondrial complex I and observed that DCA treatment did not induce ERK5 expression (Fig. 6E, left panels). In agreement with Fig. 6D, mutation in mitochondrial complex II did not inhibit DCA-induced ERK5 expression (Fig. 6E, top right panel). Cells with mutations in complex III and V, but not in complex IV, increased ERK5 expression after DCA treatment (Fig. 6D, right panels). However, complex V mutant showed lower basal ERK5 mRNA levels in agreement with in vivo experiments (Fig. 6A). Mutation in the mitochondrial tRNA Ile in the L929 cell line (mB77), which produces more ROS, did not increase ERK5 mRNA (Fig. 6E, upper right panel). This supported our results in Fig. 4 showing that de novo ROS production was not involved in ERK5 expression.

Mitochondria adapt the organization of the different complexes and supercomplexes to optimize the use of the available substrates, mainly regulating the proportion of respiratory complex III superassembled with complex I for electron transport. This is needed to avoid competition between FADH$_2$- and NADH-derived electrons. DCA, by inhibiting PDK1, activates PDH and the formation of acetyl-CoA from pyruvate. This generates 3 NADH per 1 FADH$_2$ (through succinate) molecules in the TCA cycle. Other substrates however, generate a different proportion of NADH/FADH$_2$ electrons and therefore a different demand of CI/CII dependent oxidation. Therefore, while both complexes are always delivering electrons to the ETC simultaneously, the requirement of complex I seems relatively favored by DCA. Complex II, or succinate dehydrogenase (SDH), is also part of the Krebs cycle and catalyzes the conversion of succinate to fumarate. Hence, if complex II is outcompeted by complex I activity, succinate accumulation and fumarate reduction may be induced. Both phenomena are well known in intracellular signaling. Fumarate and succinate, in their acid form, acidify culture media. Hence, we used monomethylsuccinate (MMS) and dimethylfumarate (DMF) to investigate the impact of fumarate and succinate accumulation on ERK5 expression. MMS decreased ERK5 levels (Fig. 7A). In contrast, DMF increased them (Fig. 7A). Next, we used metformin to inhibit complex I, forcing the use of complex II, and added MMS to increase complex II activity. When used together, they decreased even further ERK5 expression (Fig. 7B). This suggested that complex II activity reduced ERK5 expression, probably by inhibiting complex I activity. In summary, whereas succinate probably does not play any role per se on ERK5 expression, fumarate induces its expression. This suggested that DCA, by accumulating fumarate, induces ERK5 mRNA.

Complex I receive electrons from NADH and DCA and deliver them to CoQ. Complex II employs FADH as co-factor to deliver electrons from succinate to CoQ/FADH$_2$. Therefore, the ratio of NADH/FADH$_2$ electrons changes with different substrates and the requirement of complex I for NADH oxidation varies according to the NADH/FADH$_2$ ratio. Oxidative metabolism of one molecule of glucose generates ten NADH and two FADH$_2$, a NADH/FADH$_2$ electron ratio of 5. Fatty acids (FA), e.g. palmitate, generate a ratio of 2. Etoximor inhibits FA transport into the mitochondria and blocks fatty acid oxidation (FAO), resulting in an increase of the ratio NADH/FADH$_2$. Interestingly, etomoxir decreased basal and DCA-induced increase ERK5 mRNA (Fig. 7C). Therefore, we found no correlation between ERK5 expression and the expected changes in NADH/FADH$_2$ ratio. Etoximor and DCA were not toxic to OCI-AML3 cells, although they decreased cell proliferation (Supplemental Fig. 6B). However when combined they induced cell death suggesting that DCA treatment requires FAO for cell survival as suggested by our previous results. In summary complex I activity, through accumulating fumarate, induces ERK5 expression leading to NRF2-mediated antioxidant response.

Discussion

ROS generation is inherent to the activity of the electron transport chain, with Complex I being considered one of the main sites at which premature electron leakage to oxygen occurs and give rise to superoxide anion. We show here that complex I activity initiates an antioxidant response mediated by ERK5-induced NRF2 expression. It is interesting to note that the main generator of ROS is at the same time responsible of triggering the mechanism to eliminate them. Of relevance, ROS de novo production is not required for this response. The cell “anticipates” ROS formation and activates the pathway to avoid their uncontrolled increase. Once produced, ROS quickly originate biochemical reactions that generate damage to cell structures. Hence, it is on the cells own benefit to create the antioxidant response when ROS production is going to occur. However, new data are challenging the “only” deleterious view of mitochondrial ROS. For example ROS increase with aging, but increasing mitochondrial ROS production specifically through the respiratory complex I reverse electron transport (RET) extends Drosophila lifespan.
Figure 6. Inhibition of mitochondrial complex I and II signals ERK5 expression. (A) Erk5 and Nrf2 mRNA expression in the liver of wild-type and T/H (Tet-Off-H49R (h-IF1) mice. mRNA from 3 mice of each genotype was quantified by qPCR and represented as the % of mRNA compared to wild-type mice. (B) ERK5 and NRF2 mRNA expression in fibroblasts derived from a group of 8 healthy donors or 8 patients suffering from mitochondrial defects (Supplemental Table 1). (C) Different hematopoietic cell lines were incubated for 24 h with 5 mM metformin. mRNA expression was quantified by qPCR and represented as the % of mRNA compared to non-treated cells. ERK5 and NRF2 protein expression was analyzed in these cell lines by western blotting (lower panel). (D) Jurkat and OCI-AML3 cells were treated with 10 mM DCA and 300 µM TTFA for 24 h. NRF2 mRNA expression was quantified by qPCR and represented as the % of mRNA compared to control cells. (E) Different cell lines described in Supplemental Table 2 were treated with 20 mM DCA during 24 hours. ERK5 mRNA was quantified by qPCR and represented as the % of mRNA compared to non-mutant control cells. Experiments were done in triplicate and data represent means ± SD; statistics were performed using student t-test (A–C) or One-way ANOVA with post-hoc Tukey test (D and E); *p < 0.05, **p < 0.01, ***p < 0.001; *p < 0.05, #p < 0.01 compare to the respective control cell lines. Treatments were compared to non-treated cells (control) if not specified in the graph.
We show that mitochondrial complex I activity is required for ERK5-induced NRF2 expression. We have examined several possibilities that could account for our observation. DCA inhibits glycolysis and increases FAO as suggested by the high toxicity of the combined etomoxir plus DCA treatment ((Supplemental Fig. 6) and 29). Then, this switch would change NADH:FADH2 electron ratio. When electron flux from FAD overwhelms the oxidation capacity of CoQ:FAD, CI is degraded, releasing CIII from CI-containing complexes to receive FAD-derived electrons 35. The increased electron flux through FAD could saturate the oxidation capacity of the dedicated coenzyme Q (CoQ) pool and result in the generation of ROS 36. However, in our experiments ROS do not mediate OXPHOS-induced ERK5 expression (Figs 4 and 6). Moreover, etomoxir decreases basal ERK5 expression but it does not block DCA-induced increase. Finally, if FAO is inducing ERK5 expression, complex II inhibition should decrease it, but we found an increase with TTFA and basically no effect by genetic approaches. This suggests that another mechanism is responsible for triggering ERK5 expression.

In non-transformed cells inhibition of OXPHOS by IF1 induces AMPK activation10, which could lead to ERK5 activation17. DCA also induces AMPK12. However, our pharmacological (metformin, Fig. 6C) and genetic (siRNA, Supplemental Fig. 5) approaches suggest that AMPK is not involved on ERK5 expression during OXPHOS.

Strong mitochondrial complex I activity could decrease electron transport through complex II and the subsequent accumulation of succinate or reduced fumarate be responsible for ERK5 expression. Our results using TTFA or genetically-modified cells support this conclusion (Fig. 6). However, MMS fails to induce ERK5

Figure 7. Fumarate/succinate regulate ERK5 expression. OCI-AML3 were treated with different drug combinations and the expression of ERK5 mRNA was analyzed by qPCR. (A) OCI-AML3 cells were treated with 10 mM DCA, 5 mM MMS and/or 300 µM DMF for 24 h. (B) OCI-AML3 cells were treated with 5 mM metformin and/or 5 mM MMS for 24 h. (C) OCI-AML3 cells were treated with 5 mM DCA and/or 100 µM Etomoxir for 48 h. The data represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.005 ANOVA with post-hoc Tukey test. Treatments were compared to non-treated cells (control) if not specified in the graph.
even if complex I was blocked by metformin (Fig. 7). In contrast, we found that DMF induced ERK5 (Fig. 7). Interestingly, DMF induces Nrf2 expression through a PD98059-sensitive pathway48. Although this MAPK inhibitor was initially described as a specific MEK1 inhibitor, it also inhibits the ERK5 upstream kinase MEK549. Therefore, fumarate indeed mediates ERK5 expression. Accumulated fumarate can covalently modify cysteine residues of proteins, in an uncatalyzed process termed succinination, modifying cellular signaling50. Succination occurs on KEAP141 and results in constitutive NRF2 activation and increased expression of its target genes41. Therefore, fumarate induces the Nrf2-mediated antioxidant response by directly affecting KEAP-141 and by inducing de novo Nrf2 expression (our results).

An alternative is that succinate promotes CII activity and induces RET thereby decreasing mitochondrial membrane potential. In reverse, fumarate blocks CII thereby increasing complex I activity and this could trigger ERK5 expression.

Although it is well-established that ROS induce NRF2 activation51, recent data support that alternative pathways independent of ROS are also operative. For example, OXPHOS decreases KEAP-1 expression independently of ROS43 and NRF2 expression in AML depends on NF-κB but not on ROS52. Interestingly, ERK5 activates NF-κB in leukemic cells47. Hence, ERK5 could handle the NRF2-mediated antioxidant response by at least 3 mechanisms independently of de novo ROS generation: i) direct transcription through MEF2 (the results presented here); ii) direct transcription through NF-κB52; iii) upregulation of miR-23 and downregulation of KEAP1 mRNA53. This emphasizes the central role of ERK5 in the antioxidant response41-44.

Transcriptome analysis shows that the ERK5 pathway regulates in normoxia several genes involved in metabolic remodeling, including some controlled by hypoxia inducible factor-1α (HIF-1α under hypoxia54-55). Also, like HIF-1α, ERK5 is degraded by a process depending on the tumor suppressor von Hippel-Lindau (VHL), through a prolyl hydroxylation-dependent mechanism56. Hence, mitochondrial complex I activity through fumarate accumulation could also protect ERK5 from VHL-induced degradation. This is based on the fact that succinate and fumarate (and succinate) outcompete α-ketoglutarate, an essential co-factor of prolyl hydroxylase domain enzymes56.

How ERK5 induces Nrf2 mRNA expression is not totally elucidated. ERK5 directly phosphorylates MEF2A, C and D at different serines and threonines56-57. It activates MEF2A and D by direct interaction because ERK5 serves as a MEF2 coactivator through its signal-dependent direct association with the MEF2 MADS domain; although, at least, MEF2A-dependent transcription requires ERK5 kinase activity58-59.

Finally, forcing cells to produce energy through OXPHOS also affects cell viability and proliferation independently of ROS. This is rather related to energy depletion. In this sense OXPHOS requires mitochondrial function and DCA induces cell death in ρ0 cells, while in other cells it just inhibits growth51,52,53. In summary forcing OXPHOS in vitro is cytostatic in “normal” tumor cells and cytotoxic in cells with major mitochondrial dysfunctions.

**Experimental Procedures**

**Ethical statement.** Experimental procedures were conducted according to the European guidelines for animal welfare (2010/63/EU). Protocols were approved by the Animal Care and Use Committee “Languedoc-Roussillon” (approval number: CEEA-LR-12163). The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient prior to surgery.

**Reagents and antibodies.** DCA was from Santa Cruz Technologies. Galactose and glutamine were from GibCO. RIPA buffer to prepare protein extracts was from Euromedex. The complete protease inhibitor cocktail (Complete EDTA-free) and the phosphatase inhibitor cocktail (PhosSTOP) were from Roche. Human total RNA was from GIBCO. RIPA buffer to prepare protein extracts was from Euromedex. Táhe complete protease inhibitor cocktail

**In vivo mouse experiments.** In vivo experiments were carried out using 6 to 8 weeks/old male NSG mice. Mice were bred and housed in pathogen-free conditions in the animal facility of the European Institute of Oncology–Italian Foundation for Cancer Research (IFIRC, Institute of Molecular Oncology (Milan, Italy). For engraftment of human cells, 1 million AML cells were injected intravenously (i.v.) through the lateral tail vein in non-irradiated mice. NSG mice with established human AML tumors (day 80 post-graft) were treated with DCA (50 mg/kg, 1 dose/day by gavage, starting at day 1 for 16 consecutive days). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to visualize expression of human mRNA. In a different experiment B6 wild type mice were treated with a daily single dose of DCA (50 mg/kg/day) intraperitoneally and mouse mRNA was analyzed in spleen and liver after different times.

**hIF1 Transgenic mice.** The samples from transgenic mice containing the mutant H49K version of hIF1 have been described60. mRNA was analyzed in liver of these mice.

**Cell lines and culture conditions.** The leukemic human cell lines T Jurkat Tag, NB4 and OCI-AML3 were grown in RPMI 1640–Glutamax (GIBCO) supplemented with 5% (Jurkat) or 10% (OCI and NB4) FBS. Primary cells from a lymphoma B cell patient (BCL-P2) were grown in the same medium with 10% FBS. In certain experiments cells were grown in RPMI 1640 without glucose (GIBCO 11879) with the addition of 2 mM glutamine and 10 mM galactose (OXPHOS medium). The Jurkat Tag cells carry the SV40 large T Ag to facilitate cell transfection. HepG2C3A and HuH7 cells were grown in MEM and DMEM respectively supplemented with 10% FBS, sodium pyruvate, glutamine, penicillin and streptomycin. The HCT116 human colon cancer cells were cultured in low glucose (5 mM) DMEM medium supplemented with 10% FBS. Cellular confluence during experiments was between 80–85%.
Primary Leukemic Cells. Data and samples from patients with different hematological cancers were collected at the Oncology and Clinical Hematology Department of the CHU Montpellier, France, after patient’s informed consent. Patients were enrolled in two independent clinical programs approved by the "Comités de Protection des Personnes Sud Méditerranée I (ref 1324)” and ID-RCB: 2011-A00924-37. All samples from cancer patients were collected at diagnosis.

Human liver samples and preparation of primary human hepatocytes (PHHs) cultures. PHHs were isolated as described previously from donor organs unsuitable for transplantation or from liver specimens performed in adult patients for reasons unrelated to our research program. Liver samples were obtained from the Biological Resource Center of Montpellier University Hospital (CRB-CHUM; http://www.chu-montpellier.fr; Biobank ID: BB-0033-00031) and this study benefited from the expertise of Dr Jeanne Ramos (hepatogastroenterology sample collection) and Prof Sylvain Lehmann (CRB-CHUM manager). The procedure was approved by the French Ethics Committee and written or oral consent was obtained from the patients or their families.

Hepatocytes isolation and culture were performed as described previously. Briefly, after liver perfusion, hepatocytes were counted and cell viability was assessed by trypan blue exclusion test. A suspension of 1 × 10⁶ cells/mL per well was added in 12-well plates pre-coated with type I collagen (Beckton Dickinson) and cells were allowed to attach for 12 h. Then, the supernatant containing dead cells and debris was removed and replaced with 1 mL of serum-free long-term culture medium (Lanford medium, LNF). The number of confluent attached cells was estimated at ~1.5 × 10⁶ cells/cm².

Plasmids. The luciferase reported plasmid driven by a DNA fragment of 1.5 kb of the human Nrf2 promoter was a kind gift from Stuart Rushworth. The expression vectors for ERK5, the super vector expression vector for GFP alone or GFP plus shERK5 and the pSiren-retroQ-pur (BD Biosciences) retroviral vectors for shERK5 and control have been previously described. Control, MEF2A and C and ERK5 siRNA were ON-TARGETplus SMARTpools (mixture of 4 siRNA) from Dharmacon.

Transient transfection. Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation. In each experiment, cells were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at RT with the DNA mix and electroporated using the Gene Pulser Xcell Electroporation system (Bio-Rad) at 260 mV, 960 mF in 400 μL of RPMI 1640. Expression of the different proteins was confirmed by western blot. The transfection efficiency in Jurkat TAg cells is between 60 and 80%. OC-AML-3 cells were transfected using Amaxa D-Nucleofector Lonza Kit according to manufacturer's protocol. In HuH7 and HCT116 cells, transfection of 30–50 nM siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen), according to the manufacturer’s instructions. Adherent primary hepatocytes were transfected twice at day first and third post-seeding with 20 nM siRNA. Cells were harvested 48 to 96 h post-transfection.

RT-PCR. Total RNA was extracted using Nucleospin RNA isolation columns (Macherey-Nagel), reverse transcription was carried out using iScript™ cDNA Synthesis Kit (Biorad). Quantitative PCR was performed with KAPA
Immunoblotting. Protein analysis by immunoblotting was performed essentially as previously described\(^7\). Briefly, samples were collected, washed out with PBS and lysed with RIPA buffer. Protein concentration was determined by BCA assay (Pierce) before electrophoresis in 4–15% TGX gels (BioRad) and equal amount of protein was loaded in each well. Protein transfer was performed in TransTurbo system (BioRad) in PVDF membranes. After blocking for 1 h with 5% non-fat milk, membranes were incubated overnight at 4\(^\circ\)C in agitation with primary antibodies, washed three times with PBS-Tween 0.1% and incubated with the appropriate HRP-labeled secondary antibody for 1 h. Membranes were washed out three times with PBS-Tween 0.1% and developed with Substrat HRP Immobilon Western (Millipore). Band quantification was performed using the "ImageLab" software from BioRad and represented as the ratio between the protein of interest and a control protein i.e. actin. The value of 1 is arbitrarily given to control cells. One blot representative of several experiments is shown.

Statistical analysis. The statistical analysis is based on the mean and the standard deviation of at least three independent experiments. For statistical analysis, we used an unpaired two-tailed Student’s t-test. The results are given as the mean with the standard deviation (SD). Data in columns were analyzed using the Statistical Analysis System (SAS). P values less than 0.05 were considered statistically significant (\(p < 0.05\)).

Reagents. Unless otherwise indicated, all chemicals were of the highest quality available from Sigma-Aldrich.

SYBR Green qPCR SuperMix (Clinscience) and a CFX Connect\textsuperscript{TM} Real-Time qPCR machine (BioRad) with ERK5, NRF2, NQO1, HO-1 and actin primers. Supplemental Table 3 shows all primers used in this study. All samples were normalized to \(\beta\)-actin mRNA levels. Results are expressed relative to control values arbitrarily set at 100\(^\circ\).

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Author Contributions


Additional Information

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CHAPTER 11.

GENERAL DISCUSSION
The NK cell is an integral part of the human immune system, playing particular important roles in control of tumor development and virus infection. Indeed, evasion of immune effectors, including NK cells, is a hallmark of cancer (Hanahan and Weinberg 2011; Villalba et al. 2013) that is shared by virus. In these immuno-pathology conditions, the dynamics of NK cells to target cells (either tumor or virus infected cells) result in NK cell populations displaying distinct phenotypic imprints compared to healthy individuals. Due to the divergent nature of multiple immune evasion strategies and multiple modes of interaction between NK cell and their target cells, the pathological-related markers on NK cell population are hard to be generalized. The first part of my thesis attempted to investigate this by detailed immuno-phenotyping approaches on NK cell populations. We study different CD45 isoforms in various hematological malignancies (chapter 3-5) and in virus infection (HIV – chapter 6). The second part of the thesis provided an efficient method of generating allogeneic NK cell with highly activated potential for anti-cancer immunotherapy. These expanded NK cells can be used as a single-agent monotherapy or in combination with either clinical-approved monoclonal antibodies or metabolic modulating drugs that alter immunogenicity of tumor cells to enhance the NK-dependent anti-tumor effect. The discussion for each section of the results are presented according to the order of this framework.

8.1 GALEN study

This longitudinal study is part of a phase Ib/II clinical trial with relapsed/refractory B-NHL patients, which are followed within the timeframe of 6 cycles (in which each cycle is approximately 1 month) with combination treatment of LEN and OBZ (see Supplementary Fig 1. – Chapter 4, for treatment and sampling protocol). LEN belongs to the class of immunomodulatory drugs (IMiDs) which showed strong anti-tumor activity against multiple malignancies, especially B-cell hematological diseases (Desai et al. 2014). The immunomodulatory effects of LEN include promoting immune effector activation, especially on immune populations of T and NK cells (Gaidarova et al. 2008; Lagrue et al. 2015; Zhang et al. 2009). Notably, combination of LEN and rituximab (RTX) treatment has been shown to improve ADCC
in vitro mediated by NK cell and monocyte towards B-NHL cell lines and primary B-CLL tumor cells suggesting a beneficial synergistic effect of these two anti-tumor agents (Wu et al. 2008). In this study LEN is used with OBZ, a type II glycoengineered anti-CD20 antibody which is modified by defucosylaton for higher binding affinity to FcγRIIIA, which is expressed mainly on NK cells and macrophages. NK cell depletion followed by OBZ induction was reported in OBZ monotherapy treatment for CLL patients (García-Muñoz et al. 2018). In our cohort, we observed a transient NK depletion only at the end of the first cycle after 3 consecutive OBZ infusions (Fig 1B - Chapter 4). Nevertheless, LEN has been reported to augment NK cell proliferation in several previous studies (Gribben, Fowler, and Morschhauser 2015; Hernandez-Illizaliturri et al. 2005). Since patients in this study are co-treated with both LEN and OBZ, the observed effect of treatment on NK cell population is likely a net outcome of both single agent’s effect. Moreover, patient NK cells decreased in immature CD56br population while increased in CD56dimCD16+ population which corresponding to higher ADCC potential (Fig 2A – Chapter 4). This effect has been similarly reported by a previous study (Zhang et al. 2009). More importantly, we observed a strong reduction of the activation marker CD69 and the degranulation marker CD107a on total NK cell population at the end of final cycle upon the decrease in target cell numbers (indicated by CD19+ and CD20+ population, Fig 1C – Chapter 4). This result emphasized the close relationship between NK cell activity in vivo and the presence of target cells. More importantly, NK cell in various hematological diseases showed a distinct CD45 isoform profile: higher CD45RARO+ and CD45RO+, lower “ naïve” CD45RA+ percentage compared to HD (Chapter 3). We confirmed our previous results in this study and demonstrated that the high activation phenotypes CD45RARO+ and CD45RO+ populations are strongly reduced after target cell elimination at the end of treatment (Fig 3B – Chapter 4). Finally, we showed that CD19 expression on NK cell population is most predominant in the highly active NK subpopulations: CD45RARO+ and CD45RO+, consistently with our previous study (Chapter 3). In this study, we were not able to obtain patient samples with single treatment (either LEN alone or OBZ alone), this makes impossible to know which is relative relevance of each drug in our results.
8.2 AML study

Patients with acute myeloid leukemia (AML) are typically characterized with accumulation and rapid proliferation of immature hematopoietic myeloid precursors known as myeloblasts. The standard treatment for these malignancies is typically chemotherapy with anthracyclines and cytarabine as first-line treatments, often followed by hematopoietic stem cell transplantation (HSCT) in patients with intermediate/bad prognosis and relapsed patients. However HSCT is not always feasible. NK cell from AML patients usually displayed severed dysfunctionalities (Costello et al. 2004; Farag and Caligiuri 2006). To gain better understanding of the NK cell phenotype in AML patients after chemotherapy treatment (CT), we analysed NK cell phenotypes in 7 patients at 2 timepoints: at diagnosis and 30 days after CT (Chapter 5). To facilitate the gating process and avoid the potential mis-intepretation of CD56+ myeloid-origin cells, we exploited CD7 in the gating strategy for NK cells as this marker is restricted to the T/NK-origin lineage (Fig 5.1 – Chapter 5). 30 days after CT, the leukocyte pattern is partly restored. This correlates with a strong diminution on immature CD33+CD14- myeloblasts (Fig 5.2 – Chapter 5). Interestingly, patient’s NK cell from our study showed enhanced proportion of CD62L, CD56br NK population while reduced CD57 expressing cells. This indicates an immature phenotype after CT (Fig 5.3 C-D, Fig 5.5 B – Chapter 5). These results are somewhat similar to a previous report where a significant higher CD56br NK cell proportion after CT has been observed (Dauguet et al. 2011). Chretien and colleagues also described an accumulation of the late mature NK phenotype corresponding to CD56dim KIR+CD57+ at diagnosis, which is in agreement with our results (Chretien et al. 2015). Interestingly, we also reported a decreased in ex vivo degranulation and lower PD-1 level in the whole NK cell compartment, suggesting a less activated phenotype after decrease in tumor cell load (Fig 5.3A, Fig 5.4B – Chapter 5). Finally, we confirmed that CD45RARO+ NK cells correlate with tumor load as in our previous study (Chapter 4, Fig 5.7 - Chapter 5). Finally, CD14, but not CD33, associates strongly with the amount of CD45RARO+ NK cells (Fig 5.6 – Chapter 5). Given that CD14 is a marker specific for monocyte/macrophage populations, how the CD45RARO+ NK cells preferentially interacts with these populations but not CD33-expressing myeloid precursors
remained to be investigated. Furthermore, due to limitation of patient numbers and lack of HD controls, this study requires further investigations.

8.3 HIV study

The role of NK cell in HIV infection has been well established. Especially, the role of NK cell in ADCC-dependent cytotoxicity by anti-HIV antibodies have been connected with protective vaccine responses (Haynes et al. 2012). Immunogenetic studies also revealed certain genotypes (e.g., KIR3DS1 and HLA-Bw4 carrying individuals) that are better at delaying AIDS progression (Chapter 1.6, Alter et al. 2007; Cohen et al. 1999). Emerging studies suggest that HIV infection causes dampened NK cell homeostasis and aberrant NK cell receptor repertoire (Mikulak et al. 2017; Scully and Alter 2016). NK cells from patients who had long term exposure to HIV viremia displayed distinct phenotype with decreased CD56 level while intact CD16 (i.e. CD56negCD16+). These dysfunctional CD56neg NK cells are compromised in cytotoxicity compare to its CD56dimCD16+ counterparts. Moreover they are also defective in cytokine production, such as IFNγ and TNF-α, upon stimulation (Alter et al. 2005; Mavilio et al. 2005). To study the impact of HIV infection on NK cell population in general and in the expression of CD45 isoforms in particular, we analysed blood samples from patients with both HIV viremia and non-viremia (i.e., aviremia) (results presented in Chapter 6). In these patients, we observed a drop in percentage of both NK and NKT compartments compared to HD, which suggested HIV infection may have an impact on NK cell homeostasis (Fig 6.2 – Chapter 6). Interestingly, NK cell population from our cohort of patients showed marks of NKG2D reduction, which have not been previously reported (Fig 6.3 – Chapter 6). Since NKG2D down-modulation is likely ligand-dependent, we speculated that HIV infection resulted in higher levels of NKG2DL in patients compared to HD. NKG2DLs present in 2 forms: membrane bound (usually on stressed cells, i.e virus infected cells) or soluble. MICA, a NKG2DL, in its soluble form has been reported at high level from plasma of HIV patients. This correlates with defects in NKG2D-mediated killing by NK cells from HIV-infected patients (Mavilio et al. 2005; Nolting et al. 2010). The CD45 isoform
profile of NK cells derived from our patients revealed a distinct profile compared to HD: higher proportions of CD45RAdim and CD45RO+ NK subpopulations in patients. Most NK cells from HD displayed CD45RA+ phenotype (Fig 6.4B-C – Chapter 6). Importantly, these HIV-specific CD45RAdim and CD45RO+ NK cells expressed progressively lower level of CD16 in which CD45RO+ NK cell from both aviremic and viremic patients are mostly CD56dimCD16- (Fig 6.5 – Chapter 6). Shedding of CD16 on CD56dimCD16+ NK population upon activation via the metalloproteinase ADAM17 has been implicated as a hallmark of NK underwent prolonged stimulation (Romee et al. 2013). Therefore it is tempting to speculate that the enhanced CD45RAdim and CD45RO+ NK populations, which have low CD16 level in HIV patients, resulted from NK cell and virus infected cell interactions in vivo. However it is still possible that CD16 down-modulation is due to unknown virus-induced dysfunctional process. A further immunophenotyping on NK cells is undergoing.

NK cells are able to capture target cells antigens upon their interaction via trogocytosis. We demonstrated in vitro that allogenic primary NK cells are able to performed trogocytosis on CD4+ T cell lines (Fig 6.6 – Chapter 6). As a results of trogocytosis, NK cells carried HIV-entry receptors, i.e. CD4 and CCR5, which could sensitize them to subsequent HIV infection. HIV can infect not only CD4+ T cells but also macrophages (Honeycutt et al. 2016). Our results suggest that NK cell could become a HIV-target after trogocytosis of T cell antigens from HIV-infected cells. This is still undergoing investigation.

8.4 In vitro NK cell expansion and impact of modulating cancer cell metabolism in NK-based immunotherapy

Cancer cells prefer perform glycolysis instead of OXPHOS (i.e Warburg effect). This promotes inefficient but rapid ATP generation and breakdown of macromolecules which serve as intermediates for tumor mass and ultimately allowing them to maintain rapid a proliferation rate (Lopez-Royuela et al. 2014; Villalba et al. 2013). I have participated on several investigations leading to a better understanding of various aspects of cancer cell metabolism. Among them, we show that dichloroacetate (DCA),
a PDK1 inhibitor, promotes mitochondrial activity via OXPHOS and increases fatty acid metabolism via an ERK5-MEF2-dependent upregulation of the cholesterol receptor CD36 (Chapter 8). Metabolism and immunogenicity in cancer cells are two different aspects that has been increasingly recognized to be mutually integrated (Charni et al. 2010; Lopez-Royuela et al. 2014; Villalba et al. 2013). Modulation of cancer cell metabolism via metformin, a mitochondrial complex I inhibitor, or DCA augment several stress ligands such as MIC-A/B and ULBP1 in tumor cells. This increases NK cell recognition and enhances tumor killing, supporting the combination use of metformin and DCA with NK cell expanded in vitro for future anti-cancer treatments (manuscript in preparation).

Due tumor-mediated immunosuppression and chronic inflammation usually associated with the tumor microenvironment, endogenous immune effectors, including NK cells, are severely impaired in their cytotoxic function (discussed on Chapter 1, 1.7). It is essential to reverse this balance to favor a stronger anti-tumor immune response. In vitro generation of highly activated NK cells from UCB using NK cell promoting cytokines in combination with the EBV-LCL cell lines successfully overcomes the anti-apoptotic effect of chemo-resistant hematological cancer cells (Sánchez-Martínez et al. 2015, 2016). In this current study, we further provided basis for the application of this NK cell expansion protocol in combination with clinical-approved mAb towards a variety of tumor antigens, including CD20 (RTX and OBZ in B-cell malignancies), CD38 (daratumumab against MM), or anti-EGFR (cetuximab) and anti-HER2 (trastuzumab) against multiple solid cancer cell lines (Chapter 7). Taken together, NK cell generated in vitro using the method described in Chapter 7 provided a prospect for using NK cells in immunotherapy both as a single agent or with mAb to enhance ADCC.
8.5 Summary & final conclusions

**GALEN study:**
- NK cell from B-NHL patients after LEN and OBZ treatments reduced ex vivo activation markers, e.g. CD69, CD107a and lower CD45RO+ subpopulations.
- Furthermore, GALEN treatment did not induce exhaustion in NK cell (e.g. PD-1) while augment several activating receptors such as NKG2D, CD94 and CD137.
- NK cell from these patients performed high trogocytosis activity at diagnosis measured by carrying of CD19 and this marker decreased in NK cell compartment when target cells (CD19+ B cells) are eliminated at the end of treatment.

**AML study:**
- NK cell from AML patients undergone CT displayed immature phenotype by higher CD56br subsets and higher CD62L level while reducing CD57 maturation marker.
- The exhaustion marker PD-1 decreased in total NK cell compartment after CT treatment.
- Interestingly, the CD45RARO+ subset strongly correlates with CD14 trogocytosis marker at diagnosis indicating the interaction between NK cells versus CD14-expressing myeloid populations in these AML patients.

**HIV study:**
- Analyzing NK cell total populations revealed NKG2D down-modulation in HIV+ patients suggesting virus-induced dysfunctional hallmark.
- Moreover, NK cells from these patients exclusively include CD45RAdim and CD45RO+ subsets characterized by reduction in CD16 level.
- Lastly, trogocytosis of HIV-entry receptors (i.e. CD4 and CCR5) from in vitro co-incubation experiments suggesting NK cell can become a novel HIV target as a consequence of interaction with HIV+ CD4-target cells.

In summary, the work on analyzing CD45 isoforms on NK cell subpopulation
from various hematological cancers such as B-NHL (Chapter 4) and AML (Chapter 5) revealed evidences for NK cell dysfunction at diagnosis. Moreover, CD45 isoform profiles on NK cells from these pathologies differentiate them from healthy people. Hence, CD45RO+ NK cells are pathologically relevant and associated to clearance of tumor cells (i.e. LEN plus OBZ, Chapter 4). NK cells from HIV-infected patients contain subpopulations characterized by CD45RAdim and CD45RO+ which progressively decreased in CD16 level suggesting recent activation in vivo. These NK cells from HIV+ patients also show lower NKG2D activating receptor, which could be a virus-induced hallmark of functional impairment. Herein, within this thesis work I additionally described a new mechanism, i.e. trogocytosis, in NK cell which could play an important role in several physiological processes. This would bringing novel functions to the NK cell depending on the specific physiological context where it take places: i.e. capturing of tumor-associated antigens could potentially marks the trogocytosed NK cell to fratricide whereas interaction with virus-infected cells would facilitate transferring of virus-entry receptors to the trogocytosed NK cells which ultimately could make them as a novel virus target. These results can be clinically exploited for making patient's prognostic and perhaps also to identify NK cell fate when they uptake target's antigens.

Finally, NK cell expansion from UCB has been proven efficient in combination with several monoclonal antibodies for tumor antigens. This results in strong ADCC in vitro and in vivo. This work supports the use of expanded NK cells in immunotherapy.

8.6 Future perspectives

Phenotypic profiling of NK cell subsets, e.g. based on expression of CD45 isoforms, could detect immunopathology conditions including cancers and virus infections. This should be validated in further studies.

An efficient method of generating highly activated allogeneic NK cell in vitro from UCB could potentially overcome the endogenous immunosuppressive environment from cancer patients and regain efficient NK cell-dependent anti-tumor effect. Empirical results from Chapter 7 support the use of these in vitro activated NK
cells as either monotherapy or in combination with clinical mAbs for conferring strong ADCC cytotoxicity. This should widely study in future models.
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