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Immunosubversion du Lymphocyte Natural Killer par *Pseudomonas aeruginosa*

JURY

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ABBREVIATIONS

ADCC : Antibody-Dependent Cell Cytotoxicity
ADP : Adénosine Di-phosphate
ADPRT : ADP-ribosyltransférase
AHL : N-Acyl-Homoserine lactone
AICL : Activation-Induced C-type Lectine
AMPc : Adénosine monophosphate cyclique
ARN : Acide ribonucléique
CCR7 : The CC-chemokine receptor 7
CCL19 : C-C motif (Double pont disulfure) chemokine ligand 19
CCL4 : MIP-1β (Macrophage Inflammatory Proteins)
CCL5 : RANTES (Regulated on Activation, normal T cell expressed and secreted)
CCL3 : MIP-1α
CD : Cellule Dendritique
CMH : Complexe Majeur d'Histocompatibilité
CMV : Cytomegalovirus
CPA : Cellule Présentatrice d'Antigène
CXCL10 : C-X-C motif chemokine Ligand 10
CXCR3 : C-X-C motif chemokine Receptor 3
DAMPs : Damage-associated molecular patterns
DNAM-1 : DNAX Accessory Molécule-1, CD226
DMSO : Diméthylsulfoxyde
EFS : Etablissement Français du Sang
Exo : Exoenzyme
GAP : GTPase-activating protein
GAPDH : Glycéraldéhyde-3-phosphate déshydrogénase
GFP : Green fluorescente protein
GM-CSF : Granulocyte Macrophage Colony Stimulating Factor
HLA : Human Leucocyte Antigen
HSL : Homoserine lactone
HSV : Herpes Virus
iCD : Cellule Dentritique immature
IFN-γ : Interferon-gamma
IL : Interleukine
ILC : Innate Lymphoïd Cell
ILT2 : Human inhibitory receptor Ig-like transcript 2
ISPT : Immunosuppression post traumatique
ITAM : Immunoreceptor Tyrosine based Activating Motif
ITIM : Immunoreceptor Tyrosine based inhibitory Motif
JNK : c-jun N-terminal kinases
kDa : Kilo Dalton
KIR : Killer-cell Immunoglobulin like receptor
KpOmpA : Outer membrane protein A from Klebsiella pneumonia
LPS : Lipopolysaccharide
LT : Lymphocyte T
MB : *Mycobactérium bovis.*
MICA/MICB : MHC class I polypeptide-related sequence C (MICB)
MLL5 : Mixed-Lineage Leukemia-5
NCR : Natural Cytotoxic Receptor
NK : Natural killer
NKP : Cellule Natural Killer Précurseur
NLRC4 : NOD Like Receptor CARD-Containing
ODN : Oligodeoxynucleotides

OprF : Outer membrane protein F
PA : *Pseudomonas aeruginosa*
PA-Δ : Souche de *Pseudomonas aeruginosa* délétée
PAMPs : Pathogen-associated molecular patterns
PBMC : Peripheral blood mononuclear cells
PCNA : Proliferating Cell Nuclear Antigen
PD-1 : Programmed cell death 1
PRR : Pattern Recognition Receptor
PS : Penicillin-Streptomycin
QS : Quorum Sensing
RT-PCR : Reverse transcription polymerase chain reaction
SRIS : Syndrome de Réponse Inflammatoire
STAT : Signal Transducer and Activator of Transcription
T2SS : Système de sécrétion de type 2
T3SS : Système de sécrétion de type 3
TBI : Traumatic Brain injury
TCR : T-Cell Receptor
TLR : Toll like récepteur
TNF-α : Tumor Necrosis Factor alpha
TRAIL : TNF related apoptosis-inducing ligand
Treg : Lymphocyte T régulateur
ULBP *:* UL16 binding protein
VHC : Virus de l'hépatite C
VZV : Virus Varicelle Zona

NOMENCLATURE

CD1 : Glycoprotéine apparentée au CMH de type I, et impliqué dans la présentation d'antigènes lipidiques

CD3 : Protéine transmembranaire associée au récepteur TCR

CD4 : Glycoprotéine, Co-récepteur du TCR participant à l'interaction avec le CMH de type II

CD8 : Glycoprotéine, Co-récepteur du TCR participant à l'interaction avec le CMH de type I

CD14 : Co-récepteur avec le TLR4 et MD-2 capable de fixer le Lipopolysaccharide (LPS)

CD16 : Récepteur de basse affinité au fragment Fc des immunoglobulines

CD19 : Marqueur de cellule B disparaissant au stade plasmocyte

CD20 : Récepteur du LB impliqué dans sa maturation et l'interaction avec le microenvironnement

CD25 : Récepteur à l'IL-2 utilisé pour identifier les lymphocytes T régulateurs

CD28 : Récepteur du LT Co-activateur reconnaissant les molécules CD80 et CD86 sur la CPA

CD34 : Marqueur des progéniteurs à la phase précoce des processus d'hématopoïèse

CD56 : Glycoprotéine de la famille des immunoglobulines, présente sur les NK, LT CD4+ et LT CD8+

CD69 : Marqueur d'activation de cellules T ou NK

CD94 : Lectine hétérodimèrisée avec NKG2 reconnaissant la molécule HLA de type E

CD95 (ou FAS receptor ou TNFRSF6) : Reconnaît la molécule Fas-L et initie les voies de l'apoptose

CD107a (ou LAMP-1) : Marqueur de dégranulation lymphocytaire

CD112 : Glycoprotéine impliquée dans l'entrée du Virus HSV dans la cellule épithéliale

CD155 : Glycoprotéine transmembranaire impliqué dans les jonctions entre les cellules épithéliales.

CD226 (ou DNAM-1) : Protéine réceptrice du CD112 ou CD155

1. SITUATION DU PROBLEME

1.1. Situation du problème dans la thématique du laboratoire

En clinique humaine, les états infectieux sévères et les polytraumatismes graves sont responsables d'immunosuppression « post-traumatique » (ISPT) à court et moyen termes augmentant la susceptibilité aux infections.

L'ISPT dans sa composante innée, est caractérisée par trois anomalies principales de la réponse immunitaire. La première est une baisse de la capacité de présentation de l'antigène sur le CMH de type II par les monocytes (1). La deuxième caractéristique est l'hypo réactivité à la stimulation par les PAMPs (Pathogen-associated molecular patterns) se traduisant par une baisse des réponses cytokiniques de type TNF-α, IL-1β, IL-6, IL-8 et IFN-γ (2). Troisièmement, la réponse anti-inflammatoire (IL-10, TGF-β) participant au retour à l'homéostasie, est exacerbée et entraine une susceptibilité accrue aux infections dans les deux semaines à six mois suivant l'infection ou le traumatisme (2,3).

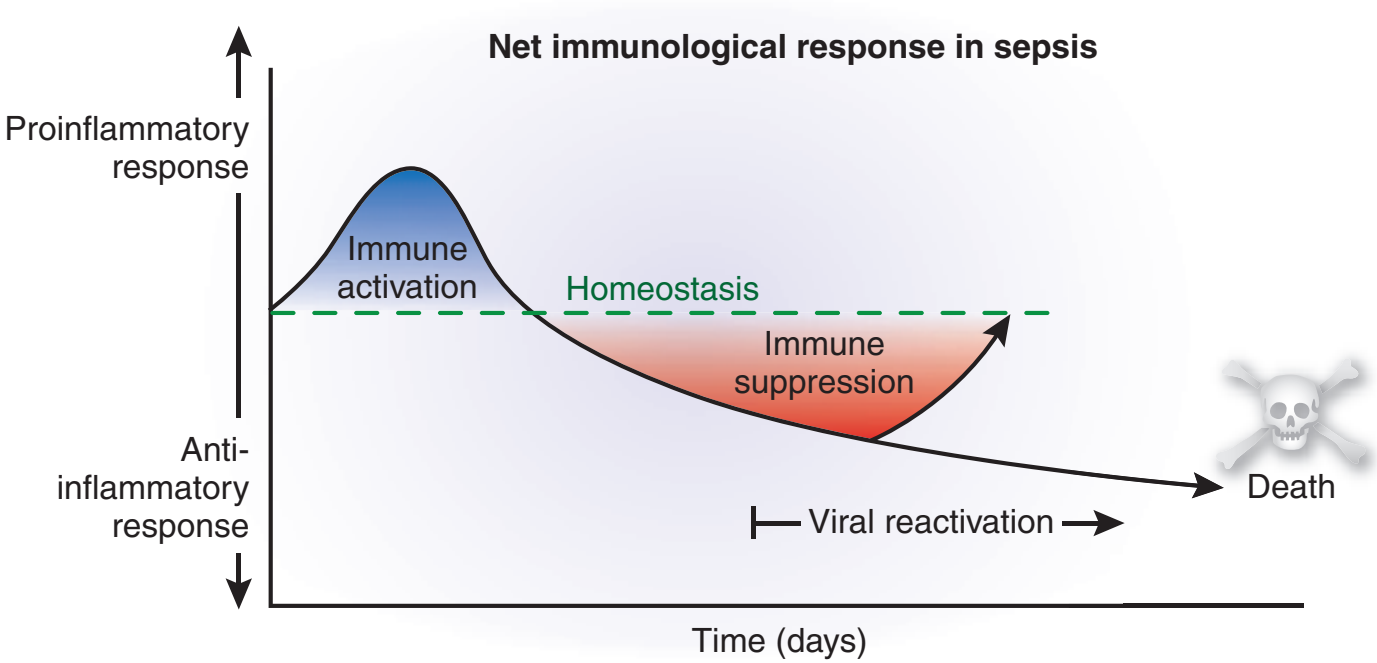


Figure 1 : Représentation schématique du concept d'immunosuppression post septique (4,5)

Parmi les ILC (Innate Lymphoid cells), on observe au cours du sepsis ou lors des traumatismes, une diminution du nombre de NK circulantes, une baisse de leurs capacités de sécrétion cytokinique (IFN-γ) en réponse au LPS et une altération de la réponse cytotoxique. La baisse d'activité IFN-γ pourrait entrainer d'une baisse de l'expression de HLA-DR provoquant une susceptibilité accrue aux infections secondaires (6,7)

Plusieurs modèles d'ISPT développés au sein de notre laboratoire mettent en évidence un rôle clé des cellules NK dans l'augmentation de la sensibilité aux infections :

- Après une hémorragie massive, le nombre de cellules NK spléniques diminuait ainsi que leur activité transcriptionnelle en ARN messager IFN-γ alors que leur activité IL-10 était augmentée (8).
- Dans un modèle murin de choc hémorragique la production d'IL-10 par les cellules NKs augmentait et entrainait l'apoptose des CD immatures (9) (**Article N°1, en annexe**).
- Dans une cohorte de patients traumatisés crâniens graves (TBI) – BIOCOLLECTION IBIS (Comité de Protection des Personnes de Nantes, N° d'autorisation AC-2008-433/FRANCE), on observait une altération de l'activité de dégranulation des cellules NK en réponse à une cible n'exprimant pas le HLA de type I (théorie du « missing-self ») entre le 1er jour et le 7^{ième} jour après traumatisme crânien. Le traitement des cellules par IL-12 restaurait partiellement l'activité de dégranulation (10) (**Article N°2, en annexe**).
- Dans la même cohorte de patients TBI, l'étude des fonctions NKT (cellule NK exprimant un récepteur de la famille des lymphocytes T, TCR) révélait une baisse de leur nombre et une augmentation de leur activité IL-10 chez les TBI par rapport aux volontaires sains. De plus, spécifiquement chez les patients TBI développant une pneumonie en réanimation, l'activité INF-γ et IL-13 des NKT était diminuée (**Article N°3, soumis, CCM, en annexe**).

La mise en évidence à la fois d'une altération de la fonction NK et d'une augmentation de la susceptibilité aux infections dans les modèles d'ISPT nous a incités à étudier spécifiquement les interactions entre la bactérie et la cellule Natural Killer (11,12). Nous avons choisi d'étudier plus particulièrement la bactérie *Pseudomonas aeruginosa* (PA) car c'est un pathogène à l'origine d'infections graves, infectant spécifiquement les patients immunodéprimés et dont l'impact en termes de morbi-mortalité est majeur.

1.2. Approche intégrée de la pneumonie à *Pseudomonas aeruginosa* dans l'immunité muqueuse pulmonaire.

Pseudomonas aeruginosa (PA) est une bactérie à gram négatif, opportuniste, responsable d'infections nosocomiales sévères et récidivantes. Grâce à son caractère saprophyte, la bactérie peut survivre sur

des surfaces inertes et métaboliser des substrats anorganiques si les conditions sont défavorables. Son implication fréquente au cours d'infections nosocomiales s'explique par ses capacités de survie dans des solutions antiseptiques, de colonisation des points d'eau hospitaliers ou du matériel chirurgical (13). En raison d'un génome hypermutable, l'acquisition de résistances aux traitements antibiotiques est très fréquente. L'incidence des souches de PA multirésistantes aux antibiotiques est d'ailleurs en constante augmentation dans le monde et à conduit l'organisation mondiale pour la santé à déclarer que la recherche de thérapeutiques ciblées sur les bactéries multirésistantes devait être une priorité (14). Selon un rapport de l'Institut National de Veille Sanitaire publié en 2016, les pneumonies à PA représenteraient 18 à 23% des pneumonies nosocomiales en France et un tiers des pneumonies à PA résistants aux antibiotiques conduirait au décès du patient (15).

En réanimation, l'infection à PA intéresse principalement l'arbre respiratoire. La susceptibilité accrue des patients de réanimation à ces infections s'explique par la présence d'une prothèse intra-trachéale perturbant la clairance muco-ciliaire, favorisant ainsi l'adhésion bactérienne. Les patients les plus touchés sont d'abords les insuffisants respiratoires chroniques (atteints de mucoviscidose ou non) et les patients immunodéprimés (situation d'immunosuppression après brûlure grave, neutropénie, néoplasie ou transplantation sous immunosuppresseurs). Concernant les patients atteints de mucoviscidose, le dysfonctionnement du canal CFTR altère la fluidité des sécrétions respiratoires et facilite également l'adhésion des agents pathogènes. PA est caractérisé par une résistance à plusieurs familles d'antibiotiques dont les amino-penicillines, aux céphalosporines de 1ère, 2^{ième} et 3^{ième} génération, au cotrimoxazole et aux tétracyclines. Les modalités et les durées de traitements des pneumonies nosocomiales en réanimation sont encore débattues notamment concernant la mono ou la bithérapie, même si les recommandations 2017 (*Pneumonies associées aux soins de réanimation, RFE SFAR/SRLF 2017*) semblent appuyer l'usage de la monothérapie pour une durée de 7 jours. Malgré un traitement adapté, les récidives sont fréquentes (16) et donc la durée de l'antibiothérapie doit probablement être adaptée au statut du patient notamment en cas de neutropénie.

A la phase aiguë de l'infection, la pneumonie à PA est fréquemment associée à une altération sévère de l'hématose. Si l'infection peut être contrôlée par la réponse immunitaire et l'antibiothérapie, elle peut également évoluer vers une atteinte marquée du parenchyme pulmonaire menant à une situation de Syndrome de Détresse Respiratoire Aigue (SDRA). La gravité des pneumonies à PA est aussi liée à ses complications potentielles à type de pleurésie purulente, d'abcès, de bactériémie ou de choc septique. La guérison sans séquelle est possible mais l'altération architecturale pulmonaire, la fibrose et le portage chronique de la bactérie sont fréquents, notamment chez les patients immunodéprimés. En conséquence, les pneumonies à PA augmentent les durées d'hospitalisation en réanimation, les durées de ventilation mécanique, le taux de mortalité et impactent considérablement les coûts d'hospitalisation. Chez le patient atteint de mucoviscidose, les infections pulmonaires successives

aboutissent à une altération progressive des paramètres respiratoires confinant à l'insuffisance respiratoire chronique dont le seul traitement reste la transplantation pulmonaire. Les mécanismes mis en œuvre par la bactérie pour persister chez l'hôte, expliquent la gravité des infections pulmonaires à PA dans cette population. L'infection aigüe initiale évolue systématiquement vers un portage chronique principalement du fait d'une altération de la clairance muco-ciliaire. Après la phase aigüe, pour persister chez l'hôte, PA procède à une modification phénotypique et adopte un métabolisme de type mucoïde. La bactérie ne synthétise alors plus de facteur de virulence impliqué dans la phase d'invasion mais produit principalement des alginates (polysaccharides) impliqués dans la formation du biofilm. Ce dernier constitue à la fois une barrière physique vis-à-vis de la réponse immunitaire et de l'antibiothérapie mais aussi une source de nutriments pour la bactérie. En conséquence, la clairance complète de l'inoculum est impossible malgré un traitement adapté et la pression de sélection des antibiothérapies successives abouti à l'émergence de souche multi-résistantes. Par ailleurs, ce switch phénotypique s'accompagne d'anomalies de réparation de l'ADN bactérien, augmentant le risque de remaniements génomiques et de résistance (17).

En plus des mécanismes de résistance multiples, ses capacités de modulation et d'adaptation à la réponse inflammatoire expliquent la fréquence des infections chroniques et la gravité des pneumonies à PA. Parmi les facteurs de virulence jouant un rôle central dans la pathogénicité de la bactérie, le système de sécrétion de type III (SSTIII) a été précisément décrit et sera abordé en détail dans le paragraphe **2.1.2.2.1**. L'infection par une souche de PA exprimant le SSTIII a d'ailleurs été associée à un risque de décès 6 fois plus important comparée à des souches ne l'exprimant pas (18). Le SSTIII permet à la bactérie d'injecter différents effecteurs (Exoenzymes S, T, U et Y) dans le cytoplasme de la cellule de l'hôte. Ces exoenzymes (Exo) perturbent les voies de signalisation intracellulaires, altèrent l'architecture du cytosquelette et peuvent mener à la mort cellulaire. L'ExoT est exprimée par plus de 95% des souches cliniques de *Pseudomonas aeruginosa* (19). Les Exoenzymes U et S semblent être les plus cytotoxiques. La cytotoxicité est une problématique centrale au cours des pneumonies car elle peut mener à l'altération définitive du parenchyme et menacer le pronostic vital du patient à court terme.

L'expression du SSTIII est régulée par le système du Quorum Sensing (QS), déterminant majeur de l'interaction hôte-pathogène au cours de l'infection à PA (description détaillée dans paragraphe **2.1.2.1**) (20). Le QS permet aux bactéries d'un inoculum de communiquer entre elles afin de coordonner l'expression de leur facteur de virulence selon la réponse immunitaire de l'hôte. Ainsi, le pouvoir pathogène est renforcé (21). Ce système repose principalement sur la synthèse et la libération de molécules de type N-Acyl-Homoserine Lactones (AHLs) par la bactérie. Les AHLs diffusent librement dans le milieu et agissent de façons autocrine ou paracrine au sein de l'inoculum.

L'accumulation des AHLs dans le cytoplasme bactérien activera également la transcription de gènes codant pour de multiples facteurs de virulence dont le SSTIII.
Pour comprendre la modulation de la réponse inflammatoire de l'hôte par le QS, 2 mécanismes doivent être considérés :

- La libération d'AHLs via les pompes d'efflux membranaires de la bactérie favorise la production d'IFN-γ par les lymphocytes de l'hôte (orientation Th1) (22).
- Le récepteur OprF de la bactérie permet d'initier la synthèse d'AHLs en reconnaissant les molécules d'IFN-γ libérées au cours de la réponse immunitaire.

En conséquence, lors d'une infection aigüe, les effets combinés du récepteur OprF et des AHLs pourront entretenir la réponse inflammatoire de type IFN-γ sans retour possible à l'homéostasie et être à l'origine de lésions parenchymateuses sévères. De la même façon, lors d'une réaction inflammatoire systémique, sous l'effet de l'IFN-γ, une souche quiescente de PA pourra augmenter sa vitesse de croissance, synthétiser de nombreux facteurs de virulence et donner lieu à une infection aigüe chez un patient porteur de PA, jusqu'ici asymptomatique (23).

L'immunité muqueuse pulmonaire présente un système de régulation unique. L'objectif est de pouvoir tolérer la présence d'antigènes et d'une flore commensale (microbiote pulmonaire) perpétuelle sans générer de réaction inflammatoire (24). La libération d'AHLs par PA peut moduler l'immunité muqueuse pulmonaire et notamment altérer la capacité de phagocytose des macrophages alvéolaires (25). De la même façon, l'ExoS du SSTIII est cytotoxique pour les neutrophiles (PNN) et les macrophages pulmonaires et limite donc leur capacité de phagocytose (26). Enfin, les ExoS et T bloquent la production de radicaux libres oxygénés par les PNN et diminuent donc leur pouvoir bactéricide (27). Ces mécanismes altèrent la clairance bactérienne et favorisent l'invasion de la bactérie.

Parmi les cellules immunitaires présentes dans le parenchyme pulmonaire, on retrouve également les cellules NK représentant 10% des lymphocytes résidents. Elles sont caractérisées par l'expression de récepteurs inhibiteurs ou KIR (Killer-cell immunoglobulin-like receptor) et activateurs ou NCR (Natural Cytotoxique receptor). Elles sont à l'interface entre immunité innée et adaptative et leurs modes de fonctionnement sera présenté en détail dans le paragraphe **2.2**. Au sein de l'immunité muqueuse pulmonaire, la réactivité des cellules NK est régulée par la balance pro (IFN de type I et II, IL-12, TNF-α) et anti-inflammatoire (TGF-β, IL-10). Les cellules NK sont d'ailleurs, elles-mêmes capables de générer une réponse pro (IFN-γ) ou anti-inflammatoire (IL-10) et participent donc à l'homéostasie pulmonaire. En l'absence d'infection, les fonctions des cellules NK sont réprimées à la fois par un microenvironnement anti-inflammatoire et par l'interaction permanente des récepteurs inhibiteurs (KIRs) avec les molécules HLA de type I dans le tissu pulmonaire. A l'inverse, en cas de

pneumonie, elles participent à la première ligne de défense antibactérienne. Elles sont notamment capables de reconnaitre des motifs bactériens spécifiques puis d'activer les macrophages, polynucléaires neutrophiles et cellules dendritiques pulmonaires pour générer une réponse inflammatoire coordonnée. Au cours de l'infection à PA, les récepteur activateurs NCR2 (NKp44) et NKG2D de la cellule NK participent respectivement à la reconnaissance (28) et à la clairance bactérienne (29,30). Si la réponse immunitaire doit être rapide et intense pour mener à une clairance bactérienne efficace, la réponse inflammatoire doit également être balancée par une réponse anti-inflammatoire permettant d'éviter l'œdème interstitiel pulmonaire, la destruction du parenchyme et l'altération de l'hématose. La régulation de la réponse immunitaire est donc primordiale dans cet organe.

Ces modalités de régulation de la réactivité des cellules NK par le microenvironnement cellulaire et cytokinique a également des conséquences majeures en pathologie non infectieuse chez les patients de réanimation. En effet, une réaction inflammatoire intense (TNF-α, IFN-γ), dite « aseptique » peut survenir après un traumatisme grave ou une lésion cérébrale et favoriser l'activation NK. Les antigènes respiratoires et la flore commensale pulmonaire jusqu'ici tolérés, sont alors reconnus comme signaux de dangers (Damage-Associated Molecular Pattern ou Pathogen-Associated Molecular Pattern). Cette reconnaissance pourra donner lieu à une réponse inflammatoire, des lésions pulmonaires et menacer le pronostic vital du patient. **Ainsi, les cellules NK jouent un rôle central dans la défense anti-infectieuse pulmonaire mais sont également source de lésions pulmonaires en cas de réponse inflammatoire exacerbée d'origine infectieuse ou non.**

En résume, la cellule NK joue un rôle central dans l'immunité muqueuse de l'arbre respiratoire. Notre équipe a d'ailleurs rapporté dans un modèle murin de pneumonie à PA, que les cellules NK étaient des acteurs clefs de la défense anti-PA. Dans ce modèle, la déplétion en cellules NK augmentait la mortalité des souris infectées à PA (31). Par ailleurs, la cellule NK étant la source principale d'IFN-γ au cours de la réponse immunitaire, la compréhension de ses interactions avec le système du QS est primordiale.
L'étude de l'interaction PA-NK peut également s'intégrer également dans la thématique émergente de l'altération de l'immunité anti-cancer secondaire à une infection. Il existe très peu de données à l'heure actuelle, mais une équipe coréenne a rapporté que l'infection à PA accélérait la diffusion métastatique du mélanome malin et que ce phénomène était secondaire à une altération de l'immunité NK médiée par PA (32). On note d'ailleurs que parmi les récepteurs activateurs de la cellule NK, NKG2D participe, à la fois, à la clairance bactérienne au cours des infection à PA (29) et à la reconnaissance de nombreux types histologiques tumoraux chez l'homme, dont le mélanome (33). Nous étudierons donc également l'influence de PA sur la reconnaissance de cellules anormales par le lymphocyte NK.

Après cette description permettant une approche intégrée de l'immunité NK au cours des pneumonies à *Pseudomonas aeruginosa,* nous poursuivrons par une présentation plus détaillée de la bactérie et des fonctions de la cellule NK.

2. INTRODUCTION : Présentation de la bactérie et de la cellule Natural Killer

2.1. La bactérie : *Pseudomonas aeruginosa.*

2.1.1. Classification, structure et habitat

La bactérie a été isolée pour la première fois en 1882 par Gessard. *Pseudomonas aeruginosa* (PA) est une bactérie de la famille des Pseudomonadaceae. C'est un bacille à Gram négatif non sporulé de moins de 5µm de longueur capable d'oxyder la forme réduite de dérivés N-méthyles du paraphénylènediamine en semi-quinone (Souche dite « Oxydase positive »). PA est capable de croître à des températures allant de +4°C à + 45°C. Son génome est entièrement séquencé depuis les années 2000 (souche PAO1) avec de nombreux gènes spécialisés dans la résistance aux antibiotiques et l'adaptation métabolique (34) facilitant sa persistance chez l'hôte.

La bactérie est protégée par une double membrane (interne et externe), hydrophobe, séparée par un espace péri plasmique hydrophile. La membrane externe est une bicouche asymétrique constituée du Lipopolysaccharide (LPS) et de phospholipides, ou s'insèrent des porines transmembranaires permettant le passage de solutés hydrophiles. PA est une bactérie ubiquitaire et saprophyte, capable de vivre indifféremment sous forme planctonique (forme mobile) ou à l'état sessile dans un biofilm, attachée à une surface inerte ou organique. Ainsi la bactérie peut survivre dans l'eau, le sol, les végétaux, les solutions antiseptiques et les surfaces inorganiques (35). Chez l'homme et les mammifères sains, la bactérie est présente dans le tube digestif.

2.1.2. Facteurs de virulence impliqués dans le pouvoir pathogène pulmonaire de PA

Lors d'une infection aigue, la bactérie produit de nombreux facteurs de virulence à l'origine de dommages tissulaires et vasculaires favorisant la dissémination de l'infection.

Parmi tous ces facteurs de virulence, certains sont donc secrétés pour une action autocrine ou paracrine sur les cellules immunitaires de l'hôte, et d'autres sont injectés directement dans la cellule. Cette dichotomie sera centrale dans la suite de notre exposé car contrairement au mode d'action paracrine, l'injection intra cytoplasmique d'effecteur nécessite un contact direct entre la bactérie et la cellule cible.

Cette présentation permettra de justifier, d'une part, les différentes hypothèses élaborées lors de la réalisation de nos travaux et, d'autre part, le choix particulier de l'étude du système de sécrétion de type III.

2.1.2.1. Le système du Quorum sensing

Ce système est le principal mécanisme de régulation de la pathogénicité de la bactérie (20). Il permet aux bactéries communiquant entre elles de coordonner leur mode de fonctionnement (21). Au sein d'un inoculum bactérien, la communication entre bactéries repose notamment sur la production de plusieurs N-Acyl-Homoserine Lactones (AHL), elles-mêmes capables d'activer la transcription de facteurs de virulences de façon autocrine chez PA (36). Pour la bactérie PA, on distingue deux systèmes de régulation :

- Le système las, régulé par deux gènes *lasR* et *lasI* codant pour les protéines LasR et LasI impliquées dans la synthèse de la protéine effectrice N-(3-oxododecanoyl)-L-Homoserine lactone (3O–C12–HSL). Cette protéine appartient à la famille des AHL. Lorsque l'inoculum bactérien est élevé, les AHLs se lient à la protéine LasR et déclenchent l'expression de nombreux gènes de virulence.
- Le système Rhl, régulé par deux gènes *rhlR* et *rhlI* codant pour les protéines RhlR et RhlI impliquées dans la synthèse de la protéine effectrice N-butyryl-L-Homoserine lactone (C4–HSL). De la même façon, le complexe RhlR-C4-HSL déclenche l'expression de nombreux gènes de virulence.

Ce système peut également être activé par la fixation de médiateurs de l'inflammation sur le récepteur OprF (Outer membrane protein F). C'est une porine de 2 nanomètres de diamètre située dans la membrane externe de la bactérie. Elle joue d'abord un rôle structural dans le maintien de l'intégrité de la paroi bactérienne dans les milieux à faible osmolarité puisque qu'elle permet la diffusion transmembranaire des espèces ioniques. Par ailleurs, elle est impliquée dans l'adhésion à l'organe cible et dans la formation du biofilm. Ainsi, la fixation d'IFN-γ sur le récepteur OprF peut activer le quorum sensing et déclencher la synthèse d'AHLs (21). Le 3O-C12-HSL pourra alors à son tour, être sécrété par la bactérie via un système de pompe à efflux type MexAB-OprM et stimuler la production d'IFN-γ par les cellules T (Figure 2).

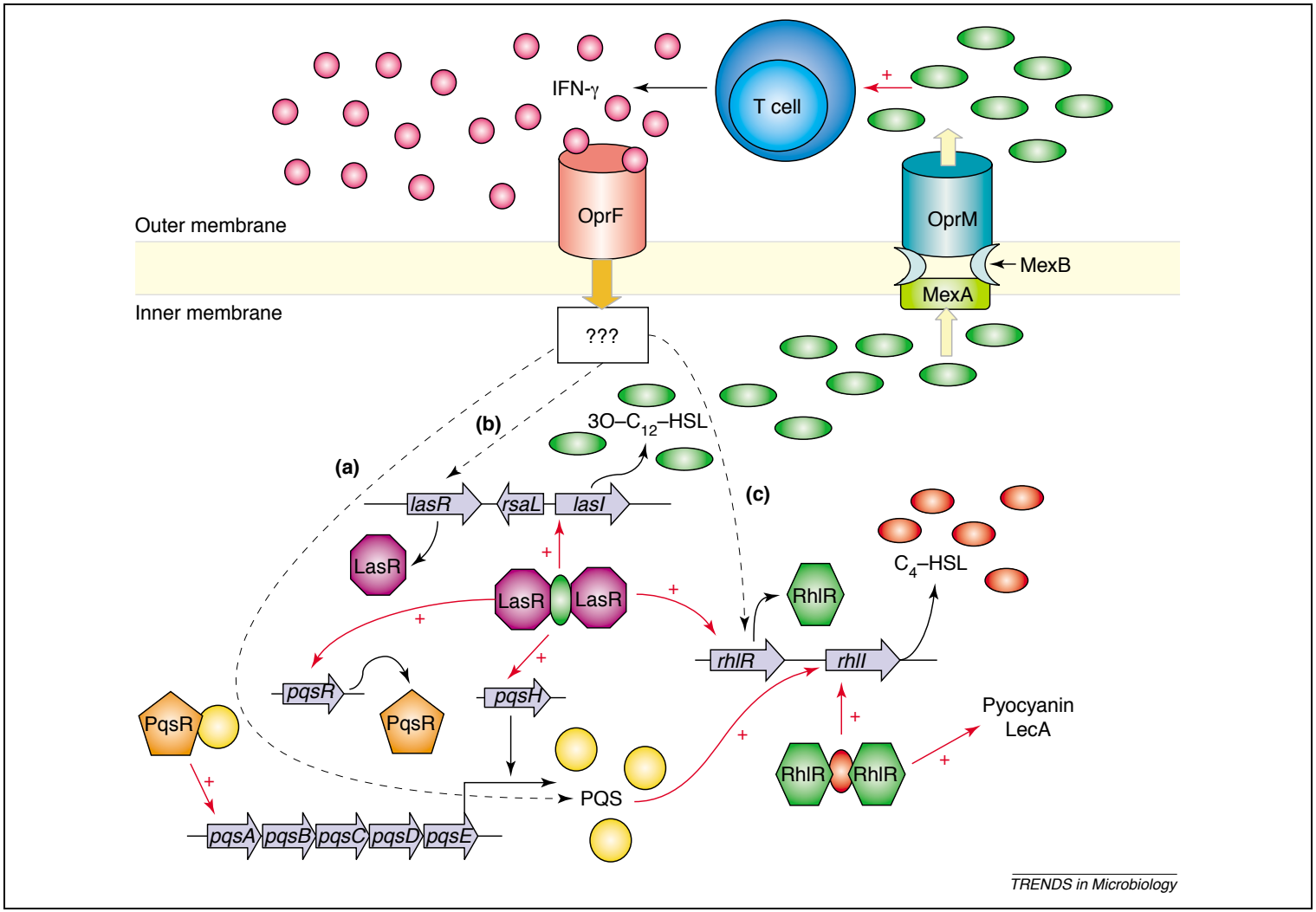
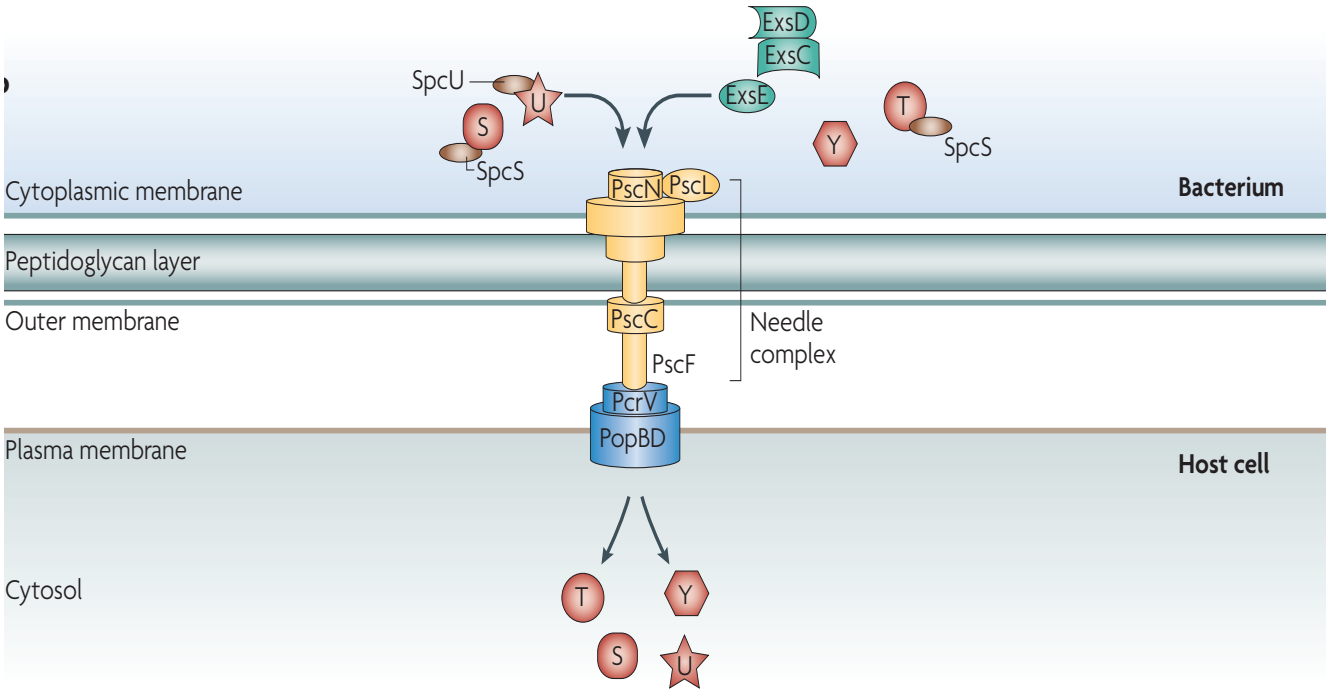


Figure 2 : Vue générale du système du Quorum Sensing, d'après Wagner et al 2006 (21)

2.1.2.2. Facteurs de virulence dépendants du quorum sensing

2.1.2.2.1. Le système de sécrétion de type 3 (T3SS) et ses effecteurs

Ce système a été caractérisé la première fois chez la bactérie Yersinia (37) et mis en évidence chez *Pseudomonas aeruginosa* en 1996 (38). Il est présent chez un nombre important de bactéries à Gram négatif notamment Salmonella, Escherichia coli, *Pseudomonas aeruginosa* et Chlamydia. Ce système a été précisément caractérisé en raison de ses implications potentielles en thérapie ciblée anti-infectieuse (Figure 3) (39). L'architecture de l'appareil de sécrétion est formée de vingt-cinq protéines codées par cinq gènes avec une importante similitude de séquence avec les composants du Flagelle (40). Cette structure est relativement conservée entre les différentes espèces bactériennes exprimant le SSTIII (41).



<u>Figure 3</u>: Vue d'ensemble des constituants du système de sécrétion de type III (39)**.**

Cet appareil est constitué d'une base d'ancrage fixée sur la membrane bactérienne, d'un injecteur et d'un translocon capables de pénétrer la membrane cytoplasmique de l'hôte et d'injecter les protéines effectrices : Exoenzymes S, T, U et Y. Voici le détail des constituants :

- La protéine PscS, « la sécrétine » est oligomérisée avec une lipoprotéine PscW. Elles forment ainsi à elles deux, le corps basal permettant l'ancrage de l'aiguille de sécrétion.
- L'aiguille d'injection est constituée d'une seule protéine polymérisée sous forme hélicoïdale, constituant un conduit nommé PscF (42). L'aiguille mesure soixante à quatre-vingt nm de

long avec un diamètre externe de 8nm (43). Le corps basal et l'aiguille d'injection forme l'injectisome. A la partie basale du « Needle complex », la protéine PscN grâce à son activité ATPase sert de socle énergétique à l'injectisome. La protéine PscL régule l'activité de PscN.

- Le translocon représente le pore protéique formé dans la membrane plasmique de l'hôte. *Pseudomonas aeruginosa* utilise trois protéines pour réaliser ce translocon : PopB, PopD et PcrV (44) PopB et PopD sont deux protéines hydrophobes sécrétées à travers l'aiguille, s'insérant ensuite dans la membrane de la cellule hôte (45). La protéine pcrV, également secrétée à travers l'aiguille, effectue le lien entre l'aiguille et le pore membranaire. Le translocon permet ainsi le passage des protéines secrétées et altère également la perméabilité membranaire cellulaire (46,47).
- Les effecteurs du système de sécrétion de type III sont les Exoenzymes sécrétées à travers l'injectisome initialement sous formes dépliées en raison du faible diamètre de l'aiguille. Les Exoenzymes (Exo) sont au nombre de quatre : ExoS, ExoT, ExoS et ExoU (48). L'expression de ces quatre Exo est inconstante et une majorité des souches bactériennes expriment S, T et Y (48). Seuls 10% des isolats de PA chez les patients atteints de Mucoviscidose expriment l'ExoU. Les ExoS et U ne coexistent qu'exceptionnellement. La souche PAO1 que nous utiliserons dans la suite de notre exposé exprime les ExoT, S et Y (34). Les Exoenzymes sont stockées dans le cytoplasme bactérien sous formes liées à des protéines chaperonnes (SpcS ou SpcU) améliorant leur stabilité et facilitant leur sécrétion par le SSTIII. L'injection des Exoenzymes directement dans le cytoplasme des cellules cibles est à l'origine d'une désorganisation du cytosquelette et des voies de signalisation intracellulaire pouvant moduler la réponse inflammatoire et/ou provoquer l'apoptose de la cellule cible.

    - ❖ Les Exoenzymes S et T sont deux toxines bi fonctionnelles avec de grandes homologies de séquence. L'ExoT est exprimée par plus de 95% de souche clinique de *Pseudomonas* et est l'un des principaux facteurs de virulence de la bactérie. (49). ExoT et ExoS ont des effets sur les GTPases de type Rho-like et provoquent des altérations du cytosquelette actinique menant fréquemment à l'apoptose de la cellule cible. Ces deux Exo possèdent un domaine N-terminal de type GTPase-activating protein (GAP) et C-terminal de type ADP-ribosyltransférase (ADPRT) tous deux semblables :
        - Le domaine ADPRT de l'ExoS cible plutôt les régulateurs du cytosquelette actinique comme les protéines de la famille Ras, alors que le domaine ADPRT de l'ExoT interagit avec la famille des protéines Crk et les phosphoglycérates kinase (50).
        - A l'opposé, les domaines GAP d'ExoS et T ciblent préférentiellement des

protéines types GTPases (51) et provoquent l'activation de la voie JNK (1/2) (52).

- L'Exoenzyme Y est une adénylate cyclase de 48kDa. Après injection de l'ExoY dans la cellule cible, la concentration intracellulaire d'AMPc augmente provoquant une désorganisation du cytosquelette actinique aboutissant à la mort cellulaire (53). Dans les modèles in vivo, le rôle d'ExoY dans la virulence du *Pseudomonas* n'est pas clairement défini.

- L'Exoenzyme U est une protéine de 74 kDa possédant une activité phospholipase A2 (54). Cette activité lipolytique altère l'un des composants principaux du surfactant pulmonaire, le dipalmitoyl phophatidylcholine, favorisant le passage des agents bactériens du tissu pulmonaire vers la circulation générale. C'est la plus cytotoxique des quatre Exoenzymes effectrices du système de sécrétion de type III expliquant la virulence accrue des souches exprimant ExoU dans les modèles murins de pneumonie (49).

Les Exoenzymes sont donc injectées dans la cellule de l'hôte par l'intermédiaire du SSTIII (55) et peuvent déclencher la mort cellulaire de la cible (19). Le rôle délétère de ce système a été rapporté en clinique humaine (56). Chez les patients atteints de mucoviscidose, le SSTIII jouait un rôle primordial dans l'invasion initiale (57) et permettait à PA de résister à la bactéricidie des polynucléaires neutrophiles humains (58).

La transcription des effecteurs est finement régulée par quatre protéines ExsA, ExsC, ExsD et ExsE : La protéine ExsA est le signal promoteur de la transcription des effecteurs. A l'état quiescent, la protéine ExsE se lie à la protéine ExsC permettant à ExsD de se fixer à ExsA prévenant ainsi sa fixation au promoteur. A l'inverse, en période d'invasion, lorsque T3SS est activée, la sécrétion des effecteurs via PscS s'accompagne d'une sécrétion de ExsE, permettant la neutralisation de ExsD par ExsC. Ainsi ExsA peut se fixer sur la région promotrice et activer l'activité transcriptionnelle (Figure 4).

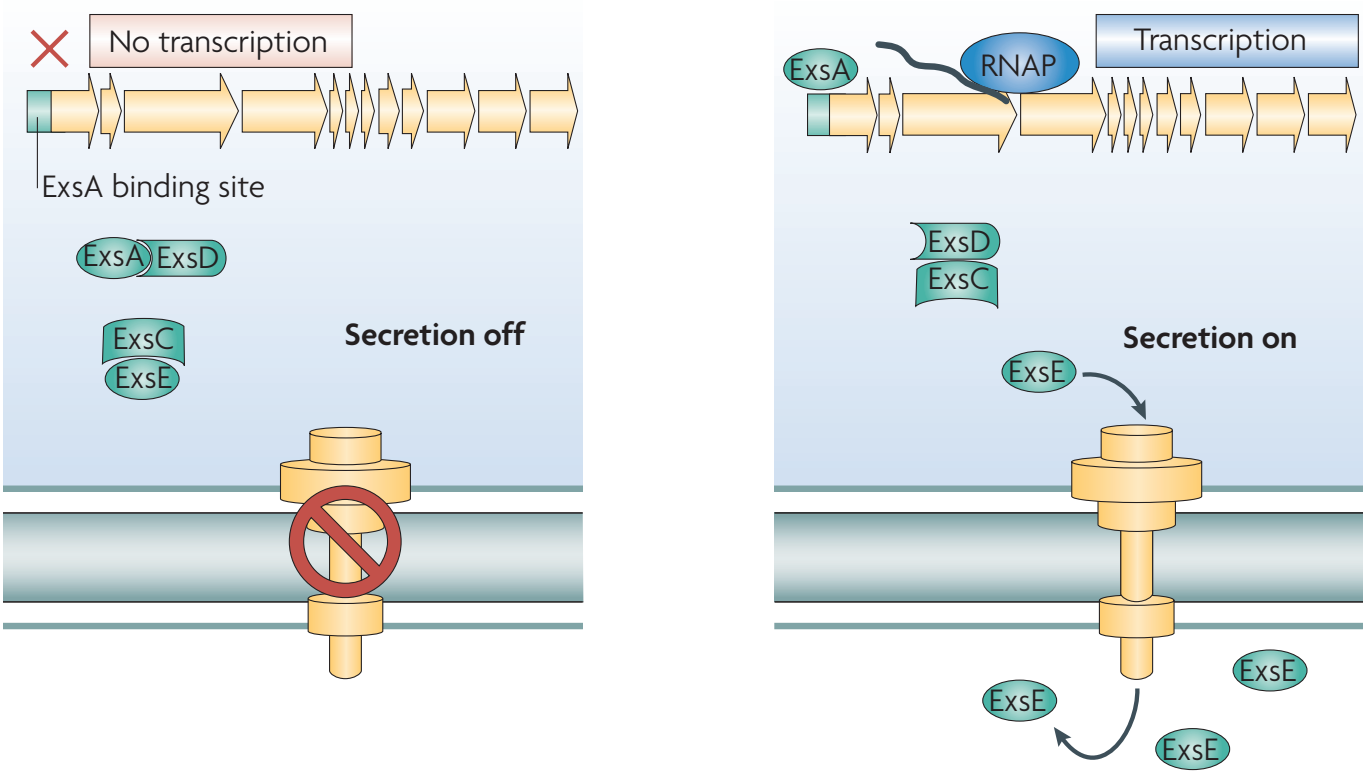


Figure 4 : Régulation de l'activité transcriptionnelle des effecteurs du T3SS par la protéine ExsA (39)**.**

2.1.2.2.2. Le système de sécrétion de type 2 (T2SS) et ses effecteurs

2.1.2.2.2.1. L'exotoxine A

L'exotoxine A est une enzyme de 66 kDa sécrétée dans le milieu. Elle est sécrétée sous la forme d'une pro-toxine inactive. Après internalisation par la cellule cible de l'hôte, l'Exotoxine A exerce une activité ADP-Ribosyltransférase inhibant la synthèse protéique et provoquant la mort cellulaire (59). Ainsi, lors d'infections *in-vitro* de sang total, cette toxine altère la sécrétion de TNF-α, IL-10, IL-6 et IL-8 par les cellules immunitaires de l'hôte (60,61). Ce mode d'invasion de la cellule hôte ne nécessite donc pas de contact physique entre la cellule cible et la bactérie pour être pathogène.

2.1.2.2.2.2. LasB, élastase et LasA, protéase.

LasB est une métallo protéase de 33 kDa, codée par le gène *lasB*, capable de cliver l'élastine et le collagène composants clés des jonctions serrées d'un épithélium (62). Ainsi au cours des infections pulmonaires la perméabilité épithéliale est accrue entrainant un œdème interstitiel. LasA est une protéase de 20kDa, codée par le gène *lasA* agissant en synergie avec l'élastase LasB (63). LasB interfère avec le canal CFTR et altère ainsi la clairance muco-ciliaire de l'hôte favorisant ainsi l'adhésion de la bactérie à l'épithélium bronchique et sa persistance. De plus, LasB altère le renouvellement de l'épithélium bronchique en interférant avec la voie de L'IL-6 majorant ainsi les lésions épithéliales générées par l'infection à PA.

2.1.2.2.2.3. La protéase IV

Il s'agit d'une protéine de 26kDa codée par le gène *pprpL.* Elle agit en synergie avec LasA et LasB et altère l'intégrité des épithéliums. Cette protéase peut également dégrader des produits de l'inflammation et les immunoglobulines sur le site de l'infection (64-66) ainsi que des composants du surfactant alvéolaire (66).

2.1.2.2.3. Facteurs de virulence impliqués dans l'adhérence et la motilité

2.1.2.2.3.1. Flagelle

Il s'agit d'un appendice impliqué dans la motilité des bactéries à gram négatif. Il est composé de protéines multiples issues de la transcription de plus cinquante gènes différents (67). *Pseudomonas aeruginosa* possède un flagelle unique lui permettant de se déplacer dans un environnement aqueux (68,69). Ce facteur de virulence est impliqué dans le système du quorum sensing car la disparition du flagelle (mutation Δflic) est à l'origine d'une anomalie de production des Homoserine lactones (70). La flagelline est un PAMP reconnu par le Toll Like Récepteur (TLR) de type 5 porté par les cellules de l'immunité innée (71-73).

2.1.2.2.3.2. Le LPS

Le Lipopolysaccharide est le principal composé de la membrane externe de PA. Il est constitué de trois éléments principaux :

- le lipide A qui est un agoniste des récepteurs TLR4 (74),
- Une chaine de polysaccharides hydrophile,
- L'antigène O déterminant le sérotype antigénique de PA.

Au cours de l'infection le LPS peut subir des mutations permettant à la bactérie de persister chez l'hôte.

2.1.2.3. Les facteurs de virulence quorum sensing indépendants

2.1.2.3.1. Les lectines

PA produit deux lectines solubles :

- PA-IL (LecA), protéine de 12kDa, codée par le gène *lecA*, reconnaissant préférentiellement des motifs galactose,
- PA-IIL (LecB), protéine de 11 kDa, codée par le gène *lecB*, spécifique du fucose. Elles sont stockées dans le cytoplasme de la bactérie. Elles sont également présentes sur la membrane externe de la bactérie (75,76).

Ces deux protéines sont impliquées dans la synthèse et la stabilisation du biofilm ainsi que dans l'adhésion de la bactérie aux cellules épithéliales. Cette protéine a également un effet cytotoxique direct sur l'épithélium respiratoire (77-79) responsables d'œdème interstitiel pulmonaire et altère la motilité ciliaire des cellules de l'arbre trachéo-bronchique.

2.1.2.3.2. Les autres systèmes de sécrétions

Pour faciliter l'excrétion des facteurs de virulence à travers sa double membrane (interne et externe), la bactérie a mis en place différents systèmes de sécrétion. Ces motifs sont conservés au cours de l'évolution dans les différents bacilles à Gram négatif (80,81). Les systèmes de sécrétion de *Pseudomonas aeruginosa* sont au nombre de six.

2.1.2.3.2.1. Le système de sécrétion de type I

Il est composé d'une protéine spécifique de la membrane externe appelée Outer Membrane Protein (OMP) qui forme un tonneau β, d'un transporteur donneur d'ATP : le transporteur ABC (ATP Binding Cassette) et d'une protéine de fusion membranaire (MFP) faisant le lien entre OMP et ABC. Certaines protéases sont notamment secrétées par ce biais (82).

2.1.2.3.2.2. Le système de sécrétion de type VI

C'est le système de sécrétion le plus récemment découvert chez *Pseudomonas aeruginosa*. Il est en fait constitué de trois ensembles H1 (Hcp Secretion Island-type I)-T6SS, H2-T6SS et H3-T6SS. Le système est régulé par les molécules du quorum sensing mais la nature des protéines sécrétées via le T6SS est mal définie (83-85).

2.2. La cellule Natural killer

Kiessling et son équipe en 1975, ont mis en évidence dans un modèle murin, une population lymphoïde capable de lyse tumorale sans immunisation préalable. Philips et Lanier en 1986 ont ensuite mis en évidence sa capacité de lyser des cellules n'exprimant pas de molécule du Complexe Majeur d'Histocompatibilité (CMH) de type I. C'est la théorie du « missing-self ».

2.2.1. Classification des Innate lymphoid cells (ILC)

Les cellules NK ont une morphologie lymphoïde, elles sont issues d'un progéniteur lymphoïde commun et possèdent toutes un récepteur commun à l'Interleukine-2 : IL-2Rγ. Elles n'expriment ni marqueur B (CD19, CD20), ni marqueur T (CD3, TCRαβ, TCRδ), ni marqueur phénotypique des cellules myéloïdes ou dendritiques. Les cellules Natural Killer représentent de 5 à 20% des PBMC (Peripheral Blood Mononuclear Cells) totaux circulant. Leur action est initiée très précocement lors de la réponse immunitaire ce qui suggère leur rôle central dans la réponse anti-infectieuse et tumorale.
Plus récemment, les cellules NKs ont été classées dans la famille des Innate Lymphoid cells (ILC). Les ILC peuvent être séparées en trois groupes définis essentiellement par l'expression d'un facteur de transcription spécifique T-bet, GATA3 ou RORγT (Figure 5) (86) :

- **Le Groupe 1 (ILC1)** est caractérisé par l'expression du facteur de transcription T-bet et la capacité de production d'IFN-γ (Cytokine de type Th1) en réponse notamment à l'IL-12 (87). Les cellules Natural Killer appartiennent au groupe des ILC1 (88),

- **Le Groupe 2 (ILC2)** est caractérisé par l'expression du facteur de transcription GATA3 et la capacité de production de cytokine de type Th2 dont l'IL-5 et l'IL-13,

- **Le Groupe 3 (ILC3)** est caractérisé par l'expression du facteur de transcription RORγt et la capacité de production de cytokine de type Th17 dont l'IL-17 et l'IL-22. Certains membres de cette famille expriment également des Natural Cytotoxic Receptor (NCR) communs avec les cellules NK : le récepteur NKp44 (NCR2), NKp46 (NCR1) et peuvent également exprimer le facteur de transcription T-bet.

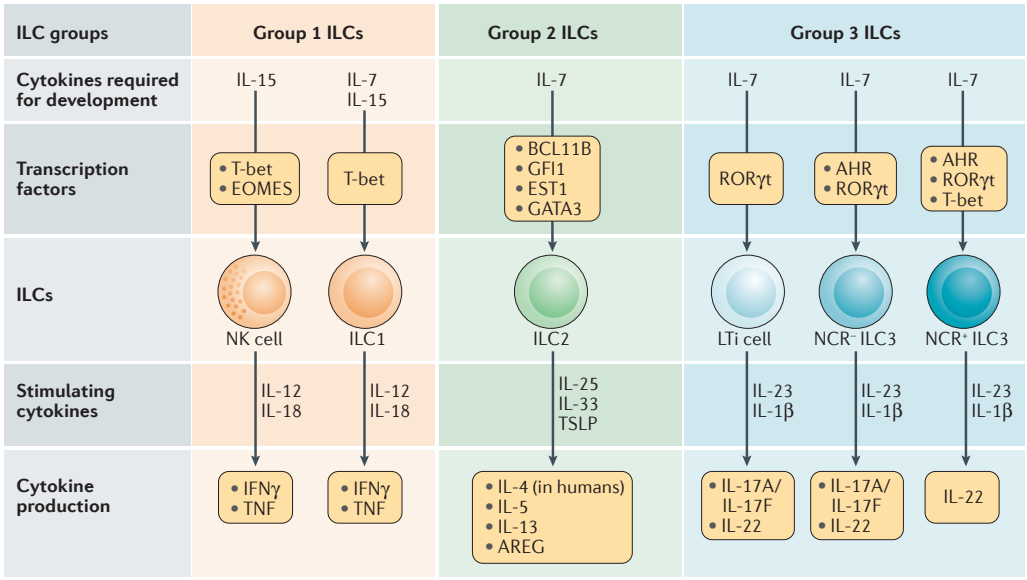


Figure 5 : Classification des Innate Lymphoid Cells (86).

2.2.2. Genèse, développement et morphologie

Selon caligiuri (89), les cellules NK sont issues d'un progéniteur $CD34^+$ et se développent majoritairement dans la moelle osseuse. Le progéniteur $CD34^+$ perd ensuite les caractéristiques des lignées myéloïdes et érythroïdes et devient un NK précurseur (NKP). Les NKP quittent ensuite la moelle osseuse puis rejoignent les organes lymphoïdes secondaires et deviennent iNK (NK immature). La cellule acquiert alors le récepteur à l'IL-2, à l'IL-7 (IL-7Rα) et à l'IL-15. L'interaction avec les cellules stromales de la moelle osseuse et les CD dans les organes lymphoïdes secondaires au cours de leur développement participe à la maturation des iNK. Notamment, la reconnaissance des ligands c-kit ligand, Flt-3 et IL-15 en périphérie ou encore la stimulation par l'IL-2 contribueront leur maturation, leur différentiation et l'acquisition de leurs caractéristiques $CD56^{bright}$ (90-92).

Contrairement aux lymphocytes T, les NK ne nécessitent pas de maturation intra-thymique mais peuvent y transiter. Néanmoins, les NK passent également par une étape d'éducation ou « licensing » afin d'obtenir une tolérance vis-à-vis des molécules du soi. Cette étape passe par une interaction entre les récepteurs Killer-cell Immunoglobulin-like Receptor (KIR) et le CMH de type I.

2.2.3. Les différents subsets de NK – Maturation et Classification

L'expression membranaire des marqueurs CD56, CD16, CD57, des récepteurs KIR et du CD94 permet classiquement de distinguer plusieurs sous-types (subsets) au sein des cellules NK.

On distingue alors quatre grands subsets : $CD56^{bright}$ ($CD94^+KIR^-CD16^-$), $CD56^{dim}$ ($CD94^+KIR^-CD16^+$), $CD56^{dim}$ ($CD94^+KIR^+CD16^+$) et $CD56^{dim}$ ($CD94^-$ KIR^+CD16^+ $CD57^+$) (Figure 6).

Ces différents stades de maturation sont marqués par une sensibilité différente à l'IL-2. La maturation $CD56^{bright}$ vers $CD56^{dim}$ s'accompagne enfin de la perte du CCR7, permettant aux cellules NK matures de quitter les relais lymphatiques et d'exercer leur activité en périphérie. La maturation, la survie et l'activation des NK sont favorisées par l'IL-12, l'IL-15 et L'IL-18 (93). La stimulation IL-2 et IL-15 participe à l'acquisition respectivement du CD16 (94) et des KIRs (95). Au contraire le TGF-β libéré par les lymphocytes T régulateurs (Treg) ou les CD inhibe la prolifération et la maturation des NKs (96).

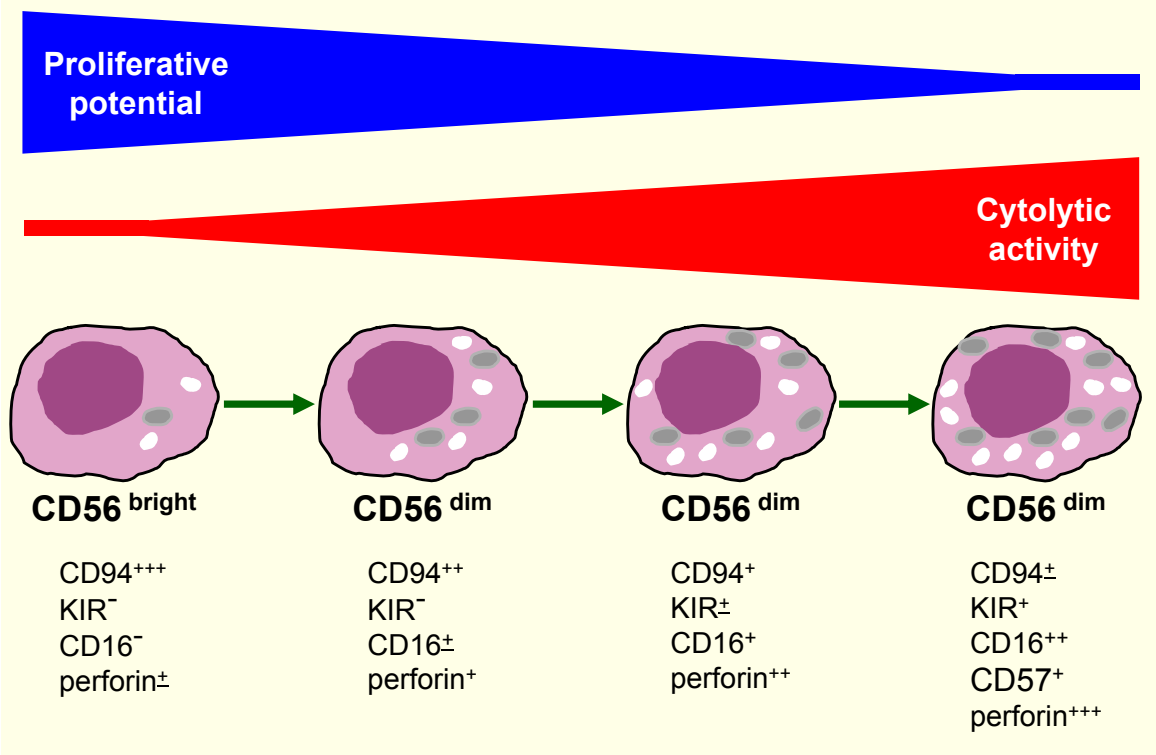


Figure 6 : Stade de maturation de la cellule Natural killer (97).

2.2.3.1. Expression sur CD56

Parmi les cellules NK on distingue classiquement les cellules $CD56^{bright}$ et $CD56^{dim}$ (Figure 7).

- Les cellules $CD56^{bright}$ sont décrites comme étant spécialisées dans la production de cytokines (IFN-γ, TNF-α, GM-CSF, IL-10 et IL-13) et sont présentes principalement dans les organes lymphoïdes secondaires.
- Les cellules $CD56^{dim}$ sont retrouvées de façon majoritaire dans le sang périphérique ou les tissus inflammatoires et sont spécialisés dans l'activité cytotoxique.

Des données récentes suggèrent qu'il existe un continuum de maturation allant des cellules $CD56^{bright}$ vers les $CD56^{dim}$. Ainsi, les NK $CD56^{bright}$ acquièrent progressivement le récepteur CD16 et deviennent $CD56^{bright}$ $CD16^{+}$ puis $CD56^{dim}$ $CD16^{+}$. Cette description est appuyée par une

augmentation de la proportion des cellules CD56^{bright} CD16^{+} et CD56^{dim} CD16^{+} par rapport au CD56^{bright} CD16^{-} chez les patients âgés (97). Au cours de leur maturation les cellules CD56^{bright} acquièrent également les récepteurs KIR (KIR^{+}) et le marqueur de maturation CD57.

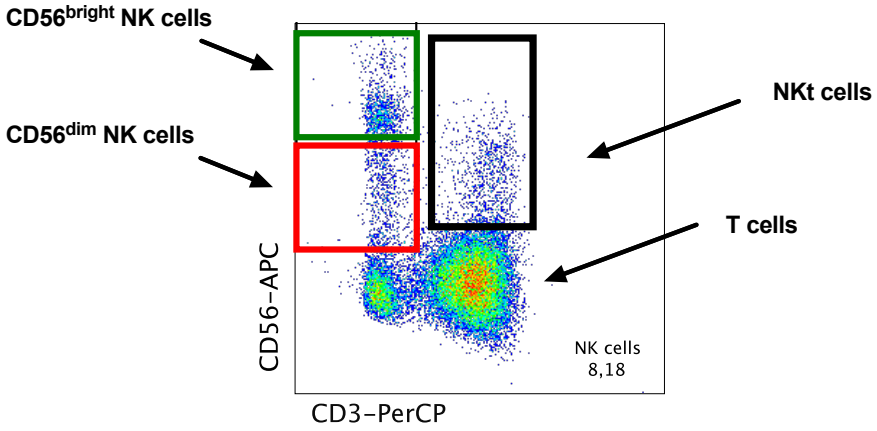


Figure 7: Profile d'expression en cytométrie du CD56 chez les cellules CD56^{+} CD3^{-} au sein de PBMC totaux de donneurs sains.

2.2.3.2. Le récepteur à l'IL-2

Au cours de développement, les iNK acquièrent successivement les sous-unités α, β et γ du récepteur à l'IL-2 (IL-2R ou CD25^{+}). Ainsi, les NK CD56^{bright} CD16^{-} expriment un récepteur à IL-2 hétérotrimérique (IL-2Rαβγ) assurant une très forte réponse proliférative après stimulation par l'IL-2. Au cours de leur maturation, les NK CD56^{dim} CD16^{+} n'expriment plus qu'un récepteur dimérique (IL-2Rβγ) ou monomérique (IL-2Rγ) expliquant leur faible réactivité à l'IL-2 (98).

2.2.3.3. Expression des KIRs

Les récepteurs KIR appartiennent à la superfamille des immunoglobulines et sont codés pour quatorze gènes très polymorphes (79). Les différents récepteurs de cette famille diffèrent par leur nombre de domaines extracellulaires (2 ou 3, d'où la nomenclature 2D ou 3D) et par la taille de leur fragment intra cytoplasmique (soit « L » pour Long, soit « S » pour Short). Chaque récepteur est spécifique d'un groupe d'allèles du CMH de classe I. Les domaines intra cytoplasmiques de type « L » sont reliés à une séquence ITIM (Immunoreceptor Tyrosine-Based Inhibitory Motif), les récepteurs correspondant seront donc dits « inhibiteurs ». A l'inverse, les domaines intra cytoplasmique de type « S » sont reliés à une séquence ITAM (Immunoreceptor Tyrosine-Based Activating Motif), les

récepteurs correspondants seront donc dits « activateurs » (Figure 8). Les KIR reconnaissent les molécules de HLA-de type I classique (A, B et C) et HLA-G (HLA type I non classique). Le détail des KIRs activateurs et inhibiteurs sera abordé dans la section dédiée aux récepteurs. Le principal signal activateur des KIRs est le « soi manquant » (absence de reconnaissance de molécule de HLA de type I à la surface de la cellule cible) aussi appelé « missing-self ».

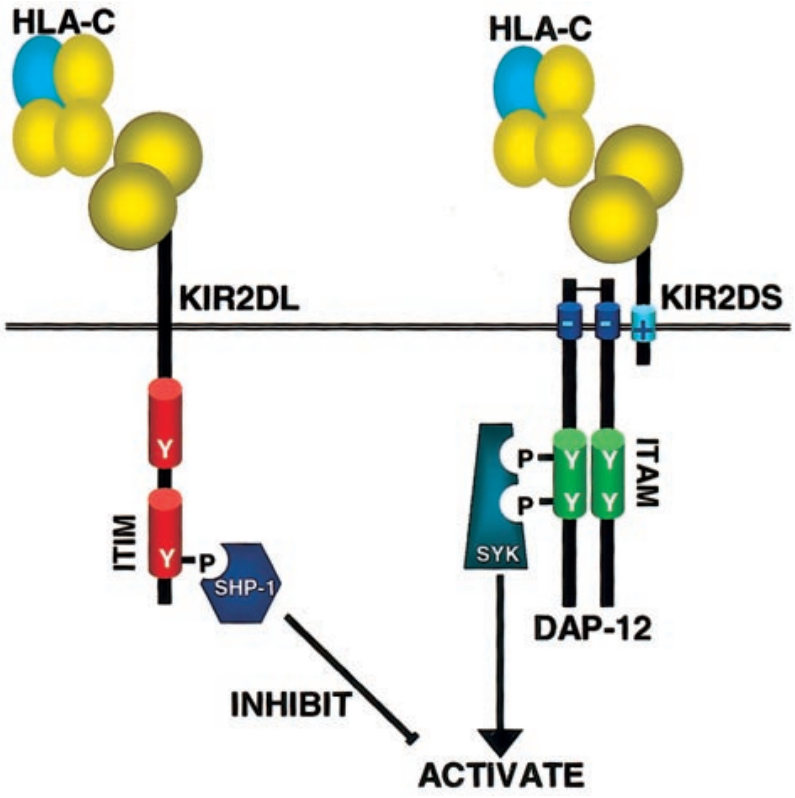


Figure 8 : Voies d'activation et de signalisation des KIRs activateurs et inhibiteurs (99).

2.2.3.4. Expression du CD16

Le marqueur CD16 aussi appelé, FcγRIII, est un récepteur de faible affinité de la partie constante Fc des immunoglobulines de type IgG. Cette famille de récepteurs est divisée en trois groupes FcγR, FcαR et FcεR reconnaissant respectivement les fragments Fc des IgG, IgA et IgE. La cellule NK exprime essentiellement le CD16 (FcγRIII). Ce marqueur est essentiellement exprimé à la surface des cellules $CD56^{dim}$ et quasi absent à la surface des NK $CD56^{bright}$. La reconnaissance du fragment Fc d'une immunoglobuline fixée à un antigène, déclenche une réponse cytokinique et cytotoxique de la NK et la destruction de la cellule cible. On parle d'opsonisation. Après reconnaissance de sa cible le récepteur CD16 signale via les tyrosines kinases Syk et ZAP70. Etant donnée la faible affinité de ce récepteur pour sa cible, une quantité importante de ligands est nécessaire à l'activation de la cellule.

2.2.3.5. Expression de CD94

La molécule CD94 est toujours associée à un membre de la famille NKG2 (A, B, C, D, E, F ou H) sous la forme d'un récepteur hétérodimérique (81). Le couple CD94/NKG2 appartient à la famille des récepteurs de type lectine-C. Ce type de récepteur est également présent à la surface des LT $CD8^+$. La nature du couple protéique définit la fonction activatrice ou inhibitrice et la nature du ligand reconnu. Leur mode de signalisation intracellulaire est semblable à celui des KIRs (Figure 9). Le détail des CD94 activateurs et inhibiteurs sera abordé dans la section consacrée aux récepteurs.

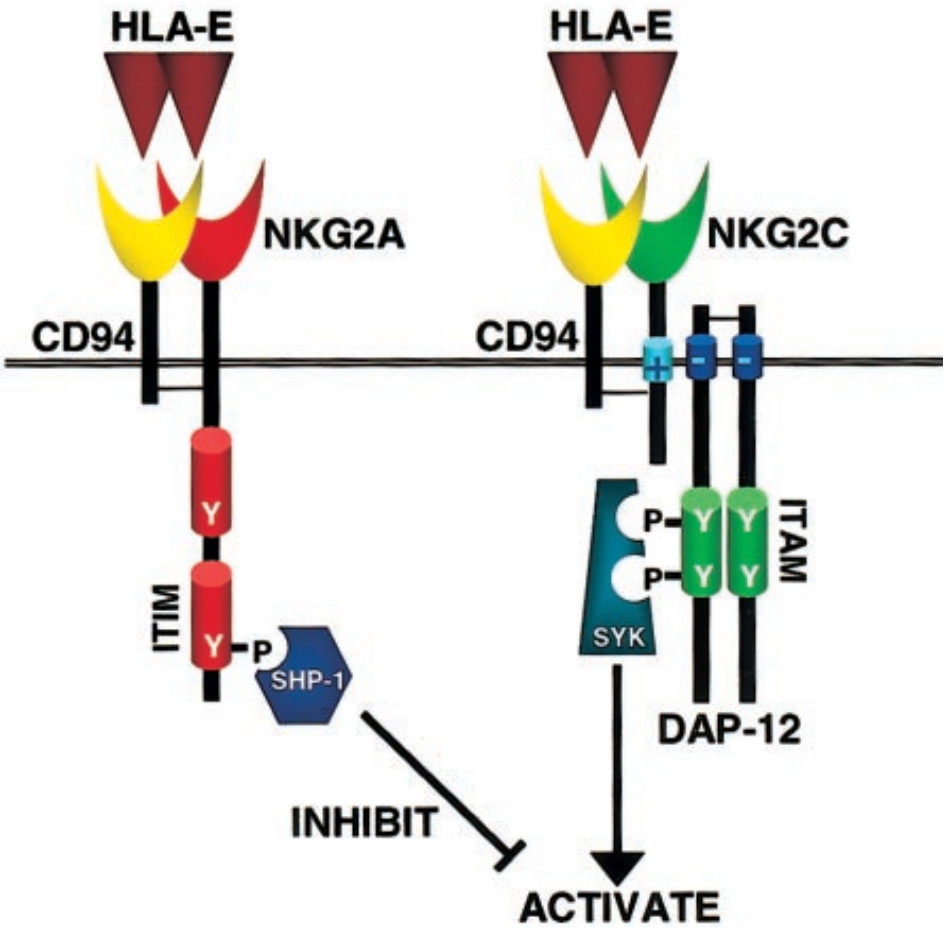


Figure 9: Voies d'activation et de signalisation des récepteurs type Lectine-C activateurs et inhibiteurs (99)

2.2.3.6. Expression du CD57

L'expression du CD57 est le marqueur d'un stade de maturation ultime de la cellule NK et d'une baisse des capacités de prolifération sans être un stigmate de senescence. Les NK exprimant fortement

le CD57 ont été rapportés comme jouant un rôle dans l'immunité antivirale après infection à CMV en raison d'un pouvoir cytotoxique intense (97). Ce marqueur est également présent sur les LT $CD8^+$.

2.2.4. Concept de balance activatrice et inhibitrice : Régulation de la réponse NK

L'activation des NK dépend de l'intégration de l'ensemble des signaux activateurs et inhibiteurs. Ainsi la cellule NK semble former une synapse immunologique avec la cellule cible. L'activation de la cellule NK peut alors résulter de (Figure 10) :

- L'absence de signal inhibiteur,
- La présence de signaux activateurs en excès par rapport aux signaux inhibiteurs (100).

Nous présenterons les récepteurs activateurs et inhibiteurs dans le paragraphe suivant.

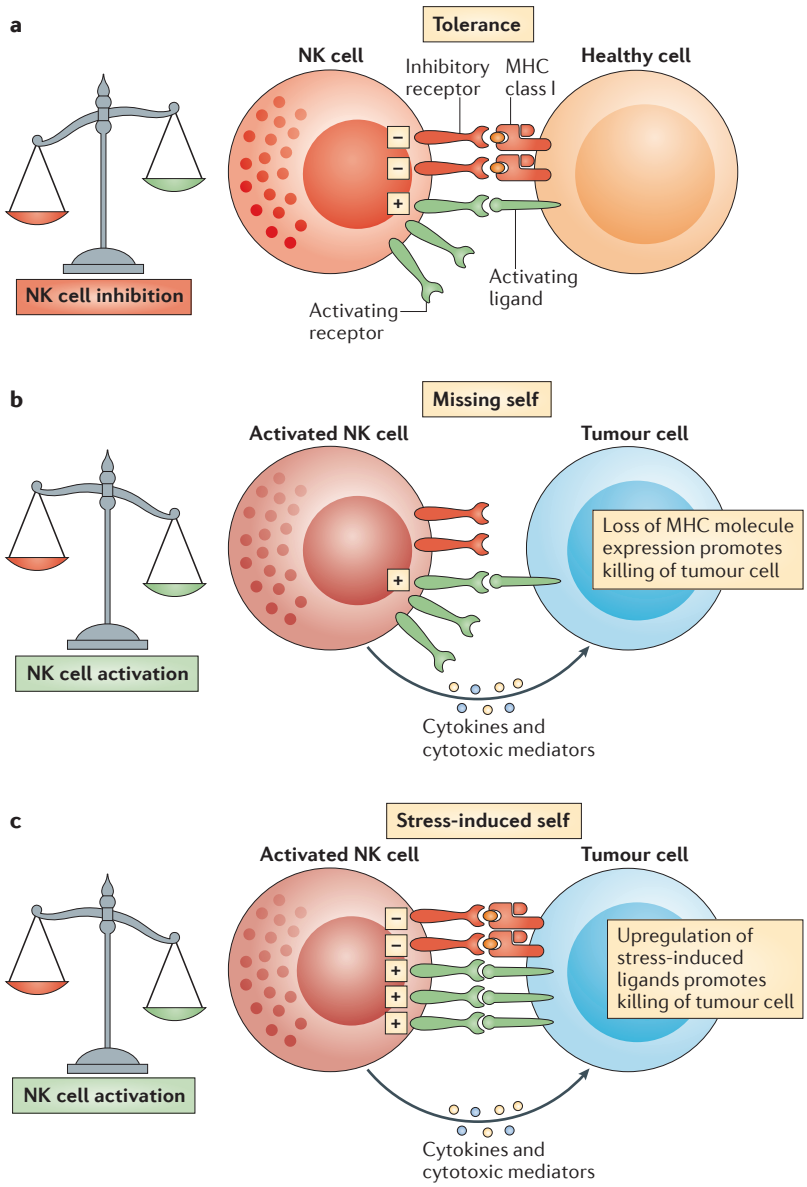


Figure 10 : Balance entre signaux activateurs et inhibiteurs déterminant la fonction des cellules NK (101).

2.2.5. Modalités de réponses de la cellule Natural Killer

On distingue deux modalités de réponses principales avec des voies de signalisations intracellulaires communes.

2.2.5.1. La fonction cytokinique :

Parmi les différents subsets précédemment décrits, les NK CD56^{bright} présentent l'activité cytokinique la plus intense. Ce subset est numériquement minoritaire dans le sang périphérique mais constitue la population NK majoritaire dans les centres lymphoïdes secondaires. Au sein des centres lymphoïdes, les NK CD56^{bright} interagissent plus spécifiquement avec les CD venant de la périphérie. Au cours de

la réponse anti-infectieuse, la NK sécrète des cytokines (TNF-α, IL-10, INF-γ, GM-CSF et de chimiokines CCL3 (MIP1-α), CCL4 (MIP-1β), CCL5 (RANTES). Ainsi la cellule NK est capable d'attirer les macrophages, les cellules CD et d'orienter la réponse inflammatoire vers les voies Th1, Th2 ou T_{reg}.

La fonction cytokinique est régulée par le microenvironnement (IL-12, IL-15, IL-18 et IL-21). L'IL-12, produite par les macrophages ou les CD, est la principale cytokine stimulant l'activité IFN-γ de la cellule NK (102,103). Le récepteur à l'IL-12, est composé de deux sous-unités β1 et β2 respectivement sites de fixation de IL-12p40 et IL12p35 (104). L'IL-12Rβ2 est présent à la surface de la cellule NK (105,106) et son niveau d'expression à la surface des lymphocytes est corrélé au degré de réponse cellulaire à l'IL-12 (107,108). La stimulation IL-12 provoque la fixation de JAK2 sur la chaine β2 du récepteur à l'IL-12. La chaine ß2 subi alors une transphosphorylation par JAK2, puis STAT4 se fixe sur la sous-unité β2 et est ensuite phosphorylée sur la Tyrosine 693 du domaine SH2. Ces événements concourent in-fine à une homodimérisation de P-STAT4 et sa migration nucléaire vers le promoteur du gène de l'INF-γ.

2.2.5.2. La fonction cytotoxique

La fonction cytotoxique est caractérisée par la libération de granules cytotoxiques (Perforine ou Granzyme A ou B) en direction de la cible. Cette fonction est principalement effectuée par les NKs $CD56^{dim}$. Elle peut être initiée soit par la reconnaissance du soi manquant (absence de signaux inhibiteurs) soit par la reconnaissance de signaux activateurs.

On distingue également de la cytotoxicité dite « naturelle » sus-décrite, la cytotoxicité induite ou ADCC (Antibody–Dependent Cell Cytotoxicity). Celle-ci est dépendante du récepteur CD16 reconnaissant le fragment Fc des immunoglobulines fixées sur une cible. La cytotoxicité peut également impliquer d'autres interactions : FAS (CD95)/FAS-L (CD95L), TRAIL (TNF-Related Apoptosis-Inducing Ligand)/TRAIL récepteur.

Les voies de signalisations des fonctions cytokinique et cytotoxique comportent de nombreuses kinases communes. Le type de réponse à un agent pathogène ou une cellule anormal dépendra donc du subset $CD56^{bright}$ ou $CD56^{dim}$ (Figure 11).

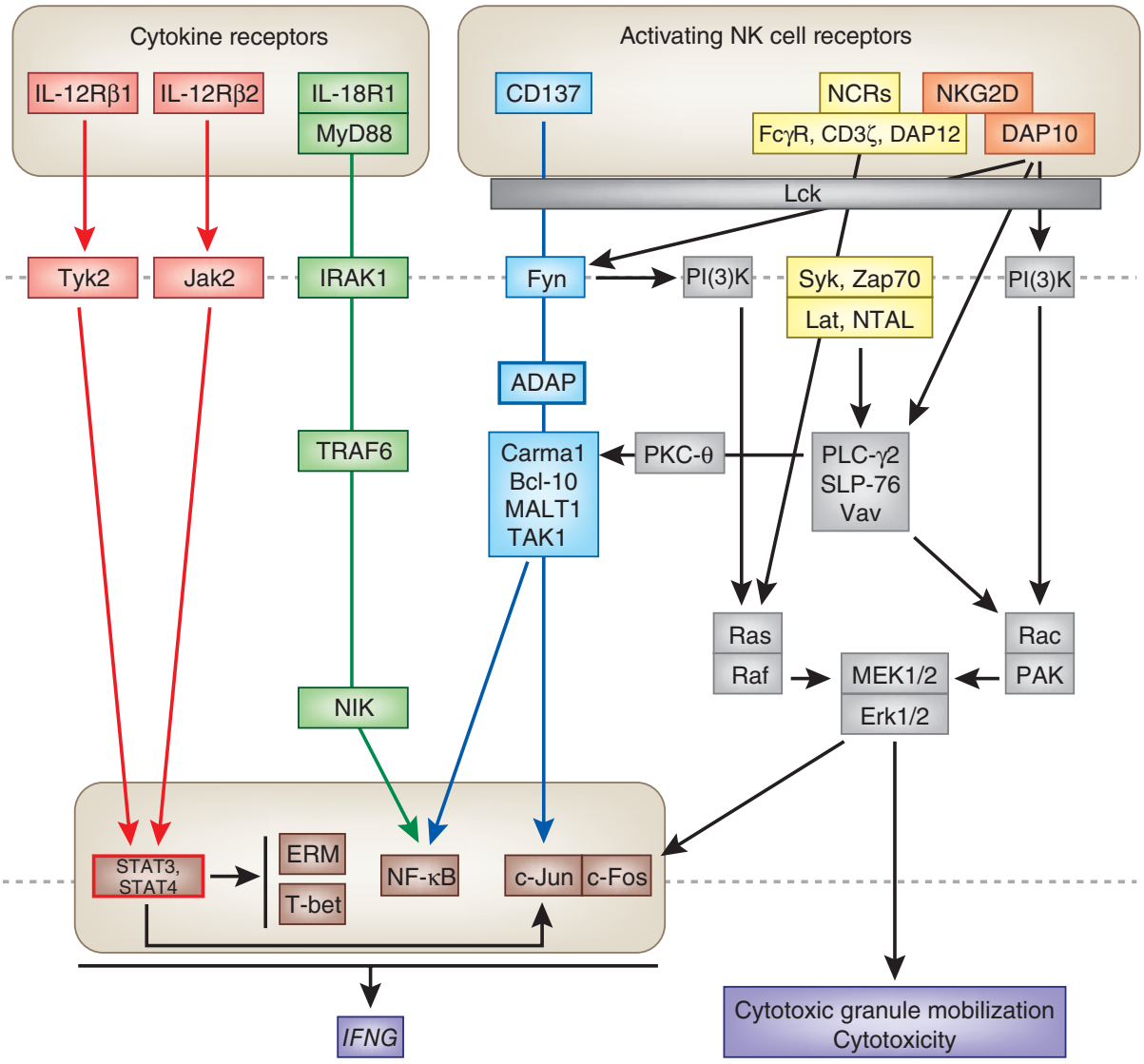


Figure 11: Modèle des voies d'activation de l'activité cytokinique IFN-γ et de réponse cytotoxique des cellules NKs (109).

2.2.6. Les récepteurs activateurs

2.2.6.1. Famille des lectines

2.2.6.1.1. Lectines couplées : CD94/NKG2C, CD94/NKG2E, CD94/NKG2F

Parmi les récepteurs de type lectine-C activateurs, on retrouve CD94/NKG2C, E et F. Ces molécules sont associées, soit à un motif intracellulaire de type ITAM (110) pour NKG2E et F soit à la molécule DAP12 (111) pour NKG2C. NKG2C reconnait les molécules HLA de type E (112,113) co-exprimées avec les molécules HLA A, B et C. Ainsi, par la reconnaissance du HLA de type E, le couple

CD94/NKG2C est garant du contrôle de l'intégrité cellulaire. Le récepteur NKG2C est impliqué dans la défense antivirale au cours de l'infection à CMV (114).

2.2.6.1.2. Lectine non couplée : NKG2D

Ce récepteur reconnaît des glycoprotéines du CMH-I non classiques structurellement proches du CMH de type I. NKG2D est exprimé à la surface de tous les lymphocytes humains sous forme d'un homo-dimère. Son expression (115) est stimulée par l'IL-15 et le TNF-α mais réprimée par le TGF-β (116). Contrairement aux autres récepteurs de type lectine, NKG2D n'est pas associé au CD94 (117). Ce récepteur activateur ne possède pas de domaine ITAM intra cytoplasmique mais est couplé à la tyrosine kinase DAP10. DAP10 signalera via PI3K et entrainera la phosphorylation de JAK2, STAT-5, ERK1/2 et MEK1/2 (118). Tous les ligands de NKG2D identifiés possèdent des domaines communs avec les chaines α1 et α2 du CMH de type I. NKG2D est ainsi impliqué dans les mécanismes de l'immunité antivirale (119) (120) et dans la défense anti bactérienne (121). Il n'y a pas de différence d'expression du NKG2D sur les subsets $CD56^{bright}$ ou $CD56^{low}$ (122).

2.2.6.2. Les KIRs activateurs

Les récepteurs activateurs sont regroupés dans les familles KIR2DS et KIR3DS. Leurs ligands respectifs ont été très peu documentés malgré une littérature abondante concernant leur implication dans la défense anti-virale (123) et les mécanismes d'allo-immunité (124). Le seul couple récepteur-ligand documenté avec un haut niveau de preuve est KIRS2DS1-HLA-C2 (125). Certains auteurs suggèrent donc que les KIRs activateurs reconnaissent des ligands distincts des motifs HLA.

2.2.6.3. Natural cytotoxique récepteur (NCR)

Leur activation est indépendante de la reconnaissance des molécules HLA de type I. Il y a quatre NCRs identifiés à ce jour : NKp30 (NCR3), NKp44 (NCR2), NKp46 (NCR1) et NKp80 (126).

Ils appartiennent à la superfamille des Immunoglobulines. Les récepteurs NCR1 et NCR2 sont également présents à la surface de certains ILC3. Chacun de ces récepteurs reconnaît des motifs spécifiques. L'activation conjointe de plusieurs récepteurs pourrait permettre d'atteindre le seuil d'activation de la cellule NK alors que pris indépendamment, le signal activateur de chaque récepteur est faible.

2.2.6.3.1. NKp46

Le récepteur NKp46 est une glycoprotéine de 46kDa présente sur les cellules NK au repos ou activées. La protéine NKp46 est associée à un motif de type ITAM et peut reconnaître plusieurs agents pathogènes : La protéine sigma1 du Réovirus (127), l'hémagglutinine du virus Influenza (128), et la bactérie *Fusobactérium nucleatum* (129). Le nombre de ligands documentés pour ce récepteur reste limité.

2.2.6.3.2. NKp44

Le récepteur NKp44 est une glycoprotéine de 44 kDa exprimée par les cellules NK activées et particulièrement par les cellules $CD56^{bright}$ $CD16^{-}$. NKp44 est associé à la molécule DAP12 signalant via un motif de type ITAM. Ce récepteur est capable de reconnaître spécifiquement des agents pathogènes comme les mycobactéries et *Pseudomonas aeruginosa* (28,130) ou des protéines virales comme l'hémagglutinine du virus Influenza. NKp44 reconnaît également la molécule MLL5 exprimée à la surface de LT CD4+ infectée par le virus de l'immunodéficience humaine (VIH).

Certains ligands de NKp44 sont également inhibiteurs. C'est le cas de PCNA (Proliferating Cell Nuclear Antigen) exprimé par des cellules pour lesquelles un processus de renouvellement de l'ADN est en cours. Les cellules tumorales peuvent exprimer ce ligand et ainsi échapper à la vigilance NK anti-cancer.

2.2.6.3.3. NKp30

Le récepteur NKp30 est une protéine de 30 kDa associée à un motif ITAM. Parmi ces ligands spécifiques on retrouve l'héparane sulfate (131) ou la protéine PP65 du virus CMV. Ce récepteur gouverne également la répression ou la promotion de la fonction des CD (132,133). Plus récemment, la molécule B7-H6 exprimée à la surface de nombreuses cellules tumorales a été identifiée comme un ligand activateur spécifique du récepteur NKp30. Ce ligand est absent des tissus sains (134).

2.2.6.3.4. NKp80

Le récepteur NKp80 est moins précisément caractérisé. Il reconnaît l'activation-induced C-type Lectine (AICL) (135) exprimé par les monocytes après stimulation des Toll Like Receptors. Ce récepteur pourrait donc participer à la régulation des populations monocytaires par la cellule NK.

2.2.6.4. Les Toll Like Receptor

Les TLRs ou PRR (Pattern Recognition Receptors) reconnaissent des motifs moléculaires conservés appelés PAMPs (Pathogen-Associated Molecular Patterns). La cellule NK présente une réponse cytokinique de type IFN-γ, IL-10 ou TNF-α après stimulation par des agonistes TLRs spécifiques : KpOmpA (agoniste TLR2), Poly I:C (agoniste TLR3), LPS (agoniste TLR4), Flageline (agoniste TLR5) ou CpG ODN (agoniste TLR9) (136).

2.2.6.5. DNAM-1

DNAM-1 (DNAX accessory molécule-1, CD226) est une protéine de la superfamille des Immunoglobulines présente sur les NKs mais aussi sur les LT, LB et sur les monocytes. Ce récepteur

reconnait notamment le CD122 et le CD155 présents à la surface des cellules tumorales mélaniques (137).

2.2.7. Les récepteurs inhibiteurs

2.2.7.1. Les KIRs inhibiteurs

Parmi les récepteurs inhibiteurs, on trouve les familles KIR2DL et KIR3DL. KIR2DL1, KIR2DL2 et KIR2DL3 reconnaissent 100% des allèles HLA de type C. KIR3DL1 reconnaît la majorité des molécules HLA de type B. KIR2DL4 reconnaît les molécules CMH de type I non classique de type HLA-G exprimées spécifiquement à la surface des cellules trophoblastiques. Les KIRs inhibiteurs ont également un rôle dans la défense antibactérienne. C'est le cas de KIR3DL2, KIR3DL1 et KIR2DL4 capables de reconnaître des motifs bactériens agonistes TLR. Ainsi KIR3DL2 servirait de protéine chaperonne pour le CpG ODN permettant son cheminement intracellulaire vers son récepteur spécifique, le TLR9, au niveau de l'endosome. Cette collaboration mène à une activité cytotoxique et cytokinique (138,139). L'absence de molécule de HLA de type I activera la cellule NK par absence de signal inhibiteur (« missing-self »).

2.2.7.2. Famille des lectines

Parmi les récepteurs de type lectine-C inhibiteurs, le CD94/NKG2A a été identifié. Cette molécule est associée à un motif intracellulaire de type ITIM et reconnait les molécules HLA de type E co-exprimées avec le HLA de type I classique (112,113). Ce récepteur est également impliqué dans l'immunité anti-virale lors d'infection à VIH (140) ainsi que dans les phénomènes d'immunosuppression post transfusionnels (141). Au cours des cycles de maturation de la cellule NK, la diminution de l'expression du NKG2A entraine également une perte progressive de l'expression du CD94.

2.2.7.3. Famille des récepteurs ILT

Dans cette famille de récepteur, seul l'ILT2 est exprimé à la surface des cellules NK (142). Il est couplé à une séquence ITIM. Ce récepteur reconnaît les molécules HLA de type I avec une faible affinité comparée aux KIRs et à CD94/NKG2A.

2.2.8. Impact du microenvironnement cellulaire et cytokinique sur les fonctions NK

Il est reconnu actuellement que les cellules NK présentent un pouvoir anti-leucémique et antiviral. (143,144). Plus récemment leur rôle dans la défense antibactérienne a été mis en évidence (12).

2.2.8.1. Interaction avec les cellules dendritiques

Les CD sont les principales cellules présentatrices d'antigènes. Elles sont impliquées dans l'activation et la maturation des lymphocytes T (LT). Après reconnaissance antigénique en périphérie, la CD acquiert le CCR7 lui permettant de migrer vers les relais lymphatiques, où elle présentera l'antigène aux LT. Du fait de l'expression constitutive du CXCR3 et CCR7 par les NK $CD56^{bright}$, ce subset prédomine dans les centres lymphoïdes. Ainsi, la co-localisation des LT et des NK permet une activation simultanée des LT et NK par la CD.

En périphérie, la sécrétion de CXCL10 par les CD activées permet de recruter des NKs sur le site de l'infection (145,146). Les CD activées induisent ensuite l'expression du CCR7 sur la cellule NK favorisant leur migration vers les centres lymphoïdes secondaires sous l'influence de la chémokine CCL19. L'activation des LT provoque leur expansion et leur sécrétion d'IL-2 favorisant l'activation et la survie des NK. Les cellules T et les CD activés expriment des ligands des récepteurs activateurs NK NKG2D, 2B4 et le CD28 (147,148) (Figure 12).

La communication CD/NK peut se faire de façon paracrine ou par contact direct par création d'une véritable synapse immunologique (149,150) avec accumulation des récepteurs KIR et CD94/NKG2A au centre de cette synapse. La libération d'IL-12, d'IL-15 (présentée par l'IL-15Rα de la CD) et d'IL-18 assure la survie des NK, leur différentiation (151) et stimule leurs fonctions cytokinique (TNF-α, IFN-γ et IL-10) et cytotoxique (152). L'IFN-α secrété par les CD de type plasmocytoïde stimule également l'activité cytotoxique de la NK. Réciproquement, la cellule NK ainsi activée libère de l'IFN-γ et du TNF-α favorisant la maturation (expression CD80, CD86, HLA-DR, CCR7) et la libération d'IL-12 (7,153) par la CD.

C'est un mécanisme d'amplification de la réponse inflammatoire. La sécrétion de GM-CSF par la NK activée, augmente également la survie et la différenciation des monocytes en CD.

2.2.8.2. Interaction avec les lymphocytes T

Les lymphocytes T $CD4^+$ sont classiquement divisés en 2 sous-populations : Les LT helper (Th) jouant un rôle central dans l'immunité adaptative et les LT régulateurs (Treg). La fonction historiquement décrite des Treg était la suppression des LT autoréactifs afin de maintenir la tolérance au soi et prévenir les réactions auto-immunes. Lorsqu'ils sont activés, les LTreg ont également un rôle suppresseur sur plusieurs populations cellulaires de l'immunité innée. Notamment, les Treg peuvent contrôler la prolifération et l'activité cytotoxique des NK par (135,136) :

- La libération TGF-β (154),
- La neutralisation des molécules d'IL-2 via leur récepteur CD25 (chaine α du récepteur à l'IL-2) et ainsi empêcher la stimulation des NKs (155),
- La diminution de l'expression NKG2D sur les cellules NK, limitant leur pouvoir cytolytique sur les Treg exprimant le NKG2DL,

- L'expression de PD-L1 et PD-L2 favorisant l'activation du récepteur PD-1 présent sur les cellules NK pouvant conduire à l'anergie ou à l'apoptose de la NK (156) (Figure 12).

Réciproquement, les cellules NK, une fois activées, ont un rôle important dans l'activation et la différentiation des LT : La production d'IFN-γ favorise la réponse de type Th1 (147). A l'inverse, les cellules NK peuvent exercer un rôle suppresseur sur les LT par :

- La synthèse d'IL-10
- La reconnaissance de ligands de NKG2D (NKG2DL) sur les LT $CD4^+$, $CD8^+$ ou LT régulateur (Treg) (148).

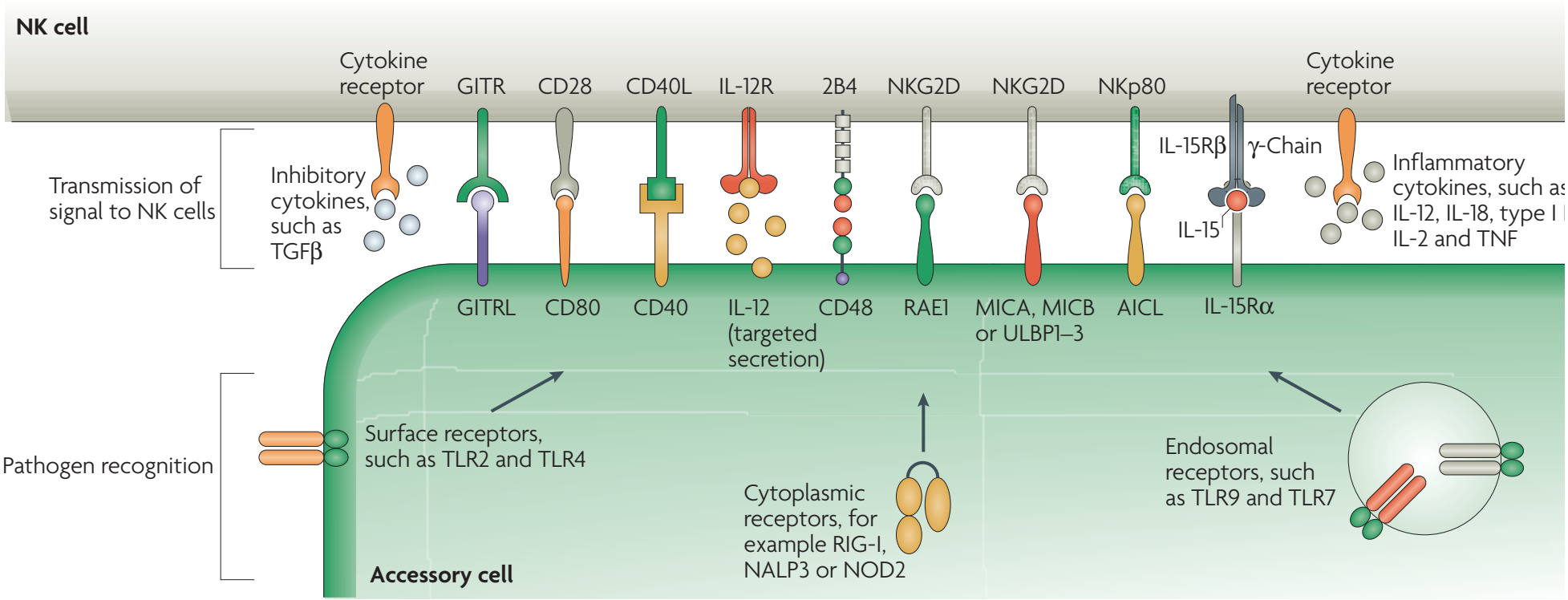


Figure 12 : Aperçu des interactions de la cellule NK avec les cellules du microenvironnement (157).

2.2.9. Traduction du déficit en NK en pathologie humain *(158)*

Le déficit en cellules NK peut concerner soit leur nombre, soit leur fonction. Le déficit de fonction peut également s'intégrer dans des syndromes de « déficit immuns combinés sévères » altérant secondairement la fonction NK (Table 1). On distingue :

- Le déficit classique en cellule Natural Killer (CNKD) : absence totale de cellule NK dans le sang périphérique ;
- Le déficit fonctionnel en NK (FNKD) : Cellules NK présentes mais non fonctionnelles.

Le déficit en cellules NKT, appartenant à la lignée T, n'est pas inclus dans cette description.

Type de déficit		Cellule NK circlantes	$CD56^{dim}$	$CD56^{bright}$	Fonction NK	Type infection	Gene muté	Transmission
CNKD								
	Type 1	NON	NON	NON	Néant	VZV, HSV, CMV, HPV	GATA 2	AD
	Type 2	NON	NON	OUI	Néant		MCM 4	AR
FNKD		OUI	OUI	OUI	Diminué	HSV, HPV, EBV	FCGR3A	AR

Table 1: Classification des déficits immuns en cellules Natural Killer**.**

Le cas le plus connu de CNKD est celui d'une jeune fille présentant de multiples infections virales sévères et disséminées à virus de la famille herpès (HSV, CMV et VZV) (159). C'est une pathologie rare, avec moins de cent patients actuellement recensés porteurs de cette anomalie.
Parmi eux, 42 % sont décédés prématurément, les autres ont présenté des infections virales sévères et récidivantes, 21% ont développé des carcinomes HPV induits, des lymphomes EBV-induits ou des leucémies. Des infections invasives fongiques ou à mycobactéries ont été relevées. Le traitement de référence reste l'allogreffe de cellules souches hématopoïétiques.

2.2.10. Rôle des cellules NK au cours des infections bactériennes

Au cours de l'infection bactérienne, les cellules NK sont capables de reconnaître l'infection grâce à un large panel de récepteurs activateurs et ainsi, donner lieu à une réponse rapide sans l'intervention d'autre cellule de l'immunité innée. La réponse cytokinique des NK permet alors d'initier et d'amplifier l'activité de phagocytose des macrophages ainsi que d'accélérer leur maturation et leur différentiation.
Au cours de l'infection, plusieurs motifs bactériens ont été rapportés comme pouvant se lier spécifiquement aux récepteurs activateurs de la cellule NK. Ainsi, le récepteur NKp44 serait capable de lier la bactérie *Mycobacterium bovis* (MB) et *Pseudomonas aeruginosa* (28,130). Cependant, la neutralisation de NKp44 ne permet pas de bloquer l'activation de la cellule NK suggérant l'existence de voies d'activation accessoires ou redondantes. En revanche, le blocage du récepteur TLR2 dans un

modèle d'infection à MB, diminuait la réponse TNF-α et IFN-γ de la cellule NK. De même, le récepteur NKp46 (NCR1) pourrait reconnaître spécifiquement la bactérie *Fusobacterium nucleatum* (129). Par ailleurs, l'expression des TLR 2, 4 ,5 et 9 a été associée à une activité IFN-γ des NK en réponse aux signaux de danger comme les DAMPs ou les PAMPs (136).

Des collaborations entre NCR et TLR ou entre KIR et TLR ont également été décrites (122) mais le rôle précis des TLR dans l'initiation de la réponse cytokinique reste débattu dans la mesure où leur inhibition spécifique ne permet pas de réduire la réponse INF-γ (160,161).

Au cours de l'infection bactérienne, l'activation des cellules NK est également indirecte et peut passer par l'activation préalable des CD ou macrophages via les TLRs stimulant la libération d'IL-12, IL-15, IL-18, IFN-α ou encore TNF-α. Enfin, à la phase tardive de l'infection, les NK jouent un rôle majeur dans la résolution de l'infection et libèrent de l'IL-10, diminuant ainsi la libération d'IL-12 par les CD (12). Cette activité anti-inflammatoire est physiologique et est indispensable au retour à l'homéostasie permettant la cicatrisation du tissu infecté. Comme évoqué en introduction, le déséquilibre de la réponse pro et anti-inflammatoire peut aboutir à une susceptibilité accrue aux infections dans le cadre d'une ISPT (162).

3. OBJECTIFS DU TRAVAIL

Les pneumonies à PA restent un challenge infectieux en réanimation et chez le patient insuffisant respiratoire chronique. En raison des capacités d'adaptation de la bactérie à son microenvironnement et du caractère hypermutable de son génome, le taux d'échec microbiologique est important. En réanimation, l'échec de traitement d'une infection pulmonaire à PA est source de morbi-mortalité élevées et se complique fréquemment de dommages tissulaires pulmonaires irréversibles.
Parmi les cellules de l'immunité innée, les cellules Natural Killer (NK) semblent particulièrement impliquées dans la défense contre l'infection à PA. Ces cellules sont également des acteurs majeurs de l'immunité anti-tumorale. Comme détaillé en introduction, les cellules NK présentent 2 fonctions principales :

- La production de cytokine : La cellule NK est la principale source d'IFN-γ de l'organisme au cours de la réponse immunitaire,
- La libération de granules cytotoxiques (Granzyme et Perforine) en réponse à des anormales ou infectées. C'est la fonction cytotoxique.

Au cours de ce travail nous donc avons étudié chacune des 2 fonctions de la cellule NK au cours de l'infection à Pseudomonas avec 2 objectifs :

- **OBJECTIF N°1 :** Evaluation des déterminants et des voies de signalisation impliqués dans la production d'IFN-γ des cellules Natural Killer lors d'une infection à *Pseudomonas aeruginosa.*

- **OBJECTIF N°2 :** Evaluation des conséquences de l'infection à *Pseudomonas aeruginosa* sur la fonction cytotoxique des cellules NK en réponse à des cellules déficientes en HLA de type I.

3.1. OBJECTIF N°1

Evaluation des déterminants et des voies de signalisation impliqués dans la production d'IFN-γ des cellules Natural Killer lors d'une infection à *Pseudomonas aeruginosa.*

Les données actuelles de la littérature suggèrent que PA pourrait détourner la réponse immunitaire qu'il génère afin de persister chez l'hôte, et notamment la réponse cytokinique de type IFN-γ via le système du Quorum Sensing (QS). En particulier, après fixation de l'IFN-γ sur son récepteur OprF (163), la bactérie augmente son pouvoir pathogène en activant la synthèse de facteurs de virulence (21,23). Il existe donc un lien singulier entre l'activité IFN-γ de l'hôte et la virulence de la bactérie.
Le lymphocyte Natural Killer est l'un des principaux producteur d'IFN-γ au cours de la réponse immunitaire anti-bactérienne et semble jouer un rôle clef dans la défense anti PA (31). L'étude des interactions PA-NK pourrait donc permettre de comprendre les modalités d'invasion et de persistance de la bactérie chez l'hôte. La réponse NK est dépendante du microenvironnement cytokinique (IL-2, IL-12, IL-15, IL-18) et cellulaire (cellules dendritiques) (164,165). Il apparaissait donc important d'intégrer ces acteurs dans l'étude de la réponse des cellules NK à l'infection à *Pseudomonas*. Nous avons formulé quatre questions pour répondre à cet objectif :

1) Quelle sous population de cellules NK est principalement impliquée dans la réponse IFN-γ après infection à PA ?
2) Quelle(s) voie(s) de signalisation mène à la production d'IFN-γ après infection à PA ?
3) Quels sont les facteurs de virulence de PA impliqués dans la réponse IFN-γ de la cellule NK ?
4) Validation de nos hypothèses dans un modèle *in vivo* de pneumonie murine à PA

3.2. OBJECTIF N°2

Evaluation des conséquences de l'infection à *Pseudomonas aeruginosa* sur la fonction cytotoxique des cellules NK en réponse à des cellules déficientes en HLA de type I.

L'étude des interactions entre infection et immunité anti-cancer est un sujet de recherche émergeant. Les cellules NKs jouent un rôle clé dans la défense anti-bactérienne et anti-tumorale. Dans un modèle de mélanome murin, Chung et coll. avaient comparé la diffusion métastatique du mélanome chez des souris infectées à PA ou non infectées. L'infection à PA quarante-huit heures avant l'injection de cellules de mélanome s'accompagnait d'une diffusion métastatique pulmonaire augmentée (166). Les auteurs mettaient en évidence que le défaut de contrôle de la maladie était expliqué par l'apoptose des cellules NK induite par l'infection à PA. Par ailleurs, dans un modèle de pneumonie murine à PA, nous avons mis en évidence au laboratoire que les cellules NK jouaient un rôle central dans le contrôle de l'infection (167). Pour ces raisons, l'étude des NK lors de l'infection à PA afin d'améliorer la compréhension des interactions entre infection et cancer apparait pertinente.

Les cellules tumorales peuvent être reconnues schématiquement de deux façons par les cellules NK :

- L'expression des molécules HLA de type I est modifiée voire absente (théorie du « missing-self » : soi manquant). L'absence de signal inhibiteur est alors reconnue par les récepteurs KIRs.
- La cellule tumorale exprime un ligand reconnu par un récepteur activateur.

Ces 2 situations mèneront à une réponse NK de type cytotoxique permettant la lyse de la cellule anormale. Ce mécanisme joue un rôle majeur dans le contrôle de la pathologie tumorale (Figure 10).

Pour expliquer les altérations de l'immunité tumorale après une infection, nous avons évalué l'influence de PA sur l'activité cytotoxique de la cellule NK. Pour cette étude, nous avons exposé les cellules NKs à des cibles (lignée cellulaire 721.221) n'exprimant pas de molécule de HLA de type I. Ces cellules initient donc une réponse NK cytotoxique intense (168) et constituent donc un bon modèle expérimental cellulaire pour étudier la reconnaissance de cellules anormales. Pour ce travail, les questions formulées étaient :

1) Influence de l'infection à PA, sur la fonction cytotoxique des cellules NK face à des cellules 721.221 déficiente en HLA de type I mimant des cellules tumorales.
2) Déterminants de l'altération de la fonction cytotoxique. Cette question se décomposait comme suit :
   2.A) Rôle des récepteurs activateurs/inhibiteurs exprimés par les cellules NK
   2.B) Rôle des cellules et des cytokines du microenvironnement
   2.C) Rôle du phénomène d'épuisement (« exhaustion ») des cellules NK

4. RESULTATS

4.1. Résultats Objectif n°1 (Article 4, Frontiers in Immunology 2017)

Evaluation des déterminants et des voies de signalisation impliqués dans la production d'IFN-γ des cellules Natural Killer lors d'une infection à *Pseudomonas aeruginosa*.

Questions formulées :

1) Quelle sous population de cellules NK est principalement impliquée dans la réponse IFN-γ après infection à PA ?
2) Quelle voie de signalisation mène à la production d'IFN-γ après infection à PA ?
3) Quels sont les facteurs de virulence de PA impliqués dans la réponse IFN-γ de la cellule NK ?
4) Validation de nos hypothèses dans un modèle *in vivo* de pneumonie murine à PA

1) Dans un premier temps, nous avons étudié la fonction NK après infection à PA de cellules mononucléés sanguines circulantes (PBMC) de volontaires sains. Nous avons montré que la réponse des cellules NK à la bactérie était principalement de type cytokinique (IFN-γ) par rapport à la réponse cytotoxique (dégranulation). **Les cellules exprimant le plus haut niveau du marqueur CD56 (CD56^{bright}) présentaient la réponse IFN-γ la plus intense après une infection à PA**. Afin de poursuivre l'étude de la réponse IFN-γ, nous avons donc étudié spécifiquement le subset (sous-population) CD56^{bright}. L'activité IFN-γ des cellules CD56^{bright} privées de leur microenvironnement cellulaire et cytokinique était très faible en l'absence de stimulation par IL-12. Ces résultats étaient en accord avec les données actuelles de la littérature puisque ce subset prédomine habituellement dans les centres lymphoïdes secondaires, lieux d'interaction privilégiés avec les cellules dendritiques (principale source d'IL-12).

2) Nous avons donc poursuivi ce travail par l'étude de l'activité transcriptionnelle et des voies de signalisation menant à la synthèse d'IFN-γ au cours de l'infection à PA. Nous avons utilisé une lignée cellulaire NK humaine (NK92) partageant les caractéristiques des cellules NK CD56^{bright} (KIR^{-}CD16^{-}). **La stimulation IL-12 était indispensable à la détection d'ARN messager d'IFN-γ. En présence d'IL-12, l'infection à PA entrainait une augmentation des ARN messager d'INF-γ par rapport à la condition non infectée.** Nous avons cherché à identifier par quels mécanismes PA augmentait la synthèse d'IFN-γ par rapport à la stimulation IL-12 seule. Considérant les niveaux de détection d'ARN messager d'IFN-γ, nous

avons formulé l'hypothèse d'une influence de l'infection à PA à un niveau transcriptionnel. Le rôle clé du facteur de transcription nucléaire P-STAT4 dans la synthèse d'INF-γ en réponse à l'IL-12 a été décrit (102,103,169). Nous avons fait l'hypothèse d'une amplification de la phosphorylation de STAT4 par l'infection expliquant l'augmentation de l'activité transcriptionnelle IFN-γ. L'étude en Western Blot ne confirmait pas cette hypothèse.

Nous avons donc exploré les voies alternatives susceptibles de participer à la synthèse d'IFN-γ. Suivant la description faite par Eric Vivier et coll. (109) (Figure 11), nous avons étudié les voies PI3k/Akt, MAPkinase P38, ERK/MEKK et NF-κB (109,170) en utilisant des inhibiteurs sélectifs. Contrairement aux trois autres voies, **l'inhibition de ERK/MEKK n'affectait pas la production d'IFN-γ en réponse à une stimulation par de l'IL-12 mais diminuait sélectivement la synthèse d'IFN-γ après infection à PA**. L'activation de ERK pouvait donc expliquer l'augmentation de la production d'IFN-γ après infection à PA par rapport à la stimulation IL-12 seule. Ces résultats ont été vérifiés sur cellules NK humaines triées issues de volontaires sains également.

3) Il restait à identifier le(s) déterminant(s) de l'activation de ERK chez la bactérie. Parmi les nombreux facteurs de virulence décrits, notre choix s'est porté sur le système de sécrétion de type III (SSTIII) et ses effecteurs car ils interagissent avec les protéines de la famille Ras, contrôlant elles-mêmes l'activation de ERK. Par l'intermédiaire du SSTIII, PA à la possibilité d'injecter directement des effecteurs (Exoenzymes S, T et Y) dans le cytoplasme des cellules de l'hôte. Nous avons utilisé des souches de PA délétées sélectivement en Exo S, en Exo T, en Exo S et T, en Exo S et T et Y ou en SSTIII. Parmi les effecteurs du SSTIII, l'Exoenzyme T était le déterminant principal de l'activité INF-γ des cellules NK après infection. Ce résultat était identique que ce soit sur la lignée NK92 ou sur des cellules NK issues de PBMC de volontaires sains. L'analyse en Western Blot confirmait la phosphorylation de ERK ExoT-dépendante. Enfin, la phosphorylation de ERK n'était pas suffisante à elle seule pour assurer la synthèse d'IFN-γ après l'infection et un priming simultané par l'IL-12 était indispensable.

4) Pour renforcer la pertinence de nos résultats, **nous avons confirmé le rôle clé de l'Exoenzyme T sur l'activité IFN-γ des cellules NK pulmonaires dans un modèle *in vivo* de pneumonie murine à PA**. Ce modèle soulignait également le rôle majeur du système du SSTIII et de ses effecteurs dans la pathogénicité de la pneumonie à PA.

Nos résultats suggèrent donc que l'expression différentielle de l'un ou l'autre des effecteurs du SSTIII au cours de l'infection à PA pourrait permettre à la bactérie de moduler la réponse inflammatoire.

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Exoenzyme T Plays a Pivotal Role in the IFN-γ Production after *Pseudomonas* Challenge in IL-12 Primed Natural Killer Cells

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Pseudomonas aeruginosa (PA) expresses the type III secretion system (T3SS) and effector exoenzymes that interfere with intracellular pathways. Natural killer (NK) cells play a key role in antibacterial immunity and their activation is highly dependent on IL-12 produced by myeloid cells. We studied PA and NK cell interactions and the role of IL-12 using human peripheral blood mononuclear cells, sorted human NK cells, and a human NK cell line (NK92). We used a wild-type (WT) strain of PA (PAO1) or isogenic PA-deleted strains to delineate the role of T3SS and exoenzymes. Our hypotheses were tested *in vivo* in a PA-pneumonia mouse model. Human NK cells or NK92 cell line produced low levels of IFN-γ in response to PA without IL-12 stimulation, whereas PA significantly increased IFN-γ after IL-12 priming. The modulation of IFN-γ production by PA required bacteria-to-cell contact. Among T3SS effectors, exoenzyme T (ExoT) upregulates IFN-γ production and control ERK activation. *In vivo*, ExoT also increases IFN-γ levels and the percentage of IFN-γ$^+$ NK cells in lungs during PA pneumonia, confirming *in vitro* data. In conclusion, our results suggest that T3SS could modulate the production of IFN-γ by NK cells after PA infection through ERK activation.

Keywords: natural killer cells, *Pseudomonas aeruginosa*, type III secretion system, innate lymphoid cells, IL-12, interferon-gamma

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INTRODUCTION

Pseudomonas aeruginosa (PA) is an opportunistic pathogen that causes lung infections in cystic fibrosis (CF) (1) as well as in intensive care unit (ICU) patients (2). In CF patients, PA infection appears after a few years and systematically becomes chronic, inducing severe pulmonary damage. In ICU patients, PA-related ventilator-associated pneumonia reduces survival and worsens outcome. The high level of PA recurrence is related to its high virulence and hypermutable genome (3), while the ability to subvert immunity may explain chronic infection.

Pseudomonas aeruginosa alters innate lymphoid cells, including natural killer (NK) cells, which play a key role in immunity against PA (4). NK cells give rise to cytokine or cytotoxic response but cytokine production prevails after bacterial infection (5). NK cells are a major source of IFN-γ, which participates in antimicrobial immunity and stimulates monocyte differentiation (6). Conversely, PA can divert cytokine response and use IFN-γ to enhance its virulence factors (7).

In order to explain how PA infection can give rise to proinflammatory response, we explored how PA can trigger IFN-γ release and especially the role of the type III secretion system (T3SS) and its effector (Exoenzyme T, S, and Y). It has been suggested that toll-like receptors (TLRs), natural cytotoxic receptors (NCRs), and killer-cell immunoglobulin-like receptors (KIRs) on NK cells can sense bacteria and trigger cytokine response (8). Alongside NK-specific pathogen recognition, antigen-presenting cells like DCs are critically involved in NK cell activation through IL-12, IL-15, IL-18, or IL-21 release (9, 10).

We sought to precisely describe the underlying mechanism of IFN-γ response in NK cells during PA infection by specifically analyzing virulence factors and pathway activation in an *in vitro* infection model. Since IL-12 is required to observe the production of IFN-γ during PA infection, we examined *in vitro* the effects of PA on the production of IFN-γ by IL-12-treated NK cells. Last, we validated our data *in vivo* in a mouse PA pneumonia model.

MATERIALS AND METHODS

Bacterial Strains

PA01 is a clinical strain of PA (no. 15692) (11) whose genome has been fully sequenced. It expresses most of the documented virulence factors, including the T3SS also known as the "needle complex" and its effectors: Exoenzymes (Exo) S, T, and Y released in targeted cells through T3SS. Three isogenic deleted strains were used: PA-ΔS (ExoS deletion), PA-ΔT (ExoT deletion), and PA-ΔT3SS (deletion of the needle complex). PA-ΔS and ΔT were a gift from Dr. Andrew Y. Koh Laboratory at the University of Texas Southwestern Medical Center in Dallas, TX, USA. PA expressing the Green Fluorescent Protein (PA-GFP) was a gift from Dr. Wu at the University of North Dakota. PA-ΔT3SS (also called ΔPscC) carries a truncated PscC gene leading to a non-functional protein. PscC is a secretin-like constitutive protein of the outer membrane forming a channel enabling needle growth. Without the functional pscC protein, the needle in the T3SS cannot protrude to the cell surface and, as a result, the bacteria cannot inject Exo in the host cell cytoplasm (12). This strain came from Dr. Donald Moir at microbiotix^{INC} in Worcester, MA, USA. The PCR study confirmed the phenotype of each deleted strain (see Figure S1 in Supplementary Material). The isogenicity between each deleted strain was confirmed by pulsed-field gel electrophoresis (see Figure S2 in Supplementary Material).

Peripheral Blood Mononuclear Cell (PBMC) from Healthy Donors, Human NK Cells Isolation, and NK92 Human Cell Line

- PBMCs (Cryopreserved Human Peripheral Blood Mononuclear Cells) were isolated from heparinized blood of healthy volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Norway). PBMCs were unfrozen and then kept in IL-2 overnight (100 UI/ml). After cell sorting, NK cells were immediately resuspended in IL-2 supplemented medium and then infected. All donors were recruited at the blood transfusion center (Nantes, France). Informed consent was obtained from all individuals and all experiments were approved by the Ethics Committee of Tours, France (2015-DC-1) (Biocollection Authorization Number DC-2014-2340), and performed in accordance with relevant guidelines and regulations.
- Human NK cells were sorted from PBMC of healthy donors with Untouch NK cell isolation kit (Miltenyi Bitoec). CD56^{bright} and CD56^{dim} NK cells were isolated from PBMC of healthy donors by Flow Cytometry Cell Sorting using CD56^{pos} and CD3^{neg} gating routinely yielded cell population with purity of 95% (FACSARIA cell sorter, BD Bioscoences). Isolated NK cells were then cultured in medium supplemented with 100 U/ml IL-2 (Proleukin, Aldesleukin, Chiron).
- NK92 is an IL-2-dependent human tumor NK cell line CD56^{bright} CD3^{neg}, expressing neither the killer cell immunoglobulin-like receptor (KIR^{neg}) nor CD16 (see Figure S3 in Supplementary Material).

Infection

- PBMC, sorted human NK cell, or NK92 cell lines were cultured at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco) containing glutamine (Gibco) with 10% fetal bovine serum (Gibco, <10 EU/ml endotoxin contamination), penicillin-streptomycin (PS), and 100 U/ml IL-2 (Proleukin, Aldesleukin, Chiron) (13). Cells were seeded in 96-well plates (250,000 per well in 200 µl).
- PA strains were grown overnight in Brain Heart Infusion medium at 37°C. Bacterial inoculum was calibrated by nephelometry. Cells and bacteria were cocultured with a 1:1 bacteria to NK cell ratio. In PBMC, we also applied a 1:1 bacteria to NK cell ratio, assuming 10% NK cells among PBMC. After 2 h of coculture in PS-free RPMI and IL-2 supplemented medium, the wells were centrifuged at 1,500 RPM for 2 min and placed in fresh IL-2 supplemented RPMI medium with PS to prevent bacteria overgrowth until the 24th hour. Non-infected wells were similarly centrifuged and resuspended in fresh medium supplemented with IL-2 and penicillin/streptomycin. During infection the medium was also supplemented with IL-2 to ensure continuous stimulation all along experiments. When mentioned, the medium was supplemented with IL-12 (Miltenyi) at a concentration of 5 ng/ml for the first 2 h. In some conditions, transparent PET membranes (filter with 0.4 µm pore size) were used in culture wells to prevent NK-bacteria contact.

Kinase Study

MEK/ERK kinase inhibitor (PD98059) were purchased from Sigma Aldrich (France). Inhibitor was diluted according to the manufacturer instructions with dimethyl sulfoxide (DMSO) at 0.04%. Before the PA challenge, NK cells were incubated for 1 h at 37°C with 10 µM of inhibitor (14). Cultures with DMSO 0.04% under inhibitor-free conditions were also prepared to control its potential effect on cytokine response.

Pneumonia Model

We used our validated pneumonia model (4, 15, 16). Six-week-old female Swiss mice (20–24 g) were anesthetized with isoflurane. A

24-G transtracheal feeding needle was inserted to inject 75 µl of bacterial suspension adjusted to 10^8 CFU/ml. The same anesthesia procedure and treatment with 75 µl of saline buffer was applied to control mice (SHAM). Mice were maintained on a 12-h light/dark cycle. All experimental protocols were approved by the Committee of Animal Ethics of the Pays de Loire (CEEA-2012-233) and all methods were carried out in accordance with the guidelines and regulations.

Cell Labeling

Antibodies were purchased from BD Biosciences unless otherwise stated. Data were collected with four-color FACSCalibur (BD Biosciences) and LSRII cytometer (Benton Dickinson, Le Pont de Claix, France) and analyzed using FlowJo 6.2 software (Ashland, OR, USA). For PBMCs, NK cell gating was performed with anti-CD56-APC (NCAM16.2, #341026), anti-CD3-PerCP (SK7, #345766), and the corresponding isotype-matched control mAb. Cytolytic activity (CD107a membrane expression) was assessed with CD107-FITC (H4A3, #555800) after 5 h of *in vitro* incubation. To study IFN-γ production, the cells were treated with Brefeldin A (Sigma) at 10 µg/ml for 5 h. IFN-γ intracellular staining was then performed with anti-IFN-γ-PE (B27, #554701) after cell permeabilization with PFA 4% (Sigma) at 4°C overnight followed by Saponin 0.1% (Sigma). Cell viability among NK cell line was assessed by APC-Fixable Viability Dye Kit eFluor 780 staining (eBioscience).

In mouse pneumonia model, cell suspensions were obtained by mechanical and collagenase D digestion (1 h at 37°C) of lungs collected 24 h postinfection. NK cell gating was performed with anti-NK1.1-BV 421 (#562921) and anti-CD3-APC (#553066). For IFN-γ intracellular staining, after red blood cells lysis (RBC lysis buffer, Ozyme), 70 µm filtered cells were cultured 5 h in RPMI 1640 medium supplemented with 2% FCS with GolgiPlug, washed twice, and then stained for surface markers. Fixation and permeabilization was performed following manufacturer instructions (BD Cytofix/Cytoperm kit, BD Bioscience). Anti-IFN-γ AlexaFluor 488 (#557724) antibody or its rat IgG1κ isotype control were incubated overnight at 4°C. Cells were washed twice before analysis on a LSRII flow cytometer (BD Bioscience).

For confocal microscopy, cells were stained with primary rabbit anti-human NCR2 antibody (#133668, Abcam) for 30 min at 4°C (1/100) and secondary goat anti-rabbit Alexa 568 antibody (#11011, Life Technologies) for 20 min at 4°C (1/400). NK92 cells were seeded onto glass cover-slips with 2-octyl Cyanoacrylate DERMABOND™, Ethicon, and underwent 2 min of centrifugation at 1,500 rpm. Infection was performed with PA-GFP immediately before confocal visualization (Nikon A1 RSi) with Plan APO 60× objective with a numerical aperture of 1.40. Stack acquisition was performed at 30-s intervals, scan size was 512 × 512, 5× zoom, pinhole 2 (Airy unit), and 2 µm step sizes. Images were not processed after acquisition.

Cytokine Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

All ELISA kits were purchased from eBioscience.

- $CD56^{bright}$, $CD56^{dim}$ NK cells, and NK92 cell lines: IFN-γ production was quantified in cell-free culture supernatant after 24 h of culture.
- Mice lungs: immediately after removal, lungs were mechanically homogenized in phosphate-buffered saline (PBS, pH 7.4), 0.1% Triton X-100 containing 1 mM protease inhibitor cocktail (Sigma). IFN-γ concentration was determined and normalized on protein concentration (BCA protein assay kit, Rockford, IL, USA) (16).

RT-PCR Analysis

Total RNA was isolated using the RNeasy kit (Qiagen) and treated for 45 min at 37°C with DNase (Promega). RNA (1 µg) was reverse-transcribed with superscript III reverse transcriptase (Life Technologies). The cDNA was subjected to RT-qPCR in a Bio-Rad iCycler iQ system using the QuantiTect SYBR Green PCR kit (Qiagen). See primer sequences in the online supplementary table (see Table S4 in Supplementary Material). Relative gene expression was normalized on GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method with samples from the IL-12 free, non-infected group as calibrators.

Western Blotting

Cell pellets were suspended in cold RIPA buffer, protease inhibitor, and 1% phosphatase cocktails (Sigma), mixed with the SDS sample buffer, boiled, and separated by SDS-PAGE (10% TGX Precast Gel, Bio-Rad). Proteins were transferred onto a Trans-Blot® Turbo™ membrane. Membranes were successively probed with primary antibodies, DyLight™ 680 or 800 secondary antibodies and revealed on an infrared imager (LICOR ODYSSEY). The following primary antibodies were used (cell signaling unless otherwise stated): anti-Phospho-Stat4 (Tyr693) (D2E4) rabbit mAb, anti-Phospho-p44/42 MAPK (Erk1/2) (9101S) rabbit mAb, anti-p44/42 MAPK (Erk1/2) (9102S) rabbit mAb or anti-Actin (A5441) mouse mAb (Sigma).

Statistical Analysis

Statistical analyses were performed with GraphPad prism software (La Jolla, CA, USA). Continuous non-parametric variables were expressed as the median (25th to 75th percentile). The Kruskal–Wallis test was used to compare multiple groups. The *post hoc* Dunn's test was used to perform multiple comparisons. Survival curves were compared to a log-rank test. $P < 0.05$ was considered to be statistically significant.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on request.

RESULTS

$CD56^{bright}$ NK Cells Are the Main Source of IFN-γ Production after PA infection

The close interaction between NK cells and PA has been reported previously (4, 17, 18). We first focused on the determinant of IFN-γ production by NK cells following PA-infection. We performed PA infection in PBMC to assess NK cells response in a physiological microenvironment. NK cells are heterogeneous with different subsets specialized in either cytokine or cytotoxic activities (8),

thus we aimed to select the main subset of NK cells specialized in IFN-γ production. After gating on $CD56^{pos}$ and $CD3^{neg}$ cells, we analyzed intracellular IFN-γ staining and CD107a membrane expression (surrogate marker of degranulation) among $CD56^{bright}$ and $CD56^{dim}$ subsets (**Figure 1A**). After PA-WT infection, the proportion of $CD56^{bright}$ NK cells increased and exhibited higher IFN-γ activity and lower cytotoxic activity compared to the $CD56^{dim}$ subset (**Figures 1B,C**). To confirm preferential IFN-γ activity of $CD56^{bright}$ subset, we sorted NK cells from PBMC by cytometry according to their subset ($CD56^{bright}$ or $CD56^{dim}$) and subsequently 24-h infected each subset with or without IL-12 stimulation. IL-12 produced by dendritic cells (DC) is critical for NK cell activation (19). As previously described, without IL-12 priming, NK cells produced low levels of IFN-γ in response to PA. After IL-12 stimulation, PA significantly increased IFN-γ as compared to non-infected cells. Moreover, $CD56^{bright}$ NK cells produced higher level of IFN-γ than $CD56^{dim}$, inciting us to study $CD56^{bright}$ subset to precise intracellular pathways leading to IFN-γ production (**Figures 1D,E**).

To further explore microenvironment influenced on IFN-γ response after PA infection, we used a human NK cells line (NK92) specialized in cytokine production and sharing the $CD56^{bright}$ NK cells receptor repertory ($CD56^{bright}$ KIR^{neg} $CD16^{neg}$) (8) (see detailed phenotype of NK92 in supplemental Figure S3). Similarly to sorted human NK cells, without IL-12 priming, NK92 cells released low level of IFN-γ after PA infection (**Figure 1F**). IL-12 triggered IFN-γ production and PA further increased IFN-γ level after IL-12 priming. As compared to IL-15 or IL-21, also reported to participate in cytokine response of NK cells, IL-12 stimulation gave rise to higher IFN-γ production (see Figure S5 in Supplementary Material).

PA Increases IFN-γ Production in a STAT-4-Independent Pathway

Our objective was to identify the pathways involved in IFN-γ production after IL-12 stimulation and the influence of PA infection on these pathways. For this purpose, PCR analysis was performed on NK92 cell line after PA-WT infection with or without IL-12 stimulation. The protein and mRNA IFN-γ followed the same trends (**Figures 1F** and **2A**) in IL-12-treated NK 92 cells infected with PA, suggesting that PA infection regulates IFN-γ at a pre-transcriptional level. STAT-4 is the main transcriptional factor involved in both IL-12 receptor (IL-12R) signaling and IFN-γ mRNA transcriptional activity (20). Thus, we compared the activation of STAT-4 after 2-h infection with or without IL-12 stimulation in NK92 cell line. Compared to IL-12-treated NK cells, PA did not affect the phosphorylation of STAT-4, which is induced by IL-12 treatment (**Figures 2B–D**). We concluded that PA could modulate the production of IFN-γ in IL-12-treated NK cells through a pathway independent from IL-12R.

T3SS and Its Effector Modulate IFN-γ Production after Direct PA-NK Binding

Live confocal microscopy recorded immediately after PA-GFP infection suggested direct bacteria-to-cell contact (Video S6 in Supplementary Material). When NK92 cells were cultured under a filter (preventing any direct contact with PA), the infection failed to increase the production of IFN-γ in IL-12-treated NK cells (**Figure 3A**). We concluded that direct bacteria-to-cell contact was involved in the cytokine activity modulation. During infection, PA uses a complex T3SS to inject effector proteins (Exoenzymes S, T, and Y) into host cells (21), these proteins

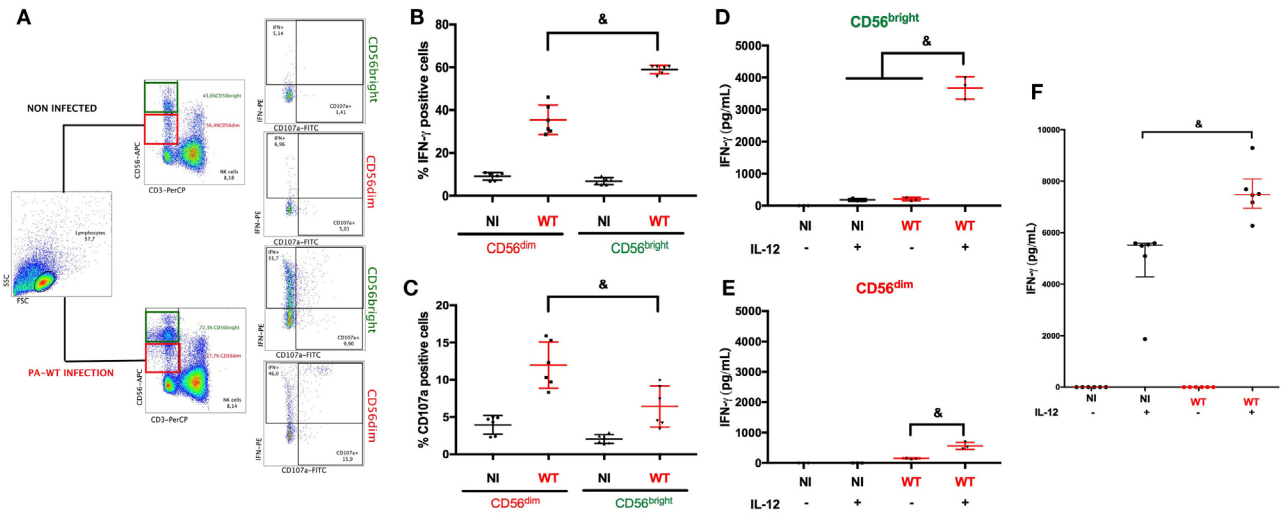

FIGURE 1 | Natural killer (NK) cells IFN-γ degranulation response after PA challenge with or without IL-12 priming. Cytokine (IFN-γ) and cytolytic (CD107a) activity of NK cells among PBMC were assessed with or without PA-WT infection **(A–C)**. Representative density plots illustrating intracellular IFN-γ and CD107a expression in $CD3^{neg}$ $CD56^{bright}$ or $CD3^{neg}$ $CD56^{dim}$ among $CD3^{neg}$ $CD56^{pos}$ cells in lymphocyte gate by flow cytometry **(A)**. Histograms of $CD3^{neg}$ $CD56^{dim}$ and $CD3^{neg}$ $CD56^{bright}$ $IFN\text{-}\gamma^{+}$ **(B)** or $CD107a^{+}$ **(C)** in NI or PA-WT-infected conditions (Representative of 6 healthy donors, 2 distinct experiments, with 3 different donors per experiments). IFN-γ concentration was measured (ELISA) in supernatant of $CD56^{bright}$ **(D)** and $CD56^{dim}$ **(E)** sorted from human PBMC (representative of three different donors) or NK 92 cells **(F)** (six distinct experiments) after a 24-h infection with or without IL-12 stimulation. Data are shown as the median and interquartile range. $^{\&}p < 0.05$, PBMC, peripheral blood mononuclear cell; NI, non-infected, WT, PA-WT infection.

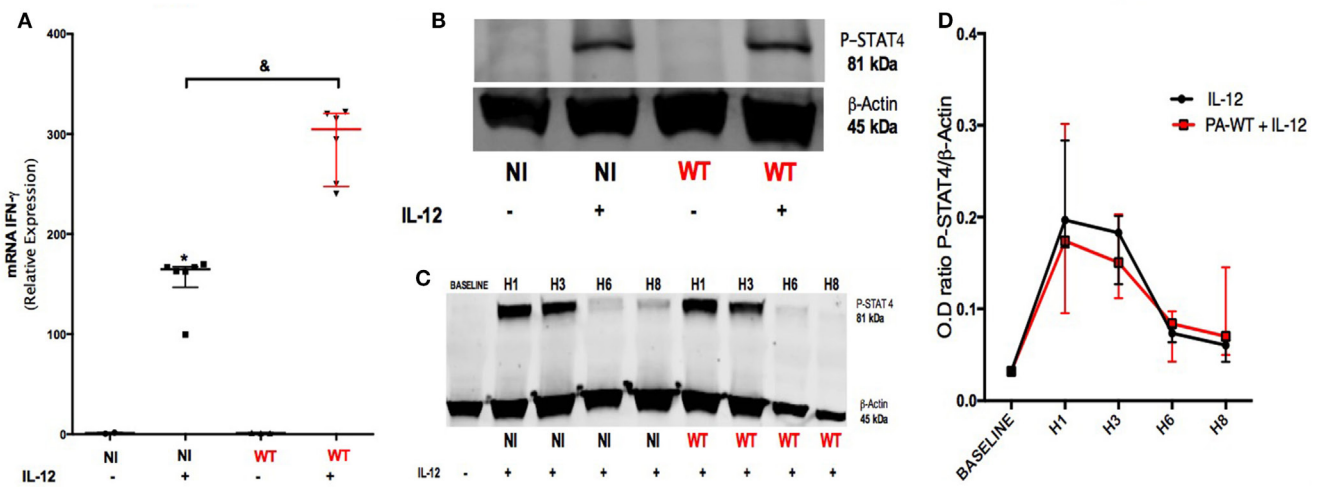

FIGURE 2 | Pathways leading to IFN-γ production in IL-12-treated natural killer cells with or without PA-WT infection. IFN-γ mRNA **(A)** was measured by RT-PCR in NK92 cell after a 6-h culture (expressed as relative expression compared to GAPDH). **(B)** Western Blot membrane of phospho-STAT-4 after a 2-h culture in non-infected (NI) or PA-WT infected (WT) NK92 with or without IL-12 priming. **(C)** Time course over 8 h of STAT-4 phosphorylation in NI or WT conditions in NK92. **(D)** Optical density (OD) of P-STAT4/β-Actin ratio evolution from baseline over 8 h after infection with IL-12 stimulation. Western Blot membranes **(B,C)** were cropped to improve the clarity and conciseness of the presentation, see the full-length membranes in Figure S7 in Supplementary Material. Data are representative of six **(A)** and three **(B–D)** independent experiments. STAT-4, signal transducer and activator of transcription-4; DMSO, dimethyl sulfoxide; NI, non-infected NK92 cells. $^{\&}p < 0.05$.

interfere with the intracellular signaling pathways (22), the function and viability of target cells (21). Exoenzymes effects on the cytokine response in NK cells had not been investigated to date. For this purpose, sorted human NK cells were challenged with three PA isogenic strains deleted for T3SS or its effectors and compared to PA-WT (**Figure 3B**) (see Isotype control for intracellular in Supplemental Figure S8). The exoenzymes deletion did not modify bacterial growth (Figure S9 in Supplementary Material) or the survival of NK cells during infection (Figure S10 in Supplementary Material). Needle complex (PA-ΔT3SS) deletion reduced IFN-γ production in IL-12-treated NK cells as compared to PA-WT infection. Contrary to PA-ΔS (expressing ExoT), infection with a strain lacking ExoT (PA-ΔT) decreased IFN-γ activity as needle complex deletion did, suggesting that ExoT is a determinant of IFN-γ activity in NK cells. These results demonstrate that the production of IFN-γ by NK cells is not solely driven by IL-12 stimulation, but that exoenzymes can also modulate cytokine production.

MEK/ERK Pathways Is Involved in ExoT-Induced IFN-γ Activity after PA Infection

Pseudomonas aeruginosa exoenzymes were already reported to interfere with Ras family proteins which control ERK phosphorylation (23). In view of the singular role of ExoT (**Figure 3B**), the next step was to investigate the pathway(s) involved in ExoT-induced IFN-γ production under IL-12 stimulation. Thus, we studied intracellular IFN-γ staining in sorted human NK cells after either PA-WT (full set of exoenzymes) or PA-ΔS (expressing ExoT but not ExoS) infection (**Figure 3C**) with or without ERK inhibitor. ERK inhibitor induced a major reduction in PA-ΔS and PA-WT whereas did not affect the percentage of IFN-γ$^+$ cells in NI condition after IL-12 stimulation. Cell viability study after infection with PA-WT or deleted strains with or without ERK inhibitor ensured that cells mortality did not explain these differences (see Figure S10 in Supplementary Material). Western Blot experiments in NK92 cell line (**Figures 3D,E**) confirmed ExoT-dependent ERK phosphorylation after 2-h infection (see the membrane with unphosphoryled form of ERK in Figure S7 in Supplementary Material). Comparable phosphorylation of ERK with or without IL-12 suggest that PA may activate NK cells through ERK phosphorylation independently of IL-12 stimulation but that IL-12 priming remains a prerequisite for IFN-γ activity. As a result, in our model, ERK is specifically involved in IFN-γ production after PA infection but not in IL-12 dependent IFN-γ production.

The T3SS Is Involved in Mouse Mortality in the PA Pneumonia Model and Influences IFN-γ Levels in Lungs

We have already reported the critical role of NK cells in controlling infection and producing IFN-γ in a lethal mouse PA-pneumonia model (4). Using the same model, we assessed the role of T3SS and its effectors on mouse mortality and IFN-γ production in mice lungs. The deletion of T3SS or its effectors reduced the mortality rate in infected mice (**Figure 4A**) irrespective of the bacterial load in lungs 24 h after infection (**Figure 4B**). These data demonstrate *in vivo* the critical role of T3SS and its effectors. The IFN-γ level in lungs followed the same trends as observed in our *in vitro* model, with significantly higher IFN-γ activity in PA-expressing ExoT (PA-WT and PA-ΔS) than in PA-ΔT pneumonia (**Figure 4C**). Cytometry analysis in lungs

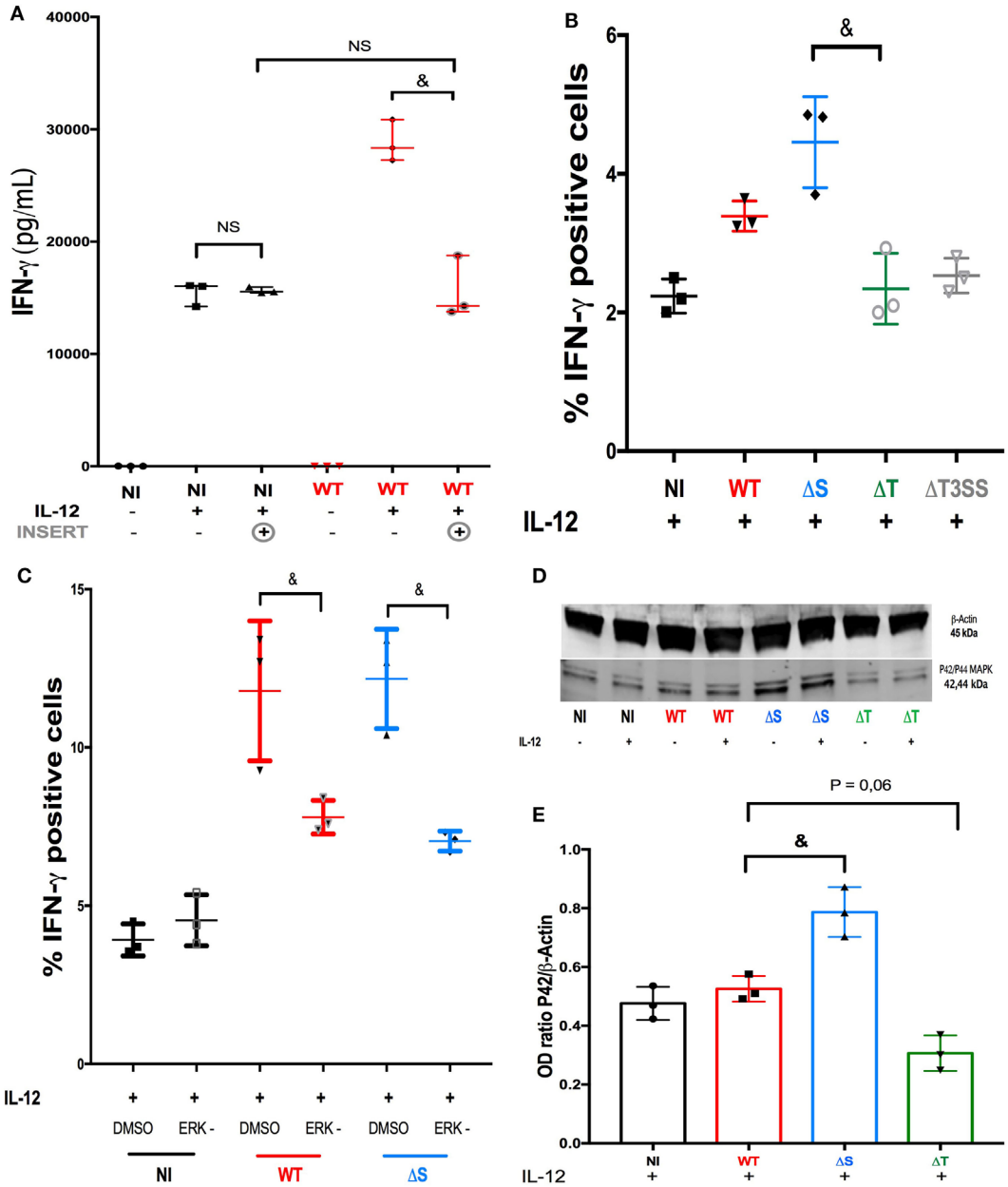

FIGURE 3 | ERK is a key regulator of IFN-γ activity after ExoT stimulation. **(A)** To determine the role of bacteria-to-NK cells direct binding on cytokine production, IFN-γ concentration was measured (ELISA) in the supernatant of non-infected (NI) or PA-WT infected (WT) NK92 cells with or without IL-12 priming and with or without an insert (three distinct experiment). Histograms representative of IFN-γ$^+$ cells among sorted human NK cells in NI or PA infected conditions after IL-12 stimulation **(B)** with or without ERK inhibitor **(C)** (representative of three distinct experiments with one different healthy donor per experiment). Western Blot membrane of p42/p44 (ERK1/2) phosphorylation **(D)** and corresponding optical density (OD) (p42 MAPK/β-Actin ratio) **(E)** analysis after a 2-h culture in NI, PA-WT, PA-ΔS, or PA-ΔT infected conditions in NK92 cells with or without IL-12 priming. Western Blot membrane **(D)** was cropped to improve the clarity and conciseness of the presentation, see the full-length membranes in Figure S7 in Supplementary Material (Representative of three distinct experiments). Data are presented as the median and interquartile range. $^{\&}p < 0.05$; NS, Non significant difference; DMSO, dimethyl sulfoxide; ERK, ERK inhibition; NI, Non-infected NK cells; WT, PA-WT infection (expressing ExoS, T, and Y); ΔS, PA deleted in ExoS (expressing ExoT and Y); ΔT, PA deleted in ExoT (expressing ExoS and Y); ΔT3SS, PA deleted in needle complex (expressing ExoS, T, and Y). MEK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase (ERK-1/2).

after PA pneumonia confirmed that IFN-γ is mainly produced by NK cells (see Figure S11 in Supplementary Material). After PA pneumonia, the absolute number of NK cells was not different compared to sham condition (**Figure 4D**) but the percentage of NK cells was reduced (**Figure 4E**). There was no difference between PA-WT and deleted strains regarding NK cells percentage. PA-ΔS (Expressing ExoT) led to higher percentage of IFN-γ$^+$ NK cells as compared to PA-ΔT (**Figure 4F**). These data confirmed the key role of ExoT *in vivo* on IFN-γ activity modulation.

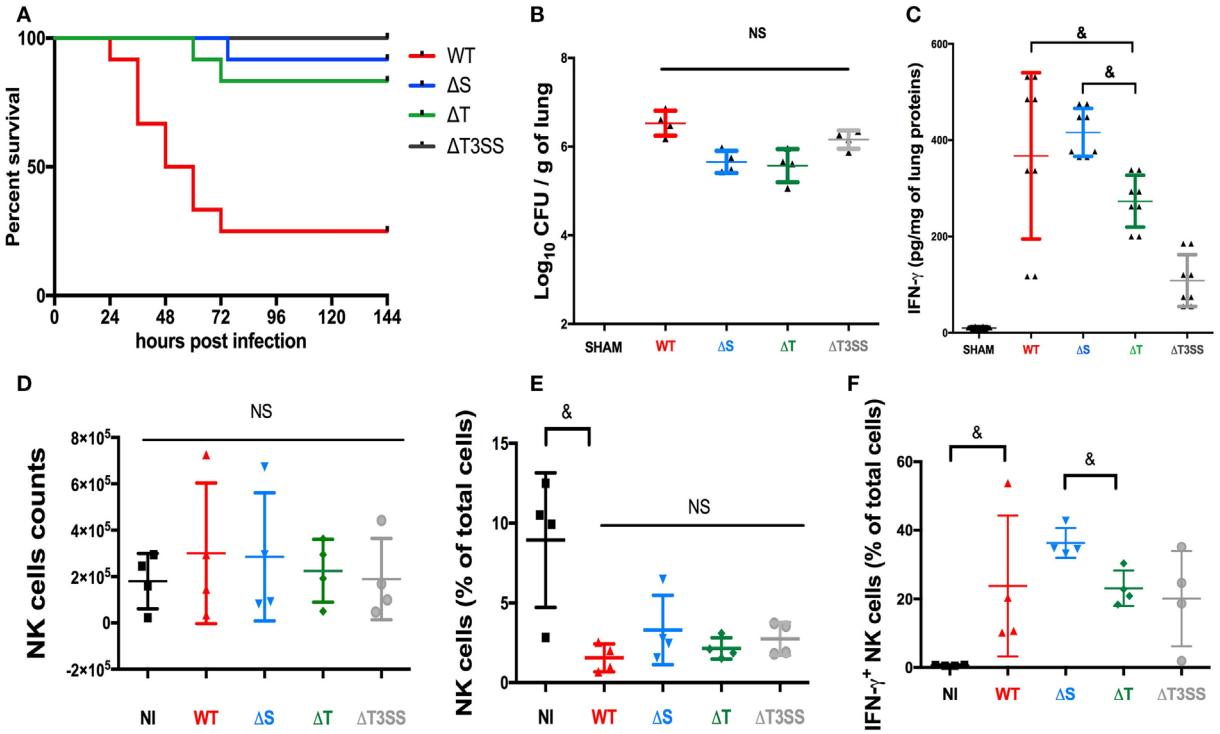

FIGURE 4 | Involvement of the type III secretion system (T3SS) in a mouse PA pneumonia model. **(A)** 6 days (144 h) survival after PA-WT pneumonia compared to three deleted strains (PA-ΔS, PA-ΔT, and PA-ΔT3SS); bacterial count **(B)** and IFN-γ **(C)** levels in lungs 24 h after infection. SHAM Swiss mice were instilled with 75 µl of saline buffer (representative of 2 independent experiments with 2 and 4 mice for each strain, respectively for the bacterial count and cytokine level). Histograms after cytometry analysis representing NK cells absolute number **(D)**, NK cells percentage among whole lung cells suspension **(E)**, and the percentage after IFN-γ+ NK cells **(F)** (two independent experiments with two mice in each group). Data are presented as the median and interquartile range. $^{\&}p < 0.05$, NS, non-significant difference; CFU, colony-forming unit.

DISCUSSION

While the treatment of NK cells by myeloid-derived cytokines (such as IL-12) is required for the production of IFN-γ, our results demonstrate that PA can directly alter IFN-γ production *via* the modulation of ERK through exoenzyme injection in NK cells. Our findings were supported and validated *in vivo* in a murine model of PA-pneumonia. IFN-γ was already documented to enhance the synthesis of virulence factor of PA. Thus, the control of NK cells IFN-γ activity by PA through Exoenzyme infection is a major concept (7).

Pseudomonas aeruginosa infection leads to an IFN-γ response that usually promotes major histocompatibility complex I and II molecule upregulation, and macrophage and CD4+ T cell activation (24). Even if inflammation is central to eliminate the pathogen in the early phase of the infection, an uncontrolled inflammatory response could lead to tissue damage, organ dysfunction, and increase the risk of further infection (25). PA has been previously reported to be capable of taking advantage of the IFN-γ response to enhance the synthesis of its virulence factors (7, 26). On the other hand, in PA-ocular infection in IL-12 knockout animals, IFN-γ reduction also resulted in unchecked bacterial growth and perforation (10).

IL-12 is the main actor in NK/DC cross talk. We have already demonstrated that in patients highly susceptible to infection, such as patients with brain injuries, IL-12 is able to restore IFN-γ production *ex vivo* in NK cells (27). Here, we confirmed the key role of IL-12/STAT4 engagement in the IFN-γ response to PA infection, specifically in CD56^{bright} NK cells (20). These data are supported by a preferential lymph node localization of CD56^{bright} NK cells, where IL-12 stimulation through NK/DC cross talk prevails (8).

We have demonstrated that a direct bacteria-to-cell contact was required to give rise to a cytokine response (**Figure 3A**). The hypothesis of PAMP recognition by NK cells through toll-like receptors has already been explored, but TLR blocking did not suppress IFN-γ response (28) suggesting alternative recognition pathways. Thus, we hypothesized that PA could release mediators directly into NK cytosol and modulate host response. Among the large arsenal of PA virulence factors, the needle complex (T3SS) allows the injection of three effectors (ExoS, T, Y) into the cytoplasm of the host cell. In a clinical setting, T3SS expression is correlated with poor outcomes in pneumonia in Intensive care Unit (29). Here, we found a pivotal role of T3SS in IFN-γ production. In particular, ExoT (expressed by more than 95% of PA strains (30)) stood out as the main trigger of IFN-γ production. These data we confirmed *in vivo*. ERK involvement in PA pathogenicity has already been reported previously as an internalization pathway for the bacteria (31). In our model, NK cells infection with PA expressing ExoT increased IFN-γ production and ERK

phosphorylation (**Figures 3C,D**). The important gap between the percentage of IFN-γ-positive NK cells among PBMC and sorted NK cells (**Figures 1A** and **3B,D**) underscores the key role of the microenvironment to initiate inflammatory response after PA infection. This is confirmed in our PA-pneumonia mice model (**Figure 4F**).

Given our results and the previous description of ExoT and ExoS functions *in vivo*, we have tried to envision how PA infection might modulate the cytokine response and ERK phosphorylation. ExoT and ExoS are bifunctional toxins with N-terminal Rho GTPase-activating protein (GAP) domains, and C-terminally encoded ADP ribosyltransferase (ADPRT) domains. ExoT and ExoS GAP domains have been reported to induce an actin cytoskeleton rearrangement leading to apoptosis. The ADPRT domain of ExoT interacts with the Crk protein (32), which binds to Cbl-b (E3 ubiquitin ligases) and undergoes rapid proteasomal degradation (33). In non-infected conditions, Cbl-b downregulates ERK phosphorylation (34, 35) and IFN-γ production (36). We can hypothesize that during PA-infection, ExoT binding to Cbl-b suppresses ERK regulation and increases IFN-γ production. Conversely, the ADPRT domain of ExoS inactivates cytoskeletal regulators, such as Ras family proteins, which can compromise ERK phosphorylation (23).

Our study presents limitations. NK92 exhibit a highly specific receptors repertory, which was not assessed in sorted NK cells, especially regarding KIR expression. Thus, the parallel with sorted human NK cells have to be tempered. siRNA or knockout cell lines would have discard the off-target effects of kinase inhibitor and increase specificity of IFN-γ pathway study. Exoenzyme detection in host cell cytoplasm after PA infection could confirm ExoT involvement in IFN-γ production. Complemented strains usually ensure a higher level of isogenicity as compared to deleted strains. Nevertheless, the constant bacterial load in the lungs of infected mice and the comparable generation time for all strains ensured that deletion did not alter bacterial growth. Finally, although PA-WT and deleted strains exhibit comparable growth, mice displayed enhanced survival when infected with PA-deleted strains as compared to PA-WT in our model. These data suggest that although each Exoenzyme triggers a singular host function, PA pathogenicity results in combined effect of the whole virulence factor apparatus.

In conclusion, without IL-12 priming, PA escapes recognition by NK cells, preventing any cytokine response. PA infection enhances IFN-γ production by NK cells through T3SS and its effectors especially ExoT. Poor outcome in PA pneumonia with strains expressing T3SS (29) could be explained by an exacerbated inflammatory response mediated by ExoT. Last, our data are in line with the current clinical and experimental research that suggests targeting T3SS or exoenzymes during PA infections (37, 38).

ETHICS STATEMENT

For PBMC: all donors were recruited at the blood transfusion center (Nantes, France). Informed consent was obtained from all individuals and all experiments were approved by the Ethics Committee of Tours, France (2015-DC-1) (Biocollection Authorization Number DC-2014-2340), and performed in accordance with relevant guidelines and regulations. For mice: mice were maintained on a 12-h light/dark cycle. All experimental protocols were approved by the Committee of Animal Ethics of the Pays de Loire (CEEA-2012-233), and all methods were carried out in accordance with the guidelines and regulations.

AUTHOR CONTRIBUTIONS

MV and KA designed all the experiments. MV, AR, GD, AB, CJ, JC, CR, and KA wrote the main manuscript text. All authors reviewed the manuscript. MV and AR participated equally. PH performed and analyzed confocal microscopy pictures. MV, AR, AB, CJ, and GD performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01283/full#supplementary-material.

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

The reviewer BS and handling editor declared their shared affiliation.

TITLE PAGE: Exoenzyme T plays a pivotal role in the IFN-γ production after *Pseudomonas* challenge in IL-12 primed natural killer cells.

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Online-only Material: Supplementary Figures, Video, Table and Legends

Supplementary Figure S1: PCR analysis of exoenzymes or PscC expression in wild-type and deleted strains

Supplementary Figure S2: Pulse-field gel electrophoresis of the wild-type, deleted and GFP strains

Supplementary Figure S3: Detailed phenotype of NK 92 cell line (Cytometry analysis)

Supplementary Table S4: PCR primers

Supplementary Figure S5: Influence of IL-12, IL-15 and IL-21 on IFN-g response after PA-WT infection

Supplementary Video S6: Live NK-PA-WT GFP interaction

Supplementary Figure S7: Full-length Western Blot gels from Figure 2B,C and 3D

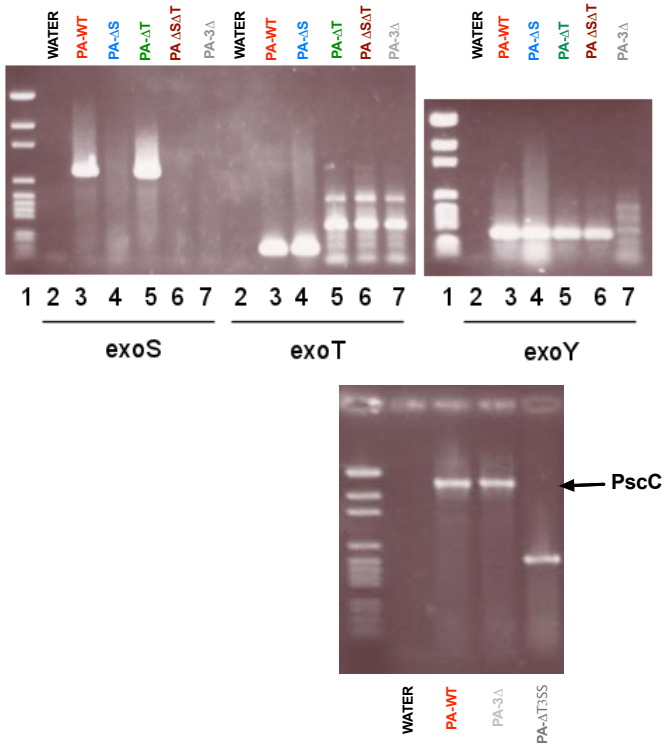
Supplementary Figure S8: IgG1 Isotype control profile for intracellular IFN-γ staining

Supplementary Figure S9: Growth curves of PA-WT and deleted strains

Supplementary Figure S10: Mortality after PA infection among sorted NK cells

Supplementary Figure S11: IFN-g source in lungs after murine PA-pneumonia.

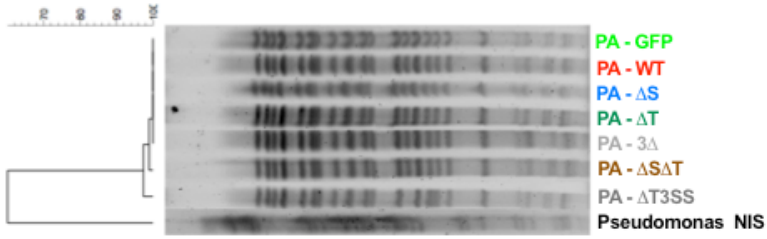
Supplementary Figure S1



<u>Title Figure S1</u>: PCR analysis of exoenzymes or PscC expression in wild-type and deleted strains

<u>Legend Supplementary Figure S1</u>: Exo: Exoenzyme, **PA-WT**: PA wild-type strain expressing ExoS, T, and Y, **PA-ΔT**: PA deleted in ExoT, **PA-ΔS**: PA deleted in ExoS, **PA-ΔT3SS**: PA deleted in needle complex but expressing ExoS, T, and Y, **PA-3Δ:** PA deleted in ExoS, T and Y, **PA-ΔSΔT**: PA deleted in ExoS and T, **PscC**; Protein secretion system.

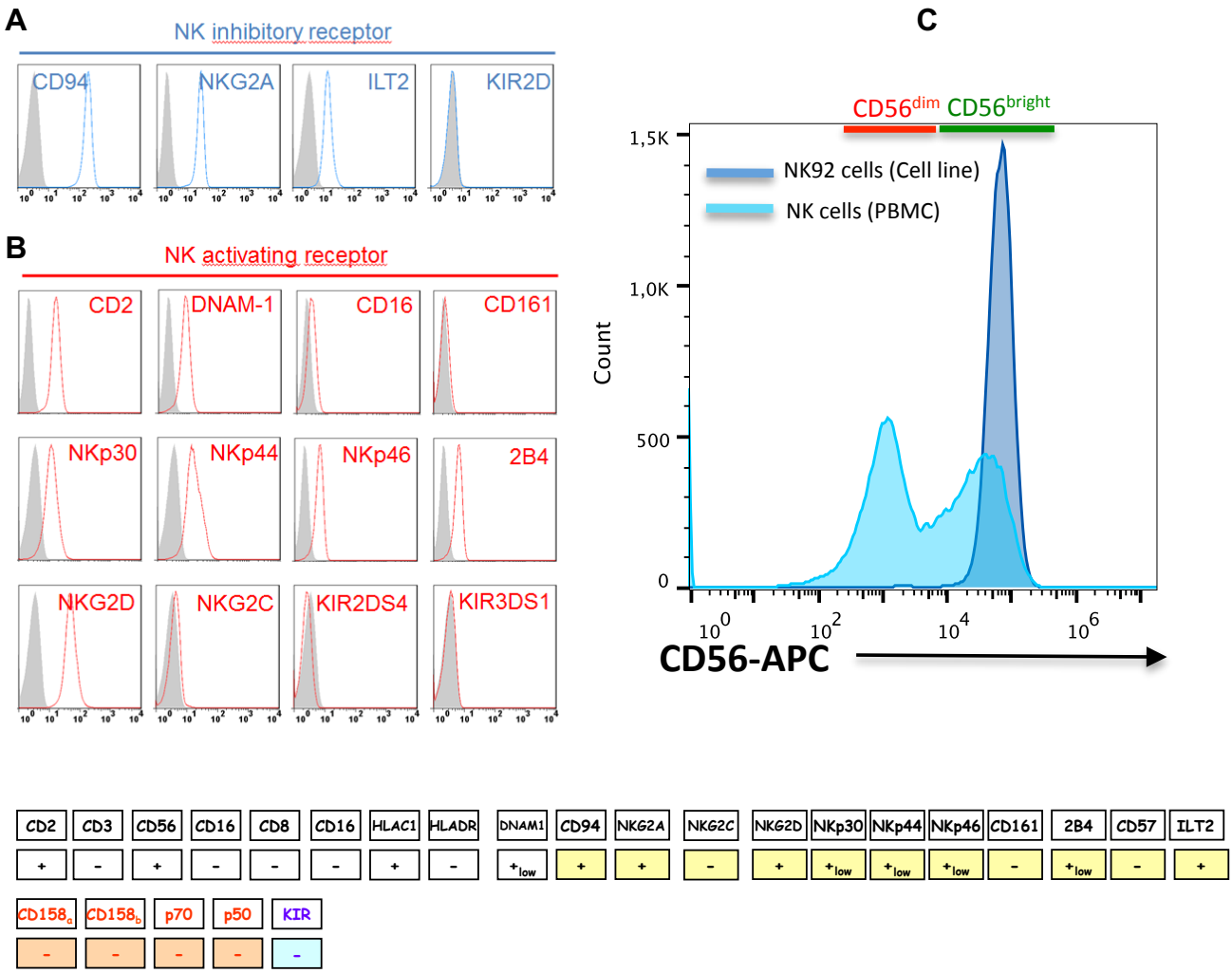
Supplementary Figure S2



Title Figure S2: Pulse-field gel electrophoresis of the wild-type, deleted and GFP strains

Legend Supplementary Figure S2: NIS: Non-isogenic PA strain, **PA-WT-GFP**: PA wild-type strain expressing ExoS, T, Y, and the Green fluorescent protein (GFP), **PA-WT**: PA wild-type strain expressing ExoS, T, and Y, **PA-ΔT**: PA deleted in ExoT, **PA-ΔS**: PA deleted in ExoS, **PA-ΔT3SS**: PA deleted in needle complex but expressing ExoS, T and Y, **PA-3Δ:** PA deleted in ExoS, T, Y, **PA-ΔSΔT**: PA deleted in ExoS and T.

Supplementary Figure S3



Title Figure S3: Detailed phenotype of NK 92 cell line (Cytometry analysis)

Legend Supplementary Figure S3: Histograms illustrating NK inhibitory receptor (**A**) and activating receptor (**B**) expression on NK92 determined by flow cytometer (The profile of IgG isotype control is shown in grey filled histogram**),** (**C**) Histograms illustrating $CD56^{pos}$ NK92 cells and $CD56^{pos}$ NK cells among PBMC (Cytometry analysis).

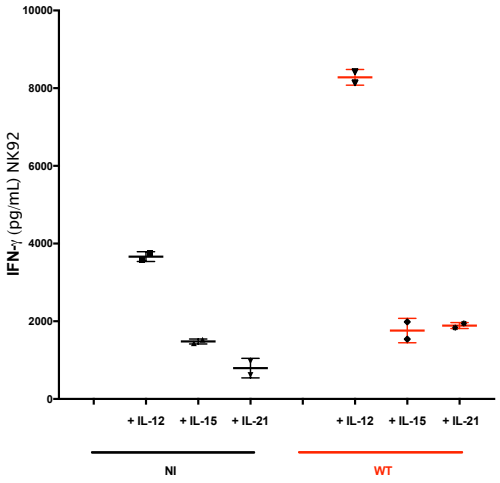
Supplementary Table S4

GAPDH	Forward primer	5′ CCCCTTCATTGACCTCAACTAC 3’
	Reverse primer	5′ GATGACAAGCTTCCCGTTCTC 3’
INF-γ	Forward primer	5’ CTAATTATTCGGTAACTGACTTGA 3’
	Reverse primer	5’ ACAGTTCAGCCATCACTTGGA 3’

Title table S4: PCR primers

Legend Supplementary table S4: GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, **IFN-g:** Interferon gamma.

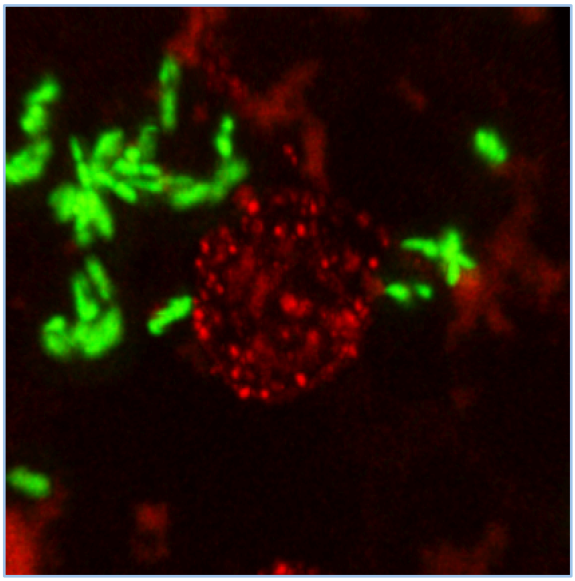
Supplementary Figure S5



Title Figure S5: Influence of IL-12, IL-15 and IL-21 on IFN-g response after PA-WT infection

Legend Supplementary Figure S5 IFN-g concentration was measured (ELISA) in supernatant of NK 92 cells after a 24-hour infection with or without IL-12 (5ng/ml) or IL-15 (50ng/mL, Miltenyi Biotec) or IL-21 (50ng/mL, Miltenyi Biotec) stimulation. WT: PA-WT 24-hour infection (Representative of 3 disctincts experiments)

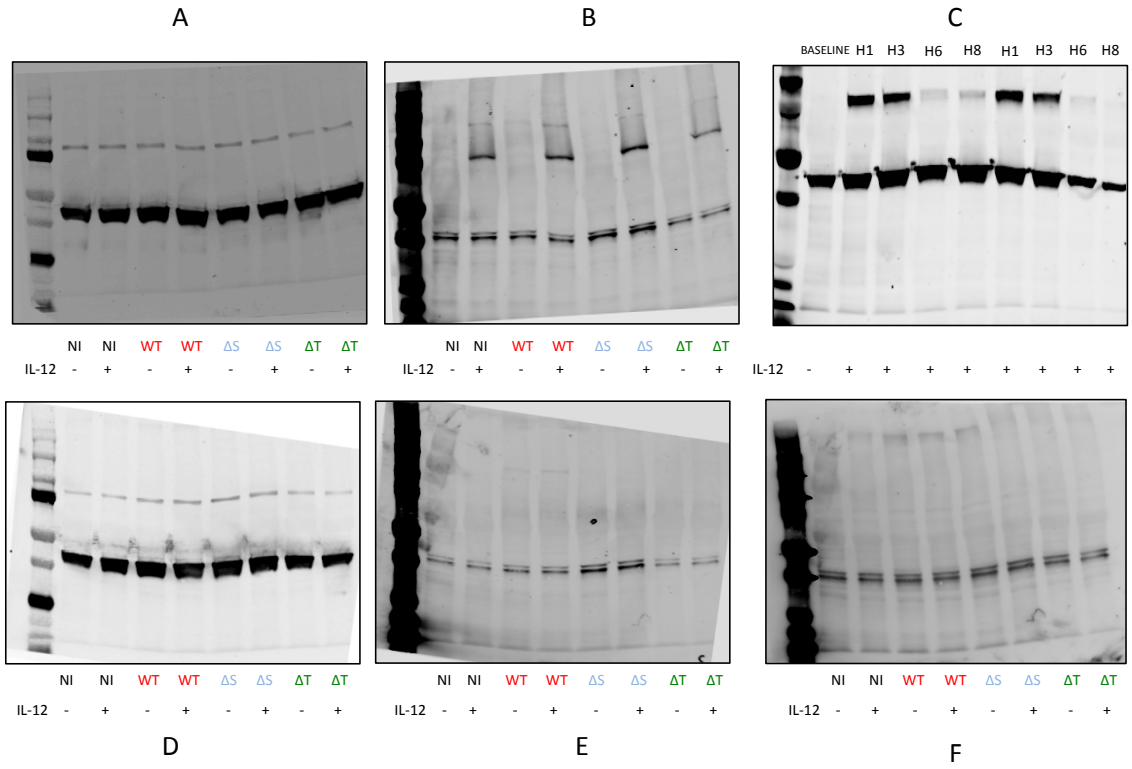
Supplementary video S6



Title video S6: Live NK-PA-WT GFP interaction

Video Legend Supplementary S6: Picture of living confocal microscopy immediately after PA-WT-GFP challenge suggesting a direct bacteria-to-cell contact: NCR2 (Nkp44) are labelled in Red and PA-WT-GFP are labelled in green. **NCR**: Natural cytotoxic receptor. **PA-WT GFP**: PA wild-type strain expressing ExoS, T, Y, and the Green fluorescent protein. Projection of 9 steps of 2 µm on Z dimension. No other processing.

Supplementary Figure S7

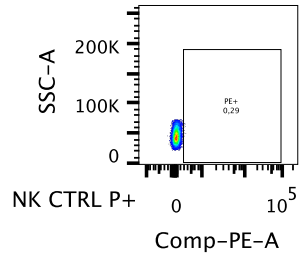


Title: Figure S7: Full-length Western Blot gels from Figure 2B,C and 3D.

Legend Supplementary Figure S7: Original gels were only cropped, flipped from right to left and rotated 180° when necessary to improve the clarity of the information. The (**A**) and (**B**) full-length gels stand for **Figure 2B**: (**A**) β-actin (45kDa) with 800nm and (**B**) P-stat 4 (81kDa) with 680 nm infrared radiation acquisition. The full-length gel (**C**) with P-stat 4 (81kDa) stands for **Figure 2C**. The (**D**),(**E**) and (**F**) full-length gels stand for **Figure 3D**: (**D**) β-actin (45 kDa) with 800 nm, (**E**) Phospho-p42/p44 (42,44 kDa) and (**F**) p42/p44 (42,44 kDa) with 680 nm infrared radiation acquisition.

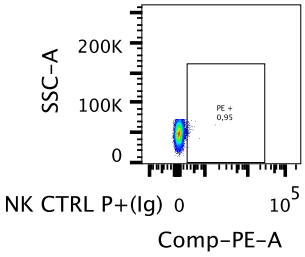
Supplementary Figure S8

A



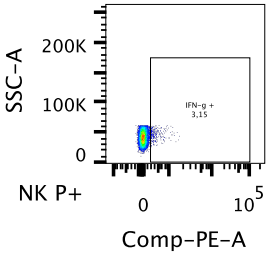
PA-WT infection
Unstained Control

B



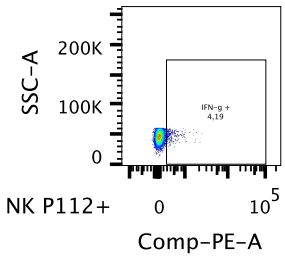
PA-WT infection
Isotype IgG1 Control

C



PA-WT infection IFN-G +

D



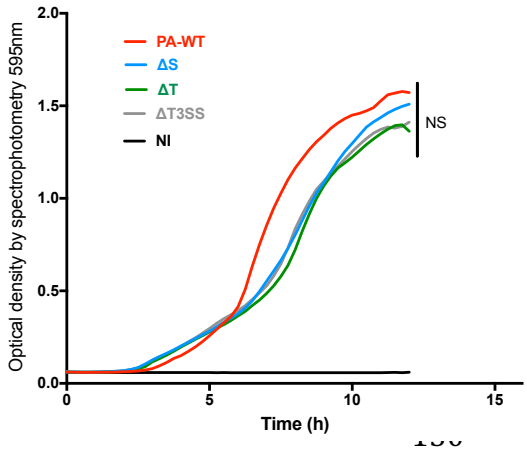
PA-deltaS infection IFN-G +

<u>Title: Figure S8:</u> IgG1 Isotype control profile for intracellular IFN-γ staining

<u>Legend Supplementary Figure S8:</u> Cytometry analysis of PBMC after 24-h PA or PA-ΔS infection. Representative density plot of Unstained control (A), intracellular staining with Isotype

control IgG1 (B), SSC/IFN-g^{+}-PE after PA infection (C) and SSC/IFN-g^{+}-PE after PA-ΔS infection (D) in sorted human NK cells.

Supplementary Figure S9

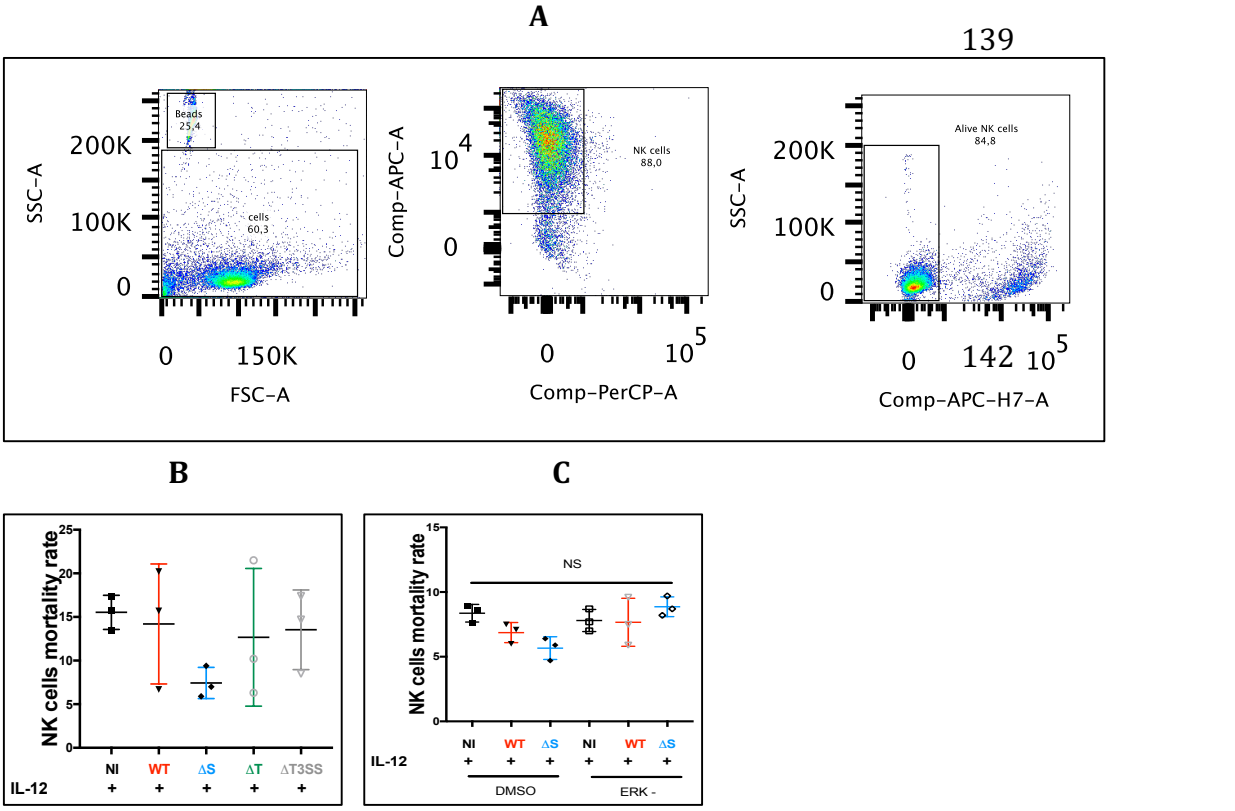


Title Figure S9: Growth curves of PA-WT and deleted strains

Legend Supplementary Figure S9: Evolution of the optical density (OD 595 nm) along 12 hours (37°C) of bacterial growth among PA-WT (WT) and deleted strains measured by spectrophotometry. Each strain was incubated in a well of a 96-well plate. The initial bacterial concentration was 1 x 10^6 UFC/mL

NS: Non-significant difference.

Supplementary Figure S10

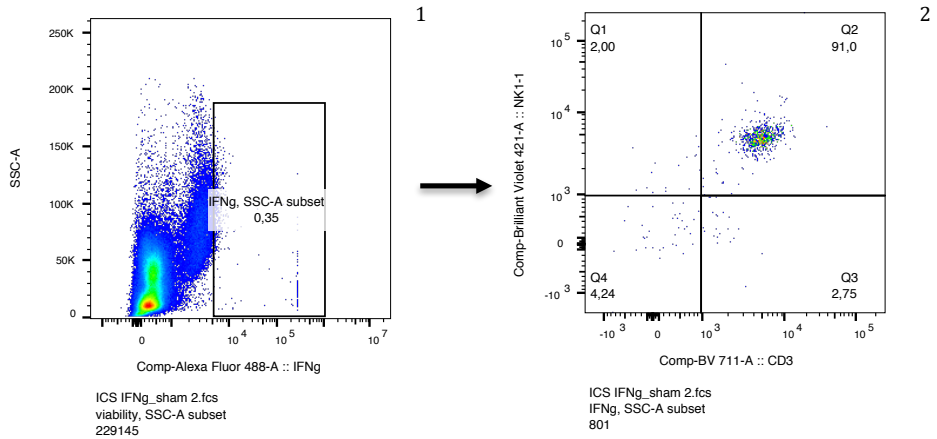


<u>Title Figure S10</u>: Mortality after PA infection among sorted human NK cells.

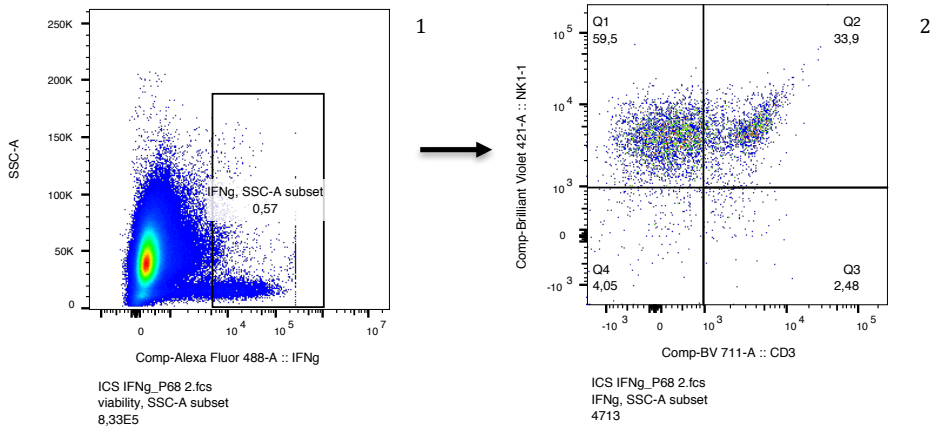
<u>Legend Supplementary Figure S10</u>: (A) Representative density plots after cytometry analysis illustrating APC H7-eFluor 780 positive among sorted human NK cell line ($CD56^+$APC/$CD3^-$ PerCP) after 2-hour PA-WT infection. (B) Representative histograms of mortality rate among sorted human NK cells after a 2-hour PA challenge with PA-WT or deleted strains after IL-12 stimulation. Data is presented as the median and interquartile range and is representative of 3 independent experiments (1 different healthy donor per experiment).

Supplemental Figure S11

A



B



<u>Title Figure S11</u>: IFN-g source in lung after mouse-PA pneumonia

<u>Legend Supplementary Figure S11:</u> Cytometry analysis in Lungs after PA-WT pneumonia. Representative density plot of SSC/ IFN-g^{+}-FITC (1) and NK1.1/CD3 (2) in SHAM (A) or PA-WT Infected (B) mice. NK cells correspond to NK1.1^{+}-BV421/CD3^{-}APC cells and NKt cells correspond to NK1.1^{+}-BV421/CD3^{+}APC cells.

4.2. Résultats Objectif n°2 (Article 5, en préparation)

Evaluation des conséquences de l'infection à *Pseudomonas aeruginosa* sur la fonction cytotoxique des cellules NK en réponse à des cellules déficientes en HLA de type I.

Questions formulées :

1) Influence de l'infection à PA, sur la fonction cytotoxique des cellules NK face à des cellules 721.221 déficiente en HLA de type I mimant des cellules tumorales.
2) Déterminants de l'altération de la fonction cytotoxique. Cette question se décomposait comme suit :
   2.A) Rôle des récepteurs activateurs/inhibiteurs exprimés par les cellules NK
   2.B) Rôle des cellules et des cytokines du microenvironnement
   2.C) Rôle du phénomène d'épuisement (« exhaustion ») des cellules NK

Les cellules NKs jouent un rôle clé dans la défense anti-bactérienne et anti-tumorale (171). Pour cette raison, l'étude de cette population cellulaire dans la compréhension des interactions entre infection et cancer apparait pertinente.

1) Afin d'évaluer la réponse cytotoxique des NK, nous avons donc exposé des PBMC de volontaires sains à une lignée cellulaire (cible) dépourvue de molécule de HLA de type I (lignée cellulaire 721.221). La fonction cytotoxique était appréciée par le marquage membranaire CD107a sur les cellules NKs, témoin indirect de la libération de granules cytotoxiques de Granzyme. Après vingt-quatre heures d'infection à PA, **l'activité cytotoxique des cellules NK était significativement réduite par rapport à l'activité des cellules non infectées**. A l'inverse, après infection à *Staphylococcus aureus* (SA), l'activité cytotoxique était augmentée. Ce résultat soulignait donc la singularité de l'interaction entre PA et la NK.

2) Pour expliquer la baisse de la cytotoxicité après infection, nous avions, à ce stade, trois hypothèses principales : Une altération des récepteurs NK après infection à PA, un effet du microenvironnement cytokinique ou cellulaire ou un phénomène « d'exhaustion » des cellules NK. Ces trois hypothèses devaient être explorées.
   2.A) Nous avons commencé par l'approche réceptorielle. Comme nous l'avons vu, pour initier une réponse cytotoxique, la NK doit reconnaitre le « soi manquant » (théorie du missing-self) via les récepteurs KIRs ou un ligand activateur via les NCRs ou NKG2D (récepteur de type lectine-C activateur). L'étude phénotypique des principaux récepteurs activateurs et inhibiteurs de la cellule NK après infection à PA révélait principalement une diminution

significative de l'expression de KIR2DS4, NKp46 et NKG2D. L'expression de KIR2DS4 était hétérogène entre les différents donneurs, alors que l'altération de la réponse cytotoxique était constante pour tous les donneurs. Son implication dans la baisse de cytotoxicité semblait donc peu probable. Pour évaluer l'implication de NKp46 et NKG2D dans la réponse cytotoxique, la mise en évidence de leurs ligands sur les cellules 721.221 était indispensable. Le phénotypage de la lignée 721.221 mettait en évidence la présence de deux ligands NKG2D (ULBP1 et 4) et l'absence de ligand NKp46. **Le récepteur NKG2D semblait donc pouvoir être impliqué dans la réponse cytotoxique des NK face aux cibles 221.** De plus, la neutralisation de NKG2D par un anticorps bloquant altérait également la réponse cytotoxique face à la lignée 721.221 dans les conditions non-infectées.

2.B) Nous avons ensuite formulé l'hypothèse d'une altération de l'expression de NKG2D et de la réponse cytotoxique médiée par les cellules accessoires au cours de l'infection. Nous avons réalisé une série de transferts adoptifs intra-donneur de cellules infectées $CD3^+$, $CD14^+$ dans des cultures de PBMC non infectés. Le résultat le plus marquant était la baisse significative de cytotoxicité après transfert adoptif de cellules $CD3^+$ infectées. Nous avons donc formulé l'hypothèse d'une participation des cellules $CD3^+$ à la baisse de cytotoxicité après infection. Le phénotypage T après infection à PA retrouvait une augmentation significative de la population T régulatrice seulement vingt-quatre heures après l'infection à PA. Ce phénomène n'était pas retrouvé après infection à SA. Au cours des processus néoplasiques, la suppression des lymphocytes T réactifs par les LT régulateurs via la libération d'IL-10 et TGF-β notamment, favorise la progression tumorale. Le rôle des LT régulateurs dans l'altération de la réponse NK devait donc être évalué dans notre modèle. Une nouvelle série de transferts adoptifs $CD4^+$, $CD8^+$ ou LT régulateur ($CD4^+CD25^+CD127^-$) a permis de mettre en évidence **que la baisse de la cytotoxicité et de l'expression du NKG2D étaient dépendantes des lymphocytes T régulateurs**.

2.C) L'exploration de l'hypothèse d'un épuisement des NK (exhaustion) après infection à PA et le phénotypage CTLA-4 et PD-1 des LT régulateurs est en cours.

La baisse de la cytotoxicité observée dans notre modèle après infection à PA peut donc être intégrée comme un mécanisme dépendant des LT régulateurs, responsables de la baisse d'expression des molécules NKG2D et donc d'une altération de la reconnaissance de cible anormale. Cette étude illustre une interaction possible entre infection et défaut de contrôle des cellules anormales.

TITLE PAGE: Pseudomonas infection impaired missing self-response of NK cells

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INTRODUCTION

Natural killer cells (NK cells) belong to innate lymphoid cells and can discriminate between self and foreign tissues thanks to killer-cell immunoglobulin-like receptors (KIR). Along infection, immunity impairment features were also reported to reduce anti-tumoral vigilance. NK cells are involved in both anti-bacterial and anti-tumoral immunity. Consequently, the study of NK cells function during infection is a relevant subject. Tumoral cells often express no or modified MHC molecule. The absence of self MHC molecules is one of the main trigger of cytotoxic function of NK cells. Nevertheless, tumoral cells also express activating ligand for Natural cytotoxic receptor (NCR) or activating receptor (NKG2D, DNAM-1) of NK cells (1). Even if the role of NK cells in the control of primary tumors remains a matter of debate, a high amount of circulating tumor-infiltrating NK cells was associated with a lower risk of metastases (2). Moreover, high levels of NK cell activating receptor expression or improved NK cell cytotoxicity have been linked to better prognosis cancers.

Pseudomonas aeruginosa (PA) is a gram-negative opportunistic pathogen that is a major causative microorganism that causes severe lung infections in cystic fibrosis (CF) and systematically becomes chronic (3). The high level of PA recurrence is related to its high virulence and hypermutable genome (4), while the ability to subvert immunity may explain chronic infection. PA was already reported to alter innate lymphoid cells (ILCs), including NK cells.

At end stage of CF, lung transplantation is the last resort. After transplantation, immunosuppressive drug, give rise to an increased susceptibility to infection and cancer. Conversely, cancer treatment often requires the interruption of immunosuppressive and often lead to graft rejection and poor prognosis. Among usual pathogen, Pseudomonas aeruginosa often early colonize the lung transplant and was associated with poor outcome. In a murine model of melanoma, Pseudomonas infection also gave rise to accelerate metastatic diffusion as compared to non-infected mouse (5). The link between PA-infection and tumoral immunity impairment has to be addressed to orient future research and development trying to reduce cancer occurrence in transplanted patient. Our study aimed at exploring the effect of PA infection on NK cells cytotoxic response after missing-self detection.

MATERIAL and METHODS

❖ Bacterial strains

PA01 is a clinical strain of *Pseudomonas aeruginosa* (no. 15692) (6) whose genome has been fully sequenced. It expresses most of the documented virulence factors, including the type III secretion system (T3SS) also known as the "needle complex" and its effectors: Exoenzymes (Exo) S, T and Y released in targeted cells through T3SS. SAMS (ATCC 29213) is a clinical *Staphylococcus aureus* (SA) methicillin-sensitive strain.

❖ PBMC from healthy donors, human NK cells isolation and NK92 human cell line

- PBMCs (Cryopreserved Human Peripheral Blood Mononuclear Cells) were isolated from heparinized blood of healthy volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Norway). PBMC were unfrozen and then kept in IL-2 overnight (200 UI/ml). All donors were recruited at the blood transfusion center (Nantes, France). Informed consent was obtained from all individuals and all experiments were approved by the Ethics Committee of Tours, France (2015-DC-1), (Biocollection Authorization Number DC-2014-2340) and performed in accordance with relevant guidelines and regulations.
- After cell sorting, NK cells were immediately resuspended in IL-2 supplemented medium and then infected.
- Human PBMC were sorted with the following Miltenyi Bitoec kit: Untouched NK cell isolation kit (), CD14 isolation kit, CD3 isolation kit, CD4 isolation kit, CD8 isolation kit and CD4+CD25+CD127- isolation kit. Isolated cells were then cultured in medium supplemented with 100U/ml IL-2 (Proleukin, Aldesleukin, Chiron).
- The 721.221 cell was a gift from EFS-PL laboratory. This line was cultured at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco) containing glutamine (Gibco) with 10% fetal bovine serum (Gibco, <10 EU/mL endotoxin contamination) and penicillin-streptomycin (PS).
- NK3.3 is an IL-2 dependent human Natural Killer cell line $CD56^{dim}$ $CD3^{neg}$ cultured in 150UI/mL IL-2 supplemented medium.

❖ **Infection**

- PBMC, sorted human NK cell, NK3.3 cell line or PBMC after CD14 or CD3 depletion were cultured at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco) containing glutamine (Gibco) with 10% fetal bovine serum (Gibco, <10 EU/mL endotoxin contamination), penicillin-streptomycin (PS), and 100U/ml IL-2 (Proleukin, Aldesleukin, Chiron) (7). Cells were seeded in 96-well plates (500,000 per well in 1 milliliter).
- PAO1 strain was grown overnight in Brain Heart Infusion medium at 37°C. Bacterial inoculum was calibrated by nephelometry. Cell and bacteria were co-cultured with a 25:1 bacteria to PBMC ratio. For sorted NK cells or NK3.3 cell line infection, we applied a 2,5:1 bacteria to NK cell ratio, assuming 10% NK cells among PBMC. After 2 hours of co-culture in PS-free RPMI and IL-2 supplemented medium, the wells PS to prevent bacteria overgrowth until the 24^{th} hour. Non-infected wells were similarly supplemented with Penicillin/Streptomycin. During infection, the medium was also supplemented with IL-2 to ensure continuous stimulation all along experiments. When mentioned, the medium was supplemented with IL-2, IL-12 or IL-15 (Miltenyi) at a concentration of 10UI/ML, 5ng/mL or 50ng/mL respectively for 5 hours during the cytotoxicity assessment.
- *Staphylococcus aureus* strain (ATCC 29213) was grown in the same conditions as PAO1

❖ **Cell labelling**

Antibodies were purchased from BD Biosciences unless otherwise stated. Data was collected with four-color FACSCalibur (BD Biosciences) and LSRII cytometer (Benton Dickinson, Le Pont de Claix, France) and analyzed using FlowJo 6.2 software (Ashland, USA). For PBMCs, NK cell gating was performed with anti-CD56-APC (NCAM16.2, #341026), anti-CD3-PerCP (SK7, #345766) and the corresponding isotype-matched control mAb. NK cells activating and inhibitory receptors phenotyping was performed with anti-NKp44-PE (clone Z231), anti-NKp30-PE (clone Z21), anti NKp46-BV650 (Clone 9E2), anti NKG2D-BV650 (1D11) Anti-KIR antibodies were purchased from Beckman Coulter: CD158a,h (anti KIR2DL1/anti KIR2DS1)-PE (Clone EB6B), CD158b1,b2,j (anti KIR2DL2, anti

KIR3DL3, anti KIR 2DS2) – PE (Clone GL183), anti CD158e1,e2 (anti KIR3DL2, KIR3DS1)-PE (Z273.7), anti CD158i (anti KIR 2DS4)-PE (FES172). NKG2D, NKp30 and NkP46 ligand were all studied with corresponding monoclonal chimeric antibodies from R&D system according manufacturer instruction.

Cytolytic activity (CD107a membrane expression) was assessed with CD107-FITC (H4A3, #555800) after 5 hours of *in-vitro* incubation during 721.221 targets exposition with a 1:1 ratio. Cells were washed twice before analysis on a LSRII flow cytometer (BD Bioscience). Cell viability among NK cell line was assessed by APC-Fixable Viability Dye Kit eFluor 780 staining (eBioscience).

❖ **Statistical analysis**

Statistical analyses were performed with GraphPad prism software (La Jolla, CA, United States). Continuous non-parametric variables were expressed as the median (25-75th percentile). The Kruskal–Wallis test was used to compare multiple groups. The post-hoc Dunn's test was used to perform multiple comparisons. Survival curves were compared to a log-rank test. $P < 0.05$ was considered to be statistically significant.

❖ **Data Availability**

The datasets generated and/or analyzed during the current study are available from the corresponding author on request.

RESULTS

PA infection altered NK cells cytotoxic response to 221 targets.

NK cells play a key role in immunity against PA (8). After bacterial infection, NK cells give rise to cytokine or cytotoxic response. We previously assessed cytokine response after PA infection and found a direct modulation of the IFN-γ response by the bacteria in IL-12 primed NK cells. Thus, we explored the consequence of PA infection on NK cells cytotoxic response. Cytotoxic response was assessed in response to HLA Class-I-Deficient 721.221 cells, referred as 221 cells. As a surrogate marker of degranulation, we analyzed CD107a membrane expression that correspond to Perforin and Granzyme B release (9). NK cells cytotoxic response after PBMC infection PA infection was very low with less than 10% CD107a positive NK cells (**Figure 1, A&B**). After exposure to 221 targets, PA infection (PA-PBMC) significantly altered the ratio of $CD107a^{+}$ NK cells among PBMC compared to non-infected condition (NI-PBMC). At the opposite, when the infection was performed with SAMS strain, NK cells cytotoxic response to 221 targets was increased (**see supplementary eFigure S1**).

NKG2D membrane expression is altered after PA infection.

Trying to understand how PA infection can modulate 221 targets response in NK cells after a 2 hours infection, we first assessed whether PA infection could modulate main activating or inhibitory receptor involved in 221 targets cytotoxic response (10,11). Notably, 221 cells line were previously reported to express specific ligand of Natural Cytotoxic receptor (NCR) such as NKp44 ligand expression (10). Moreover, the lack of type I HLA molecule on 221 targets may trigger Killer-cell immunoglobulin-like receptor (KIR). After PA infection, NKG2D, Nkp46 and CD158i (KIR2DS4) expression significantly decreased (**Figure 2**). KIR2DS4 expression varied between donors. In patients lacking KIR2DS4, cytotoxicity impairment was constant. These data did not support KIR2DS4 involvement in NK cells' response to 221 targets. We did not detect NKp46 ligand on 221 targets. Nevertheless, we detected ULBP1 and ULBP4 corresponding to NKG2D ligand (**Figure 3,A**). NKG2D is critical for NK cell activation in Host defense against Pseudomonas aeruginosa respiratory infection (12). NKG2D activating receptor is constitutively expressed on the surface of circulating NK

cells (13). NKG2D activation stimulates cytotoxic effects against virally infected or stressed. Pseudomonas is a potent inducer of NKG2D ligand in pulmonary epithelial cells following infection and NKG2D blocking impaired PA pulmonary clearance. To further investigate NKG2D involvement in NK cell cytotoxicity toward 221 cell line we blocked NKG2D with neutralizing antibody (**Figure 3,B**). As compared to isotype control, neutralizing NKG2D receptor with anti-NKG2D antibody significantly decrease CD107a activity in non-infected condition.

Accessory cells are involved in NK cells cytotoxicity impairment after PA infection

Accessory cells are largely reported to participate in NK cells function. To investigate their participation in NK cell cytotoxicity impairment, we first assessed the cytotoxicity among isolated NK cells in PA-infected and non-infected (NI) conditions. Degranulation among NK cells deprived from accessory cell was significantly reduced as compared to whole PBMC in non-infected conditions (**Figure 4,A**). As compared to non-infected sorted NK cells, PA infection did not significantly reduce CD107a activity of sorted NK cells. In the same way, when assessing a human NK cell line cytotoxicity (NK3.3 lineage), as compared to whole PBMC infection, PA-infection in NK 3.3 did not significantly alter cytotoxicity (**Figure 4,B**).

In parallel, contrary to whole PBMC infection, PA infection of isolated NK cells did not reduce NKG2D expression suggesting an accessory cells-dependent regulation of NKG2D in our model (**Figure 4,C**). To figure out accessory cells' influence on CD107a activity during PA infection, we assessed cytotoxicity of NI PBMC after an intra-donor adoptive transfer of PA-infected non-NK cells. Before transfer, infected cells were treated with high doses of PS and Gentamycin to ensure the lack of residual bacteria. Adoptive transfer of 6-hour PA infected non-NK cells among NI PMBC led to significant cytotoxicity impairment as compared to NI PBMC without adoptive transfer (**Figure 4,D**). As a result, there was increasing evidence of accessory cells' involvement in cytotoxicity reduction after PA infection.

Among accessory cells, DC are central in controlling NK cell functions. DC were previously reported to be involved in PA immunity (14) while PA could also reduce IL-12 release by DC (15). We supplemented the medium with IL-12 or IL-15 to mimic dendritic cell or monocytes stimulation

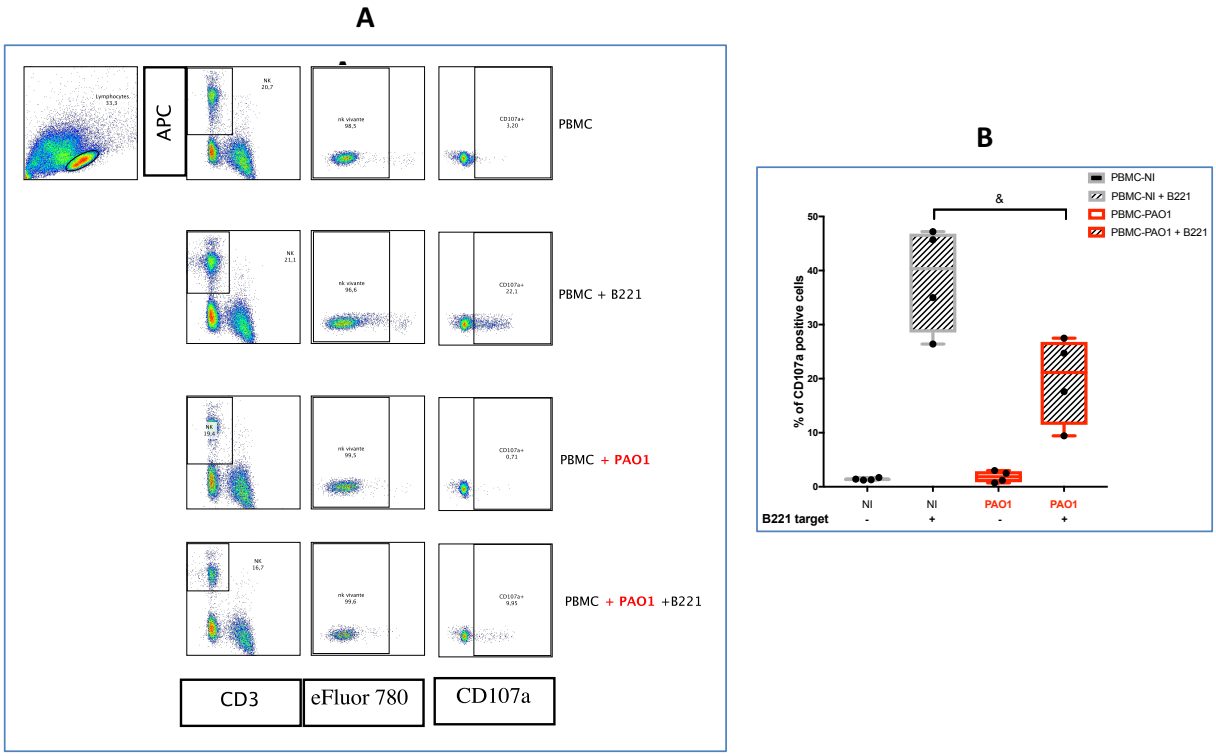
without any effect on CD107a activity in infected or NI condition (**see supplementary eFigure S2,A**). These results suggested that cytotoxicity reduction was not likely to be related to IL-12 or IL-15 secretion impairment.

We then performed intra-donor adoptive transfer with PA-infected $CD14^+$ or $CD3^+$ cells. $CD3^+$ adoptive transfer among NI PBMC dramatically decreased CD107a activity as compared to $CD14^+$ transfer (**Figure 4,E**). In parallel, intra-donor adoptive transfer of 6-hour PA infected $CD3^+$ reduced NKG2D expression on NK cell (**Figure 4,F**). These data suggested a T cell-dependent impairment of NK cells' cytotoxicity and modulation of NKG2D expression after PA infection.

$CD4^+CD25^+CD127^-$ T cells play a pivotal role in NK cells' cytotoxicity impairment after PA infection

To further investigate T cells involvement in cytotoxicity reduction, we determine T cell differentiation after a 24-hour PA infection. We differentiate Th1, Th2, Th17 and Treg orientation through IL-2, IL-4, IL-17 intracellular staining and CD4, CD8, CD25 and CD127 expression study. Among $CD3^+$ cells, PA infection did not affect the ratio of CD4/CD8 T cells, but the percentage of $CD4^+CD25^+CD127^-$ T cells increased (**Figure 5,A**). This subset usually corresponds to Regulatory T cells. At the opposite, after SA infection, the percentage of Regulatory T cells was stable (**see supplementary eFigure S2,C)**. Thus, we hypothesized that Regulatory T cells could be responsible for NK cells' cytotoxicity impairment after PA-infection. In our model, similarly to PA infection in whole PBMC, intra-donor adoptive transfer of 6-hour infected regulatory T cells both reduce cytotoxicity and NKG2D expression on NK cells (**Figure 5,B&C**). Thanks to CD25 (IL-2Rα), regulatory T cell can deprive $CD4^+$ T, DC or NK cells from IL-2 stimulation. In our model, IL-2 supplementation did not increase CD107a after PA infection (**see supplementary eFigure 2,B**). NK cells exhaustion after PA infection and CTLA-4, PD-L1 and TNFR2 expression on Regulatory T cells, are under investigation.

FIGURES and LEGENDS



<u>Figure 1</u>: PA infection impaired NK cells cytotoxicity in response to 221 cell line

<u>Legend Figure 1</u>: NK cells cytolytic (CD107a) activity among PBMC was assessed with or without PAO1 infection and with or with 221 targets. Representative density plots illustrating CD107a expression in NK cells (PerCp-CD3^{neg} APC-CD56^{pos}) in lymphocyte gate by flow cytometry (**A**). Histograms of CD107a^{+} in NI or PAO1 infected conditions with or without 221 targets (**B**).

Data is shown as the median and interquartile range of 4 distinct healthy donors

&: $p < 0.05$, **NS**: Non-significant difference**, NI:** Non-infected**, PBMC**: peripheral blood mononuclear cell, **PAO1**: PBMC after 24-hours infection with PAO1 strain, **APC-H7 efluor 780**: viability assessment, **B221 target:** 721.221 cell 5-hour exposition with a PBMC:221 ratio of 1:1.

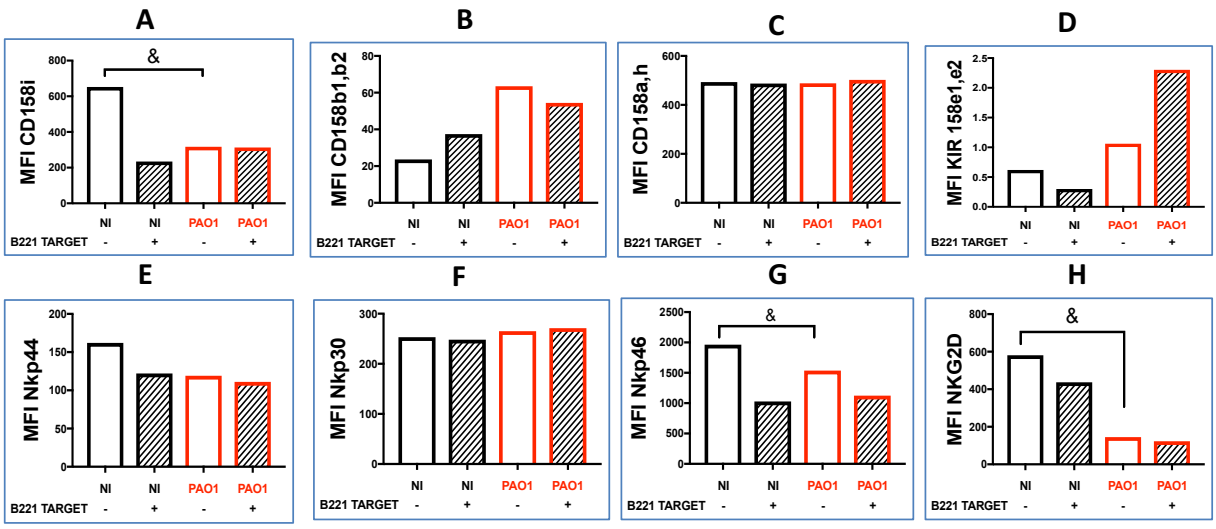


Figure 2: PA infection influence on activatory and inhibitory receptor

Legend Figure 2: Representative Histograms of NK cells expression of NCRs, NKG2D and KIR in infected and NI PBMC with or without 221 targets. NK cells were analysed in lymphocyte gate by flow cytometry after PerCp-CD3^{neg} APC-CD56^{pos} staining.

Data is shown as the median and interquartile range of 4 distinct healthy donors

&: $p < 0.05$, **NS**: Non-significant difference**, NI:** Non-infected**, PAO1**: PBMC after 24-hours infection with PAO1 strain, **APC-H7 efluor 780**: viability assessment, **B221 target:** 721.221 cell 5-hour exposition with a PBMC:221 ratio of 1:1.

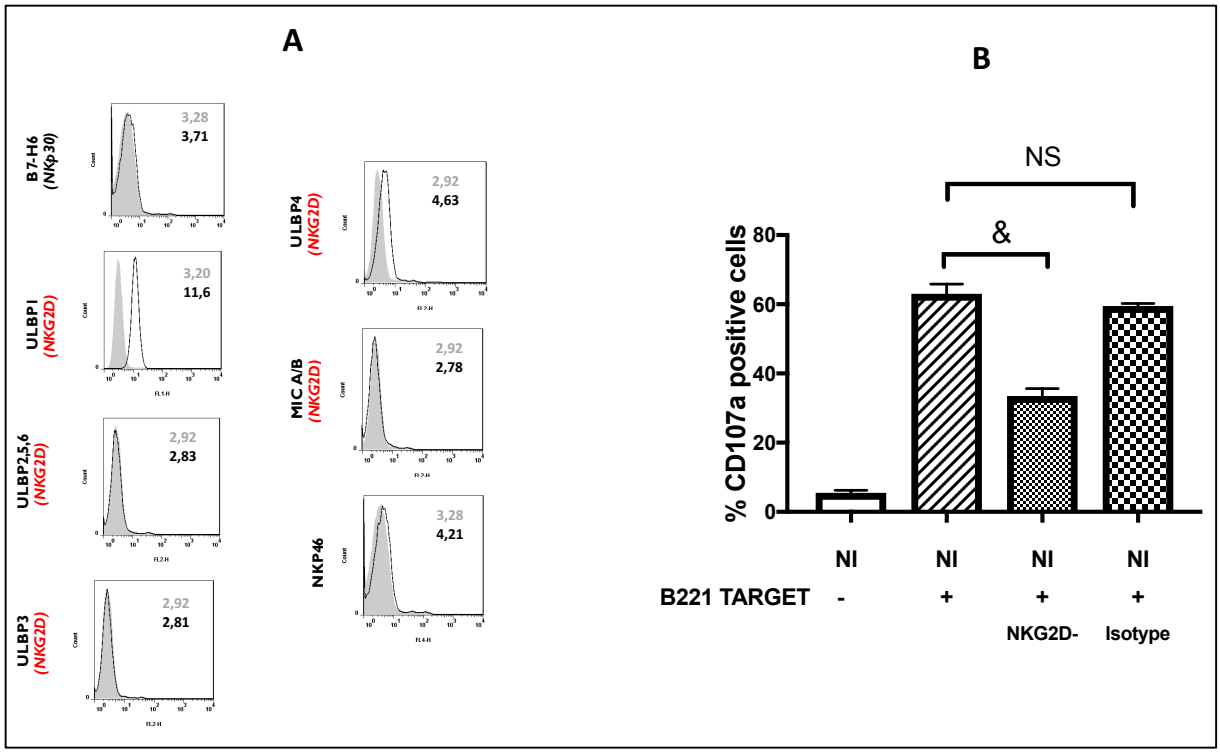
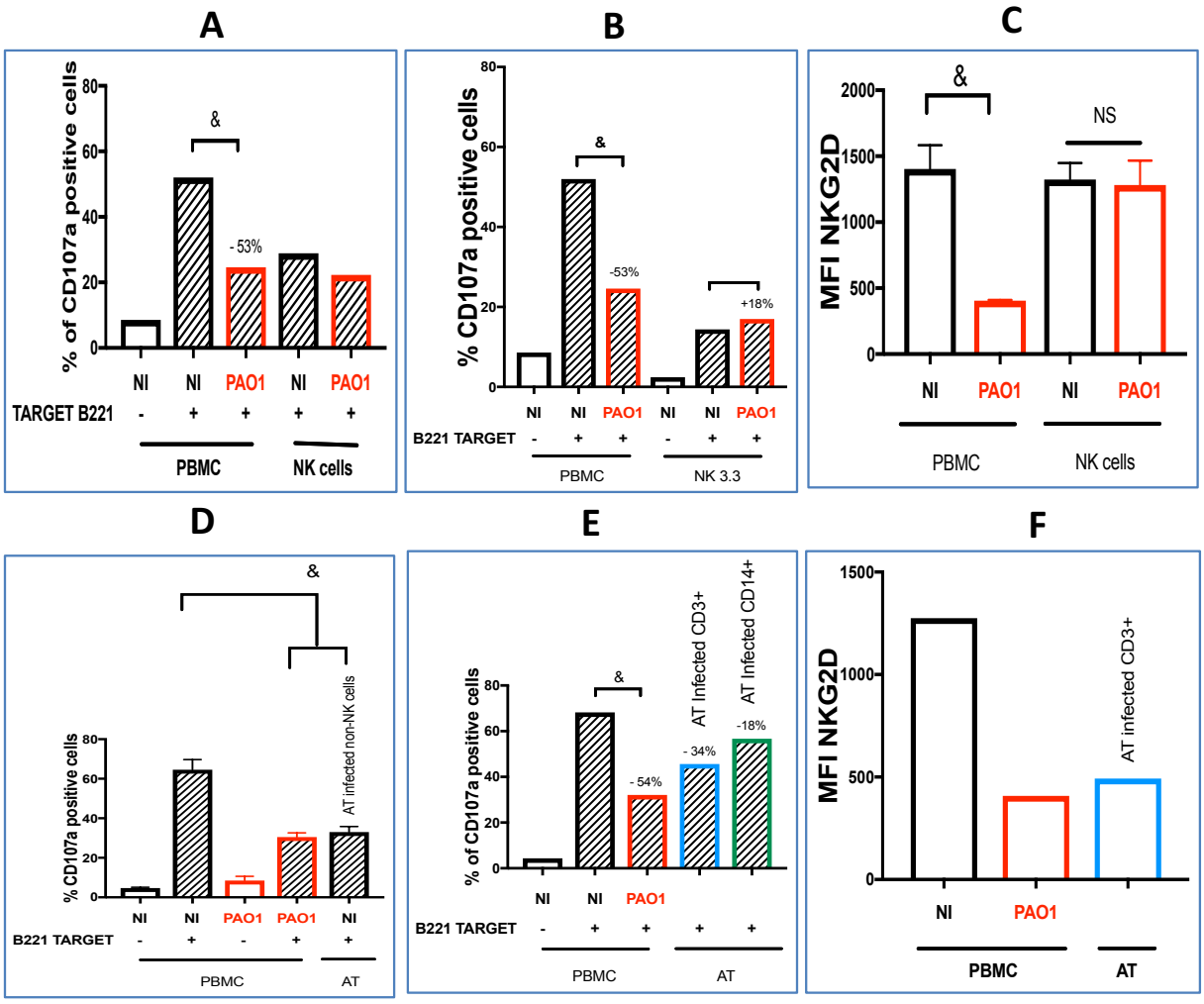


Figure 3: NKG2D involvement in NK cells cytotoxic response to 721.221 target

Legend Figure 3: Histograms illustrating the expression for NKG2D, NKp46 and NKp30 ligands (**A**) on 721.221 determined by flow cytometer. The profile of IgG isotype control is shown in grey filled histogram. Representative histograms of NK cells cytotoxic response to B221 target with or without 1-hour pre-treatment of PBMC with anti-NKG2D neutralizing antibody (**B**).



<u>Figure 4:</u> Microenvironment and accessories cells influence on NK cells cytotoxicity and NKG2D expression.

<u>Legend Figure 4</u>: PBMC were sorted to study NK cells cytotoxic activity with or without infection among isolated NK cell (**A**), PA infection impact on the cytotoxicity of NK 3.3 cell line was also assessed (**B**). Effect of intra-donor adoptive transfer of infected non-NK cells (**D**), CD3+ or CD14+ cells (**E**) on NK cell cytotoxicity. Before transfer cells suspension were sterilized with high concentration of antibiotics and the lack of residual bacteria was controlled. NKG2D expression in mean Fluorescence Intensity (MFI) was assessed after isolated NK cell infection (**C**) and after $CD3^{+}$ cells adoptive transfer (**F**). Data is shown as the median and interquartile range of 3 distinct healthy donors.

&: $p < 0.05$, **NS**: Non-significant difference**, NI:** Non-infected**, PAO1**: PBMC after 24-hours infection with PAO1 strain, **B221 target:** 721.221 cell 5-hour exposition with a PBMC:221 ratio of 1:1, **AT**: adoptive transfer, **NK^+**: NK cells after PBMC sorting with untouched NK cells isolation kit (unlabelled cells), **Non-NK cells**: Cells suspension obtained after PBMC sorting with untouched NK cells isolation kit by flushing out the plunger into the column to expulse magnetically labelled cells, **$CD3^+$ cells:** cells suspension after positive selection of $CD3^+$ cells among PBMC, **$CD14^+$ cells:** cells suspension after positive selection of $CD14^+$ cells among PBMC.

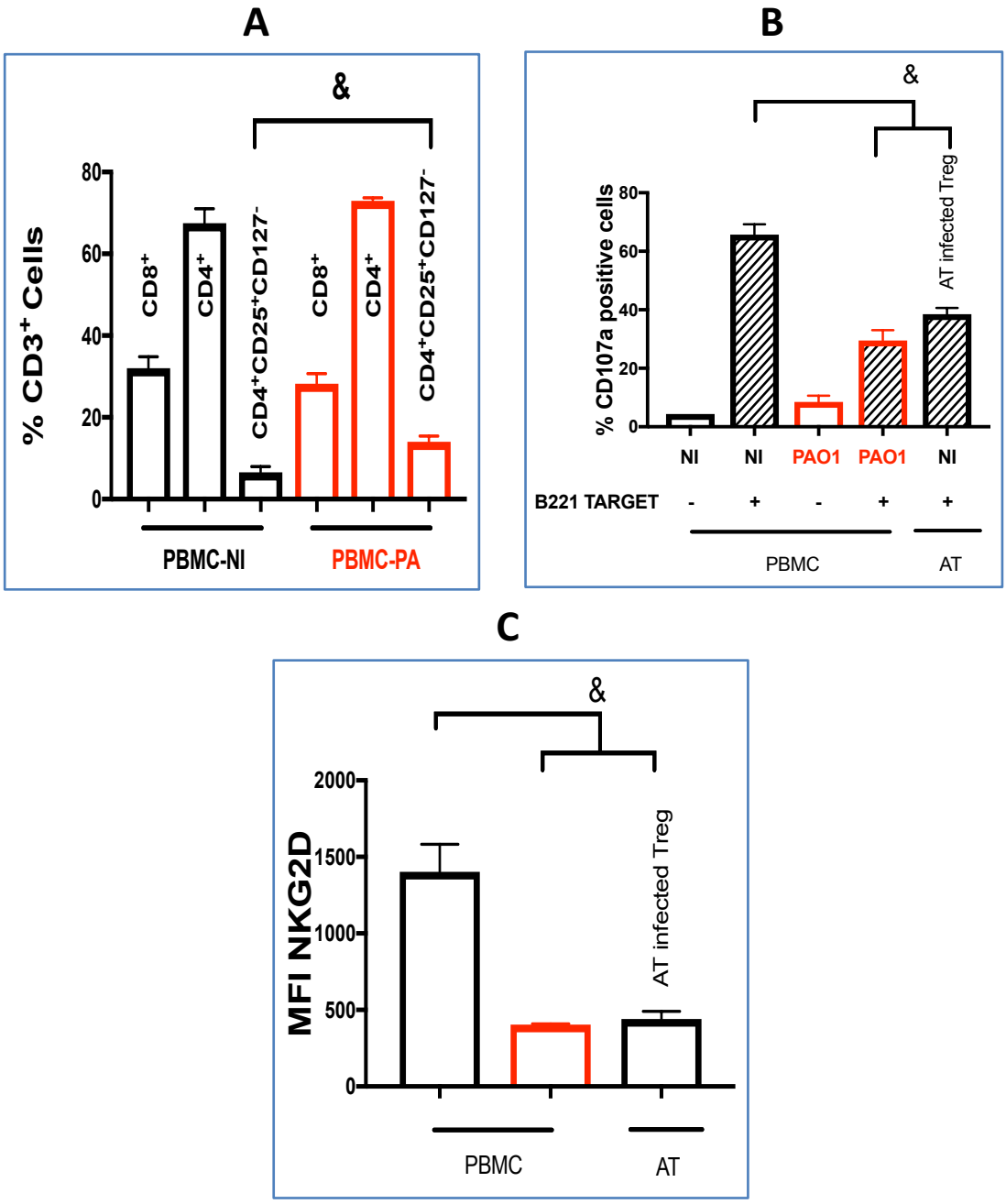


Figure 5: Involvement of $CD4^+CD25^+CD127^-$ Regulatory T Cells in PA-induced cytotoxicity impairment in NK cells.

Legend Figure 5: (**A**) Representative histogram of $CD4^+$, $CD8^+$ and $CD4^+CD8^+CD127^-$ in $CD3^+$ cells among infected or non-infected PBMC (Lymphocytes gate). Representative histogram $CD107a^+$ NK cells in response to 221 cell line (**B**) and NKG2D expression (**C**) with (PAO1) or without PA-infection

and with or without infected Treg adoptive transfer. (Before adoptive transfer Treg cells with high concentration of PS and gentamycin to ensure no residual bacteria).

&: $p < 0.05$, **NS**: Non-significant difference**, PBMC-NI:** Non-infected**, PBMC-PA**: PBMC after 24-hours infection with PAO1 strain, **B221 target:** 721.221 cell 5-hour exposition with a PBMC:221 ratio of 1:1, **AT:** adoptive transfer, **Treg**: Regulatory T cells ($CD4^{+}CD25^{+}CD127^{-}$).

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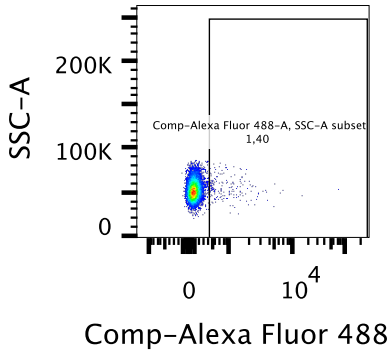
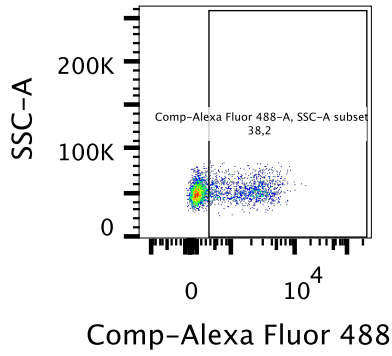
Online-only Material: Supplementary Figures, Video, Table and Legends

Supplementary Figure S1



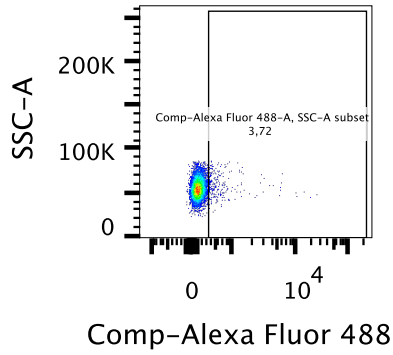
PBMC

1,4% NK CD107a⁺



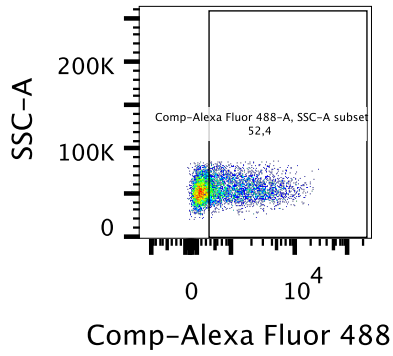
PBMC + B221

38,2% NK CD107a⁺



PBMC + SAMS

3,72% NK CD107a⁺



PBMC + SAMS + B221

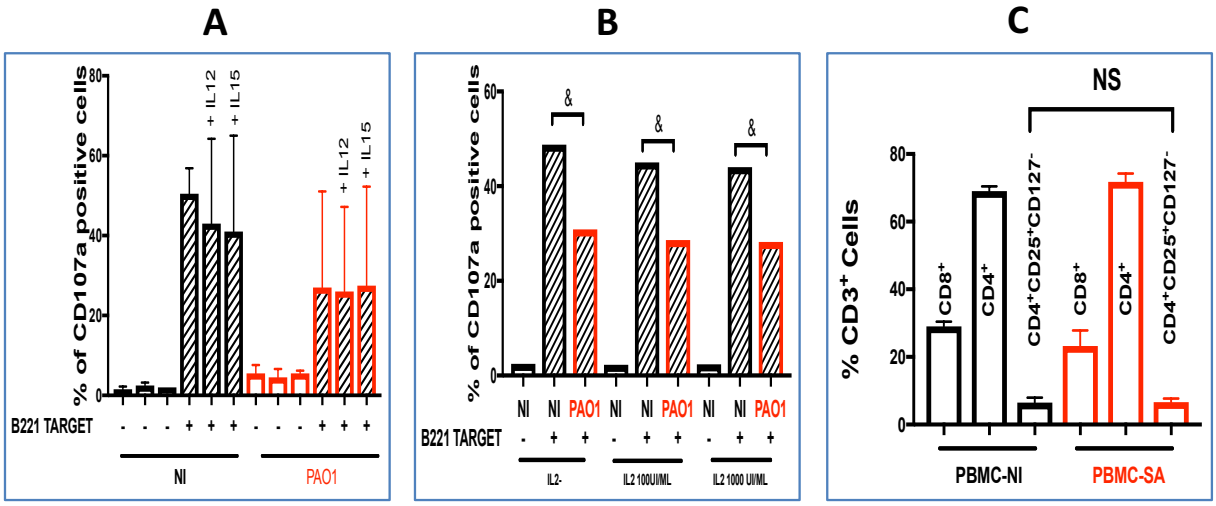
52,4% NK CD107a⁺

Title Figure S1: SAMS infection increases NK cells cytotoxicity in response to 221 cell line.

Legend Figure S1: NK cells cytolytic (CD107a) activity among PBMC was assessed with or without SAMS infection and with or with 221 targets. Representative density plots illustrating CD107a expression in NK cells (PerCp-$CD3^{neg}$ APC-$CD56^{pos}$) in lymphocyte gate by flow cytometry.

PBMC: peripheral blood mononuclear cell, **SAMS**: PBMC after 24-hours infection with SAMS strain, **B221 target:** 721.221 cell 5-hour exposition with a PBMC:221 ratio of 1:1.

Supplementary Figure S2



Legend Figure S2: To determine the role of IL-12, IL-15 (**A**) and IL-2 (**B**) in our model, PBMC were treated with 5ng/mL IL-12, 50ng/mL IL-15 or IL-2 (100UI/mL or 1000 UI/mL) in infected and NI conditions with or without 221 targets. Representative histograms of T cell phenotype ($CD4^+$,$CD8^+$ or $CD4^+CD25^+CD127^-$) with or without SA infection (**C**)

5. DISCUSSION

5.1. Discussion Objectif n°1

L'IL-12 est la cytokine principale de la communication entre la CD et la cellule NK. Elle est indispensable à sa réponse IFN-γ. Après stimulation IL-12, l'infection à *Pseudomonas* augmentait la réponse IFN-γ de la cellule NK par rapport à la stimulation IL-12 sans infection. Cette augmentation d'activité IFN-γ ne semblait pas médiée par STAT-4 mais par une voie accessoire nécessitant l'activation de ERK. En particulier, nous avons identifié que l'Exoenzyme T, effecteur du SSTIII, était un des principaux déterminants de l'activation de ERK. Enfin, l'IL-12 n'avait pas d'influence sur l'activation de ERK, mais la synthèse d'IFN-γ ERK-dépendante par la bactérie nécessitait une stimulation IL-12 préalable.
Nos hypothèses de travail ont été confirmées par un modèle *in vivo* avec une augmentation du niveau de synthèse pulmonaire d'IFN-γ après infection à PA exprimant l'ExoT. Nous également confirmé que la cellule NK était la source principale d'IFN-γ dans le poumon au cours d'une infection à PA. Le concept de régulation de la réponse pro-inflammatoire par le pathogène est important considérant que l'IFN-γ stimule la synthèse de facteur de virulence de PA en se fixant sur son récepteur OprF (21,23).

L'IFN-γ augmente l'expression des molécules de CMH de type I et II et favorise l'activation des macrophages et des lymphocytes T $CD4^+$ (172). Cette réponse inflammatoire est indispensable pour initier la réponse immunitaire anti-infectieuse à la phase précoce. A l'inverse, une réponse inflammatoire non contrôlée par une balance anti-inflammatoire déficitaire peut être délétère et engendrer des lésions tissulaires pulmonaires, d'autres défaillances d'organes, et augmenter la susceptibilité aux nouvelles infections (173).
côté de ces effets sur la synthèse des facteurs de virulence de PA, l'IFN-γ participe également à la défense antibactérienne et au contrôle de l'infection dans des modèles murins d'infection oculaire à PA (165).

Notre équipe a récemment rapporté que chez les patients présentant une susceptibilité accrue aux infections comme les patients traumatisés crâniens, le traitement par IL-12 était capable de restaurer les capacités de sécrétion d'IFN-γ des cellules NK (10). Nos résultats confirment également le rôle clef de l'activation de la voie IL-12/STAT4 dans la réponse de type IFN-γ de la cellule NK au cours de l'infection à PA et ceci, spécifiquement pour subset de NK $CD56^{bright}$ (104). Ces données sont appuyées par la localisation préférentielle de ces cellules dans les centres lymphoïdes secondaires, lieux privilégier de communication avec la CD via l'IL-12, notamment (122).

Nous avons mis en évidence qu'un contact direct entre la bactérie et la cellule était requis pour initier la réponse IFN-γ de la lignée NK 92 après infection à PA. L'inhibition sélective des TLRs et NCRs n'altérait pas la réponse IFN-γ dans notre modèle. Parmi les acteurs impliqués dans l'interaction directe cellules-bactéries, le rôle du SSTIII dans la réponse IFN-γ devait être évalué. Le SSTIII permet à la bactérie d'injecter trois effecteurs (Exoenzymes S, T et Y) dans le cytoplasme de la cellule hôte. Chez l'homme, au cours de l'infection pulmonaire à PA, l'expression du T3SS par la bactérie a été rapportée comme un élément de mauvais pronostic (56).
Dans notre étude nous avons également mis en évidence un rôle central du T3SS dans la régulation de la réponse IFN-γ de la cellule NK aussi bien *in vitro* que *in vivo*. Notamment, l'Exoenzyme T (exprimée par plus de 95% de souche de PA (19), apparait comme le déterminant principal de l'activité IFN-γ. Cette activité est dépendante de la phosphorylation de ERK mais nécessite une stimulation préalable par l'IL-12. Le rôle central de ERK dans le pouvoir pathogène de PA avait déjà été rapporté (174). La participation des voies P38 MAPK, PI3K/Akt and NF-κB dans la production d'IFN-γ en réponse à la stimulation IL-12 (109,170) avait également été rapportée. Notamment, l'effet stabilisateur de MAPKinase P38 sur l'ARN messager IFN-γ était connu (170).

Suite à ces résultats, plusieurs hypothèses peuvent être formulées sur les modalités de régulation de l'activité IFN-γ par le SSTIII de PA. Les Exoenzymes T et S sont des protéines à deux fonctions avec un domaine N-terminal possédant une Rho GTPase-activating protein (GAP) et un domaine C-terminal codant pour une ADP ribosyltransférase (ADPRT). Les domaines GAP des Exoenzymes T et S peuvent perturber l'architecture du cytosquelette actinique cellulaire et mener à l'apoptose cellulaire. Dans une situation non-infectée, la phosphorylation de ERK et la production d'IFN-γ sont réprimées par le complexe Cbl-b (E3 Ubiquitin ligases) (175-177). Après liaison au domaine ADPRT de l'ExoT, la protéine Crk (50) se lie à Cbl-b et le complexe formé subi une dégradation dans le protéasome (178). Nous pouvons donc supposer qu'au cours de l'infection, la présence de l'ExoT dans le cytoplasme de la cellule hôte provoque la dégradation de Cbl-b. La phosphorylation de ERK n'est alors plus réprimée expliquant l'augmentation de synthèse de l'IFN-γ. A l'inverse, l'ExoS via son domaine ADPRT inactive les protéines de la famille Ras impliquées dans l'activation de ERK (179).

Notre étude présente plusieurs limites. Les cellules NK92 utilisées pour les analyses en PCR et les Western Blot présentent un répertoire spécifique de récepteurs probablement incomplètement partagé par les cellules NK humaines de volontaires sains triées sur colonnes. En effet, la culture des PBMC dans un milieu supplémenté en IL-2 est connue pour modifier l'expression des KIRs à la surface des cellules NKs de volontaires sains. Concernant l'utilisation des inhibiteurs de kinase, le risque d'inhibition de cible non spécifique n'est pas maitrisé dans le modèle *in vitro*. L'utilisation de lignée cellulaire KO ou de miRNA aurait pu améliorer le niveau de preuve concernant l'étude des voies de

signalisation. Par ailleurs, nous n'avons pas contrôlé la réalité de l'injection intra-cytoplasmique des Exoenzymes dans notre modèle. De même, l'utilisation de souches délétées plutôt que complémentées représente également une limite. Cependant, les charges bactériennes pulmonaires étaient constantes après vingt-quatre heures d'infection et les courbes de croissance bactérienne étaient comparables entre les différentes souches testées.

En conclusion, sans une stimulation préalable par IL-12, la cellule NK est incapable de sécréter de l'IFN-γ en réponse à l'infection à PA. L'infection à PA majore la réponse pro-inflammatoire type IFN-γ de la cellule NK via l'Exoenzyme T, enzyme effectrice du système de sécrétion de type III. Le mauvais pronostic des pneumonies à PA exprimant le T3SS (56) pourrait alors être en partie expliqué par une réponse pro-inflammatoire exacerbée, aboutissant à un état d'immunosuppression post septique (173) (Figure 1). Enfin, nos résultats appuient les recherchent actuelles évaluant des thérapeutiques ciblant le T3SS ou ses effecteurs au cours des pneumonies à PA (180,181).

5.2. Discussion Objectif n°2

Au cours des maladies respiratoires chroniques, comme la mucoviscidose, la transplantation pulmonaire représente le dernier recours thérapeutique. Après la transplantation pulmonaire, les traitements immunosuppresseurs indispensables à la tolérance de la greffe donnent lieu à une susceptibilité accrue aux infections et au développement de tumeurs. Parmi les pathogènes colonisant ou infectant habituellement le greffon pulmonaire, on retrouve *Pseudomonas aeruginosa.* Cette bactérie a déjà été rapportée comme altérant l'immunité anti-cancer et le contrôle de la diffusion métastatique du mélanome dans un modèle murin (166). L'interaction entre infection et immunité anti-cancer est donc un sujet central chez les patients transplantés et la prévention du risque infectieux dans cette population est donc un challenge. Pour ces raisons, nous avons choisi d'évaluer l'influence de l'infection à PA sur la fonction cytotoxique de la cellule NK.

Nous avons mis en évidence que l'infection à *Pseudomonas* de PBMC de donneurs sains était à l'origine d'une altération profonde de l'activité cytotoxique des cellules NK, en réponse à une cible déficiente en molécule HLA de type I. L'altération de la réponse cytotoxique des NK lors de l'infection était médiée par les LT avec une augmentation de la population de LT régulateurs au cours de l'infection à *Pseudomonas* contrairement à ce qu'on observait après une infection à *Staphylococcus aureus*. Le transfert adoptif de LT régulateurs infectés vers des PBMC non infectés reproduisait la baisse de cytotoxicité et d'expression de NKG2D observées après infection de PBMC totaux. L'étude de NKG2D est particulièrement intéressante dans la problématique infection et cancer car de multiples types histologiques tumoraux expriment des ligands NKG2D (NKG2D-L) y compris le mélanome, certains lymphomes et une majorité des adénocarcinomes chez l'homme.

Il restera deux questions principales à traiter :

- La baisse d'expression du récepteur NKG2D peut-elle expliquer la baisse de cytotoxicité observée face aux cellules 721.221 à elle seule ?
- Comment les LT régulateurs interviennent-ils sur la fonction NK ? Est-ce par la libération de cytokines immunosuppressives (IL-10, TGF-β) ou par une interaction récepteur-ligand via CTLA-4 ou PD-1 ?

Voici les éléments de la littérature pouvant nous aider à répondre à ces questions et que nous sommes en train d'évaluer.

5.2.1. NK et allo immunité

Certaines équipes considèrent que l'infection bactérienne est à l'origine d'une diminution du nombre de LT régulateurs au sein du greffon, permettant alors aux LT activés d'exercer leur activité cytotoxique menant au rejet du greffon (182). Dans leur publication, Young JS et coll. rapportaient chez des souris transplantées cardiaques initialement tolérantes à leur greffe, que l'infection à *Listeria*

monocytogène (LM) provoquait le rejet du greffon à la phase aigüe de l'infection chez un tiers des souris. L'analyse des greffons rejetés après infection retrouvait une baisse de la population T régulatrice et une augmentation de l'infiltration des LT $CD8^+PD\text{-}1^+$ et $CD4^+FOXP3^-PD\text{-}1^+$. PD-1 (Programm cell death 1) est un membre de la famille B7-CD28 reconnaissant le ligand PD-L1. Les LT régulateurs sont porteurs du ligand PD-L1. L'interaction de ce récepteur avec son ligand inhibe l'activation et la prolifération de la cellule portant PD-1. Au cours des phénomènes de tolérance de greffe, l'inhibition des T $CD4^+$ et $CD8^+$ par les LT régulateurs est assurée par cette interaction notamment et le traitement par anti PD-L1 s'accompagne d'un rejet systématique du greffon. Dans ce modèle, la baisse du nombre de LT régulateurs compromet l'interaction PD-1/PD-L1 et permet aux LT activés de persister et d'exercer leur action cytotoxique sur le greffon.

Parmi les médiateurs capables d'augmenter l'expression PD-L1 au cours de l'infection, on retrouve notamment l'IFN-γ dont nous avons démontré qu'il s'agissait d'une cytokine clef de la réponse inflammatoire lors d'une infection à *Pseudomonas*. Les cellules NK expriment également PD-1. Dans notre modèle, par analogie avec ces résultats, l'augmentation de PD-L1 sur les LT régulateurs secondaire à la production d'IFN-γ, pourrait alors expliquer l'altération de fonction des NKs via l'interaction PD-1/PD-L1. Cette hypothèse devra être explorée.

5.2.2. NK et immunité anti-tumorale

Les cellules Natural Killer jouent un rôle central dans la vigilance anti-cancer. Dans la thématique relations entre infections et cancers, une équipe a déjà rapporté que dans un modèle de mélanome murin (32), des souris infectées à *Pseudomonas aeruginosa* présentaient une diffusion métastatique accélérée du mélanome comparées aux souris non infectées.

Si l'implication des cellules NK dans la reconnaissance et le contrôle des tumeurs est bien documentée, les ligands et les récepteurs impliqués sont multiples et encore incomplètement identifiés. Nous avons donc fait l'hypothèse que cette baisse observée de cytotoxicité face à la lignée 721.221 pouvait être secondaire à l'altération d'un récepteur activateur sur la NK au cours de l'infection. Esin et al en 2008 avaient rapporté que la cellule NK reconnaissait spécifiquement la bactérie *Pseudomonas aeruginosa* par l'intermédiaire du récepteur NKp44. Dans cette thématique, l'équipe de Vincent Vieillard avait également rapporté que la reconnaissance de la lignée 721.221 par la NK passait par le récepteur NKp44 (NCR2) (183). L'interaction de *Pseudomonas* avec ce récepteur aurait donc pu expliquer la baisse de cytotoxicité observée dans notre modèle. Pour explorer cette hypothèse, nous avions mis en évidence par microscopie confocale, que PA pouvait se lier spécifiquement au NKp44 (Figure 13). Néanmoins, l'inhibition de cette interaction par anticorps neutralisant anti-NKp44 ne modifiait pas l'activité cytotoxique des NK (données non présentées). De plus, contrairement à l'équipe de Vincent Vieillard, nous n'avons pas non plus mis en évidence de ligand NKp44 sur notre

lignée 721.221 mais préférentiellement des ligands du récepteur NKG2D. Ces résultats contradictoires peuvent s'expliquer par la très faible affinité des anticorps commerciaux (type NKp-Fc) pour leur ligand et les dérives des lignées de laboratoire au cours de leur culture.

Le rôle des NK dans l'immunité anti-cancer ne peut pas être restreint à une interaction récepteur-ligand déclenchant une réponse lytique, mais doit s'intégrer dans une réponse immunitaire globale. Par exemple, un très haut niveau de LT régulateurs circulant est associé à un mauvais pronostic chez le patient atteint de cancer. Les LT régulateurs exercent une action inhibitrice sur les cellules NK via la libération de TGF-β ou d'IL-10 ou via l'interaction de PD-L1 ou CTLA4 avec leur ligand. Nous avons vu le mécanisme d'action de PD-L1 ci-dessus. CTLA-4 interagit habituellement avec les molécules CD80-86 des CD prévenant ainsi leur fixation sur le CD28, nécessaire à l'activation des lymphocytes T. L'interaction CTLA-4/CD80-86 augmente la synthèse d'IDO (Indoleamine 2,3 dioxygenase) par la CD et réprime la fonction NK. Ce mécanisme d'action des LT régulateurs nécessite donc la présence des cellules accessoires.
Nous analyserons ces différentes fonctions dans notre modèle. Il faudra également déterminer le mécanisme aboutissant à la baisse d'expression de NKG2D sur la cellule NK. Certains auteurs décrivent que ce phénomène est dépendant de l'activité TGF-β des LT régulateurs. Une autre piste de réflexion est la présence de ligands du récepteur NKG2D sur les lymphocytes T régulateurs (33).

En conclusion, les altérations de la réponse cytotoxique observées semblent multifactorielles avec un rôle important du microenvironnement et des lymphocytes T régulateurs tant sur la fonction que sur le phénotype NK. Il reste à déterminer les modes d'actions des LT régulateurs dans notre modèle. Nous attendons beaucoup de l'étude des check-points immunologiques PD-1 ou CTLA4.

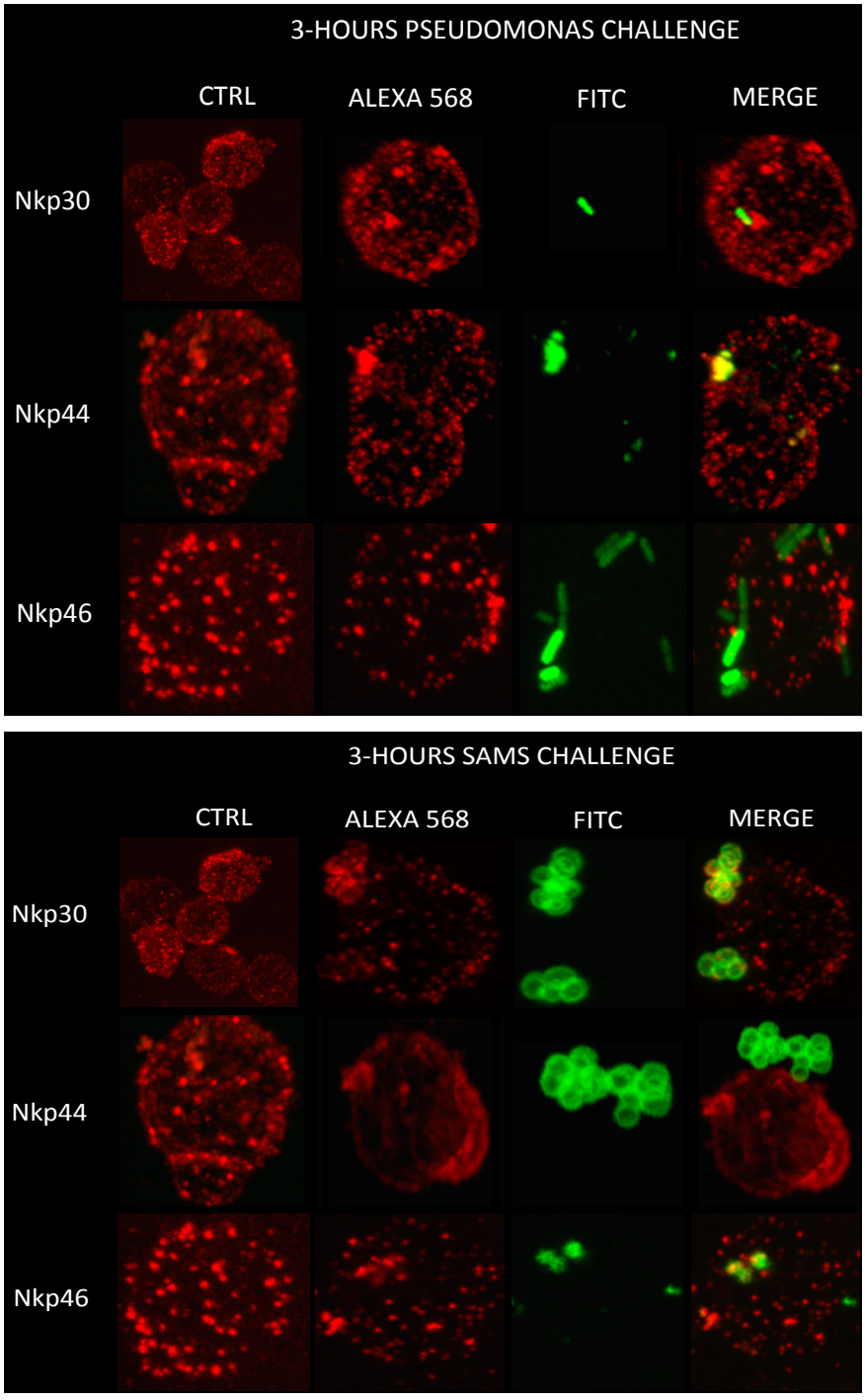


Figure 13 : Etude en Microscopie confocale de la colocalisation des bactéries PA et Staphylocoque avec les récepteurs NKp30, NKp44 et NKp46.

Marquage rouge (Alexa-568) : NKp44-568**, marquage vert :** PA-GFP ou SAMS-GFP.

6. CONCLUSION

L'interaction entre la bactérie *Pseudomonas aeruginosa* et le lymphocyte Natural Killer est donc singulière à plusieurs égards. Par l'intermédiaire de ses facteurs de virulence, et plus spécifiquement le SSTIII, PA est capable de moduler la réponse cytokinique de type IFN-γ de l'hôte par une voie dépendante de ERK. Par ailleurs, l'infection du microenvironnement cellulaire, particulièrement des LT, mène à une altération de la réponse cytotoxique de la cellule NK. L'infection à PA module donc les 2 principales fonctions de la cellule NK.

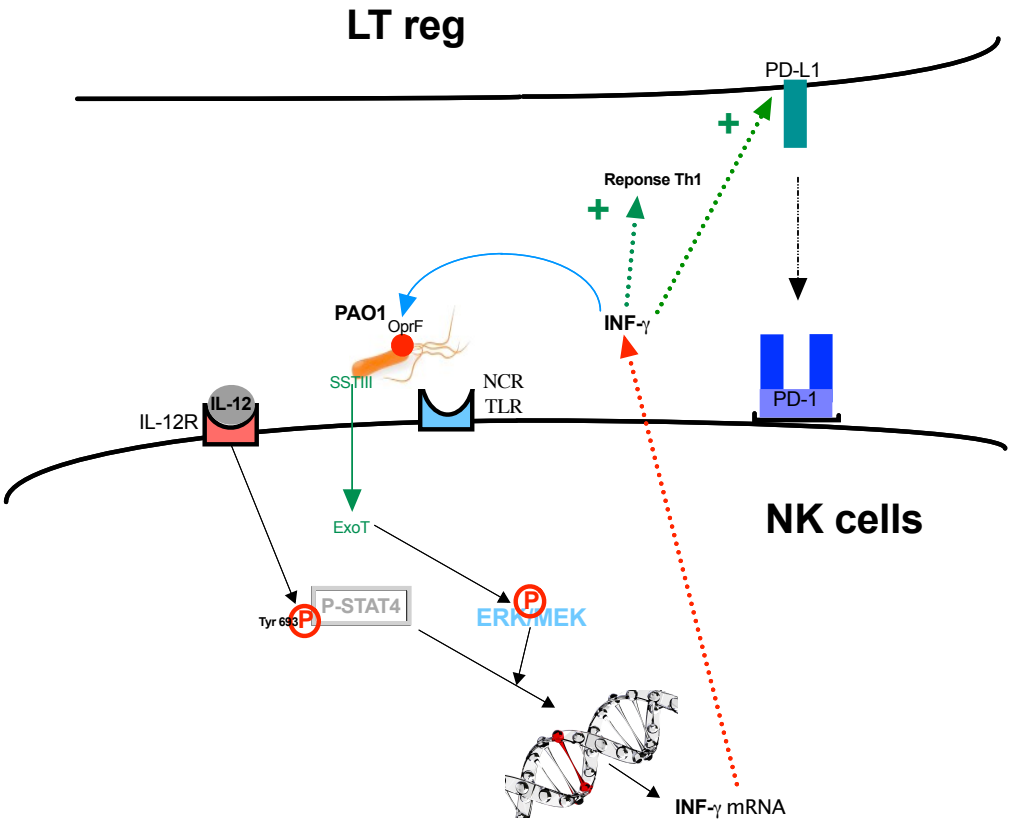


Figure 14 : Vue générale des interactions possibles entre Pseudomonas et la cellule NK pouvant mener aux altérations observées.

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8. ANNEXES

Article 1 - Hydrocortisone Prevents Immunosuppression by Interleukin-10+ Natural Killer Cells After Trauma-Hemorrhage, *Critical Care Medicine 2014*

Antoine Roquilly, MD, PhD; Alexis Broquet, PhD; Cédric Jacqueline, PhD; Damien Masson, Pharm D, PhD; Jean Pierre Segain, PhD; Cecile Braudeau, PhD; **Mickael Vourc'h**; Jocelyne Caillon, Pharm D, PhD; Frédéric Altare, PhD; Regis Josien, MD, PhD; Christelle Retière, PhD; Jose Villadangos, PhD; Karim Asehnoune, MD, PhD

Article 2 –Rôle of IL-12 in overcoming the low responsiveness of NK cells to missing self after traumatic brain injury, *Clinical Immunology 2015*

Antoine Roquilly, Gaëlle David, Raphael Cinotti, **Mickaël Vourc'h,** Helene Morin, Bertrand Rozec, Christelle Retière, Karim Asehnoune.

Article 3 –Alterations of the iNKT cell compartment in traumatic brain injury, *Submitted Critical Care Medicine, 2017*

Allan Patinec, PhD, Jézabel Rocher, **Mickael Vourc'h, MD**, Antoine Roquilly, MD, PhD, Karim Asehnoune, MD, PhD, Jacques Le Pendu, PhD.

Hydrocortisone Prevents Immunosuppression by Interleukin-10+ Natural Killer Cells After Trauma-Hemorrhage

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Antoine Roquilly, MD, PhD[1,2]; Alexis Broquet, PhD[1]; Cédric Jacqueline, PhD[1]; Damien Masson, Pharm D, PhD[3]; Jean Pierre Segain, PhD[4]; Cecile Braudeau, PhD[5,6]; Mickael Vourc'h[1,2]; Jocelyne Caillon, Pharm D, PhD[1]; Frédéric Altare, PhD[7]; Regis Josien, MD, PhD[5,6]; Christelle Retière, PhD[8]; Jose Villadangos, PhD[9,10]; Karim Asehnoune, MD, PhD[1,2]

Objective: Trauma induces a state of immunosuppression, which is responsible for the development of nosocomial infections. Hydrocortisone reduces the rate of pneumonia in patients with trauma. Because alterations of dendritic cells and natural killer cells play a central role in trauma-induced immunosuppression, we investigated whether hydrocortisone modulates the dendritic cell/natural killer cell cross talk in the context of posttraumatic pneumonia.

Design: Experimental study.

Settings: Research laboratory from an university hospital.

AQ3 **Subjects:** BALB/cJ mice (weight, 20–24 g).

Interventions: First, in an a priori substudy of a multicenter, randomized, double-blind, placebo-controlled trial of hydrocortisone (200 mg/d for 7 d) in patients with severe trauma, we have measured the blood levels of five cytokines (tumor necrosis factor-α, interleukin-6, interleukin-10, interleukin-12, interleukin-17) at day 1 and day 8. In a second step, the effects of hydrocortisone on dendritic cell/natural killer cell cross talk were studied in a mouse model of posttraumatic pneumonia. Hydrocortisone (0.6 mg/mice i.p.) was administered immediately after hemorrhage. Twenty-four hours later, the mice were challenged with *Staphylococcus aureus* (7×10^5 colony-forming units).

Measurements and Main Results: Using sera collected during a multicenter study in patients with trauma, we found that hydrocortisone decreased the blood level of interleukin-10, a cytokine centrally involved in the regulation of dendritic cell/natural killer cell cluster. In a mouse model of trauma-hemorrhage–induced immunosuppression, splenic natural killer cells induced an interleukin-10–dependent elimination of splenic dendritic cell. Hydrocortisone treatment reduced this suppressive function of natural killer cells and increased survival of mice with posthemorrhage pneumonia. The reduction of the interleukin-10 level in natural killer cells by hydrocortisone was

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TABLE 1. Patient Characteristics

	Hydrocortisone Group	Placebo Group	*p*
n	39	40	
Male, *n* (%)	31 (79)	31 (78)	1.00
Age, median (IQR)	36 (18)	35 (18)	0.96
Injury severity score, median (IQR)	31 (23–38)	29 (22–37)	0.76
Hemorrhagic shock and/or received blood product for active bleeding on admission, *n* (%)	29 (74)	31 (78)	0.80
Hospital acquired pneumonia, *n* (%)	9 (23)	16 (40)	0.11
Duration of mechanical ventilation (d), median (IQR)	13 (12)	16 (12)	0.06
Duration of ICU hospitalization (d), median (IQR)	19 (14)	24 (13)	0.05
Death in ICU, *n* (%)	3 (8)	2 (5)	0.68

IQR = interquartile range.

partially dependent on the up-regulation of glucocorticoid-induced tumor necrosis factor receptor-ligand (TNFsf18) on dendritic cell.

Conclusions: These data demonstrate that trauma-induced immunosuppression is characterized by an interleukin-10–dependent elimination of dendritic cell by natural killer cells and that hydrocortisone improves outcome by limiting this immunosuppressive feedback loop. (*Crit Care Med* 2014; XX:00–00)

Key Words: dendritic cells; hydrocortisone; interleukin-10; natural killer cells; pneumonia; trauma

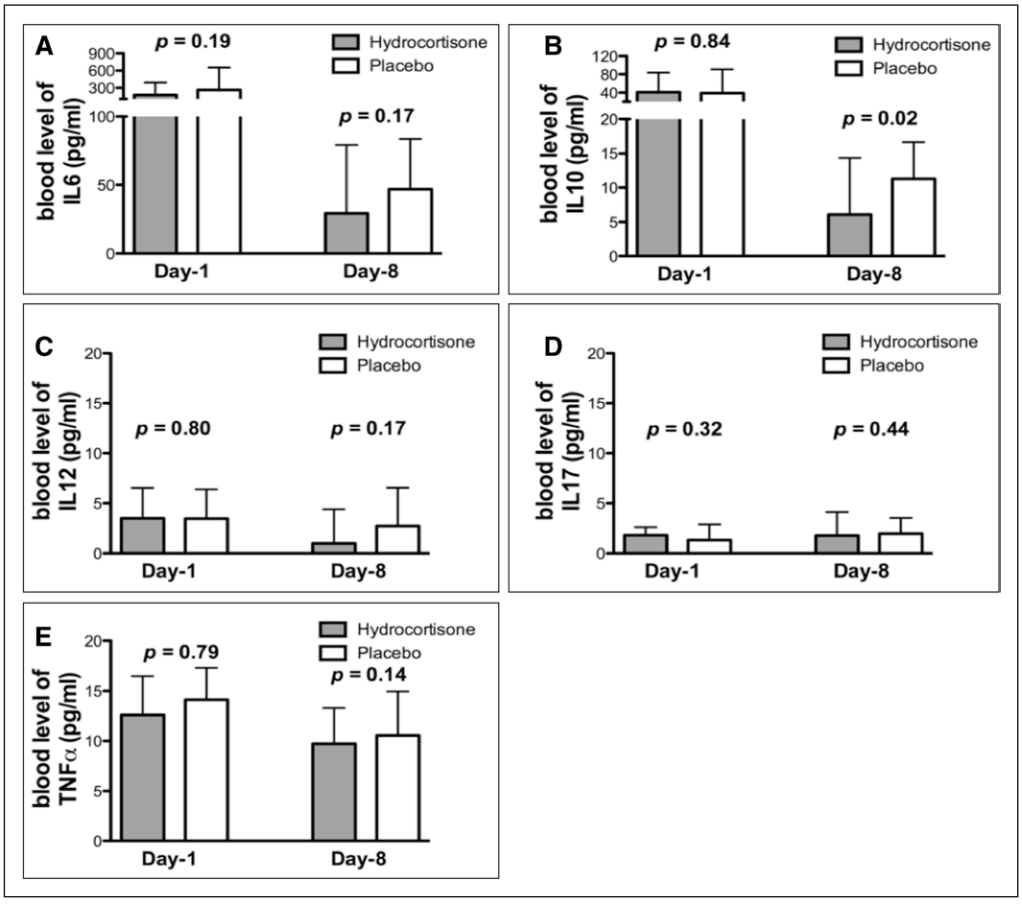

Figure 1. Stress dose of hydrocortisone decreases serum interleukin-10 concentration on day 8 in human patients with trauma. Serum concentrations of **(A)** interleukin (IL)-6, (**B**) IL-10 (**C**) IL-12, (**D**) IL-17, and (**E**) tumor necrosis factor (TNF)-α were assessed on day 1 and day 8 in 79 patients with trauma randomized to hydrocortisone (*n* = 39; continuous intravenous infusion: 200 mg/d) or placebo (*n* = 40) from day 1 to day 7. Gray bars for hydrocortisone group, white bars for placebo group (median ± interquartile range).

Bacterial hospital acquired pneumonia (HAP), which develops in up to 40% of mechanically ventilated patients, increases the duration of hospitalization and the rate of death in this population (1). A state of acquired immunosuppression, described in critical conditions such as trauma (2), brain-injuries (3), or sepsis (4), is responsible for this high susceptibility to infection of critically ill patients.

Alterations of dendritic cells (DC) and of natural killer (NK) cells have been associated with nosocomial infection during brain injury- or sepsis-induced immunosuppression (5–7). During inflammatory critical condition–induced immunosuppression, a cascade of events occurs, allowing DC to shut down antigen uptake, down-regulate antigen presentation, and decrease their ability to induce T-cell

proliferation (8). NK cells are innate lymphocytes that regulate the DC populations that gain access to secondary lymphoid organs by increasing their maturation or by eliminating tolerogenic DC (9). In particular, NK cells are able to eliminate autologous immature DC (10). This NK cell–mediated quality control of DC populations ensures the development of an effective antiinfectious immune response.

Interleukin (IL)-10 has been associated with immunosuppression in patients with sepsis (11) and with the development of bacterial infection in patients with trauma (12). During infection, the production of IL-10 by NK cells is central to the observed inability of DC to produce IL-12, a cytokine critically involved in the clearance of the pathogen by the host (13). IL-10 enhances the lysis of DC by NK cells during chronic viral infection and is involved in exhaustion of myeloid DC during inflammation (14, 15). Finally, IL-10 release is a common mechanism for critical disease-induced immunosuppression, but data regarding its effects on subsequent infection are sparse.

After severe trauma, hypercortisolemia contributes to immune homeostasis, but patients with trauma frequently develop adrenal insufficiency that alters immunity (16, 17). We have demonstrated that hydrocortisone decreased the rate of bacterial pneumonia in ventilated patients with trauma (18).

Therefore, we hypothesized that hydrocortisone could decrease the trauma-induced immunosuppression, and we investigated whether hydrocortisone modulates the DC/NK cell cross talk in the context of posttraumatic pneumonia.

MATERIALS AND METHODS

Clinical Study Design and Blood Levels of Cytokines

Using a luminex assay (Bio-Rad, Marnes-la-Coquette, France), the levels of five cytokines (IL-6, IL-10, IL-12, IL-17, and tumor necrosis factor [TNF]-α) were investigated in sera collected in patients from the HYPOLYTE study with available samples (18). The study was approved by an Institutional Review Board (Comite de Protection des Personnes de Angers, Angers, France; Nb 2006/25). HYPOLYTE was a multicenter, randomized, double-blind, placebo-controlled study in which with patients with severe multiple trauma were randomly assigned to an intravenous infusion of either hydrocortisone (200 mg/d for 7 d) or a placebo.

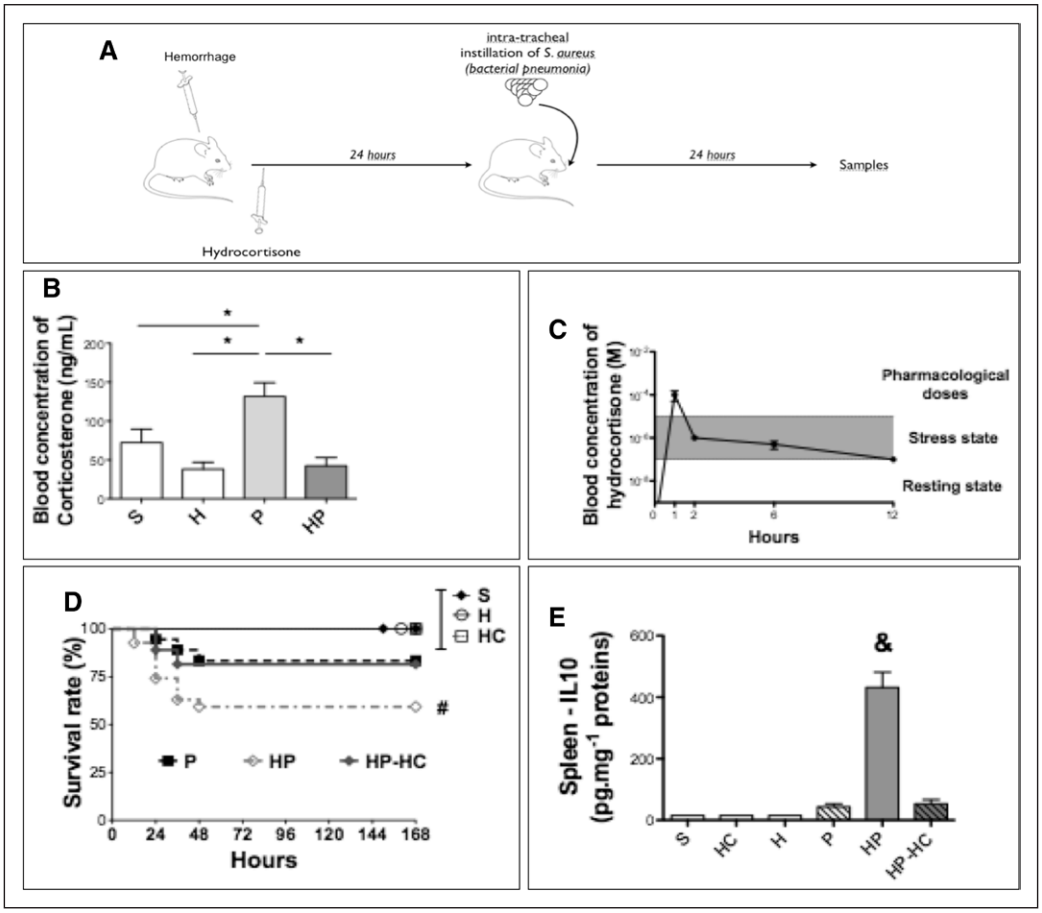

Figure 2. Hydrocortisone decreases splenic interleukin (IL)-10 level in posthemorrhage pneumonia. **A**, Six groups of mice were studied: sham-operated (S), hydrocortisone alone (HC), hemorrhage alone (H), *Staphylococcus aureus* pneumonia alone (P), posthemorrhage *S. aureus* pneumonia (HP), and hydrocortisone-treated mice with posthemorrhage *S. aureus* pneumonia (HP-HC). **B**, Blood level of corticosterone was assessed in the four untreated groups ($n = 6$ per group; median ± interquartile range [IQR]). **C**, Blood level of hydrocortisone was assessed in HP-HC group ($n = 6$; median ± IQR). **D**, Survival rates of mice are expressed as percentage. *Dashed red line* for HP group, *blue line* for HP-HC group, *dashed black line* for P group ($n \geq 16$ per group). **E**, Concentration of IL-10 was assessed in spleen homogenates ($n \geq 6$ mice per group, median ± IQR). Samples were harvested 24 hours after tracheal instillation of *S. aureus* (except when otherwise stated). Figures are representative of two independent experiments. $p \leq 0.05$ compared with all other groups; #$p \leq 0.05$ compared with HP-HC, *$p \leq 0.05$.

Animal Care. Experiments were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication No 86-23, revised 1985). The Institutional Review Board of the University of Nantes approved all animal experiments. Male BALB/cJ (20–24 g) were purchased from Janvier Laboratories, Laval, France.

Posthemorrhage Pneumonia. One third of the blood volume (0.3 mL/10 g body weight) was withdrawn by cardiac puncture during general anesthesia with isoflurane (Baxter, Maurepas, France). The collected blood was restored by a retroorbital plexus injection after 90 minutes. Hydrocortisone (0.6 mg/mice i.p.) was administered immediately

after the reinjection of the blood. Twenty-four hours later, 7×10^5 colony-forming units of a methicillin-susceptible *Staphylococcus aureus* strain (ATCC 29213) were instilled in the trachea of anesthetized mice (for description of the study groups see Fig. E1, Supplemental Digital Content 1, http://links.lww.com/CCM/B92) (19, 20).

Blood Concentrations of Corticosterone and of Hydrocortisone

Corticosterone and hydrocortisone concentrations in blood were determined using IDS Corticosterone EIA kit (Immunodiagnostic System, Paris, France) and on Roche Cobas 6000 immunoassay system (Roche Diagnostics, Meylan, France) respectively.

Reagents. Hydrocortisone was purchased from Serb (Paris, France). The monoclonal antibodies used were obtained from eBiosciences (San Diego, CA): anti-CD3 (17A2), anti-CD11c (N418), anti-CD49β (DX5), anti-CD69 (H1.2F3), anti-CD122 (TM-b1), anti–glucocorticoid-induced tumor necrosis factor receptor/tumor necrosis factor receptor superfamily, member 18, clone DTA-1 (GITR/TNFRSF18), and GITR-Ligand/TNFsf18 (clone YGL386). Anti-CD40 (3123), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-IAd (class II major histocompatibility complex [MHC], AMS-32.1) were obtained from BD Biosciences (Le Pont de Claix, France). DAPI (D1306) was obtained from Invitrogen (Saint Aubin, France). AQ5

Phenotypic Analysis. DC subsets were defined by specific membrane markers: $CD3^{neg\ CD49\beta neg\ CD11c high}$ for conventional DC. NK cells were characterized as $CD3^{neg}\ CD49\beta^{pos}\ CD122^{pos}$ cells. Data were analyzed using FlowJo software (Treestar, Ashland, OR). Cell sorting was performed on a FACS Aria (BD Biosciences) and routinely yielded cell populations with purity up to 95%.

Real-Time Quantitative Polymerase Chain Reaction. Analysis was performed as previously described (20). GAPDH was used to normalize gene expression. Relative gene expression was calculated by the $2^{-\Delta\Delta}$ Ct method using samples from S group as calibrator (for primer sequences, see **Table E1**, Supplemental Digital Content 1, http://links.lww.com/CCM/B92).

DC/NK Cell Cocultures. Sorted splenic NK cells and DC were cultured at effector/target ratios of 1:10, 1:20, and 1:50 in culture media (RPMI 1640 containing 10% SVF, 2 mM *L-glutamine, penicillin 50 IU/mL penicillin, IL-2 50 U/mL), in the presence or absence of an IL-10R–blocking antibody (BD Bioscience Pharmingen, San Diego, CA).* AQ6

Adoptive Transfer of NK Cells. Sorted-NK cells (5×10^5 cells per mouse) were IV administered immediately before tracheal instillation of *S. aureus*.

In Vivo Monoclonal Antibody Treatment. Mice were IV injected with 100 µg of mouse anti–IL-10R (clone 1B1.3a), mouse anti-GITR-ligand/TNFsf18 (clone 337122) or isotype-matched IgG1 (clone R3-34). Anti-GITR ligand was purchased from R&D System (Lille, France).

Statistical Analysis. GraphPad prism (La Jolla, CA) software was used for statistical analysis. Continuous nonparametric

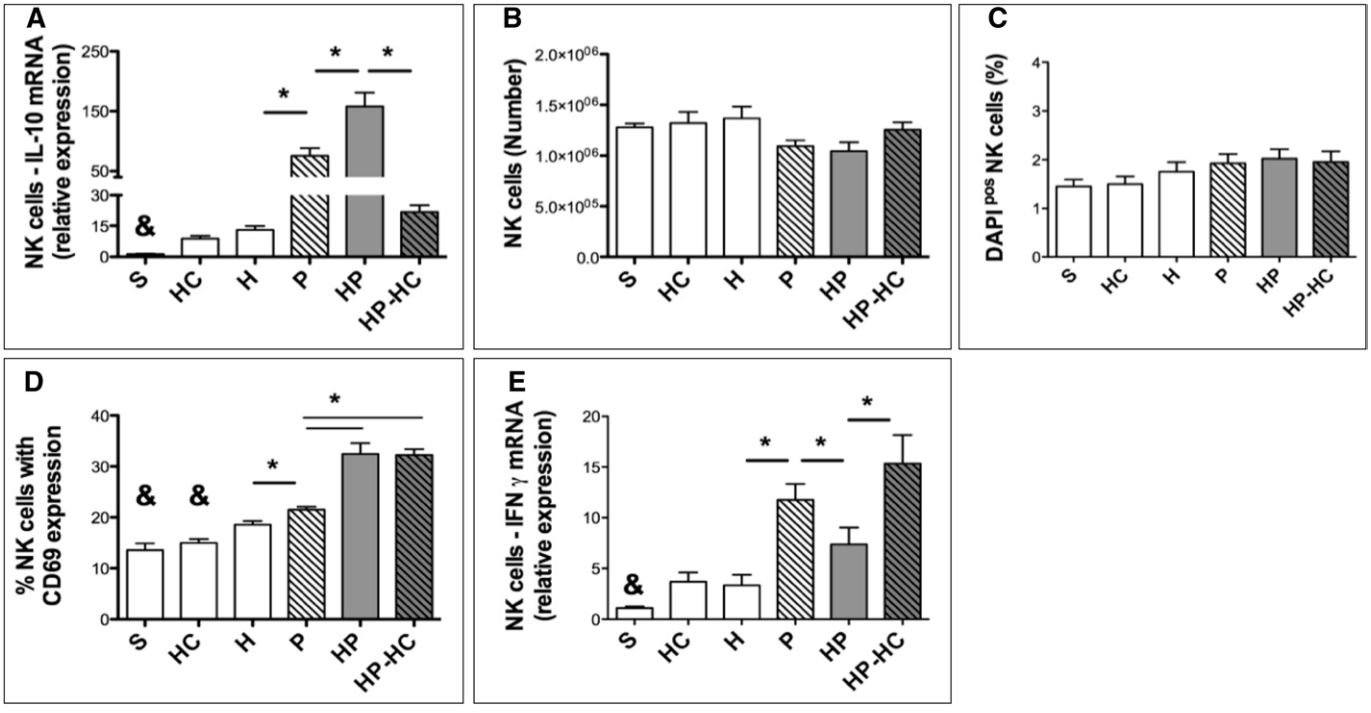

Figure 3. Hydrocortisone decreases the interleukin (IL)-10 production by natural killer (NK) cells in posthemorrhage pneumonia. Six groups of mice were studied: sham (S, first white bar), hydrocortisone alone (HC, second white bar), hemorrhage alone (H, third white bar), *Staphylococcus aureus* pneumonia alone (P, hatched white bar), posthemorrhage *S. aureus* pneumonia (HP, gray bar), and hydrocortisone-treated mice with posthemorrhage *S. aureus* pneumonia (HP-HC, hatched grey bar). **A**, Splenic IL-10 mRNA levels were assessed 24 hours after pneumonia onset in NK cells. At the studied timepoint, NK cells were the major source of splenic IL-10 mRNA and we assessed NK cells (**B**) numbers, (**C**) percentage of nonviable cells, (**D**) membrane expressions of CD69, and (**E**) interferon-γ mRNA levels. Histograms represent median with interquartile ranges issue from three independent experiments ($n \geq 4$ mice per group). $p \leq 0.05$ compared with all other groups; $^*p \leq 0.05$.

variables were expressed as median (interquartile range) and were compared using the Mann–Whitney test, or Kruskal–Wallis test with the Dunns test (post-hoc test) was used for intergroup comparison. For the comparison of cytokines in human samples, a Friedman test was used to compare day 1 and day 8 levels in each group; a Mann–Whitney test was used to compare the two groups at day 1 and at day 8. Survival curves were compared with a log-rank test. $P \leq 0.05$ was considered to be statistically significant. See supplemental appendices (Supplemental Digital Content 1, http://links.lww.com/CCM/B92) for additional method description.

RESULTS

Hydrocortisone Decreases Blood IL-10 Level in Patients With Severe Trauma

We assessed whether hydrocortisone treatment altered cytokine serum levels using samples from ventilated patients with trauma included in a multicenter, double-blind randomized trial study (18). Patient characteristics and outcomes are shown in **Table 1**. HAP was diagnosed in nine patients (25.6%) in the hydrocortisone group and in 16 patients (40%) in the placebo group. Serum levels of IL-6, IL-10, IL-12, IL-17, and TNF-α were assessed immediately before a 7-day infusion of hydrocortisone or placebo (day 1) and 24 hours after stopping the treatment (day 8). Cytokine blood levels were similar in the two groups on day 1 (**Fig. 1 A–E**). On day 8, only IL-10 was significantly decreased in the hydrocortisone group when compared with that in the placebo group (Fig. 1*B*). We then aimed to delineate the mechanisms explaining this IL-10 decrease, and we used a previously described mouse model of posthemorrhage pneumonia (19, 20).

Stress Dose of Hydrocortisone Enhances the Lung Response to Posthemorrhage Pneumonia

Hydrocortisone was administered after hemorrhage and 24 hours before performing the bacterial challenge (**Fig. 2A**). First, the blood concentration of corticosterone, the major glucocorticoid hormone in mice, was increased in infected mice (P group) but not in mice with posthemorrhage pneumonia (HP group) (**Fig. 2B**). Second, the blood levels of hydrocortisone reached the concentrations observed at stress-state in humans (10^{-7} to 10^{-5}M) (21) (**Fig. 2C**). Third, hydrocortisone (HP-hydrocortisone group) increased the survival rate of mice with posthemorrhage pneumonia (**Fig. 2D**). Hydrocortisone did not alter the bacterial load in the lungs or in the spleen but decreased the lung endothelial lesions and the lung production of inflammatory cytokines when compared with the mice left untreated (**Fig. E2 A–E**, Supplemental Digital Content 1, http://links.lww.com/CCM/B92). The effects of hydrocortisone on the splenic IL-10 level was maximal at H24 in the HP group (**Fig. E3**, Supplemental Digital Content 1, http://links.lww.com/CCM/B92). At this timepoint, the splenic IL-10 level was dramatically increased in mice with posthemorrhage pneumonia (HP group) when compared with the P group (**Fig. 2E**). Hydrocortisone massively decreased the splenic IL-10 level when compared with the mice left untreated (Fig. 2*E*), mimicking the results observed in the blood of patients with trauma (Fig. 1*B*).

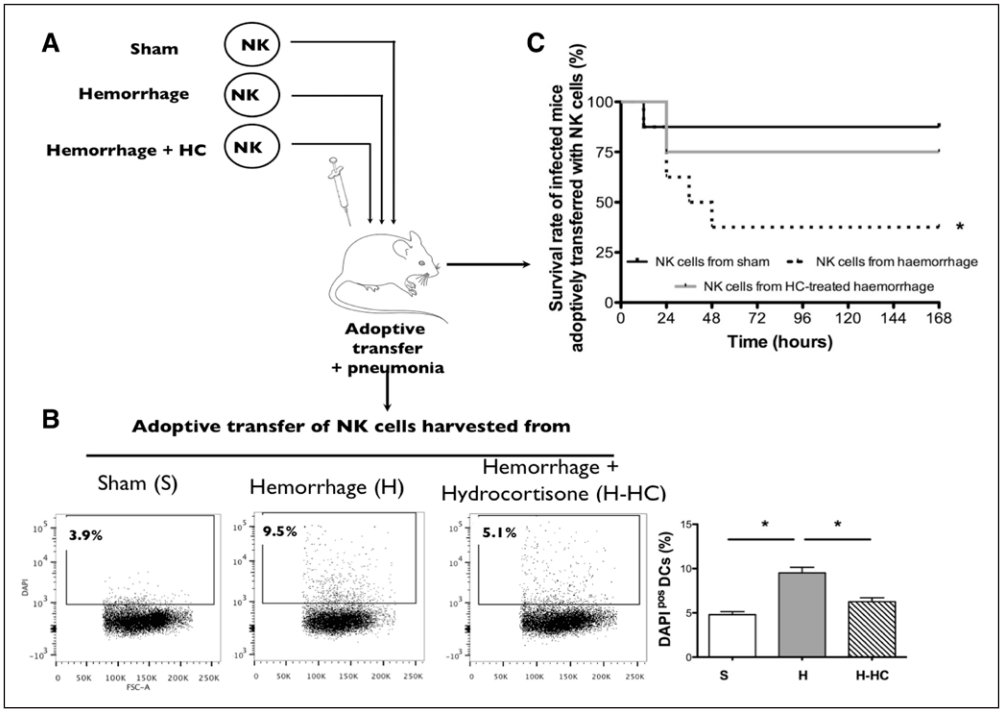

Figure 4. Hydrocortisone prevents the elimination of dendritic cells by natural killer (NK) cells in hemorrhaged mice. **A**, Splenic NK cells harvested from sham (S group), hemorrhaged (H group), or HC-treated hemorrhaged mice (H-HC group) were transferred to mice subjected to pneumonia. **B**, The rate of splenic nonviable DC (DAPI positive DC) was assessed 24 hours after pneumonia onset in mice treated by adoptive transfer ($n \geq 3$ mice per group, representative of two independent experiments). The S group is represented by the white bar, the H group by the gray bar, and the H-HC group by the hatched gray bar. **C**, The survival rate of these three groups was assessed more than 168 hours ($n = 8$ mice per group, representative of two independent experiments). The S group is represented by the black line, H group by the dotted line, and H-HC group by the gray line. *$p < 0.05$.

Stress Dose of Hydrocortisone Decreases IL-10 Transcription in Splenic NK Cells in Posthemorrhage Pneumonia

The main cellular source of IL-10 after trauma has not been documented. Therefore, we screened splenocytes for IL-10 transcriptional activity. The level of IL-10 mRNA in splenic T cells and DC was similar in all groups of mice (variation between any two means < 10%, data not shown). The IL-10 mRNA level was dramatically elevated in the NK cells from the HP group when

compared with the NK cells from P and sham groups. Hydrocortisone treatment prevented this elevation in IL-10 mRNA in the splenic NK cells (**Fig. 3A**) without modifying NK cell numbers (**Fig. 3B**), survival (**Fig. 3C**), or expression of the activation marker, CD69 (**Fig. 3D**). It has been demonstrated that IL-10 production by NK cells correlates with low capacity to produce interferon-γ (22). Strikingly, NK cells from mice having hemorrhage with subsequent pneumonia (HP group) also expressed lower levels of interferon-γ transcripts when compared with the mice without hemorrhage (P group), and this down-regulation was averted with hydrocortisone treatment (HP-hydrocortisone group) (**Fig. 3E**). Overall, these results demonstrate that hydrocortisone limits the differentiation of NK cells into IL-10–producing cells.

Stress Dose of Hydrocortisone Dampens the Elimination of DCs by NK Cells in Hemorrhaged Mice

IL-10–producing NK cells were shown to eliminate mature DC in patients with chronic viral infection (14). Because hydrocortisone decreases the transcription of IL-10 in NK cells, we hypothesized that hydrocortisone could provide resistance to posttraumatic infection by decreasing the deleterious effects of NK cells on DC.

We first aimed to confirm that NK cells contribute to posttraumatic immune suppression and that hydrocortisone modulates their effects. After inducing *S. aureus* pneumonia (P), we adoptively transferred splenic NK cells purified from sham (S), hemorrhage (H), or hydrocortisone-treated hemorrhaged mice (H-hydrocortisone group) (**Fig. 4A**). The percentage of nonviable ($DAPI^+$) splenic DC was higher in mice receiving NK cells from hemorrhaged mice (H group) than in mice receiving NK cells from sham or from hydrocortisone-treated animals (**Fig. 4B**). The survival rate decreased from 88% for mice that received NK cells from the sham group to 38% for recipients of NK cells from hemorrhaged mice (**Fig. 4C**). However, the survival rate of mice that received NK cells harvested from hydrocortisone-treated hemorrhaged mice was unaltered when compared with those receiving NK cells from the sham group (Fig. 4*C*). These data demonstrate that NK cells contribute to the posthemorrhage susceptibility to infection and that hydrocortisone prevents NK cells from acquiring immunosuppressive functions after hemorrhage.

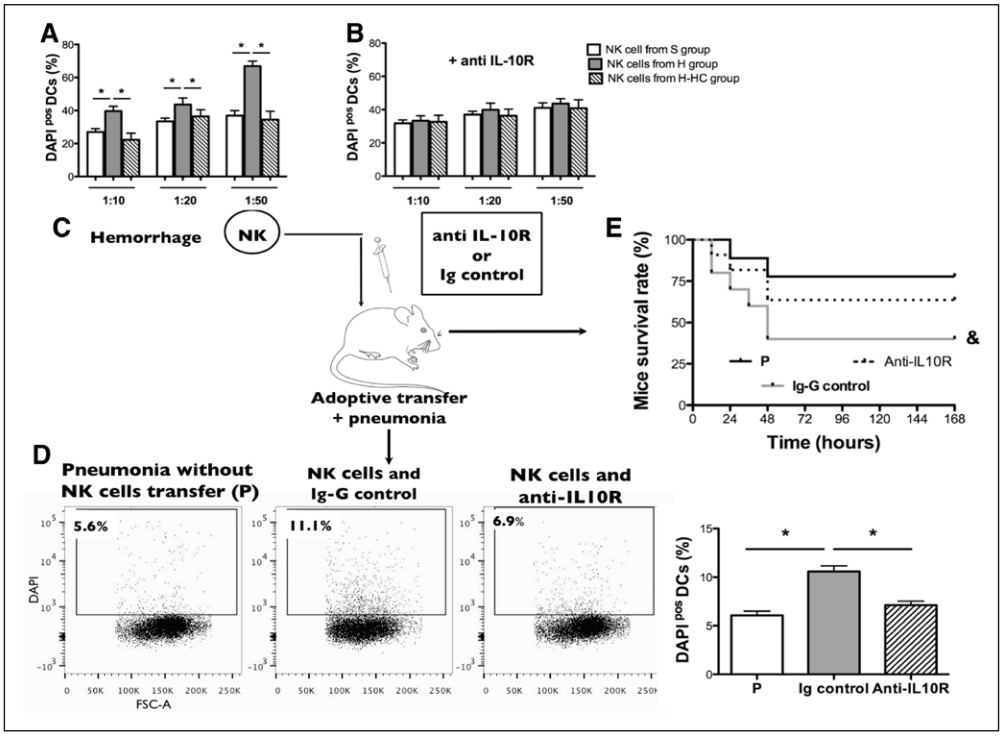

Figure 5. The elimination of dendritic cells (DC) by natural killer (NK) cells is dependent from interleukin (IL)-10 in hemorrhaged mice. Splenic DC were harvested from naive mice and cocultured overnight with either splenic NK cells harvested from sham (S group), hemorrhaged (H group), or HC-treated hemorrhaged mice (H-HC group) in the absence **(A)** or in the presence **(B)** of an anti–IL-10 receptor (IL-10R)-blocking antibody. Histograms represent the percentages (median ± interquartile range) of nonviable DC at 1:10, 1:20, or 1:50 effector/target ratios with NK cells (n=3). The S group is represented by the white bars, the H group by the gray bars, and the H-HC group by the hatched grey bars. **C**, Splenic NK cells that were harvested from hemorrhaged mice were transferred to infected mice treated with anti–IL-10R-blocking antibody or control isotype. **D**, The rate of splenic nonviable DC (DAPI positive DC) was assessed 24 hours after pneumonia onset in treated mice ($n \geq 3$ mice per group, representative of two independent experiments). The P group is represented by the white bar, NK cells with IgG control by the gray bar, and NK cells with anti–IL-10R by the hatched gray bar. **E**, The survival rate of these three groups was assessed more than 168 hours ($n = 8$ mice per group, representative of two independent experiments). The P group is represented by the black line, NK cells with IgG control by the gray line, and NK cells with anti–IL-10R by the dotted line. $^*p \leq 0.05$.

The Elimination of DCs by NK Cells Is Dependent on IL-10 in Hemorrhaged Mice and Hydrocortisone Decreases DC Death

We investigated whether the suppressive functions of NK cells were specifically dependent on IL-10. We first performed in vitro cocultures of naive DC with sorted-NK cells harvested from sham (S), hemorrhage (H), and hydrocortisone-treated hemorrhage mice (hydrocortisone-H) (**Fig. 5, A** and **B**). In the cocultures, NK cells from the H group induced a higher rate of DC death when compared with the NK cells from the S group. In contrast, cocultures with NK cells from hydrocortisone-H mice did not induce DC death (Fig. 5*A*). The differences among the three groups were no longer apparent in the presence of a blocking IL-10R antibody, indicating a crucial role for IL-10 in NK cell–induced DC death (Fig. 5*B*). To investigate whether IL-10 played a similar role in vivo, we adoptively transferred NK cells from hemorrhaged mice into

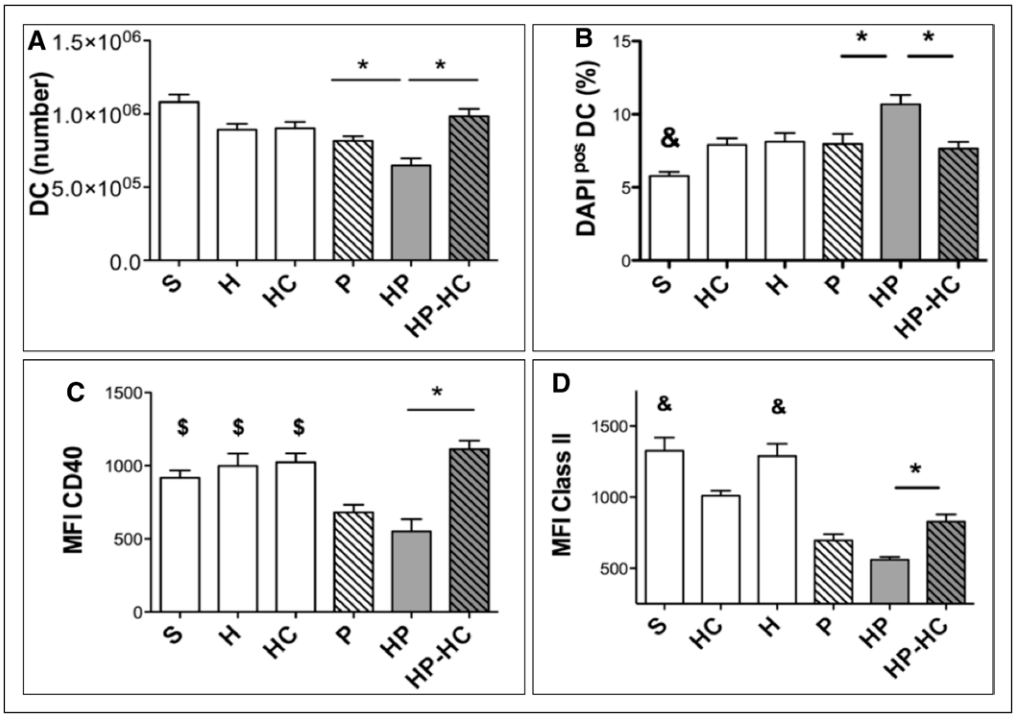

Figure 6. Stress dose of hydrocortisone reduces the rate of nonviable dendritic cells (DCs) and restores the maturation of splenic DCs in mice undergoing posthemorrhage pneumonia. Six groups of mice were studied: sham (S, first white bar), hemorrhage alone (H, second white bar), hydrocortisone alone (HC, third white bar), *Staphylococcus aureus* pneumonia alone (P, hatched white bar), posthemorrhage *S. aureus* pneumonia (HP, gray bar), and hydrocortisone-treated mice undergoing posthemorrhage *S. aureus* pneumonia (HP-HC, hatched gray bar). **A**, The number of DCs was assessed in the spleen 24 hours after pneumonia onset. **B**, The percentage of nonviable DCs and the membrane expressions of (**C**) CD40 and (**D**) class II major histocompatibility complex were assessed in splenic DCs. Histograms represent median ± interquartile ranges and were issued from three independent experiments ($n \geq 4$ mice per group). $p \leq 0.05$ versus all other groups, $\$p \leq 0.05$ versus P and HP groups; $^*p \leq 0.05$.

animals having pneumonia, which were then left untreated or were injected with an IL-10R–blocking antibody (**Fig. 5C**). Anti–IL-10R decreased the induction of DC death by these NK cells (**Fig. 5D**) and increased the mice survival rate (**Fig. 5E**). These experiments indicate that NK cells induce DC death by an IL-10–dependent mechanism.

Hydrocortisone Improves Survival and Restores the Maturation Level of Splenic DC in Mice With Posthemorrhage Pneumonia

Hydrocortisone treatment (HP-hydrocortisone group) prevented a drop in splenic DC number and reduced the percentage of nonviable DC that was observed in mice with posthemorrhage pneumonia (HP group) (**Fig. 6, A** and **B**). In addition, hydrocortisone treatment restored the maturation status of splenic DC as evidenced by the up-regulation of CD40 and MHC-II expressions (**Fig. 6, C** and **D**).

The Modulation of the GITR/TNFRsf18-GITR-Ligand/TNFFSF18 Pathway by Hydrocortisone Is Involved in the Down-Regulation of IL-10 Expression by NK Cells

Finally, we aimed to characterize the molecular pathway underlying the effect of hydrocortisone on regulation of NK/DC cell cross talk. Signaling through the GITR pathway was shown to abrogate NK cell–mediated immunosuppression in inflammatory conditions and to modulate DC/NK cell cross talk during glucocorticoid treatment (23). Importantly, hydrocortisone treatment induced the expression of GITR-ligand/TNFSF18 on DC (**Fig. 7A**). Membrane GITR/TNFRSF18 expression on NK cells was higher in mice treated with hydrocortisone (HP-hydrocortisone group) when compared with mice left untreated (HP group) (**Fig. 7B**). We further determined the role of GITR/TNFRsf18-GITR-ligand/TNFsf18 pathway in the IL-10 expression by NK cells. In mice undergoing posthemorrhaged pneumonia and treated with hydrocortisone, GITR-ligand/TNFsf18 blockade increased the IL-10 mRNA level in NK cells but at a lower level than that observed in the HP group (**Fig. 7C**). Altogether, these in vivo results suggest that hydrocortisone-induced up-regulation of the GITR/TNFRSF18 pathway decreases IL-10 production by NK cells.

DISCUSSION

NK cells play a beneficial role during acute pneumonia in mice (24), and defects in NK cells functions have been associated with infections in critically ill patients (25). This study demonstrates that the involvement of NK cells in posthemorrhagic immunosuppression is not limited to a loss of these functions but is also associated with the acquisition of IL-10–dependent suppressive functions, which can be limited by hydrocortisone treatment.

Our results suggest that IL-10 is immunosuppressive after trauma-hemorrhage, resembling long-term immunosuppression observed in patients with malaria (26). However, in a mouse model of peritonitis, van der Poll et al (27) demonstrated that the endogen production of IL-10 reduces the mortality rate, probably through the inhibition of proinflammatory cytokines (28). However, effects of IL-10 on the immune response are highly dependent of the timing and in healthy volunteers challenged with endotoxin, IL-10 displays antiinflammatory properties when it is administered early, but proinflammatory effects when it is injected late (29–31). Recently, Carles et al (32) demonstrated that heat shock proteins increase the lung injury associated with posttraumatic pneumonia via an IL-10–dependent mechanism. These results are in line with ours because we found an association between high levels of IL-10 after hemorrhage and an overwhelming inflammatory lung response during subsequent pneumonia. We thus propose that high IL-10 levels before infection are deleterious for the immune response.

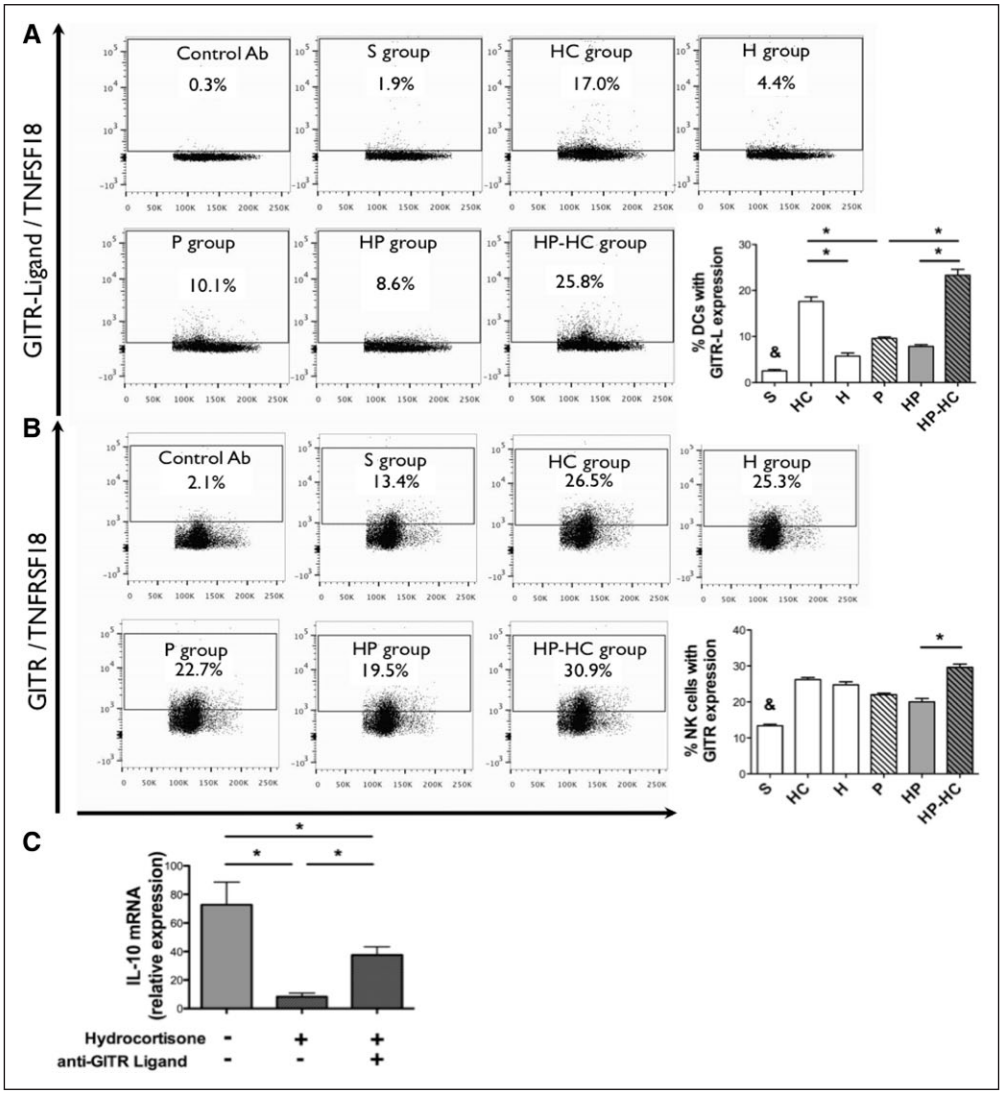

Figure 7. Hydrocortisone-induced up-regulation of glucocorticoid-induced tumor necrosis factor receptor/tumor necrosis factor receptor superfamily, member 18, clone DTA-1 (GITR/TNFRSF18)-GITR-ligand/TNFSF18 pathway reduces interleukin (IL)-10 transcription in natural killer (NK) cells. Six groups of mice were studied: sham (S, first white bar), hydrocortisone alone (HC, second white bar), hemorrhage alone (H, third white bar), *Staphylococcus aureus* pneumonia alone (P, hatched white bar), posthemorrhage *S. aureus* pneumonia (HP, gray bar) and hydrocortisone-treated mice with posthemorrhage *S. aureus* pneumonia (HP-HC, hatched gray bar). **A**, The percentage of GITR-ligand/TNFSF18 ^{pos}DC and of (**B**) GITR/TNFRSF18 ^{pos}NK cells were assessed in the spleen 24 hours after pneumonia onset. **C**, The IL-10 mRNA level was assessed in NK cells of HP groups treated with HC with isotype control or with anti-GITR ligand antibody. Histograms represent median ± interquartile ranges and were issued from two independent experiments ($n \geq 4$ mice per group). $^{*}p < 0.05$.

An up-regulation of the IL-10 pathway in the peripheral blood mononuclear cells of patients with trauma has been previously associated with complicated clinical recovery (33). Immature NK cells, so-called NK2 cells, produce antiinflammatory cytokines (IL-4 and IL-13) but not IL-10. Cell-to-cell contacts of NK2 cells with IL-12–producing DC induce their maturation into interferon-γ–producing NK cells (so-called NK1 cells) (22). In vitro, the production of IL-10 by NK cells has been described only after prolonged stimulation and occurred at a later stage of NK1 cell maturation (34). In vivo, the production of IL-10 by NK cells appeared at the end of the maturation process and was achieved in systemic but not in local *Toxoplasma gondii* infection (13). The ability of NK cells to produce IL-10 at a late stage of maturation fits with the proposed role of IL-10, which is to terminate inflammatory responses ultimately. We propose that the increase in inflammatory lung response and in the dissemination of the bacteria observed in hemorrhaged mice could boost the maturation of NK cells toward the transcription of IL-10.

In the present experiments, hydrocortisone reduces the levels of IL-10 within posthemorrhage pneumonia. In patients with sepsis, hydrocortisone treatment reduced the blood level of IL-10 when compared with placebo (35). However in healthy volunteers intravenously challenged with endotoxin, hydrocortisone administered immediately before endotoxin increased the plasma level of IL-10 (36). Of note, hydrocortisone had no effect on IL-10 level when it has been administered more than 6 hours before the endotoxin. These data were confirmed in a recent issue of the journal (37). The authors demonstrated that early or late initiation of hydrocortisone treatment induces different patterns of TNF-α production and impact the mortality rate. Overall, these data underline the importance of the timing when treating critically ill patients with hydrocortisone.

The hydrocortisone-associated decrease in IL-10 production by NK cells partially depends on the GITR/TNFRSF18-GITR-ligand/TNFsf18 pathway. The engagement of GITR induces interferon-γ production in NK cells and abrogates immune tolerance (38, 39). Expression of GITR-ligand decreases the susceptibility of immune cells to the suppressor activity of Treg (40), and we propose that it limits the elimination of DC by suppressive NK cells. This result demonstrates that hydrocortisone does act not only by limiting the preconditioning of NK cells by an overwhelming inflammation such as that encountered after trauma/hemorrhage but also by stimulating the GITR-GITR-L pathway.

Synthetic glucocorticoids are widely used as immunosuppressants. However, low doses of hydrocortisone can be used in

patients with severe septic (41), and we have recently described its use for the prevention of infection in patients with trauma (18, 42). These counterintuitive results may be explained by the immunological effects of hydrocortisone during inflammatory response. In patients with severe sepsis, hydrocortisone enhanced the phagocytic capacities of neutrophils (43) and restored the blood levels of some cytokines involved in the defense of the host against infections (35). Moreover, the inhibition of the cortisol release by IL-10 reinforces the rational for the hydrocortisone treatment in critical diseases (29, 30). We bring new insights by demonstrating that hydrocortisone prevents the development of posttraumatic immunosuppression characterized by high production of IL-10 by NK cells.

Our study has limitations. First, we used hydrocortisone in mice, whereas the natural hormone in rodents is the corticosterone. However, the use of hydrocortisone in the model was consistent with the hydrocortisone treatment in the HYPOLYTE trial (18) (Fig. 1). Acknowledging that corticosterone may signal independently of cortisol (44), its use should be investigated in models of acute stress. Second, the study was focused on the role of NK cells on DC, whereas NK cells could exhibit some other important functions participating in the posttraumatic immunosuppression.

CONCLUSIONS

Finally, IL-10–producing NK cells has been proposed to participate in a physiological feedback loop aiming to counteract overwhelming inflammatory response (45). Our study demonstrates that hydrocortisone treatment decreases the susceptibility to posttraumatic pneumonia by inhibiting IL-10–producing NK cells.

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Role of IL-12 in overcoming the low responsiveness of NK cells to missing self after traumatic brain injury

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ABSTRACT

Blood samples from 32 patients with severe Traumatic brain injury (TBI) were studied and compared with 11 cardiac surgery patients, and 29 healthy controls. A dramatic decreased expression of HLA class I molecules on monocytes was associated with increased KIR+ NK cell frequency in TBI patients. Overall, the phenotype of TBI NK cells marked by KIR and CD57 expression and lower level of NKp46 and DNAM-1 reflected a differentiated state. The NK-cell response to missing self was marked by lower degranulation and lower IFN-γ production after stimulation with HLA class I deficient cell line. In contrast, the NK-cell ADCC was not altered. IL-12 was able to restore both IFN-γ production and the cytotoxicity capacities of NK cells. This study provides the first extensive description of the phenotype and functions of NK cells in TBI patients. Further evaluation of IL-12 treatment to overcome immunosuppression-induced nosocomial infections is warranted.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and prolonged disability worldwide [1–3]. After brain injury, severe acquired immunosuppression with impairment of both innate and adaptive immunity is observed [4]. Immunosuppression is critically involved in the occurrence of nosocomial pneumonia which is the main complication recorded in the ICU following TBI [1,5]. Pneumonia alters outcome and neurological recovery [4,6]. Improving nosocomial pneumonia prevention by overcoming immunosuppression therefore remains a critical issue. In the setting of immunosupression, we showed that circulating dendritic cell functions are severely impaired in patients with aneurysmal subarachnoid hemorrhage [5,7]. Recent data from other groups have indicated that innate lymphoid cells have crucial roles in regulating immune response after BI [6,8]. Accordingly, we recently demonstrated that BI patients exhibit a maturation defect in the ex vivo granulomatous response involving innate lymphocytes: γδ T cells as well as natural killer (NK) lymphocytes. Interestingly, a severely decreased recruitment of NK cells into the granulomatous structure was correlated with the occurrence of secondary pneumonia [7,8].

NK cells represent 4% to 15% of blood lymphocytes and do not express the antigen-specific-receptor expressed by B and T cells. NK-cell functions are regulated by a broad panel of activating and inhibitory receptors [8–11]. These cells are naturally cytotoxic by granule polarization and exocytosis of various proteins including perforin or granzymes and by producing high amount of pro-inflammatory cytokines (IFN-γ, TNF-α). NK cells also express Fcψ receptor III3 known as CD16. This receptor recognizes antibody-coated target cells through their Fc region. Fc-CD16 binding mediates antibody-dependent cytotoxicity (ADCC) and IFN-γ production. An absence of HLA class I expression on monocytes (target cells) leads to NK cell activation. This phenomenon is called "missing self" and is marked by the absence of inhibitory NK receptor engagement with self HLA class I molecules allowing NK cells to eliminate cells with low or absent expression of HLA class I molecules [7,8]. This sophisticated pattern provides a robust line of defense against cancer and infections.

Several lines of evidence suggest that NK cells play a role in the defense of the immunocompromised host in the ICU [6,9–11]. In

Abbreviations: TBI, Traumatic brain injury; CP, Cardiac surgery patients; HC, Healthy control; GCS, Glasgow Coma Score.

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particular, data from our lab [7,12,13] and from other groups [6,14–16] suggest that NK cells are critically involved in brain-injured host defense. These cells are also important players in the setting of bacterial pneumonia [4,12,13], probably through the production of IFN-γ, a cytokine that is able to enhance immune response against bacterial infections and improve patient outcome [14–17].

To date, the phenotype of NK cells in ICU-acquired immunosuppression has not been thoroughly described. Moreover, the mechanisms leading to these alterations are still not understood. NK cells engage in crosstalk with other immune cells such as monocytes. We hypothesized that HLA class I molecules can be downregulated on monocytes, therefore participating in alterations of NK cells. We simultaneously investigated monocytes and NK cells as potential crosstalkers in TBI patients. Since TBI significantly increases susceptibility to infections by brain specific mechanisms [2,4], we used samples from cardiac surgery patients as positive controls.

2. Materials and methods

2.1. Ethics

This work belongs to a global study on immune dysfunctions in ICU. An institutional review board for human experimentation approved the protocol (Comité de Protection des Personnes de Nantes, authorization number AC-2008-433/French). Written informed consent from next-of-kin was required for enrollment. When possible, retrospective consent was obtained from patients. Critically ill patients were enrolled from January 2013 to December 2013 in two French surgical ICUs at a university hospital.

2.2. Study population

The brain-injured cohort was made up of traumatic brain-injured patients (Glasgow Coma Scale (GCS) below or equal to 12 aged 18 years or older, hospitalized in ICU and requiring mechanical ventilation. The major surgery cohort was composed of patients aged 18 years or older who were scheduled for elective coronary artery bypass or aortic valve replacement with cardiopulmonary bypass. Exclusion criteria for the two cohorts were previous immunosuppression, cancer in the previous 5 years, treatment with corticosteroids before hospitalization for brain injury, and pregnancy. Control samples were collected from matched healthy blood donors (age ± 10 years, sex, race). All blood donors were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals.

2.3. Sample collection

EDTA-anticoagulated blood samples were withdrawn after ICU admission (<48 h after admission, day 1) and on day 7 after TBI and immediately sent to the laboratory. For cardiac surgery, samples were collected immediately before and 6 h after the end of surgery. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation, frozen at −80 °C and stored until investigation of NK cell phenotype and functions.

2.4. Follow-up

The following data were recorded: general characteristics including demographics, medical history, severity of traumatic brain injury assessed according to the Glasgow Coma Scale, infections, duration of ventilator support and ICU hospitalization and death at day 90. During the 28-day follow-up period, clinical assessments were performed twice daily in the ICU.

2.5. Cells

Peripheral blood mononuclear cells (PBMC) were isolated from citrate-phosphate-dextrose blood from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield, PoC AS, Oslo, Norway). P815 murine cell line and HLA class I deficient 7221.221 (221) cell line were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies) and supplemented with 10% human serum (EFS, Nantes) for P815 cell line and 10% FBS (Life Technologies) for 221 cell line.

2.5.1. Phenotype and functional assays by flow cytometry

PBMCs were preincubated with anti-CD107a (H4A3; BD Biosciences, San Jose, CA). NK-cell degranulation was assessed after incubation with media for 5 h (negative control), with 721.221(221) (E: T ratio of 1:1) or with P185 cell line after a preincubation with CD16 specific mAb or IgG control at 10 μg/mL. For the last 4 h of incubation, the cells were treated with brefeldin A (Sigma) at 10 mg/mL to block trans-Golgi transport and allow the intracellular accumulation of IFN-γ. The cells were cell surface stained and then permeabilized before intracellular IFN-γ staining with PE-conjugated anti-human IFN-γ (B27, BD Biosciences). For some experiments, PBMC were cultured overnight with IL-12 10 ng/mL before the functional assays.

PBMC were stained with Abs against CD3(SK7), CD56 (NCAM16.2), CD16 (NKP15), CD8 (HIT8a), CD161 (DX12), ILT2 (GH1/75), CD57 (HNK-1), DNAM-1 (DX11), NKp46 (9E2), Granzyme A (CB9), perforin (γG9) (BD Biosciences), NKG2C (134,591) (R&D Systems), NKp44 (Z231), NKp30 (Z25), NKG2A (Z199), KIR2DL1/S1 (EB6), KIR2DL2/3/2DS2 (GL183), KIR3DL1/S1 (Z27), HLA-DR (Immu357) (Beckman Coulter, Fullerton, CA) and KIR2D (1A6) [3] CD14 (RMO-52) (EFS, Rennes), HLA-A, −B, −C (F41-IE3) (EFS, Nantes). Flow cytometry was performed using a FACSCalibur apparatus with CellQuest software (BD Biosciences) and analyzed using FlowJo 7.6.1 software (Tree Star, Ashland, OR) [18].

2.6. Statistical analysis

All statistical analyses were performed with Prism-6 software (GraphPad Software). The one-way analysis of variance (ANOVA) test was used for comparisons of multiple groups. Dunnett's multiple comparisons test was used as a *post hoc* test for intergroup comparisons. Continuous nonparametric variables are expressed as medians (interquartile range). Significance was defined as P less than 0.05. * $P \ll 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

3. Results

3.1. Population

Healthy donors, traumatic brain-injured (TBI) and major surgery patients are described in Table 1. The median Glasgow Coma Scale score was 7 (6–9) in the brain-injured patients. All TBI patients were mechanically ventilated. During ICU hospitalization, 19 (59%) patients developed hospital-acquired pneumonia. Four (13%) brain-injured patients died in the ICU. In the major surgery population, no patient developed hospital-acquired infection. We used samples from cardiac surgery patients as positive control because this condition represents a reproducible acute condition in which severe systemic inflammatory response syndrome is followed by IS [17,19].

3.2. Monocytes and T cells are altered after TBI

The proportion of monocytes ($CD14^+$ cells in the monocyte gate) was significantly higher in the TBI patients on days 1 and 7 compared with the healthy controls (HC) and major surgery control patients

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Table 1
Demographic characteristics.

	Healthy controls (n = 29)	Traumatic brain-injured patients(n = 32)	Major surgery (n = 11)
Age, years, *median (25–75th percentiles)*	35 (23–57)	52 (25–60)	78 (66–80)
Male, *number* (%)	18 (62)	24 (75)	6 (55)
Medical history, *number* (%)	NA		
Immunosuppression		0 (0)	0 (0)
Diabetes mellitus		0 (0)	2 (18)
Chronic heart failure		0 (0)	0 (0)
Chronic renal failure		0 (0)	0 (0)
Alcoholism		6 (19)	0 (0)
Nicotine addiction		10 (31)	2 (18)
Severity on admission, *median (25–75th percentiles)*	NA		
Apache-II		40 (34–53)	NA
SOFA score		8 (7–10)	NA
Glasgow Coma Scale		7 (6–9)	NA
ASA score		NA	3 (3–3)
Trauma severity			
Injury Severity Score		20 (15–39)	NA
Abbreviated Injury Score			
Head–neck		4 (4–5)	NA
Face		1 (0–3)	NA
Thorax		0 (0–1)	NA
Abdomen		0 (0–1)	NA
Extremities		0 (0–1)	NA
Skin		0 (0–1)	
Duration of extracorporeal circulation, min, *median (25–75th percentile)*	NA	NA	58 (53–78)
Hospital acquired infection, yes, *number* (%)	NA	21 (66)	0 (0)
Site of hospital acquired infection, *number* (%)			
Pneumonia		19 (59)	0 (0)
Meningitis		0 (0)	0 (0)
Bacteremia		1 (3)	0 (0)
Urinary tract infection		7 (21)	0 (0)
Invasive fungus infection		1 (3)	0 (0)
Time on mechanical ventilation, days, *median (25–75th percentiles)*	NA	12 (7–17)	1 (0–1)
ICU length of stay, days, *median (25–75th percentiles)*	NA	13 (10–22)	1 (1–2)
Death in ICU, *number* (%)	NA	4 (13)	0 (0)
Death at day 90, *number* (%)		4 (13)	0 (0)

ASA: American Society of Anesthesiology, ICU: intensive Care Unit, NA: non-applicable, ND: not done, SOFA: sequential organ failure assessment.

(CP) postoperatively (Fig. 1A). In contrast, the mean fluorescent intensity (MFI) of CD14 was significantly lower on monocytes from TBI patients on days 1 and 7 compared with HC and CP pre- and postoperatively (Fig. 1B–D). We found a significant down-expression of HLA-DR molecules on TBI monocytes on days 1 and 7 compared with HC and CP preoperatively (Fig. 1C–D). In parallel, the frequency of NK cells determined as $CD3^-$ $CD56^+$ cells in lymphocyte gate (Fig. 1E) was similar in TBI patients and HC regardless of the post-BI kinetic. Moreover, T lymphocyte ($CD3^+$ $CD56^-$) frequency was significantly lower in TBI patients on days 1 and 7 compared with HC (Fig. 1E). The expression of granzyme A (Fig. 1F–H) and perforin (Fig. 1G–H) in T lymphocytes was significantly lower in TBI patients compared with HC on days 1 and 7. These results demonstrate that both monocytes and T cells compartments are severely impaired in TBI patients.

3.3. The expression of HLA class I molecules on monocytes was dramatically decreased in TBI patients

In this immune-depressed context marked by HLA-DR down-regulation and T cell alterations, we investigated the expression of HLA class I molecules. These molecules are not only essential to trigger T cell lymphocytes to defend the organism against pathogens but also crucial to NK cell activation which recognize altered or absent HLA class I molecules in different contexts (stress, viral infection, tumoral process and allogeneic cell/organ transplantation). Interestingly, HLA class I molecules, which are highly expressed on HC monocytes, were dramatically down-expressed on TBI monocytes on days 1 and 7 (Fig. 2A). In contrast, the expression of HLA class I molecules (Fig. 2A) was not altered in CP before and after surgery and the down-expression of HLA class I was specific to TBI (Fig. 2A–B). As expected, NK cells were activated, and the frequency of $CD69^+$ NK cells (19.5 ± 4.1, $n = 16$) was significantly increased in TBI patients compared to the control counterpart (7 ± 1.1, $n = 14$, $p = 0.01$). However, no correlation between $CD69^+$ NK cell frequency and HLA class I MFI on monocytes was observed in our study.

3.4. HLA class I deficiency on monocytes is associated with an increased KIR^+ NK cells frequency in TBI patients

Specific inhibitory receptors interact with HLA class I molecules to prevent attack of normal cells by NK cells whereas cells with altered HLA class I molecule expression will be destroyed. Different types of inhibitory receptors are described: the KIR family of receptors and the CD94/NKG2A or ILT2 molecules. In an attempt to determine whether HLA downregulation impacts the immunobiology of TBI NK cells, we evaluated the expression of the different HLA specific inhibitory NK cell receptors by flow cytometry. Interestingly, $KIR2D^+$ (KIR2DL1/2/3/S1/2) NK cell frequency was inversely correlated with HLA class I expression on monocytes (Fig. 2C) whereas no correlation was apparent in CP patients (Fig. 2D). We observed a trend toward an increase of $KIR2D^+$ NK cell frequency in TBI patients compared with HC (Fig. 2E–F). However, a significantly higher frequency of NK cells co-expressing KIR and NKG2A (Fig. 2E–G), two HLA specific inhibitory receptors, was observed in TBI patients without increased frequency of $NKG2A^+$ NK cells in TBI patients (data not shown). In contrast, no significant differences were observed for ILT2 expression in TBI patients (data not shown).

3.5. The NK cell repertoire is characterized by a higher frequency of late differentiated NK cell subset

The next step was to investigate different markers of differentiation leading to better characterization of the NK cell repertoire in TBI patients. NK cells in TBI patients express CD57 with a significantly higher frequency on day 1 than HC subjects (Fig. 3A). Concerning the activating NK receptors, we highlighted a significantly decreased frequency of $CD16^+$ NK cells (Fig. 3B), a lower expression of NKp46 (Fig. 3C) and finally a decreased expression of DNAM-1 on TBI NK cells compared with HC (Fig. 3D) as illustrated in Fig. 3E–F for representative individuals. No significant differences were noted in the studied groups concerning other studied NK receptors such as CD161, NKG2C, NKp30, NKp44 and NKG2D (data not shown). Overall, the phenotype of TBI NK cells marked by KIR and CD57 expression and lower level of activating NKp46 and DNAM-1 reflects a differentiated state [2,20].

3.6. The cytotoxic capacities of NK cells are impaired in TBI patients

In order to follow up the phenotypic study of the functional abilities of TBI patient NK cells, we evaluated ex vivo cytotoxic enzyme (perforin and granzyme A) expression by flow cytometry. Perforin expression in NK cells was significantly lower not only in TBI on days 1 and 7 but also in CP compared with HC (Fig. 4A). However, we observed a significantly decreased expression of granzyme A in the NK cells which was specific to TBI patients (Fig. 4B). It should be noted that in the TBI patients, granzyme A level expression in the NK cells was correlated

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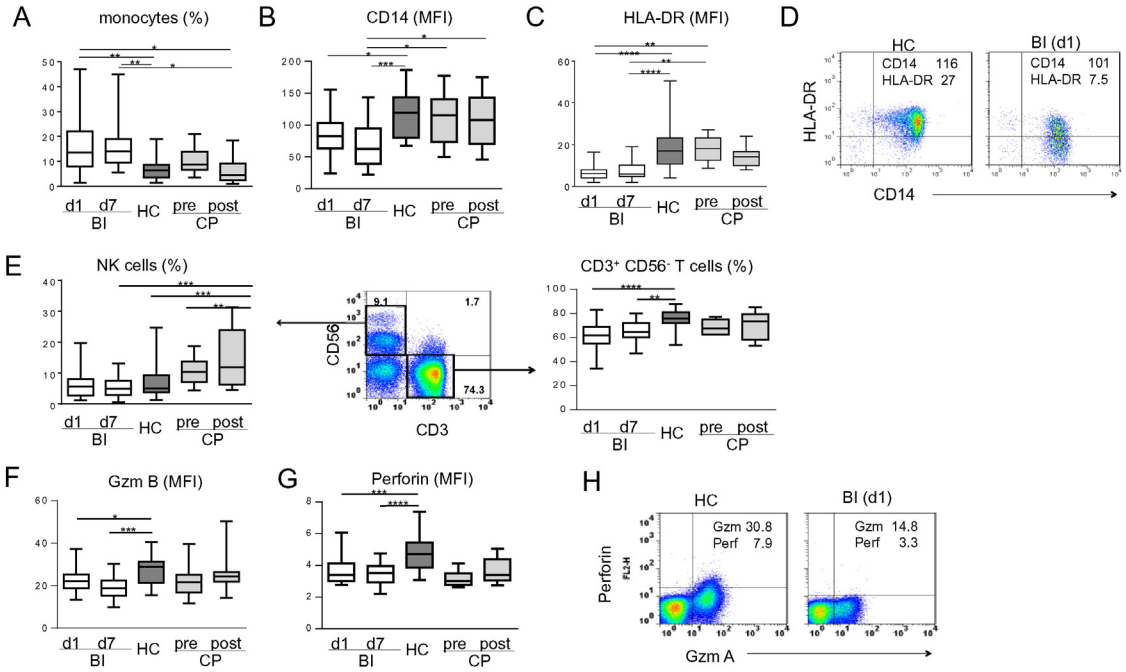

Fig. 1. The immune profile of TBI patients resembles sepsis-induced immunosuppression. Summary box and whisker plot summarizing (A) the percentages of monocytes, (B) the CD14 MFI and (C) HLA-DR-MFI on monocytes in TBI on days 1 (n = 17–23) and 7 (n = 17–23), HC (n = 21–23) and CP pre- (n = 9) and postoperatively (n = 9). Top and bottom whiskers represent the values of the top and bottom 25% of cases, respectively; boxed area, interquartile range. (D) Representative density plots illustrating CD14 and HLA-DR expression on monocytes from HC and BI patient at day 1. The MFI for each marker is indicated on the density plots. (E) Representative density plot illustrating both $CD56^+$ $CD3^-$ NK cells and $CD3^+$ $CD56^-$ T lymphocytes within lymphocyte population gated following FSC/SSC characteristics. Summary box and whisker plot summarizing the percentages of NK cells and T lymphocytes in TBI on days 1 (n = 23) and 7 (n = 22–23), HC (n = 23) and CP pre- (n = 10–11) and postoperatively (n = 10–11). Summary box and whisker plot summarizing (F) granzyme A and (G) perforin MFI in T lymphocytes ($CD3^+$ $CD56^-$) from TBI on days 1 (n = 23) and 7 (n = 21), HC (n = 21) and CP pre- (n = 10) and postoperatively (n = 10). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (H) Representative density plots illustrating granzyme A and perforin expression on $CD3^+$ T lymphocytes from HC and BI patient at day 1. The MFI for each marker is indicated on the density plots.

with $CD16^+$ NK cell frequency (Fig. 4C) but it was inversely correlated with KIR^+ NK cell frequency on days 1 and 7 (Fig. 4E); no correlation was apparent in CP patients (Fig. 4D and F). These data suggest a different impact of the impaired granzyme A phenotype on antibody-dependent cytotoxicity (ADCC) or on spontaneous lysis.

3.7. NK cells from TBI patients are hyporesponsive in response to missing self

Based on our phenotypic study of NK cells, we hypothesized that the shaping of the NK-cell repertoire in TBI could lead to hyporesponsiveness

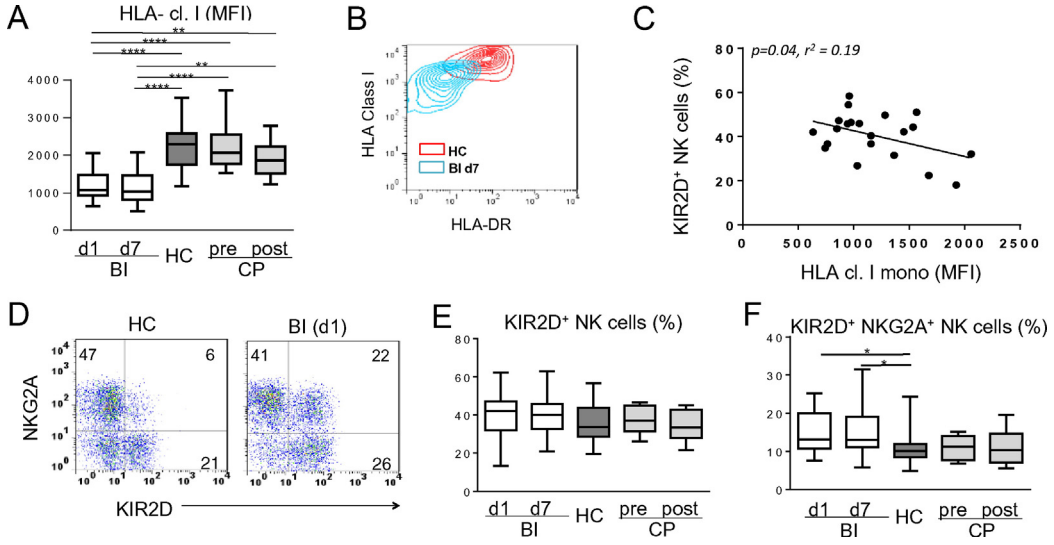

Fig. 2. HLA class I deficiency on monocytes is associated with an increased KIR^+ NK-cell frequency in TBI patients. Summary box and whisker plot summarizing (A) HLA class I MFI on monocytes in TBI on days 1 (n = 22) and 7 (n = 22), HC (n = 21) and CP pre- (n = 9) and postoperatively (n = 9). Top and bottom whiskers represent values of the top and bottom 25% of cases, respectively; boxed area, interquartile range. (B) Representative staining of HLA class I and HLA-DR molecules in NK cells from HC and TBI patients on day 7. (C) Correlation between expression of KIR^+ (KIR2D) NK cells (%) in NK cells and HLA class I expression (MFI) on monocytes from 18 TBI patients on day 1 and (D) from CP pre- (n = 11) and postoperatively (n = 11). (E) Representative density plots illustrating KIR2D and NKG2A expression on NK cells from HC and BI patient at day 1. Summary box and whisker plot summarizing (F) the percentages of $KIR2D^+$ NK cells and (G) $KIR2D^+$ $NKG2A^+$ NK cells in NK cells in TBI on days 1 (n = 22–23) and 7 (n = 22), HC (n = 22–23) and CP pre- (n = 10) and postoperatively (n = 10). $^*P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$.

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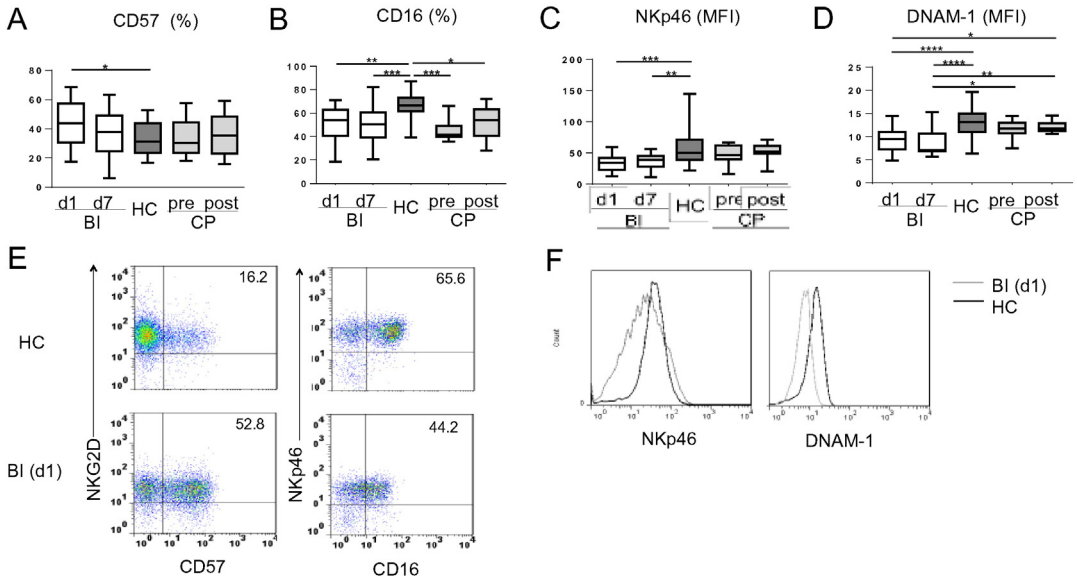

Fig. 3. The NK-cell repertoire is characterized by a higher frequency of late differentiated NK cell subset. Summary box and whisker plot summarizing the percentages (A) of $CD57^+$ NK cells (B) $CD16^+$ NK cells, (C) NKp46 MFI and (D) DNAM-1 MFI in NK cells from TBI on days 1 (n = 21–23) and 7 (n = 21–23), HC (n = 22–23) and CP pre- (n = 10–11) and postoperatively (n = 11). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (E) Representative density plots illustrating CD57, NKG2D, CD16 and NKp46 expression and (F) representative histograms illustrating NKp46 and DNAM-1 expression on NK cells from HC and BI patient at day 1.

of TBI NK cells to HLA class I deficient cells. To evaluate the functional ability of NK cells, we determined their (i) cytotoxic capacities (assessed by the CD107 expression, a surrogate marker of degranulation) and (ii) IFN-γ production after activation of spontaneous lysis (Fig. 5A–C) and reverse ADCC (Fig. 5D–F). For this purpose, we stimulated NK cells with 2 cell lines deficient in HLA class I expression (221 and Fc^+ P815 cell lines). Degranulation was significantly decreased in the TBI patients on days 1 and 7 compared with HC (Fig. 5C) in contrast to IFN-γ production (Fig. 5B) which was only decreased in TBI patients on day 7 compared with HC and CP postoperatively. In contrast, the functional abilities to produce IFN-γ (Fig. 5E) and to degranulate (Fig. 5F) after stimulation with specific CD16 antibody (ADCC) were not impaired in the TBI patients. Overall, the hyporesponsiveness of TBI NK cells was only associated with spontaneous lysis and not with reverse ADCC.

3.8. IL-12 restored IFN-γ production and degranulation of TBI NK cells

In NK crosstalk with monocytes and dendritic cells (DCs), the IL-12 produced by both cells triggers IFN-γ production and NK cell functions [19,21,22]. It has been previously observed that depressed IL-12 production by monocytes correlates with altered lymphocyte functions in trauma patients [4,20]. Thus, since IL-12 treatment restores cytokine production and cytotoxicity by NK cells [21–23], we evaluated the impact of IL-12 preincubation on TBI and HC NK cell function cells after activation of spontaneous lysis (Fig. 6A) and reverse ADCC (Fig. 6B) using the HLA class I deficient 221 and Fc^+ P815 cell lines respectively. IL-12 significantly triggered the IFN-γ and degranulation of TBI NK cells against HLA deficient 221 cells, spontaneously (via inhibitory receptors) (Fig. 6A) and via the ADCC pathway (Fig. 6B). The impact of IL-12 in

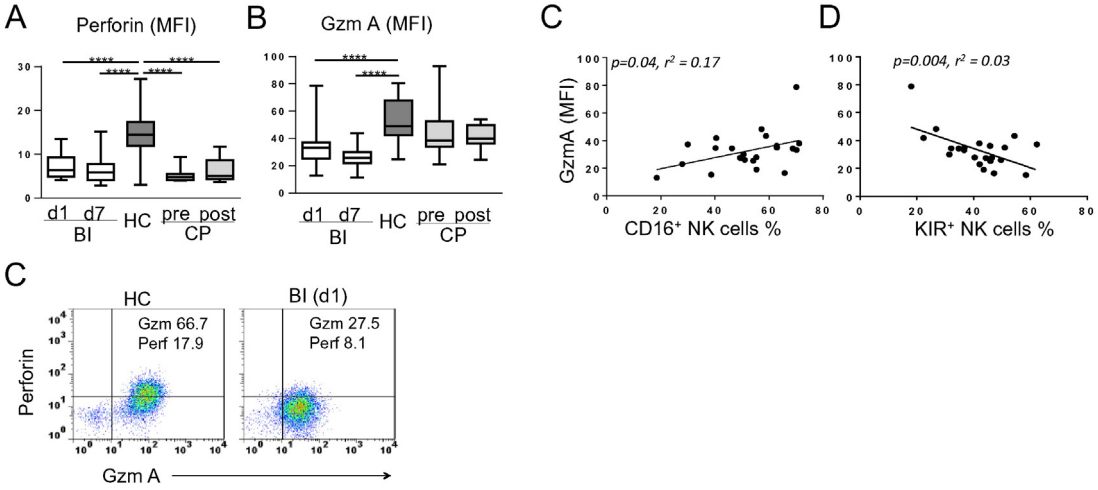

Fig. 4. Cytotoxic capacities of NK cells are impaired in TBI patients. Summary box and whisker plot summarizing (A) perforin and (B) Granzyme A MFI in NK cells from TBI on days 1 (n = 23) and 7 (n = 21), HC (n = 21) and CP pre- (n = 10) and postoperatively (n = 10). Top and bottom whiskers represent the values of the top and bottom 25% of cases, respectively; boxed area, interquartile range. (C) Representative density plots illustrating granzyme A and perforin expression on NK cells from HC and BI patient at day 1. The MFI for each marker is indicated on the density plots. Dot representation of granzyme A MFI in NK cells **(D)** as a function of $CD16^+$ NK cell frequencies NK cells in TBI patients (n = 23) and **(E)** in CP pre- (n = 11) and postoperatively (n = 11); (F) as a function of KIR^+ ($KIR2D^+$) NK-cell frequencies NK cells in TBI patients (n = 22) and (G) in CP pre- (n = 11) and postoperatively (n = 11). $^{****}P < 0.0001$.

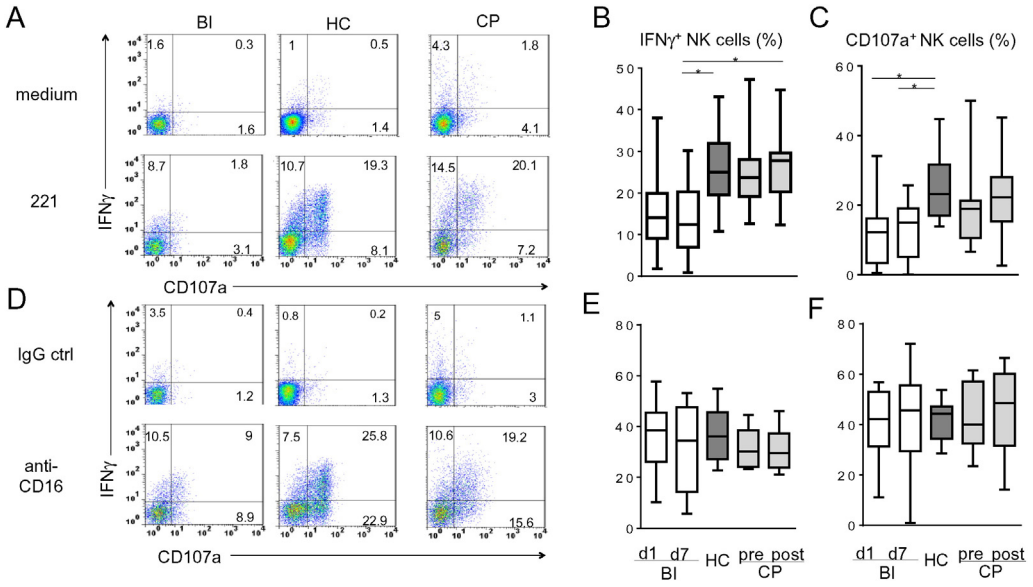

Fig. 5. TBI patient NK cells are hyporesponsive in response to missing self. (A) Representative density plots illustrating CD107a expression and IFN-γ production by NK cells from one representative TBI, HC and CP individual in a 5-h functional assay following the PBMC: target ratio of 1:1 with medium and 221 target cell line to determine spontaneous lysis. NK cells were gated as $CD56^+$ $CD3^-$ cells in the lymphocyte gate. Summary box and whisker plot summarizing the percentages of (B) $IFN\gamma^+$ NK cells and (C) $CD107a^+$ NK cells following spontaneous lysis from TBI on days 1 (n = 13) and 7 (n = 12), HC (n = 14) and CP pre- (n = 11) and postoperatively (n = 11). (D) Representative density plots illustrating CD107a expression and IFN-γ production by NK cells from one representative TBI, HC and CP individual in a 5-h functional assay following the PBMC: target ratio of 1:1 with medium and Fc^+ P815 cell line with IgG control or CD16 specific mAb to determine the reverse ADCC from TBI on days 1 (n = 13) and 7 (n = 12), HC (n = 16) and CP at pre- (n = 11) and postoperatively (n = 11). Summary box and whisker plot summarizing the percentages of (E) $IFN\gamma^+$ NK cells and (F) $CD107a^+$ NK cells following the reverse ADCC pathway. $^*P < 0.05$.

triggering NK-cell functions was evaluated by comparison of the fold increased $IFN\gamma^+$ $CD107a^+$ NK cell frequency determined for TBI on days 1 and 7 versus HC via the spontaneous lysis and reverse ADCC pathways (Fig. 6C). Interestingly, IL-12 treatment was particularly efficient on hyporesponsive TBI NK cells via spontaneous lysis when compared with HC NK cells which is consistent with the impaired missing-self recognition of TBI NK cells previously highlighted in our study.

4. Discussion

In the present study performed on severe TBI patients, we observed for the first time a significantly decreased expression of HLA class I on monocytes as well as severe impairment of NK-cell functions. Most of these alterations lasted 7 days. Pre-incubation with IL-12 was able to restore IFN-γ production and the cytotoxicity capacities of NK cells. This cytokine may therefore be considered as a potential treatment candidate in TBI patients with IS.

We first monitored HLA-DR expression on monocytes, the landmark of immunosuppression after BI [4,23–25]. HLA-DR is probably the most studied and accurate biomarker of IS currently available in ICU patients [24,26]. Despite an increased number of circulating monocytes, their membrane expression of CD14 and HLA-DR were severely impaired after TBI compared with healthy volunteers and with cardiac surgery patients.

During their development, NK cells acquire functional capacities via the engagement of their inhibitory KIR with cognate HLA ligands [25,27]. This specific interaction allows NK cells to be "licensed" to become functionally competent and to acquire effector functions [2,26]. This functional education is also essential to maintain self-tolerance. In the present experiment, the drastically decreased expression of HLA class I molecules observed on monocytes was correlated with an increased expression of $KIR2D^+$ NK-cell frequency in TBI patients, and NKG2D may play a role in inflammatory diseases [28]. It has been previously shown that the absence of HLA ligand favors the outgrowth of KIR NK cells [27,29] and it is conceivable that the NK repertoire is skewed by the decreased expression of HLA class I ligands in a TBI context. Even though the frequency of KIR2D + NK cells was not significantly increased in the TBI patients compared with the controls, the NK-cell subset co-expressing KIR2D and NKG2A (HLA specific inhibitory receptors) was preferentially represented in the TBI patients. Of note, NK cells from TBI patients present a late state of differentiation marked by CD57 and KIR expression and a lower expression of activating receptors such as

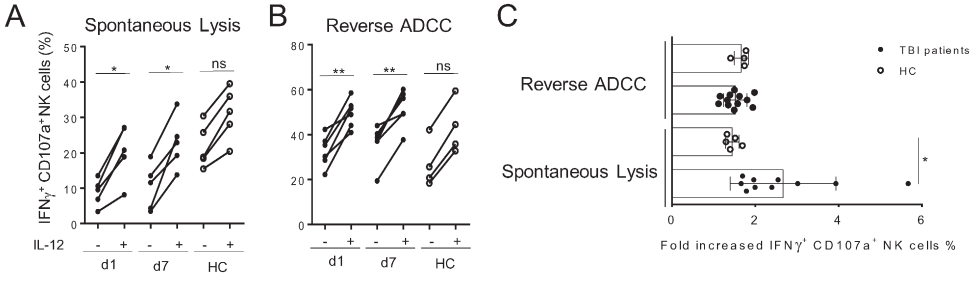

Fig. 6. IL-12 restored IFN-γ production and degranulation of TBI NK cells. Scatter plots representing $IFN\gamma^+$ $CD107a^+$ NK cells in a 5-h functional assay following the PBMC: target ratio of 1:1 to determine (A) spontaneous lysis with and without O/N treatment with IL-12 of PBMC of TBI on days 1 (n = 5) and 7 (n = 5) and HC (n = 5) and (B) reverse ADCC with and without O/N treatment with IL-12 of PBMC of TBI on days 1 (n = 6) and 7 (n = 6) and HC (n = 4). (C) Bars indicate the mean of the fold increased $IFN\gamma^+$ $CD107a^+$ NK cell frequencies (with IL-12/without IL-12 ratio) for spontaneous lysis (10 TBI and 5 HC) and reverse ADCC (12 TBI and 4 HC). $^*P < 0.05$.

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NKp46 and DNAM-1 [2,30]. Interestingly, a higher frequency of this CD57 + KIR + NKG2A + NK cell subset was described in TAP (Transporter associated with Antigen Processing) deficient patients who express a low level of HLA class I molecules [29,31]. Overall, the phenotypic alterations described in TBI patients suggest an impaired functional activity of NK cells from TBI patients.

Since TBI NK cells show alterations in the expression of both inhibitory and activating receptors, we evaluated the two major effector functions of NK cells which are: (i) cytotoxicity associated with degranulation and extracellular release of cytolytic enzymes (granzyme and perforine) and, (ii) production of cytokines. The functional profile of TBI NK cells is characterized by poor IFN-γ response and reduced degranulation in response to HLA deficient target cells. These results are line with those of Souza-Fonseca-Guimaraes et al. [2,30] demonstrating that the ex vivo production of IFN-γ by NK cells is impaired in septic patients. However, the low level of cytotoxic granule molecules cannot completely explain the hyporesponsiveness of TBI NK cells since they had good ADCC against the cell target P815. Indeed, although there was a decreased frequency of CD16 + NK cells in TBI patients, degranulation and IFN-γ production following stimulation with anti-CD16 were similar to those observed in control individuals. This observation could be explained by a consistent level of CD16 on differentiated CD57 + KIR + NK cells which confer enhanced responsiveness [31]. Another explanation for the lower responsiveness against HLA deficient target could be associated with the higher frequency of late differentiated NK cells in TBI patients. Indeed, it has been previously shown that the differentiated status of NK cells is associated with a loss of functionality [2,10,11]. It was proposed that the more differentiated (CD57 + KIR +) NK-cell subset expresses lower levels of major signaling unit for IL-2, IL-15 and IL-18 [2–4] linked to hyporesponsiveness after cytokine stimulation. On the contrary, in other acute conditions, patients displayed a high ADCC of their NK cells [9–11] underlining the fact that the NK-cell functional impairment observed in TBI patients is somehow specific compared with other ICU patients.

Antigens presenting cells positively interact with NK cells through the production of cytokines such as IL-12, IL-15, and IL-18 [9,22]. In the present experiments, the strong response to ex vivo IL-12 therapy shows that the IL-12 pathway remains functional in circulating NK cells. IL-12 increases the production of IFN-γ by NK cells, stimulates cytotoxicity of activated NK cells, and enhances ADCC against abnormal cells [22,32]. In the setting of bacterial infections, it was demonstrated that NK cells naturally internalize the bacterial pathogen-associated molecular pattern muramyl dipeptide and the adjunction of IL-12 stimulates the production of IFN-γ [32,33] Moreover, in conjunction with IL-15, IL-12 is responsible for the non-antigen-specific IFN-γ production in CD8 T cells in response to *Listeria monocytogenes* infection [33,34]. It was recently demonstrated that human NK cells exhibit memory-like functions (see reference (34,35) for review). Interestingly, memory-like NK cells are able to produce more IFN-γ than naive NK cells [5,35] and this phenomenon appears to be highly dependent on IL-12 signaling. This critical feature provides a new rationale for using reactivation with IL-12 in NK-cell immunotherapy protocols. DCs, particularly conventional DC, are the main producers of IL-12. We previously found that the number and functions of DC were impaired in BI patients with subarachnoid hemorrhage [5,36–39]. In particular, the production of IL-12 by conventional DC through TLR3/4 stimulation was dramatically decreased compared with that of healthy donors. These data strengthen the need for an exogenous administration of IL-12 since endogenous production is impaired.

Our study has several strengths including a global characterization of both the phenotype and effector functions (cytotoxicity and IFN-γ production) of NK cells. In addition, when a single early sampling time was reported in other studies, our kinetic suggested that NK-cell impairment lasts during the period in which nosocomial infections occur (within the first week of ICU hospitalization). Moreover, to underscore the specific features of TBI-induced immunosuppression, we used samples from cardiac surgery patients as positive controls. Finally, we propose IL-12 as a new potential treatment available to overcome NK cell alterations.

Some limitations should be mentioned. First, we studied a limited number of patients, and the clinical consequences of the immunological impairment described here could not be assessed. In particular, our preliminary results cannot support a direct correlation between a decreased class I expression and the neurological evolution after traumatic brain injury. Furthermore, the analyses were performed on frozen samples and not on fresh cells. However, considering the large number of analyses and the validated methods used [36–40], technical biases should be limited. We did not explore patients for genetic NK-cell immunodeficiency but the patients included had no history of severe viral infections, notably in childhood. Treatment with exogenous cytokine may induce side effects or immune deregulation, and further studies are needed before proposing IL-12 treatment in TBI patients. Finally, we explored circulating NK cells but the status of NK cells from tissues could be different [40].

In conclusion, this study provides the first extensive description of the phenotype and functions of NK cells in TBI patients. We found that TBI-induced immune suppression is characterized by a terminally differentiated phenotype of NK cells (high expression of inhibitor receptors and dampened cytotoxic ability). Finally, our results advocate for a further evaluation of IL-12 treatment to overcome IS-induced nosocomial infections in TBI patients.

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Authors' contributions

Contribution: A.R. and G.D. designed and performed the majority of experiments, analyzed data, and wrote the manuscript. R.C. and M.V. analyzed data, and wrote the manuscript. HM, JBP, and BR provided patient materials and analyzed data. C.R. and K.A. developed and supervised the entire project, designed experiments, interpreted data, and wrote the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Alterations of the iNKT cell compartment in traumatic brain injury

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ABSTRACT

Objective: Brain injury (BI) induces a state of immune depression (IS) leading to pneumonia. The mechanisms of this IS are not well understood. We investigated immunological parameters of brain-injured patients, focusing on the iNKT cell compartment.

Design: observational study.

Setting: Two surgical ICUs of a single institution and a research laboratory.

Subjects: Severe brain-injured patients (n=33) requiring mechanical ventilation and sex- and age-matched healthy donors (n=40).

Intervention: none.

Measurements and Main results: Using BI patients' blood samples collected within 24h after trauma, we observed the presence of IL-10 in serum, a loss of IFNγ and IL-13 secretions by PBMCs following IL-2 stimulation and a down-regulation of HLA-DR expression on both monocytes and B cells. Inversely, CD1d, the HLA class I-like molecule involved in antigen presentation to iNKT cells, was over-expressed on patients' monocytes and B cells. The antigen presenting activity to iNKT cells of PBMCs was increased in the patients who developed pneumonia, but not in those who remained free of infection. Frequencies of iNKT cells among PBMCs were severely decreased in patients regardless of their infection status (0.01% [range:0.02%-0.3%]) compared with healthy donors (0.9% [range:0.7x%-1.2%]). Following amplification, an increased frequency of CD4+ iNKT cells producing IL-4 was noticed in the group of patients who did not become infected compared with those who became infected and to healthy donors. Finally, serum from BI patients inhibited the iNKT cells specific response as well as the non-specific IL-2 stimulation of PBMCs and expression of beta-2 adrenergic receptor was elevated on the surface of patients T lymphocytes.

Conclusions: Brain-injured patients experience an immunodepressive state associated with early alterations in the iNKT cells compartment, including enhanced capacities for antigen presentation to iNKT cells and the presence of inhibitory serum factors.

Key words: brain injury, nosocomial pneumonia, iNKT cells, CD1d, immunodepression, beta2 adrenergic receptor

INTRODUCTION

In intensive care units, nosocomial infections, mainly pneumonia[1–3], are associated with a poor outcome and increased morbidity and mortality after an acute brain injury (BI)[4]. It has become clear that susceptibility to infections is critically correlated with a post-traumatic immunodepression (IS)[5,6]. A better understanding of the mechanisms involved in the IS should contribute to the identification of early biomarkers allowing to select patients requiring prophylactic treatment.

A similar impairment of systemic immune responses is found in several situations of sterile burning infection, such as sepsis, stroke or traumatic brain injury[7–10]. With the exception of sepsis, this systemic IS may be at least partially explained by long distance neuroimmune communication through the release of neurotransmitters in blood[11,12]. Using a mouse stroke model, Wong and al. reported data indicating that invariant Natural Killer T cells (iNKT) may play a major role[13]. These authors observed that iNKT cells produced more anti-inflammatory cytokines (IL-10) after induction of brain injury, whereas their secretion of pro-inflammatory cytokines decreased (IFN-γ, IL-12p70). An efficient anti-infectious immunity could be restored when iNKT cells were specifically stimulated with their canonical ligand α-GalactosylCeramide (α-GalCer). Moreover, either blocking the catecholamine pathway with a specific antagonist (propranolol) or using iNKT-deficient mice prevented the switch of cytokines secretion from a T_h1 to a T_h2 profile and restored clearance of infection after stroke. These results clearly highlighted the involvement of both catecholamines and iNKT cells in the appearance of IS. iNKT cells are a discrete subset of T lymphocytes characterized by an invariant T cell receptor[14]. TCR recognition is restricted by the monomorphic MHC class-I-like molecule CD1d that is expressed by professional antigen presenting cells (APC)[15]. Several subsets of iNKT cells have been described based on their expression of the CD4 or

CD8 molecules. They can be $CD4^+/CD8^-$, $CD4^-/CD8^+$ or $CD4^-/CD8^-$[16], but in contrast to conventional T cells which recognize peptides, the iNKT TCR reacts to self or foreign lipid antigens (bacterial lipids) loaded on CD1d. Activation of iNKT cells leads to a quick and massive release of both pro-inflammatory (T_h1) and anti-inflammatory (T_h2) cytokines[17]. iNKT cells are highly versatile cells that can contribute to various types of immune responses, including anti-microbial and anti-cancer responses, but also inflammatory and autoimmune diseases. They are particularly efficient to drive the first stages of innate responses[17].

In the present work we focused on the first 24h after trauma. We hypothesized that iNKT lymphocytes may be good candidates to early discriminate BI patients prone to pneumonia. After confirming the severe immunodepression state of our cohort of BI patients, we observed an up-regulation of CD1d expression on APCs that was associated with an increased ability to present a glycolipid antigen in BI patients who developed severe pneumonia later on. Frequencies and responsiveness of patients' iNKT cells were also severely affected. Finally we noticed the presence of serum factor(s) that decreased iNKT cell activity as well as the response of PBMCs to a non-specific IL-2 stimulation. Taken together, this analysis provides new original information pointing out the possibility to discriminate patients based on their pattern of iNKT cells functional profile.

MATERIALS AND METHODS

Patients and healthy volunteers

This work is part of a global study on immune dysfunctions in ICU. An institutional review board for human experimentation approved the protocol (Comité de Protection des Personnes de Nantes, authorization number AC-2008-433/French). Written informed consent from next-of- kin was required for enrollment. Whenever possible, retrospective consent was obtained from patients.

Patients were enrolled from January 2013 to November 2013 in two French surgical ICUs of one university hospital. Control samples were collected from healthy blood donors at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) after obtaining informed consent.

Tables 1 and S1 show the patients information . Nosocomial pneumonia was defined as pneumonia occurring 48h or more after admission and not incubating at the time of admission. Eighteen patients were diagnosed for pneumonia during the first 11 days, whereas the other 13 did not declare nosocomial infection. Pneumonia diagnosis was always confirmed by culture from lower respiratory tract samples obtained by endotracheal aspirate, bronchoalveolar lavage or with a blind-protected specimen catheter (significant threshold: 10^6 colony-forming units/mL, 10^4 colony-forming units/mL, 10^3 colony-forming units/mL, respectively).

Sample collection

Blood samples were collected after ICU admission within 24h following trauma. Peripheral blood mononuclear cells (PBMCs) were obtained by gradient centrifugation following standard protocol and serum were isolated by centrifugation and stored in liquid nitrogen or at −80 °C until investigation, respectively.

Flow cytometry

PBMCs and other cells were stained with anti-human mAbs: anti-CD3 FITC, anti-CD4 BV605, anti-CD8 BV421, anti-CD14 BV711, anti-CD19 BV605, anti-CD1d APC anti-HLA-DR BV421, IFN-γ PE and IL-4 PE (all from BD Biosciences, Vienna, Austria), APC-labelled human CD1d tetramers loaded with the α-GalCer analogue PB557 were from the MHC Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Anti-human ADRB2 (AbD Serotec, Oxford, UK) was coupled with alexa fluor647 fluorochrome by using a protein labeling kit (Life Technologie, Paisley, UK). Viability was assed with Zombie Nir viability dye (BioLegend, London, UK) or with fixable viability dye eFluor506 (eBiosciences, Vienna, Austria). The corresponding isotype control mAbs were used to assess staining specificity.

Generation of iNKT cells

iNKT cells were enriched from PBMCs by positive selection of Vα24-Jα18 cells by magnetic beads separation (MACS Miltenyi, Paris, France). Purified cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated human pooled serum from 40 donors, 2mM glutamine, 50U/ml penicillin, 50µg/ml streptomycin (Gibco BRL), PHA 1µg/ml (Sigma-Aldrich, Schnelldorf, Austria), and IL-2 300 U/ml (PeproTech, USA) in presence of irradiated allogenic PBMCs for 1 week. Purified iNKT cells were then maintained in culture in the same medium without PHA and irradiated feeder up to three months. Purity of iNKT cells was assessed by flow cytometry after staining the cells with mAbs specific for CD3 and with CD1d PBS57-loaded tetramers.

Generation of Namalwa-CD1d cells

Human Namalwa (Nam) Burkit lymphoma cell line was obtained from Dr. J. Wiels (Villejuif, France) and was then transduced with a lentiviral vector containing a human CD1d insert in

142 order to generate high expression of cell surface CD1d and efficient stimulation of iNKT cells
143 following loading with specific glycolipid agonists, as previously described[18]. These antigen-
144 presenting cells were cultivated in RPMI 1640, with 10% heat-inactivated fetal calf serum
145 (FCS), 2mM glutamine, 50U/ml penicillin, 50µg/ml streptomycin.

146 **Cytokine secretion assays**

147 PBMCs alone were cultured at cells density 1 X 10^6/ml in 96-well culture plates at 37°C.
148 Mitogenic stimulation of PBMCs was performed with IL-2 (200 U/ml) for 48h. In some
149 experiments, stimulation of PBMCs was carried out in the presence of 10^{-5} M epinephrine.
150 IFN-γ and IL-13 secretions in cell supernatants were then quantified by ELISA (eBioscience).
151 For iNKT specific activation with α-GalCer, antigen-presenting cells (APC) were needed.
152 Nam-CD1d or PBMCs were respectively plated at 30.000 or 300.000 per well on 96-well
153 culture plates in complete RPMI containing healthy volunteers' pooled sera and were loaded
154 overnight at 37°C with 0.1µM α-GalCer (Sigma-Aldrich). Cells were then washed twice in
155 RPMI alone. When Nam-CD1d cells were used as APCs, 15.000 iNKT cells were added, and
156 when PBMCs were used as APCs, 50.000 iNKT cells were added. In both conditions, APCs
157 and iNKT cells were co-incubated at 37°C in complete RPMI containing pooled sera from
158 healthy volunteers or pooled sera from BI patients, depending on experiments. Cytokines
159 secretion in supernatants was quantified by ELISA (eBioscience) after 48h stimulation.

160 **Amplification of iNKT cells from PBMCs**

161 PBMCs from human volunteers (HV) or from patients were cultured at cells density 1 x
162 10^6/ml in 24-well culture plates at 37°C under mitogenic stimulation with IL-2 (300 UI/ml)
163 and in the presence of α-GalCer (0.1µM) for 10 days. Half of the medium was renewed every
164 3 days. At day 10, cytokine secretion was blocked with brefeldine A for 6 hours, and then
165 cells were collected to analyze intracellular cytokines by flow cytometry.

166 **Statistical analysis**

167 All statistical analyses were performed with Prism-6 software (GraphPad Software).
168 Continuous nonparametric variables are expressed as medians (interquartile range). For 2
169 groups comparisons, the Man-Whitney *U* test was used. The one-way analysis of variance
170 (ANOVA) test was used for comparisons of multiple groups. Dunnett's multiple comparisons
171 test was used as a post hoc test for intergroup comparisons. Significance was defined as P less
172 than 0.05.

173

RESULTS

Clinical characteristics of patients

A total of 33 traumatic brain-injured patients were enrolled in the study. Their general characteristics are described in Table 1. Severity of brain injury was quite variable with GCS ranging from 3 to 15, in accordance with a variable duration of ICU stay ranging from 2 to 99 days (mean = 15 days). Nosocomial pneumonia occurred in 54% of patients during their ICU stay and 18% died while in ICU. The main agent of pneumonia was methicillin-sensitive *Staphylococcus aureus*, followed by *Streptococcus pneumoniae* and *Escherichia coli*, as indicated in Table S1.

Evidence of immunodepression in BI patients

As traumatic brain injury is known to induce an IS associated with a high level of anti-inflammatory cytokines[5], we compared the cytokine levels in serum from HV and BI patients. IL-10 was detected in sera from patients but not in HV (**Fig S1**). We next compared the cytokine secretion of PBMCs from healthy volunteers and from patients following a non-specific stimulation with IL-2. We observed that IFN-γ and IL-13 secretions were markedly depressed in BI patients (**Fig. 1A**).

IS following severe brain injury[19] or sepsis is also characterized by a decreased capacity of monocytes to present antigens as assessed by the loss of HLA-DR on monocytes. Phenotypes of both monocytes and B lymphocytes were compared to those of healthy donors (**Fig. 1B**). Firstly, a major decrease of HLA-DR expression on monocytes of BI patients as compared to HV was observed (**Fig. 1C**). Furthermore, a similar drop in HLA-DR expression was observed on patients B cells (**Fig. 1C**). Since our major aim was to look for a potential involvement of iNKTs we next looked for CD1d expression as it corresponds to the molecule that presents glycolipids antigens to iNKT cells. Interestingly, results were the exact opposite

of those obtained for HLA-DR expression since CD1d was strongly overexpressed on both monocytes and B cells in BI patients (**Fig. 1D**), suggesting a potential impact on iNKT antigen-specific activation.

Specific activation of iNKT cells by PBMCs from BI patients

As we observed an elevated level of CD1d on APCs from patients, we suspected an increased ability of BI patients PBMCs to activate iNKTs. We thus investigated IFN-γ and IL-13 secretions induced by activation of iNKT cells isolated from HV after a 48h stimulation with PBMCs from either HV or BI patients that had been loaded with α-GalCer. The secretion of IFNγ from cells stimulated by α-GalCer-loaded-PBMCs from patients did not differ from that obtained using PBMCs from healthy volunteers, although they showed a somewhat increased secretion of IL-13. We noticed a large dispersal of the values obtained from the patients group, which prompted us to split the patients group into those who declared pneumonia and those who did not. Strikingly, we then observed a much stronger concentration of both secreted cytokines when PBMCs from BI patients with pneumonia were used (**Fig. 2**). This occurred despite the fact that CD1d was over-expressed on PBMCs of patients regardless of their infection status. It indicates that the iNKT antigen-specific response can differentiate the two subgroups of patients. These results also demonstrate that the decreased capacity to present antigens is not a generalized phenomenon because whereas the expression of HLA-DR molecules is decreased on APCs, the capacity for presenting glycolipids through CD1d expression is higher in patients who are expected to be the most immunocompromized, that is the infected patients.

Alterations of iNKT cells in BI patients

After having observed alterations in patients' iNKT antigen presenting cells, we investigated the patients iNKT cells themselves. We first quantified circulating lymphocytes from patients and observed that their numbers remained within the normal range despite a trend toward lymphopenia in our cohort at 1 day post-BI. It was not significant likely due to the large individual variation and the limited number of patients of our study (**Fig. S2**). However, when focusing on iNKT cells among PBMCs from patients(**Fig. 3A**), we observed drastically decreased frequencies compared to those of healthy volunteers (**Fig. 3B**). Considering the small number of iNKT cells recovered from patients, functional studies did not appear feasible. Thus we decided to amplify the remaining small number of iNKT cells prior to analysis of their phenotype. To this aim, PBMCs from patients and HV were cultured for 10 days in the presence α-GalCer. Compared to day 0, day 10 iNKT cells were increased in both groups, but their frequency in BI remained significantly lower than in HV after amplification (**Fig. 3C**). It was then observed that the frequencies of iNKT cells secreting IFN-γ was higher in patients than in HV following expansion and activation (**Fig. 3D**). IFN-γ secretion is a marker of iNKT cells activation since it is secreted by all types of iNKT cells regardless of CD4 and CD8 expression. To determine if frequencies in the iNKT subtypes might be altered in patients, we next focused on their CD4 and CD8 expression. We observed that the CD4+/CD4- ratio differed between patients and healthy volunteers iNKT cells and furthermore that the higher CD4+/CD4- ratio of the cells from BI patients was entirely resting on the subgroup of patients who did not develop pneumonia. (**Fig. 3E**). Since CD4+ iNKT cells are known to have a T_h2 phenotype characterized by a strong IL-4 secretion[20], we looked for intracellular IL-4. Consistent with their high CD4+/CD4- ratio, iNKT cells from patients who did not develop pneumonia presented a higher frequency of IL-4 positive cells (**Fig. 3F**).

Patients' serum factors decrease the specific activity of iNKT cells

We then aimed at determining the presence of factors in sera from BI patients that may alter the specific activity of iNKTs cells. To this aim we first co-cultured purified iNKTs from HV with CDd1-transduced Namalwa cells loaded with α-GalCer (**Fig. 4A**). The cells were co-cultured either in the presence of culture medium containing pooled serum from 40 healthy volunteers or in the presence of pooled serum from 10 BI patients. In these conditions, we observed that iNKT cells activation in the presence of BI patients' serum led to a significantly weaker secretion of pro-inflammatory (IFN-γ and IL-2) and anti-inflammatory cytokines (IL-10 and IL-13). The same experiment was performed using PBMCs from 8 individual healthy donors as APCs instead of the Nam-CD1d cells since PBMCs may be more relevant APCs (**Fig. 4B**). Similar results were obtained, indicating the presence of one or several factors in the serum of patients that decrease the specific activation of iNKT cells. However, we did not observe differences between BI patients with or without pneumonia in these experiments.

Finally, we explored the potential involvement of catecholamines as serum factors able to induce the immunosupression. PBMCs from healthy volunteers were thus stimulated with a mitogenic signal, IL-2, in the presence of pooled serum from patients or of epinephrine as positive control. In both cases we observed a decrease of IFN-γ and IL-13 (**Fig. S3**). Inhibition of catecholamines was ascertained through addition of propanolol, a specific antagonist of adrenergic receptors. While propranolol was able to revert the decrease induced by epinephrine, it did not when using serum from patients. (**Fig. S3**), indicating that the inhibition of cytokines secretion induced by the patients' sera was not directly dependent or solely dependent on catecholamines. Nonetheless, we observed a clear increase of adrenergic receptor B2 on the surface of T lymphocytes (**Fig. 4C**) from BI patients compared to its very low expression on those of healthy volunteers (**Fig. 4D**).

DISCUSSION

BI-induced immunodepression has been characterized through three major features: an imbalance favoring anti-inflammatory cytokines (including IL-10)[21]; a decreased capacity of leukocytes to produce pro-inflammatory cytokines *in vitro* after LPS stimulation[22]; and a decreased capacity of APCs to present antigen due to a major down-regulation of HLA-DR[23] . In our study we used samples collected within 24h after trauma in order to analyze early events that may contribute to the occurrence of nosocomial pneumonia, focusing on the iNKT cell compartment. We first aimed at controlling the PTI status of our patient's cohort. As expected, we found the presence of IL-10 in the serum of BI patients, but not of healthy volunteers. We additionally observed that the patients PBMCs presented a near complete loss of cytokine production after an mitogenic stimulation with IL-2. Interestingly, the effect was visible for both IFN-γ, a pro-inflammatory or T_h1 cytokine, and IL-13, an anti-inflammatory, or T_h2 cytokine, consistent with an early and deep alteration of the overall response. We next confirmed the strong down-regulation of HLA-DR expression on patients' monocytes[24] and we additionally observed that this down-regulation also existed in the B cell compartment.

Focusing on the iNKT compartment, we then unexpectedly observed a clear over-expression of CD1d both in monocytes and B-cells. It was visible regardless of whether the patients would later develop pneumonia. This was the exact opposite of what was found for HLA-DR. CD1d is the non-classical MHC-I molecule involved in antigen presentation to iNKTs, suggesting a potential increased potency of patients APCs to activate iNKT cells in an antigen-dependent manner. In agreement with this, following specific activation of iNKT cells using PBMCs from patients or healthy volunteers as APCs, a significant increase of cytokines secretion IFN-γ and IL-13 was indeed observed, but only in BI patients that contracted pneumonia several days after trauma. This also indicates that the APCs of patients who are

294 able to control infection possess other characteristics that modulate specific iNKTs activity
295 despite increased CD1d expression on their APCs.
296 We then sought to analyze the fate of patients' iNKT cells and observed a major decrease of
297 their frequency in the periphery. An earlier study highlighted a decrease up to 40% of the
298 major lymphocytes subsets one day after stroke[31]. The near complete disappearance of iNKT
299 cells among PBMCs is all the more important when compared to the relatively contained
300 lymphopenia observed on our cohort. After expansion in the presence of the specific
301 glycolipid α-GalCer, patients' iNKT cells were able to produce IFN-γ that represents an iNKT
302 activation marker. To determine if iNKT cells from patients can secrete as much IFN-γ
303 following specific activation as iNKT cells from healthy donors, a detailed kinetic analysis
304 would be required since the lower IFN-γ secretion that we observed for the healthy donors in
305 our experimental conditions might be due to an exhaustion after a 10 days stimulation.
306 Regardless, the phenotype of these amplified iNKT cells differed between the two subgroups
307 of patients. We observed an increased frequency of $CD4^+$ iNKT cells in the case of patients
308 who did not develop infection. This cell population is considered as a T_h2 sub-population[16], in
309 agreement with the high level of $IL\text{-}4^+$ iNKT cells also observed for this sub-group of patients
310 as compared to BI patients who developed pneumonia or to healthy volunteers. Beyond the
311 type of response (pro- or anti-inflammatory), the current results clearly indicate that an altered
312 reactivity of iNKT cells to their usual ligands is probably tightly related with the occurrence
313 of secondary infections.
314 Brain injury is largely reported to induce activation of the sympathetic nervous system leading
315 to a release of numerous molecules in circulation that impair immune function[25]. Several
316 studies focused on the central implication of catecholamines to promote systemic
317 immnosuppression[13,25]. In our study, although having highlighted serum factors able to down
318 regulate cytokines secretion, we failed to revert the phenomenon after treatment with

antagonist of catecholamines, propranolol. The serum factors responsible for the down-regulation of the immune response thus remain to be characterized. Nonetheless, involvement of the adrenergic pathway cannot be excluded. Indeed, analysis of the cell surface expression of the beta-2 adrenergic receptor on T lymphocytes revealed a marked overexpression in BI patients. Our results suggest that this could represent a potent stress marker of the lymphocytes of BI patients and potentially of patients with related IS states.

Overall, our results indicate that iNKT cells are conditioned from the first day after injury and can participate to the post trauma immune response, although this will require confirmation from studies on larger cohorts of patients. We showed here that CD1d and beta-2 adrenergic receptor may be new candidates markers, showing increased, rather than decreased, expression. They should be of potential interest both to understand the mechanisms underlying PTI and in the quest of early prognosis markers allowing prediction of the occurrence of severe infection after traumatic brain injury.

ACKNOWLEDGEMENTS

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FIGURE & LEGENDS

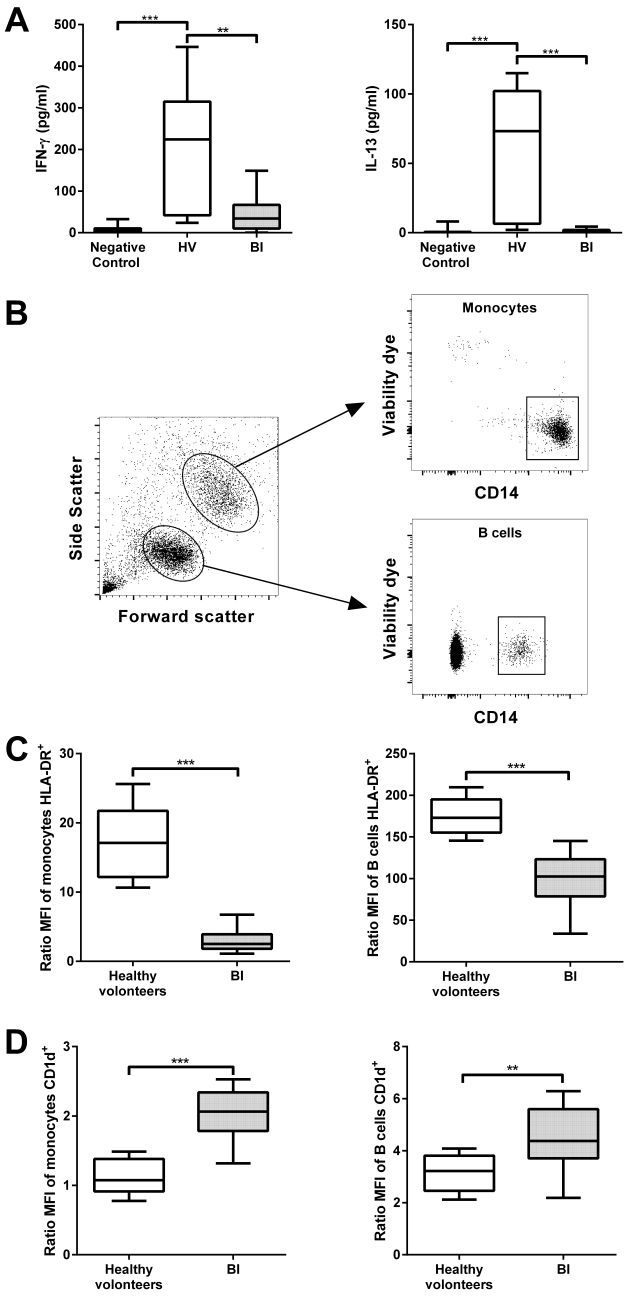
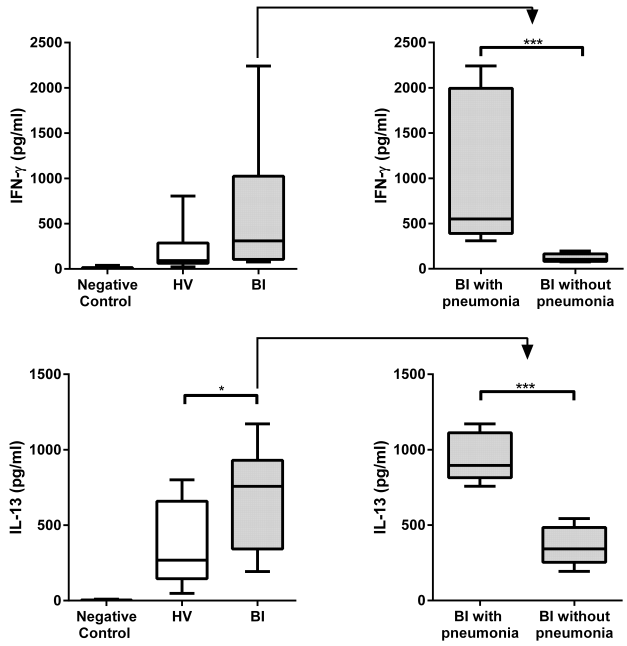


Figure 1. Alteration of PBMCs response and phenotype in BI patients. (**A**) PBMCs from 10 HV or from 19 BI patients were cultured in complete medium containing 10% pooled sera from 40 healthy donors. Concentrations of IFN-γ and IL-13 in culture supernatants were measured by ELISA after a 48h stimulation with 200 µg/ml recombinant IL-2. Data are shown as the concentration in pg/ml of cytokines, and are representative of 2 independent experiments. Negative controls represent results obtained in absence of IL-2. (**B**)

Representative density plots illustrating the gating strategy used to analyze phenotypes of $CD19^{+}$lymphocytes and CD14+monocytes. Expression of HLA-DR (**C**) and CD1d (**D**) were analyzed on stored cells from 10 individual healthy volunteers (HV) and 20 individual BI patients. Data are shown as the ratio of median fluorescence intensity (MFI) obtained with the specific antibodies to those obtained with their associated control isotypes. Top and bottom whiskers represent the extreme values; boxed area represent the 25th percentile, the median and the 75th percentile. (*p< 0.05, **p<0.01, ***p<0.001; statistical analysis, Mann-Whitney test).



418

419 **Figure 2.** Cytokine secretion of iNKT cells activated by α-GalCer loaded on PBMCs from BI
420 patients. iNKT cells isolated from a healthy donor were stimulated by PBMCs loaded with α-
421 GalCer from 10 healthy volunteers (HV) (white boxes) or from BI patients (grey boxes), 10
422 with pneumonia and 9 without pneumonia. Cytokines secretions were analyzed by ELISA
423 after 48h of stimulation. IFN-γ upper panel, IL-13, lower panel. Data are shown as the
424 concentration of cytokines in pg/ml and are representative of two independent experiments.
425 Negative controls indicate cytokine secretion in absence of α-GalCer. Right panels show the
426 data obtained for BI patients when split into those who declared pneumonia and those who did
427 not. Top and bottom whiskers represent the extreme values; boxed area represent the 25th
428 percentile, the median and the 75th percentile. (*p< 0.05, **p<0.01, ***p<0.001; statistical
429 analysis, Mann-Whitney test).

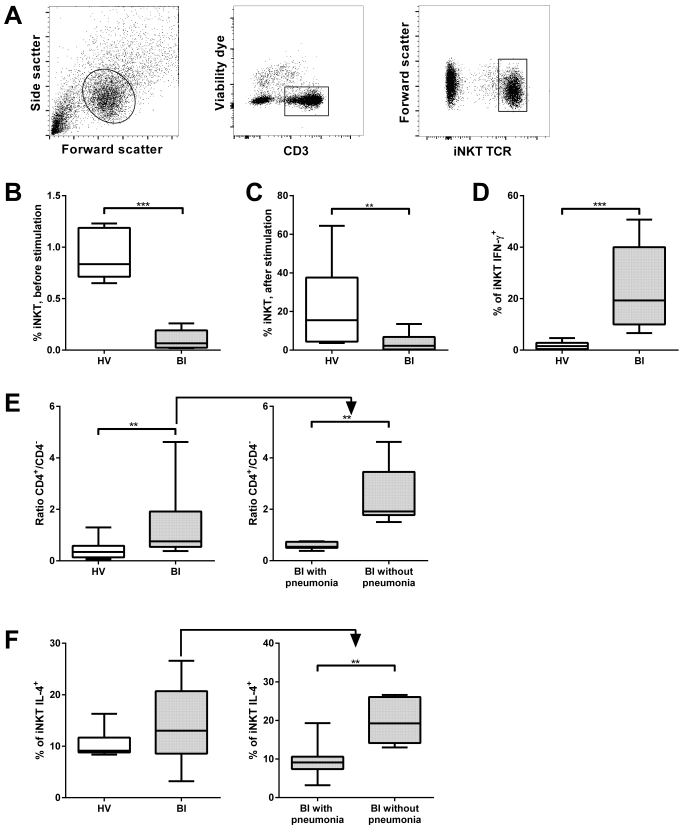
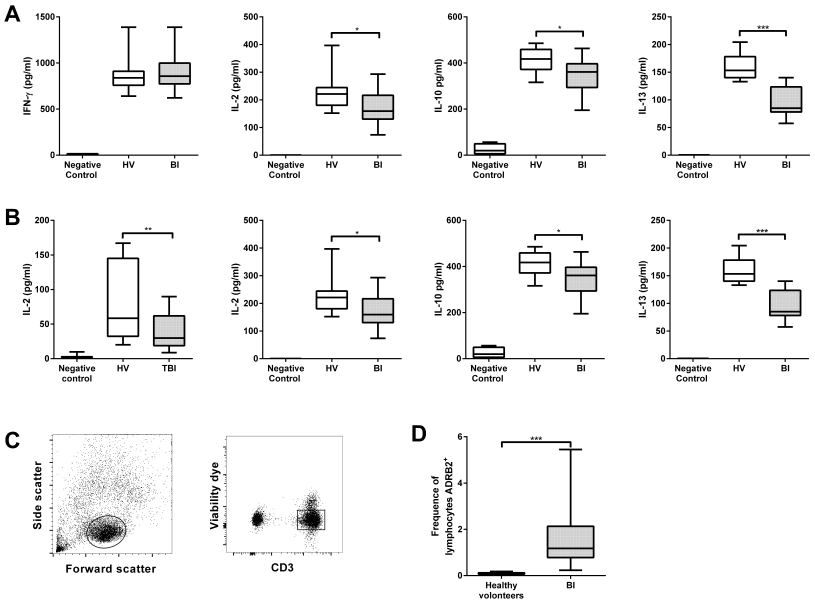


Figure 3. Comparison of iNKT cells populations after α-GalCer expansion. PBMCs from 10 healthy volunteers (HV) (white boxes) or PBMC from BI (grey boxes), 9 with pneumonia and 8 without pneumonia were cultured in medium containing α-GalCer and IL-2 for 10 days to induce iNKT cells expansion. (**A**) Representative density plot illustrating the gating strategy used to characterize iNKT cells and their phenotypes. Summary boxes and whisker plots summarizing the percentage of iNKTs in the $CD3^+$ compartment before stimulation (**B**) or after stimulation (**C**), the percentage of iNKT IFN-γ^+ (**D**), the ratio of $CD4^+/CD4^-$ iNKT cells (**E**) and the percentage of iNKT IL-4^+ (**F**) are shown. In **E** and **F,** right panels show the data obtained for BI patients when split into those who declared pneumonia and those who did not. Top and bottom whiskers represent the extreme values; boxed area represent the 25th percentile, the median and the 75th percentile. (*p< 0.05, **p<0.01, ***p<0.001; statistical analysis, Mann-Whitney test).



443

444 **Figure 4.** Cytokine secretion after specific activation of iNKT cells in BI patients' sera. iNKT
445 cells isolated from a healthy donor were stimulated with α-GalCer loaded on Nam-CD1d (**A**),
446 or on PBMCs from 8 healthy volunteers (HV) (**B**), in medium containing pooled serum from
447 40 HV or sera from 20 BI patients. Cytokines were measured in supernatants after 48h by
448 ELISA. Data are shown as the concentration of cytokines in pg/ml and are representative of at
449 least two independent experiments. Negative controls represent results obtained in absence of
450 α-GalCer. (**C**) Representative density plots illustrating the gating strategy of $CD3^+/ADRB2^+$
451 cells. (**D**) Expression of the adrenergic receptor β2 (ADRB2) on the surface of $CD3^+$
452 lymphocytes. Data are shown as their percentage for 5 HV and 10 BI patients. Top and down
453 whiskers represent the 25th percentile, the median and the 75th percentile. (*p< 0.05,
454 **p<0.01, ***p<0.001; statistical analysis, Mann-Whitney test).

455

456

Table 1

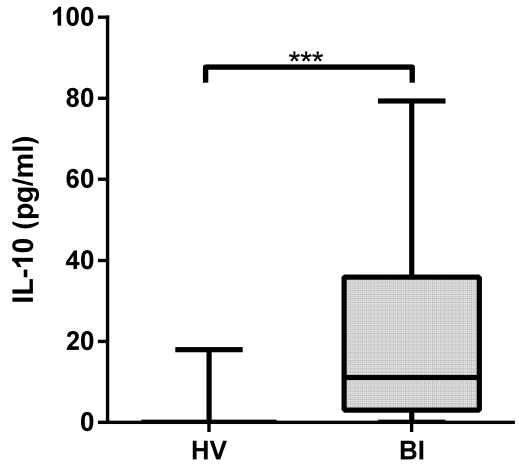
Clinical characteristics of brain-injured patients

	Total (n=33)	BI with pneumonia (n=18), 55%	BI without pneumonia (n=15), 45%
Age (years)	46 [26-56]	49 [30-60]	43 [23-54]
Male, *n*	26 (79%)	14 (78%)	12 (80%)
Initial GCS	6 [3.55-8]	5 [3.8-7.3]	7 [3-8]
Barbiturate, *n*	7 (21%)	3 (17%)	4 (27%)
Corticotherapy, *n*	1 (3%)	1 (6%)	0 (0%)
Nosocomial Pneumonia, *n*	18 (55%)	18 (100%)	0 (0%)
Acute respiratory distress syndrome, *n*	6 (18%)	5 (28%)	1 (7%)
Decompressive Cranectomy, *n*	2 (6%)	0 (0%)	2 (13%)
Aneurysmal subarachnoid haemorrhage, *n*	9 (27%)	7 (39%)	2 (13%)
Duration of mechanical ventilation (days)	11 [6.5-20]	18 [11-27]	7 [5-15]
ICU lenght of stay (days)	15 [10-29]	23 [14-35]	11 [7-17]
Death in ICU, *n*	6 (18%)	1 (6%)	5 (33%)

Data are given as the median [interquartile range] or *n* (%)

ICU : intensive care unit; GCS : Glasgow Coma Scale

462 **SUPPLEMENTAL DIGITAL CONTENT**



463

464 **Figure S1.** Presence of IL-10 in serum from individual BI patients. IL-10 was measured in
465 serum from 10 healthy volunteers (HV) and 20 BI patients by ELISA. Results are shown as
466 the concentration of cytokine in pg/ml. IFN-γ, TGF-β, IL-2, IL-4, IL-10, IL-12 and IL-13
467 could not be detected in the serum of either patients or healthy volunteers.

468

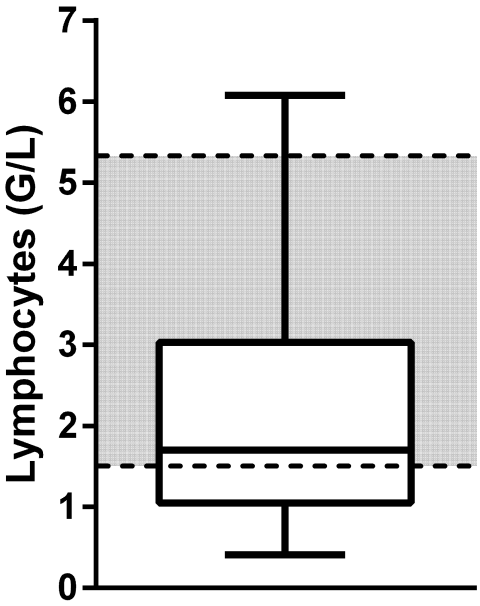


Figure S2. Summary box and whisker plot summarizing the number of lymphocytes in BI patients. Top and bottom whiskers represent the 25th percentile, the median and the 75th percentile, respectively. The grey area shows the normal range (1-4 x10^9 cells/L).

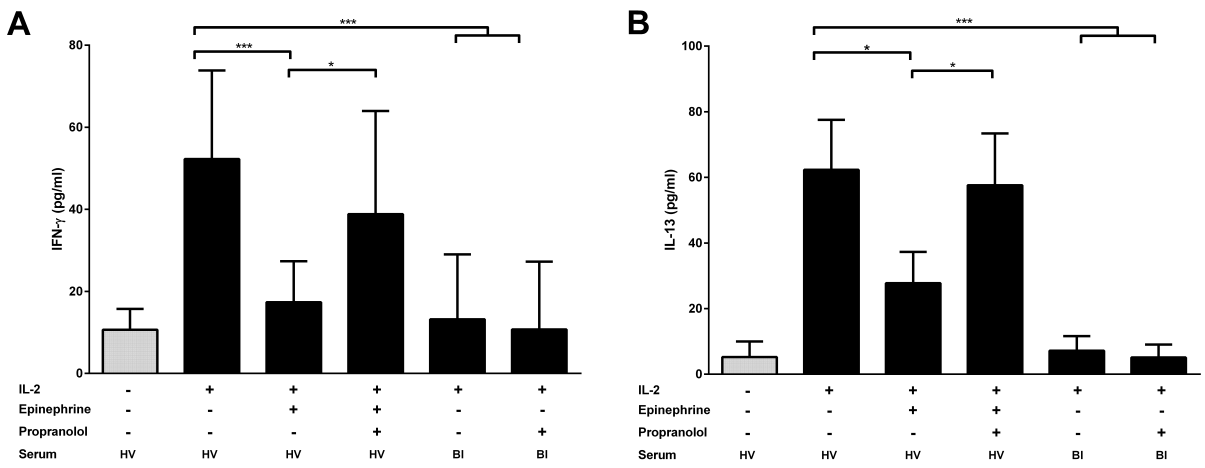


Figure S3. Role of catecholamines in the serum-induced IS. Total PBMCs from healthy volunteers were incubated with 200 U/ml recombinant IL-2 for 48h. Concentrations of IFN-γ (**A**) and IL-13 (**B**) in culture supernatants were measured by ELISA. PBMCs were cultured in medium containing 10% pooled sera from 40 healthy donors with or without 10^{-5} of epinephrine, or in medium containing 10% pooled sera from 20 TBI patients. β-adrenergic blocker propranolol at 10^{-5} M was added in culture medium when indicated. Using propranolol alone did not significantly affect the cytokine secretion after IL-2 stimulation (not shown). Data represent values obtained from 4 independent healthy PBMC donors (mean ± SD), and are representative of 3 independent experiments. (*p< 0.05, **p<0.01, ***p<0.001; statistical analysis, Mann-Whitney test).

Table S1

Pathogens involved in nosocomial pneumonia

Pathogens involved, n (%)	Nosocomial pneumonia (n=18)
Methicillin-sensitive *Staphylococcus aureus*	5 (28%)
Streptococus pneumoniae	3 (17%)
Escherichia coli	3 (17%)
Haemophius influenzae	2 (11%)
Pseudomonas aeroginosa	1 (6%)
Proteus mirabilis	1 (6%)
Other Gram negative bacteria	1 (6%)
Polymicrobial pneumonia	3 (17%)
Unknown	1 (6%)

Among the 33 brain-injured patients, there were 20 episodes of nosocomial pneumonia involving a total of 18 patients.

UNIVERSITE BRETAGNE LOIRE

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Mickael VOURC'H

Immunosubversion du lymphocytes Natural Killer par *Pseudomonas aeruginosa*

Résumé

Pseudomonas aeruginosa (PA) est un pathogène opportuniste responsable d'infections pulmonaires chez le patient immunodéprimé. Parmi ses facteurs de virulence, PA exprime le système de sécrétion de type III (SSTIII) et ses effecteurs (Exoenzymes S, T et Y). Les cellules Natural Killer (NK) jouent un rôle clef dans la défense antibactérienne et particulièrement anti-PA. Leur activation est dépendante du microenvironnement myéloïde. Les NKs présentent 2 fonctions principales : La sécrétion de cytokine et la libération de granules cytotoxiques capables de lyser les cellules anormales. Nous avons étudié l'effet de PA sur ces 2 aspects. Les manipulations *in-vitro* ont été réalisées sur cellules de volontaires sains (PBMC), NK triées à partir de PBMC et 2 lignées NK humaines (NK92 et NK3.3). Un modèle de pneumonie murine à PA nous a permis de confirmer nos hypothèses *in vivo*. L'activité cytotoxique a été évaluée par exposition des NKs à des cibles déficientes en HLA de type I (lignée 721.221).
Au cours de l'infection, la NK nécessite une stimulation IL-12 pour synthétiser de l'IFN-γ. PA augmente la réponse IFN-γ comparée à la stimulation IL-12 en condition non infectée. Cette modulation nécessite un contact direct entre PA et la cellule. Parmi les effecteurs du SSTIII, l'ExoT régule la réponse IFN-γ via un mécanisme dépendant de ERK. Concernant la fonction cytotoxique, l'activité de la NK diminuait de façon importante après infection à PA. Cette altération de fonction est multifactorielle avec notamment une modification du répertoire activateur (NKG2D) de la cellule NK et une influence du microenvironnement en particulier des lymphocytes T.

Mots clés : Cellules Natural Killer, *Pseudomonas aeruginosa*, Système de sécrétion de type III, Immunité innée, IL-12, Interferon-gamma, cytotoxicité, NKG2D.

Abstract

***Pseudomonas aeruginosa* (PA) is an opportunistic pathogen that causes lung infections in immunosuppressed patients. Among its virulence factor PA expresses the type III secretion system (T3SS) and effector Exoenzymes (ExoS, T and Y). Natural killer (NK) cell plays a key role in anti-bacterial immunity especially after PA infection. Their activation is highly dependent on their microenvironment especially on myeloid cells. NK cell exhibits two main functions: Cytokines production and cytotoxicity toward stressed or abnormal cells. We studied PA influence on these two main functions. We used peripheral blood mononuclear cells (PBMC), sorted human NK cells and two human NK cell lines (NK92, NK3.3) for *in vitro* experiments and a PA-pneumonia mouse model to validate our hypothesis *in vivo*. Degranulation was assessed by cytotoxicity assay, exposing NK cells to 721.221 targets lacking HLA-A, B and C class I antigens.**
NK cells required IL-12 priming to produce IFN-γ in response to the infection and PA increased IFN-γ activity as compared to IL-12 stimulation in non-infected conditions. The modulation of IFN-γ production after PA infection required bacteria-to-cell contact. Among T3SS and its effector, ExoT is the key regulator of IFN-γ activity through a ERK dependant signalisation. Our hypotheses were confirmed *in vivo*. Alongside with cytokine function, CD107a activity, a surrogate marker of degranulation (Cytotoxic function), dramatically decreased after NK cells infection with PA. Cytotoxicity impairment could be explained by the modification of NK cells receptor expression after infection (notably NKG2D) or accessory cells, especially T cells.

Key words: NK cells, *Pseudomonas aeruginosa*, Type III Secretion System, Innate lymphoid cells, IL-12, Interferon-gamma, Cytotoxicity, NKG2D