Action immunitaire de chimiothérapies anticancéreuses : un exemple à travers les modes d'actions des cellules "Interferon-producting Killer Dendritic Cells".

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Action immunitaire de chimiothérapies anticancéreuses : un exemple à travers les modes d'actions des cellules "Interferon-producting Killer Dendritic Cells".

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Le manque de réflexion n'est pas bon.

Quand on va trop vite, on fait des erreurs.

(Proverbes 19 :2)

Une théorie qui n'est réfutable par aucun évènement qui se puisse concevoir est dépourvue de caractère scientifique.

(Karl Popper, *Conjectures et réfutations*)

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Liste des abréviations

ADCC	Antibody Dependent Celluler Cytotoxicity
ADN	Acide DesoxyRibonucléique
AP	Antigen presenting
ARN	Acide RiboNucléique
BrdU	Bromodeoxyuridine
CTL	Cytotoxic T lymphocyte
CMH	Complexe Majeur d'Histocompatibilité
CPA	Cellule Présentatrice d'Antigène
CpG ODN	CpG Oligodeoxynucléotide
DAMP	Danger Associated Molecular Pattern
DC	Cellule Dendritique
DISC	Death-Inducing Signaling Complex
DR	Death Receptor
DTR	Diphteria Toxin Receptor
IFN	Interferon
IKDC	Interferon-producing Killer Dendritic Cell
IL	Interleukine
IM	Imatinib Mesylate
IP-10	Inducible Protein 10
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
KDC	Killer Dendritic Cell
KIR	Killer Inhibitory Receptor
KO	Knocked-Out
LAK	Lymphokine-Activated Killer
LMC	Leucémie myéloïde Chronique
LPS	lipopolysaccharide
NK	Natural Killer
NKG2D	Natural Killer Group 2 member D
NLR	Nucleotide binding and oligomerization domain-Like Receptors
Ova	Ovalbumine
PAMP	Pathogen Associated Molecular Pattern
pDC	Cellule dendritique plasmacytoïde
PRR	Pattern Recognition Receptor
TAP	Molécule de transport associé au protéasome.
TCR	T-cell receptor
TGF	Transforming Growth Factor
Th	Helper T, T auxiliaire
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand

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2 Introduction

2.1 Le cancer

Le cancer est devenu au fil des années une cause majeure de mortalité dans le monde (deuxième cause de décès après les maladies cardio-vasculaire selon l'OMS). En France, en 2003, le cancer était la cause de la mort de plus d'une femme sur cinq et près d'un homme sur trois (22,2% et 31,9% respectivement, chiffres Institut National du Cancer).

2.1.1 Une maladie de la cellule

La cellule tumorale est, par définition une cellule qui échappe aux mécanismes de régulation de son tissu d'origine. Par un phénomène darwinien (Nowell 1976), elle va acquérir un avantage prolifératif sur ses voisines, qui va s'amplifier jusqu'à lui permettre d'envahir l'organisme de l'hôte, suivant un principe simple : plus une cellule se reproduit rapidement, plus la probabilité que le patrimoine génétique de sa descendance mute augmente. Les anomalies vont donc s'accumuler avec le temps qui passe, et la cellule transformée va progressivement acquérir sept caractéristiques (Hanahan and Weinberg 2000; Zitvogel, Tesniere et al. 2006):

- Echappement à l'apoptose ;
- Désorganisation des lames basales, invasion et métastase ;
- Induction d'une néoangiogénèse ;
- Acquisition de l'immortalité ;
- Affranchissement vis-à-vis des facteurs de dépendance de son tissu d'origine ;
- Résistance aux signaux de contrôle de la croissance ;
- Echappement à la réponse immunitaire.

La tumeur est constituée de cellules tumorales. Cette lapalissade cache une vérité essentielle de cette pathologie : chaque cellule tumorale représente une menace légèrement différente de ses voisines. Ceci signifie que la masse tumorale est hétérogène, et que la multiplicité des

clones tumoraux pose le problème de la thérapie. L'échappement aux mécanismes de contrôle de la réplication de l'acide désoxyribonucléique (ADN) rend cet ADN des cellules tumorales hypermutable. Les traitements médicamenteux se heurteront donc à un problème de même ordre que ceux rencontrés par les épidémiologistes dans le cadre des maladies infectieuses : leur cible est mouvante, floue, et évolue sans cesse pour survivre.

2.1.2 Une maladie de l'hôte

La présence d'une tumeur cancéreuse va provoquer diverses perturbations dans l'organisme. Naturellement, la tumeur va physiquement perturber le fonctionnement des organes, par compression, détournement du flux sanguin, consommation des nutriments, également production de déchets métaboliques en grande quantité.

Mais les tumeurs sont également capables de modifier les réponses immunitaires globales du patient en perturbant fortement les réactions inflammatoires (Clevers 2004; Ben-Baruch 2006; Kim, Emi et al. 2006). Ceci leur permet d'échapper à l'action du système immunitaire, et participe également à la fragilisation de l'organisme(Kiessling, Wasserman et al. 1999).

2.1.3 Les traitements du cancer

Les traitements classiques du cancer suivent essentiellement un principe : éliminer physiquement les cellules tumorales. Pour ce faire, différents moyens sont utilisés.

La chirurgie consiste à enlever les tissus transformés et leur voisinage. Elle permet l'ablation des tumeurs détectables par les moyens d'imagerie, mais ne permet pas d'éliminer les micrométastases ; de plus, dans certains cas, du fait de la taille et de la localisation de la tumeur, l'exérèse n'est pas réalisable.

La radio/curiethérapie consiste à utiliser sur les cellules tumorales des rayonnements ionisants, qui, en provoquant des lésions graves de l'ADN, vont entraîner la mort des cellules irradiées. Naturellement, le contrôle de l'irradiation est primordial pour éviter de léser les tissus sains.

La chimiothérapie antitumorale vise le plus souvent une action cytotoxique ou antiproliférative : en provoquant la mort des cellules en division rapide, ou en ralentissant le cycle cellulaire, on cible les tumeurs, qui seront fragilisées. Certains médicaments, tels les sels de platine, endommagent l'ADN des cellules (agents dits alkylants). Celles qui sont en cycle rapide n'ont pas le temps de le réparer avant leur prochaine mitose, ce qui conduit à leur mort.

D’autres, tels les inhibiteurs de topoisomérases (topotécan, étoposide), ralentissent le cycle, ce qui laisse le temps aux mécanismes intracellulaires de régulation de se mettre en place, provoquant également la mise en apoptose. Certains médicaments anticancéreux visent spécifiquement un mécanisme particulier, propre à un type tumoral donné, c’est le cas de l’imatinib mésylate (voir plus loin).

2.2 La mort cellulaire

Les traitements chimiques du cancer visent à provoquer la mort des cellules tumorales. Mais comment qualifier cette mort ? On fait classiquement une différence entre la nécrose, traumatique, mort cellulaire non-programmée, passive, causée par une lésion majeure de la cellule, telle que la rupture des membranes, la dessiccation ou la cuisson ; et la mort cellulaire programmée, liée à un stress, et qui est un comportement actif de la cellule. S’il existe plusieurs modalités de mort cellulaire programmée (Broker, Kruyt et al. 2005) (Kroemer, Galluzzi et al. 2009), une nous intéresse particulièrement : l’apoptose.

2.2.1 Nécrose

La nécrose est largement considérée comme un phénomène subi par la cellule suite à un stress physico-chimique intense, que ce soit un choc thermique ou un choc oxydatif brutal, mais aussi l’interaction entre le Tumor Necrosis Factor (TNF) et son récepteur. Les mécanismes moléculaires sont moins connus que ceux de l’apoptose, mais le rôle du calcium et des espèces réactives de l’oxygène semble avéré (Festjens, Vanden Berghe et al. 2006). La nécrose est caractérisée par une rupture rapide des membranes cellulaires. La cellule morte va se vider et libérer son contenu dans l’espace intercellulaire. Ceci provoquera une inflammation, du fait de la présence dans les molécules nucléaires ou cytosoliques de motifs associés au danger (danger-associated molecular patterns, DAMP) (Seong and Matzinger 2004; El Mezayen, El Gazzar et al. 2007).

Il est important de noter que les autres types de morts cellulaire, y compris l’apoptose, sont toujours suivis par une nécrose secondaire. De même, dans certaines conditions qui provoqueraient l’apoptose des cellules, l’inhibition des caspases favorise la mort par nécrose (Vandenabeele, Vanden Berghe et al. 2006). Enfin, des cellules tumorales restent sensibles à la nécrose, même lorsqu’elles sont résistantes à l’apoptose. Ceci permet des stratégies anti-tumorales par hyperthermie, visant à provoquer la nécrose par choc thermique, que ce soit par micro-onde, bain chaud ou ultrasons (Storm and Morton 1983), ou encore par administration

d’éthanol dans le traitement de tumeurs ou métastases hépatiques de petite taille (Giovannini 2002).

2.2.2 Apoptose

L’apoptose est un sujet de recherche très étudié depuis plus de 35 ans (Kerr, Wyllie et al. 1972). Elle correspond à un phénomène complexe par lequel une cellule mourante va se désagréger de manière la plus « silencieuse » possible. Son ADN et ses protéines seront fragmentés, et ses organelles séparées les unes des autres dans des vésicules issues de la membrane plasmique : les corps apoptotiques, qui seront aisément pris en charge par les cellules phagocytaires (Barker, Erwig et al. 1999).

Ce phénomène est primordial dans le développement et le fonctionnement de l’organisme, tant durant la morphogénèse animale (Hengartner and Horvitz 1994; Domingos and Steller 2007) que la régulation des populations cellulaires, telle que la contraction clonale ou la mort induite par hyperactivation des lymphocytes (Donjerkovic and Scott 2000; Krammer, Arnold et al. 2007).

Le fait pour une cellule de pouvoir résister à la mise en apoptose est une des étapes indispensable de la carcinogénèse (Hanahan and Weinberg 2000).

2.2.3 Régulation et mécanisme de l’apoptose

L’apoptose est donc un phénomène actif de réponse à un stress. Ses acteurs majeurs sont les mitochondries (Cheng, Leach et al. 2008) et une famille de protéines nommées caspases (Hengartner and Horvitz 1994; Salvesen and Riedl 2008). Pour éviter une mort cellulaire intempestive ou au contraire la survie prolongée d’une cellule inutile, ce phénomène est finement régulé dans une cellule saine.

L’apoptose dite intrinsèque est déclenchée par un stress interne à la cellule, tel qu’une lésion de l’ADN non réparée. Dans ce cas, la protéine p53 (Crawford, Pim et al. 1981), impliquée dans le contrôle du cycle cellulaire (Mercer, Avignolo et al. 1984), provoquera l’inhibition des protéines inhibitrices de l’apoptose de la famille de Bcl-2 (Tsujimoto, Cossman et al. 1985; Haupt, Berger et al. 2003; Heiser, Labi et al. 2004). La mort, ou plutôt le signal de mort, suivra alors la voie mitochondriale : les protéines de la famille Bax provoqueront le relargage du cytochrome c dans le cytosol, qui lui-même formera avec Apaf-1 et la caspase 9

l’apoptosome, un complexe enzymatique qui activera les caspases effectrices, les caspases 3, 6 et 7 (Green and Reed 1998; Bao and Shi 2007; Kumar 2007; Jeong and Seol 2008).

L’apoptose extrinsèque est provoquée par la liaison d’un ligand sur un récepteur de mort, de la famille du récepteur au TNF, que sont le TNF-R, le Fas et les récepteurs du TRAIL (TNF-related apoptosis inducing ligand). Cette voie de déclenchement apoptose est mise en jeu dans les réactions immunitaires. La transduction du signal, via les domaines de mort FADD (Fas-associated death domain) et la formation du DISC (death-inducing signaling complex), provoquera l’activation des caspases initiatrices : les caspases 8 et 10 (Ashkenazi and Herbst 2008).

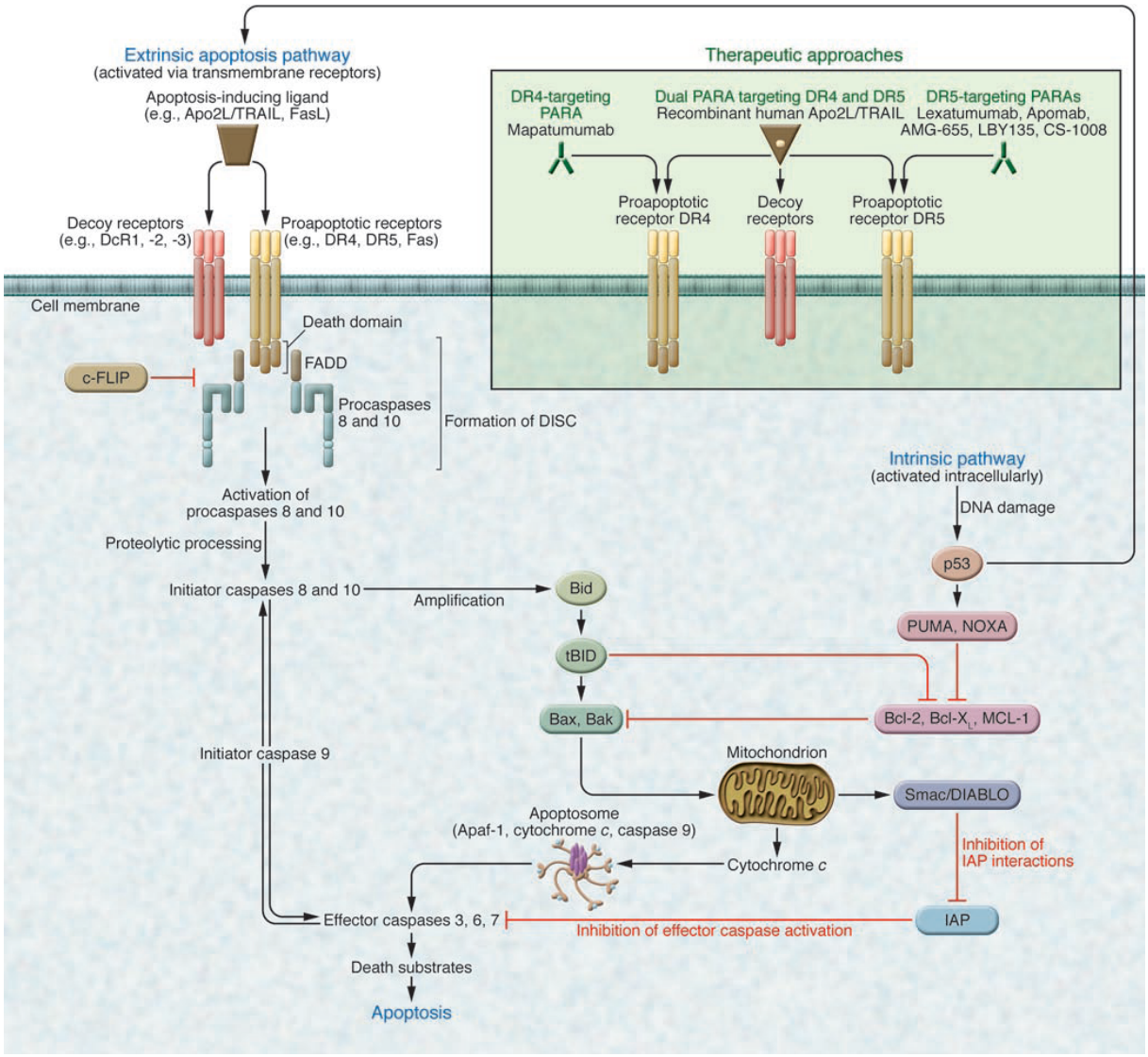

Figure 1 : Induction de l'apoptose intrinsèque ou extrinsèque (Ashkenazi and Herbst 2008).

Deux cas peuvent alors se trouver. Dans certaines cellules il existe une forte activité de la caspase 8, et celle-ci sera alors capable d'activer les caspases effectrices (type I, indépendant des mitochondries), ou bien le DISC n'a produit que peu de caspase 8 active, et une amplification du signal sera nécessaire pour provoquer l'apoptose de la cellule (type II, dépendant des mitochondries). L'amplification passera par la protéine Bid qui, une fois clivée, provoquera, de manière similaire à la voie intrinsèque, l'inhibition des molécules de type Bcl-2 (anti-apoptotique) et l'activation des molécules de type Bax (pro-apoptotique). Ces deux actions permettront le relargage de cytochrome c des mitochondries dans le cytosol, entraînant l'activation des caspases effectrices (Haupt, Berger et al. 2003; Kumar 2007).

Le but des traitements anticancéreux cytotoxiques est de provoquer l'activation de la voie intrinsèque de l'apoptose. Malheureusement, les cellules tumorales semblent capables de s'adapter à cette pression de sélection : ainsi les cellules tumorales peuvent devenir porteuses de mutations inactivatrices pour p53 (un cancer sur deux porte une mutation de p53 (Lee and Bernstein 1995)) et/ou Bax (LeBlanc, Lawrence et al. 2002), et exprimer de grandes quantités de Bcl-2 (anti-apoptotique), rendant de moins en moins efficace la voie intrinsèque de l'apoptose, mais également la voie extrinsèque des cellules de type II, ce qui diminue d'autant leur sensibilité aux traitements classiques du cancer (Strasser, Harris et al. 1994; Soengas, Alarcon et al. 1999). Pareillement, l'accumulation de mutations portant sur les gènes codant pour les protéines de la famille Bcl-2 entrent en synergie avec des mutations d'autres pro-oncogènes tels c-myc pour l'induction de cancers (Strasser, Harris et al. 1990). Il est alors tentant de considérer la possibilité de provoquer la mort par la voie extrinsèque de type I, c'est-à-dire une apoptose qui n'est pas régulée par Bcl-2. C'est dans cette optique que différentes études montrent un intérêt de ligands de récepteurs de morts (Death Receptor, DR) DR4 et DR5 (Ashkenazi and Herbst 2008), tels que des protéines recombinantes (TRAIL soluble) ou des anticorps monoclonaux anti-DR4 (mapatumumab) ou anti-DR5 (lexatumumab). C'est aussi un intérêt majeur des cellules immunitaires capables d'exprimer TRAIL : voir plus loin.

Les mécanismes de l'apoptose sont donc essentiels à la fois à la protection contre les néoplasies et au bon fonctionnement du système immunitaire.

2.3 Immunité anti-tumorale

Le système immunitaire dans son aspect le plus général correspond à l'ensemble des mécanismes par lequel l'organisme se défend contre les agressions. Nous nous concentrerons ici sur les acteurs impliqués au site tumoral.

2.3.1 Généralités

L'idée selon laquelle le système immunitaire est impliqué dans la lutte contre les tumeurs spontanées, n'est pas nouvelle (Burnet 1957; Ehrlich (1909)), mais est toujours sujette à polémique de nos jours (Qin and Blankenstein 2004). D'une part, certains considèrent qu'aucune pression de sélection ne peut favoriser un tel système, puisque la plupart des cancers surviennent à un âge avancé, c'est-à-dire après que les patients ont eu leurs enfants (Blankenstein 2007). D'autres soutiennent l'idée que la formation spontanée de tumeurs est

tenue en échec tout au long de la vie par le système immunitaire (Dunn, Old et al. 2004a; Dunn, Old et al. 2004b). Cette immunosurveillance s'articulerait sur trois phases :

- L'élimination : les cancers spontanés sont reconnus et détruits par le système immunitaire.
- L'équilibre : les cancers spontanés sont sous contrôle du système immunitaire. Cette phase consisterait en un équilibre dynamique, pendant lequel le système immunitaire modifierait, par la pression de sélection qu'il induit, les caractéristiques de la tumeur. C'est le concept d'édition de la tumeur par l'immunité.
- L'échappement : une tumeur évolue suffisamment pour échapper au contrôle exercé par le système immunitaire. La maladie cancéreuse débute alors.

Il y a quelques arguments expérimentaux en faveur de l'immunosurveillance du cancer (Shankaran, Ikeda et al. 2001; Koebel, Vermi et al. 2007; Waldhauer and Steinle 2008) ; cependant les opposants de cette hypothèse considèrent que les modèles utilisés ne permettent pas de conclure à une action favorable du système immunitaire dans la lutte contre les cancers spontanés (Willimsky and Blankenstein 2007). On peut cependant remarquer qu'il existe des maladies auto-immunes induites par une tumeur (lors d'un syndrome paranéoplasique), ce qui est parfois interprété comme la preuve que le système immunitaire parvient à produire une réponse immunitaire efficace, mais que la tumeur y échappe (Thirkill 2006).

2.3.1.1 Immunité innée

L'immunité innée correspond aux mécanismes qui préexistent à la menace. Ce sont des cellules capables de réagir à un stimulus précis, qui peut être partagé par de nombreux types de menaces, et qui n'ont besoin que de peu de stimulation avant d'être efficaces. Elles reconnaissent des motifs moléculaires associés au danger (Matzinger 1994), les DAMPs, ou aux organismes pathogènes, les PAMPs (pathogen-associated molecular patterns). Les récepteurs de ces motifs sont nommés récepteurs de reconnaissance de motifs (Pattern Recognition Receptor, PRR) (Medzhitov and Janeway 1997).

L'immunité innée compte dans ses rangs des cellules myéloïdes phagocytaires, capables de prendre en charge les débris, les particules (tels des virions ou des bactéries) et les cellules mourantes, mais également de gérer les réactions inflammatoires. Il s'agit des granulocytes et

des macrophages, ainsi que les cellules présentatrices de l'antigène (CPA), dont les cellules dendritiques (DC, dendritic cells). Ils jouent un grand rôle dans la lutte contre les infections. Lorsqu'une CPA engage ses PRR avec des DAMP, elle s'active et devient capable d'activer à son tour les cellules de l'immunité adaptative (Medzhitov and Janeway 1997).

Parmi les acteurs de l'immunité innée se trouvent également des cellules lymphoïdes, capables de réagir très vite face à une cellule vivante anormale : les lymphocytes tueurs naturels (NK). Subtilement régulée, leur activation obéit à un ensemble d'interactions cellulaires que nous développeront plus loin.

2.3.1.2 Immunité adaptative

L'immunité adaptative représente l'ensemble des mécanismes qui s'éduquent face à la menace de manière dépendante d'un antigène. Il s'agit de cellules lymphocytaires, qu'il est possible en résumé de répartir en trois populations :

- Les cellules B sont capables de réagir à un antigène natif, c'est à dire de détecter une conformation tridimensionnelle. Une fois sélectionnés par leur antigène, les lymphocytes B deviennent des plasmocytes, qui produiront de grandes quantités d'anticorps spécifiques de l'antigène, qui participeront à l'élimination de la menace qui y est liée (LeBien and Tedder 2008).

- Les cellules T $CD8^+$ sont capables de réagir à un antigène présenté dans les molécules du complexe majeur d'histocompatibilité de classe I (CMH-I). Elles sont, après sélection et activation, capable de provoquer la lyse des cellules présentant ce même antigène, présenté dans les molécules du même CMH-I (Lin, Selin et al. 2000).

- Les cellules T $CD4^+$ fonctionnent sensiblement de la même façon que les cellules T $CD8^+$, mais reconnaissent un antigène présenté dans les molécules du CMH de classe II. Leur rôle principal est d'aider la mise en place d'une réponse immunitaire efficace et durable. En effet, leurs sécrétions cytokiniques moduleront la réponse immunitaire adaptative en fonction du type de menace détectée ; et l'interaction entre les cellules T $CD4^+$ et les cellules T $CD8^+$ ou les cellules B permettra la formation d'une mémoire immunitaire, qui permettra à l'organisme qui a survécu à la menace de la combattre plus efficacement si elle se reproduit (Zhu and Paul 2008).

L'orientation de la réponse immunitaire passe par différents types de cellules T auxiliaires (Mosmann, Cherwinski et al. 1986; Zhu and Paul 2008) (T $CD4^+$ helpers, Th). Il est classiquement considéré que la réponse immunitaire peut être de 2 types (Mosmann, Cherwinski et al. 1986) :

- Le type 1 qui est adapté à la lutte contre une menace cellulaire ou intracellulaire, telle des cellules infectées par un virus ;
- Le type 2 qui correspond à une menace extracellulaire, telle une infestation parasitaire.

Dans le cas du cancer, il semble naturellement plus intéressant pour le patient qu'il existe une réaction de type 1 dirigée contre les cellules tumorales. Et en effet, la présence de T effecteurs $CD8^+$ cytotoxiques (CTL) est cliniquement associée à une meilleure survie et une dissémination réduite dans les cancers colorectaux (Pages, Berger et al. 2005; Galon, Costes et al. 2006).

On note les deux types décrits ci-dessus Th1 et Th2, pour T helpers 1 et T helpers 2. L'induction de ces réponses dépend des signaux reçus par les CPA à l'origine de la sélection ; ainsi l'interleukine (IL) -12 conditionnera une DC à induire une réponse Th1, et donc des cellules T cytotoxiques sécrétant de l'IFN-gamma (Hsieh, Macatonia et al. 1993) ; et réciproquement l'exposition d'une DC à l'IL-4 induit la différentiation de cellules Th2, très sécrétrices (entre autre d'IL-4) (Le Gros, Ben-Sasson et al. 1990), favorisant les réponses humorales.

Parmi les régulations, il faut noter que la présence d'IL-4 empêche la différentiation des cellules Th1, et l'IL-12 celle des cellules Th2. Il convient également de signaler la présence de deux autres groupes de cellules T auxiliaires importants : les lymphocytes T régulateurs (Sakaguchi, Sakaguchi et al. 1995) et les Th17 (Harrington, Hatton et al. 2005; Park, Li et al. 2005), qui sont impliqués respectivement dans la tolérance périphérique et la lutte contre certains infections (bacterienne et fongiques) (Weaver, Harrington et al. 2006). Les cellules Th17 sont également impliquées dans des pathologies inflammatoires auto-immunes (telle l'encéphalomyélite auto-immune expérimentale (Thakker, Leach et al. 2007)).

2.3.1.3 La présentation de l'antigène

L'immunité adaptative, pour déclencher une réponse spécifique d'un antigène, nécessite une présentation de l'antigène. Les lymphocytes T naïfs sont en effet incapables de détecter un antigène natif en conditions normales.

Chaque clone de cellule T possède un récepteur de cellule T (T Cell Receptor, TCR) unique, capable de reconnaître un épitope, une séquence peptidique précise dans un contexte de molécules du CMH précis. Mais la simple rencontre de cette combinaison peptide/CMH ne suffit pas à provoquer une réaction des lymphocytes naïfs. Il faut en effet que la cellule portant ce complexe moléculaire soit une cellule présentatrice d'antigène dite « professionnelle ».

Ces CPA professionnelles sont en effet capables, quand elles sont pleinement activées, non seulement de présenter l'antigène, mais également d'exprimer des molécules de co-stimulation, qui activeront les cellules T, et de produire des cytokines inflammatoires (IL-1, IL-6, Interféron (IFN) …) et des cytokines de contrôle de l'induction des réponses effectrices (IL-4, IL-5, IL-10, IL-12, TGF-beta) (Medzhitov and Janeway 1997).

Suivant les stimuli qui ont accompagné la captation de l'antigène et son apprêtement (processus par lequel une CPA dégrade une protéine en peptides et les associe à des molécules du CMH), les cellules T reconnaissants l'antigène seront activés plus ou moins fortement, cette activation allant de l'anergie (les cellules T ne réagiront plus à leur antigène) à la mort par hyperactivation en passant par l'expansion clonale de lymphocytes T effecteurs et mémoires. Selon les molécules de co-stimulation et les cytokines produites par la CPA, les lymphocytes T seront induits dans différentes modalités de réponse effectrice. Schématiquement, on considère donc qu'il existe deux grands types de réponse : les réponses de type 1 (Th1, Tc1, pour helper T cells et cytotoxic T cells) se composent de cellules cytotoxiques, et sont adaptées aux menaces intracellulaires, comme une infection virale ou une transformation cancéreuse ; les réponses de type 2 (Th2, Tc2) se composent pour leur part de cellules produisant de grandes quantités de facteurs solubles, encourageant les réponses anticorps, et sont adaptées à des menaces extracellulaires, comme une infection bactérienne ou une toxine.

Pour caractériser une CPA professionnelle, on peut relever différentes propriétés (Medzhitov and Janeway 1997) :

- Capacité de présentation croisée aux cellules T $CD8^+$, c'est-à-dire : présentation en CMH-I des antigènes exogènes ;
- Capacité de présentation aux cellules T $CD4^+$, c'est-à-dire expression des molécules de CMH-II ;
- Capacité d'induire une réponse lymphocytaire primaire, c'est-à-dire d'induire l'expansion clonale et l'activation de cellules T naïves.

L'archétype de CPA professionnelle est la cellule dendritique.

2.3.1.4 Les cellules dendritiques

Les cellules dendritiques (DC) sont des cellules de l'organisme spécialisées dans la captation des antigènes périphériques et leur présentation aux lymphocytes dans les organes lymphoïdes secondaires.

Ainsi, elles sont capables de capter des antigènes solubles ou particulaires, de les dégrader dans leurs endosomes et de présenter les peptides générés dans des molécules des CMH-I et CMH-II (Savina and Amigorena 2007).

2.3.1.4.1 Captation de l'antigène

Les DC immatures sont capables de prendre en charge des échantillons de leur environnement : protéines solubles via la macropinocytose (Sallusto, Cella et al. 1995), ainsi que des particules, bactéries et corps apoptotiques notamment (Albert, Sauter et al. 1998) par la phagocytose (Reis e Sousa, Stahl et al. 1993). Les DC sont également capables d'endocytose des complexes immuns, via l'expression des récepteurs aux fragments constant des anticorps de types IgE et IgG (Sallusto and Lanzavecchia 1994).

2.3.1.4.2 Dégradation de l'antigène

Une fois dans le compartiment endosomal, les protéines captées vont subir une protéolyse lente et relativement limitée. Contrairement aux granulocytes neutrophiles ou aux macrophages, chez lesquelles le pH des endosomes varie rapidement et considérablement,

chez les DC le pH des endosomes se maintient autour de pH=7 à 7.5, en produisant des radicaux oxygénés de manière continue par l'enzyme NADPH oxidase, NOX2 (Savina, Jancic et al. 2006). Dans ces conditions de pH, la plupart des enzymes protéolytiques des lysosomes sont inactives ; la dégradation des protéines se fait essentiellement via la cathepsine S. Le but de la protéolyse dans les DC est de générer des fragments peptidiques capables de se lier aux molécules du CMH.

2.3.1.4.3 Présentation de l'antigène

Les molécules du CMH-I sont présentes dans le réticulum endoplasmique (RE) de la cellule. Dans la plupart des cellules, ces molécules du CMH-I sont chargées avec des peptides endogènes, issus de la dégradation de protéines endogènes par le protéasome (Townsend, Bastin et al. 1988; Brown, Driscoll et al. 1991; Michalek, Grant et al. 1993; Grant, Michalek et al. 1995).

Le CMH-II est présent dans le compartiment endosomal ; sa promiscuité avec les antigènes captés par la cellule permet d'assurer le chargement. Lors de la maturation des DC, le pH endosomal va chuter, permettant la digestion, sous la dépendance de la cathepsine S, à la fois des antigènes et de la chaperone des molécules du CMH-II, permettant leur chargement (Driessen, Bryant et al. 1999; Shi, Villadangos et al. 1999) -et conduisant à la présentation des débris dans les molécules du CMH-II (Trombetta, Ebersold et al. 2003).

Mais en ce qui concerne la présentation croisée, c'est-à-dire la présentation via les molécules du CMH-I d'antigènes exogènes, le mécanisme semble plus complexe : les protéines dégradées dans les endosomes par les protéases actives à pH=7 génèrent des peptides de plusieurs dizaines d'acides aminés. Ces polypeptides sont exportés dans le cytosol, probablement par la protéine Sec61 (Savina and Amigorena 2007), où ils sont pris en charge et dégradés par le protéasome, et suivent la même voie que les peptides endogènes. Ils sont donc injectés via les molécules de transport associées au protéasme (TAP) dans le réticulum endoplasmique, où ils sont chargés sur les molécules du CMH-I (Van Kaer, Ashton-Rickardt et al. 1992; Shastri, Cardinaud et al. 2005).

Il est important de noter que selon les cellules, le protéasome ne produira pas les mêmes peptides à partir des mêmes protéines. Les cellules dendritiques et les autres cellules présentatrices d'antigène, de même que des cellules soumises à l'action de l'IFN-gamma

(Tanaka 1994), possèdent ainsi un immunoprotéasome et les peptides produits ne seront pas les mêmes que dans un autre type cellulaire. Ainsi, des CTL spécifiques d'un antigène ubiquitaire pourront-ils reconnaitre des cellules tumorales rénales mais pas des cellules B (Morel, Levy et al. 2000).

2.3.1.4.4 Maturation

Selon leur état de maturation et les signaux qui les auront influencées, les DC ne porteront pas le même message en accompagnement des antigènes qu'elles présentent. En effet, un des importants rôles des DC est de préserver la tolérance périphérique, tout autant que de déclencher des réactions immunitaires.

D'un point de vue phénotypique, une DC mature augmentera l'expression de ses molécules de CMH, de même que l'expression membranaire de molécules de co-stimulation. Cette maturation s'accompagne aussi de la dégradation des antigènes captés (Trombetta, Ebersold et al. 2003) et de l'arrêt des fonctions de capture de l'antigène. Une DC mature ne fait plus que présenter les antigènes qu'elle a déjà acquis (Schuler and Steinman 1985; Romani, Koide et al. 1989), tandis que la présentation par une cellule immature est nettement moins efficace (Romani, Koide et al. 1989), car elle n'exprime pas de molécules co-stimulatrices.

L'expression des molécules de co-stimulation est en effet déterminante. En effet, la « simple » liaison d'un TCR sur un complexe peptide/CMH ne suffit pas à induire la réaction du lymphocyte T (van der Merwe and Davis 2003). S'il n'y a que ça, les cellules T spécifiques de cet antigène deviennent anergiques par défaut d'activation. Par contre, s'il y a un dialogue entre les cellules T et les DC impliquant les molécules de co-stimulation, la réponse des cellules T pourra être modulée en rapport avec le danger initiateur.

Le phénomène de co-stimulation implique des protéines du coté lymphocytaire et dendritique, chacune pouvant après engagement transduire un signal. Ainsi, la liaison CD28/CD80 ou CD28/CD86, une des interactions étudiées depuis longtemps, permet l'activation des cellules T (Linsley and Ledbetter 1993). Ceux-ci vont, entre autre, exprimer la molécule CD40-L, tandis que les cellules dendritiques vont exprimer CD40. L'interaction CD40-L/CD40 conduira à la pleine activation des deux cellules : les cellules T pourront débuter leur prolifération clonale et maturation d'affinité, ainsi que leur différenciation (Bennett, Carbone et al. 1998; Grewal and Flavell 1998), tandis que la DC sera pleinement mature (et

augmentera encore l'expression des molécules CD80 et CD86) et pourra activer plus rapidement et plus fortement d'autres cellules T qui reconnaitraient les antigènes qu'elle présente (Caux, Massacrier et al. 1994; Grewal and Flavell 1998; Zang and Allison 2007).

La maturation des DC dépend des signaux qu'elle reçoit dans sa phase immature. Ainsi, l'IL-10 l'inhibera-t-elle (Koppelman, Neefjes et al. 1997), tandis que d'autres cytokines l'accéléreront (tel l'IL-1 ou le TNF-alpha) (Banchereau, Briere et al. 2000). Les récepteurs de signaux de danger (PRR) sont également des facteurs de maturation, parmi lesquels les récepteurs de type NLR (nucleotide binding and oligomerization domain-like receptors) (Franchi, Park et al. 2008), et les Toll-like receptors (TLR), codés par des gènes homologues de Toll, décrits chez la Drosophile comme impliqués dans la lutte contre les infections fongiques (Lemaitre, Nicolas et al. 1996). Ces homologues chez les mammifères font partie des PRR et sont spécialisés dans la reconnaissance de PAMP, tels que le Lipopolysaccharide (LPS), la flagelline, les ARN doubles brin, ou encore les motifs CpG non méthylés (reconnus respectivement par les TLR-4, -5, -7/8 et -9) (Iwasaki and Medzhitov 2004), mais également par des produits endogènes. On a ainsi décrit que certaines protéines de choc thermique, de même que les LDL oxydées, la fibronectine ou l'alarmine HMGB1 (high-mobility-group box-1) étaient des ligands de TLR-4 (Seong and Matzinger 2004).

La présence de ligands de TLR induit la maturation des cellules dendritiques, et la nature de ces ligands les influencera grandement. Ainsi, selon la nature du danger, le profil des TLR engagés changera, et modifiera la réponse lymphocytaire induite (Iwasaki and Medzhitov 2004). On peut ainsi décrire des synergies entre ligands de TLR conduisant à une maximisation de profil Th1 (Napolitani, Rinaldi et al. 2005).

L'interaction entre les DC et d'autres cellules de l'immunité innée peut également conduire à leur maturation (Munz, Steinman et al. 2005). Les cellules NK, T gamma-delta, NKT sont ainsi, comme les cellules T, capables d'établir un dialogue avec les DC, menant à la maturation des DC et à l'activation des lymphocytes impliqués. Une cellule dendritique en cours de maturation produira ainsi de l'IL-2 (Schartz, Chaput et al. 2005), de l'IL-12 (Macatonia, Hosken et al. 1995), de l'IL-15 (Jonuleit, Wiedemann et al. 1997) et de l'IFN-alpha (Eloranta, Sandberg et al. 1997), qui sont toutes des cytokines importantes pour l'activation des lymphocytes. Ainsi, le dialogue entre les DC et les cellules NK implique de nombreuses modalités (Moretta 2002; Borg, Jalil et al. 2004; Zitvogel, Terme et al. 2006;

Terme, Chaput et al. 2008), et participe à l'activation de ces deux populations, ou à la lyse des DC immatures par les NK (Wilson, Heffler et al. 1999). Par ailleurs, des études ont montré que les NK dépendent de la présence des DC pour leur homéostasie, via l'IL-15 (Hochweller, Striegler et al. 2008), et que cette interaction était en partie contrôlée par les Treg (Terme, Chaput et al. 2008).

2.4 Immunogénicité de la chimiotherapie

2.4.1 Concept

La notion prédominante concernant le système immunitaire quand on traite un patient cancéreux, c'est que la plupart des chimiothérapies agissent seules et ont tendance à détruire non seulement les cellules tumorales, mais également d'autres cellules, telles les cellules épithéliales de l'intestin, les cellules des follicules pileux ou les cellules hématopoïétiques, entrainant des effets secondaires nombreux et invalidants. Cette action intrinsèque, toxique sur les cellules à prolifération rapide, exclu à priori toute action bénéfique du système immunitaire du patient lors de la chimiothérapie, car on vise comme objectif thérapeutique de traiter le patient avec la dose maximale tolérable, afin de profiter au mieux de l'effet toxique direct sur la tumeur. Dans ces conditions, les traitements cytotoxiques sont à priori considérés comme immunosuppresseurs (Mackall 2000).

L'utilisation du système immunitaire dans le traitement des tumeurs pourrait néanmoins se montrer d'une efficacité réelle : autogène, évolutif et adaptable, contrairement aux médicaments nécessairement xénobiotiques, sélectifs et finis. D'où l'interrogation : peut-il y avoir un effet à médiation immunitaire de la chimiothérapie anticancéreuse ?

L'action positive du système immunitaire lors du traitement du cancer est déjà connue. Il est également admis que la plupart des cancers se protègent contre cette action. Une hypothèse courante est que des versants régulateurs et inhibiteurs de l'immunité prennent le pas sur les bras cytotoxiques lors du développement des tumeurs. Une des idées est donc de considérer que la sensibilisation du système immunitaire a bien eu lieu, mais que l'immunosuppression induite par la tumeur la masque. Il existe dans ce cadre deux stratégies actuellement en développement : l'une concerne les lymphocytes T régulateurs, l'autre les cellules myéloïdes suppressives. Les premiers sont inhibés par de faibles doses de cyclophosphamide (Ghiringhelli, Menard et al. 2007), les seconds par de la gemcitabine (Suzuki, Kapoor et al.

2005), deux médicaments de chimiothérapie anticancéreuse bien connus. Ces deux stratégies permettraient de diminuer l'influence des phénomènes inhibiteurs, révélant ainsi l'action antitumorale spontanée du système immunitaire. De fait, il a été mis en évidence dans des modèles murins une synergie entre l'administration de gemcitabine et de médicaments immunostimulants (anticorps anti-CD40L) (Nowak, Robinson et al. 2003). Plus récemment, une étude a montré un effet immunomodulateur du docétaxel, qui, sans agir sur les lymphocytes T régulateurs, augmente les capacités des lymphocytes T $CD8^+$ (Garnett, Schlom et al. 2008).

Une autre démarche consiste à considérer que le système immunitaire peut profiter de l'action des médicaments sur la tumeur elle-même. Que ces médicaments provoquent un stress, l'apoptose, ou une sélection de variants, le système immunitaire pourrait en tirer parti. Connaître les modalités de ce phénomène pourrait ouvrir la voie à de nouvelles stratégies thérapeutiques, visant l'optimisation des réponses immunitaires.

2.4.2 Notions expérimentales

La notion expérimentale selon laquelle la chimiothérapie antitumorale pouvait agir en partie grâce à l'hôte, en dehors de son activité toxique intrinsèque sur la tumeur, n'est pas nouvelle. En effet, dès 1973, il a été montré que la doxorubicine, bien que moins active que la daunorubicine *in vitro* sur une tumeur donnée (la leucémie P-288 dans des souris DBA/2), l'est davantage *in vivo* (Schwartz and Grindey 1973). Ils montrèrent également que cet effet disparait chez des souris immunodéprimées. Ceci impliquait une activité du médicament via l'hôte, et plus spécifiquement via son système immunitaire. Puis, en 1977, Orsini montra que le traitement par les anthracyclines augmentait la cytotoxicité des splénocytes de souris contre la tumeur, ce qui indiquait une action directe de ces médicaments sur les cellules du système immunitaire (Orsini, Pavelic et al. 1977).

Par la suite, d'autres études apportèrent de nouvelles notions : il existe une action antitumorale des anthracyclines indépendante de leur effet cytotoxique direct. Ainsi, une étude montra qu'une lignée tumorale, résistante *in vitro* à la doxorubicine, y était sensible *in vivo* (Maccubbin, Wing et al. 1992). Maccubbin et al montrèrent une variété d'action de la doxorubicine sur diverses fonctions effectrices du système immunitaire : cytotoxicité augmentée des cellules T, macrophages, lyse augmentée via l'ADCC (antibody dependent

cellular cytotoxicity), activité diminuée des NK et des LAK (Salup, Back et al. 1987; Maccubbin, Cohen et al. 1990; Maccubbin, Wing et al. 1992).

Par la suite dans les années 1990, essayant de profiter de cette action immunomodulatrice des anthracyclines, des essais furent conduits qui montrèrent la synergie entre des anthracyclines et d'autres médicaments ciblant l'immunité : l'IL-2 (Lumsden, Codde et al. 1992; Ho, Maccubbin et al. 1993; Lumsden, Codde et al. 1996), l'IL-12 (Zagozdzon, Golab et al. 1998), le TNF-alpha (Ehrke, Verstovsek et al. 1998; Ehrke, Verstovsek et al. 2000). Ces études permirent de montrer un rôle des cellules T $CD8^+$ et une corrélation entre l'activité CTL et la régression tumorale, chez la souris. Quelques essais cliniques associant l'épirubicine et le BCG, l'IL-2 ou l'IFN de type I montrèrent un intérêt dans le traitement du cancer de la vessie, mais l'index thérapeutique trop étroit de ces produits ne permit pas leur développement (Naglieri, Gebbia et al. 1998; Le Cesne, Vassal et al. 1999).

Il fallu attendre 2005 et l'étude par Casares *et al* pour une première approche mécanistique de l'effet hôte-dépendant des anthracyclines (Casares, Pequignot et al. 2005). Ces auteurs purent ainsi montrer que la doxorubicine induisait une mort tumorale « immunogène », c'est-à-dire que les cellules mortes induisaient une réponse lymphocytaire, et que c'était le type de mort induit par les anthracyclines qui déterminait cet effet, plus que la présence du médicament lui-même. Par des expériences d'inhibition des caspases, les auteurs purent montrer que la mort par apoptose induite par les anthracyclines permet aux cellules tumorales d'être phagocytées par les DC de l'hôte, ce qui conduit à l'induction d'une réponse lymphocytaire T $CD8^+$, capable de rejeter une tumeur de manière antigène-spécifique (voir Annexes).

D'autres modalités d'action d'un traitement par chimiothérapie font intervenir spécifiquement les NK : c'est le cas de l'imatinib mesylate, développé plus loin.

2.5 Antigen-presenting-NK

2.5.1 Les cellules natural killer

2.5.1.1 Généralités

Les cellules NK sont capables de lyser leurs cellules cibles sans activation préalable, au contraire des cellules T qui doivent auparavant avoir été induites. Cette activation relève

cependant d'une régulation fine. Les cellules NK sont en effet pourvues de récepteurs inhibiteurs et de récepteurs activateurs.

Les récepteurs inhibiteurs utilisent un mécanisme commun : ils comportent tous dans leur domaine intracytoplasmique une séquence ITIM (Immunorécepteur Tyrosine-based Inhibition Motif) (Olcese, Cambiaggi et al. 1997; Vely and Vivier 1997). Lorsqu'un récepteur à ITIM (appelé KIR, pour Killer Inhibitory Receptor) est engagé, il induit l'activation de protéines de la famille Src, qui vont phosphoryler les sites ITIM. Ceux-ci vont alors recruter les phosphatases intracellulaires SHP-1 et/ou SHP-2 et/ou SHIP responsables de l'inhibition du lymphocyte NK (Binstadt, Brumbaugh et al. 1996; Burshtyn, Scharenberg et al. 1996; Olcese, Cambiaggi et al. 1997). Ces phosphatases vont provoquer l'inactivation des protéines activatrices, telles que Vav-1(Stebbins, Watzl et al. 2003). Les principaux ligands des KIR sont les molécules du CMH de classe I (Karlhofer, Ribaudo et al. 1992).

Les récepteurs activateurs sont généralement constitués par une association de deux protéines : l'une possède un domaine intracellulaire court sans activité de transduction, et l'autre possède au contraire un domaine intracytoplasmique contenant une séquence ITAM (immunorécepteur tyrosine-based activation motif). Lors de l'engagement des récepteurs, les séquences ITAM seront phosphorylées par des protéines de la famille Src ; par la suite des protéines de la famille Syk/zap70 sont recrutées et activées.

Un récepteur activateur très important dans la lutte contre les tumeurs est NKG2D (natural killer group 2 member D), dont les ligands sont souvent présents sur les cellules tumorales. Les souris déficientes en NKG2D souffrent d'un défaut d'immunosurveillance des tumeurs (Guerra, Tan et al. 2008). Chez la souris, les récepteurs CD161c (NK1.1), CD16/CD32 et NKP46 sont également des récepteurs activateurs.

L'activation des NK qui rencontre une cible potentielle résulte d'une intégration des signaux activateurs et inhibiteurs. Dans le cas où la « cible » est une cellule normale, la présence de molécules du CMH-I à la surface engage les KIR des NK, et il n'y a pas de ligands de récepteur activateur : la cellule n'est pas lysée. Dans le cas de certaines cellules tumorales (ou de cellules infectées par un virus), la présence de ligands de récepteurs activateurs, liée à la diminution de l'expression des molécules du CMH-I, permet l'activation des NK, et la lyse des cibles. Dans de nombreux cas cependant, les cellules tumorales expriment à la fois des

ligands de KIR (comme des molécules du CMH), et des ligands de récepteurs activateurs, comme les ligands de NKG2D, tels MIC-A et MIC-B chez les humains (Bauer, Groh et al. 1999), et les protéines Rae-1 (Cerwenka, Baron et al. 2001) ou ULBP-16 chez la souris (Carayannopoulos, Naidenko et al. 2002; Diefenbach, Hsia et al. 2003), conduisant à un équilibre entre les effets activateurs et inhibiteurs, dont le résultat est que la cellule NK demeure inactive.

2.5.1.2 Action anti-tumorale

L'activation des NK se traduit par leur dégranulation : ils vont relarguer au contact de leur cible des protéines qui visent la mort de cette dernière par apoptose. Mais il peut se produire également la sécrétion de cytokines ou l'expression membranaire de ligands de récepteurs de mort.

2.5.1.2.1 Perforine et granzymes

La dégranulation permet le relargage de perforine et de granzyme, la première protéine étant destinée à former des pores dans les membranes de la cible (Liu, Perussia et al. 1986; Young, Liu et al. 1986), et la seconde étant une sérine protéase qui permettra la fragmentation de l'ADN de la cellule cible, et donc sa mise en apoptose par la voie intrinsèque (Trapani, Davis et al. 2000).

La perforine est l'arme cytotoxique principale des cellules NK (Kagi, Ledermann et al. 1994). En effet, des tumeurs dont le rejet est NK-dépendant (RMA-S, EL-4, MBL-2) chez la souris progressent plus vite dans des souches perforine$^{-/-}$ (van den Broek, Kagi et al. 1995; van den Broek, Kagi et al. 1996). Le rôle de la perforine est également primordial dans le rejet de tumeurs portant des ligands de NKG2D (Hayakawa, Kelly et al. 2002), dans la limitation des métastases (Smyth, Thia et al. 1999), dans la sensibilité à des carcinogènes chimiques (van den Broek, Kagi et al. 1996) et dans l'immunosurveillance des tumeurs spontanées (Smyth, Thia et al. 2000).

Quant aux granzymes, leur importance est discutée. Si on a décrit une synergie entre perforine et granzymes (Trapani 1995), des équipes utilisant le même modèle RMA-S ont décrit, l'une que les souris invalidées pour le gènes du granzyme A et/ou B ne rejetaient pas différemment la tumeur que les souris sauvages (Davis, Smyth et al. 2001), l'autre que les granzymes sont nécessaires au même titre que la perforine (Pardo, Balkow et al. 2002).

2.5.1.2.2 TNF-R

Une autre arme consiste en l'expression de ligands de récepteurs de mort de la famille du TNF que sont Fas-L et TRAIL. Si certaines populations de NK expriment constitutivement TRAIL, notamment dans le foie (Takeda, Hayakawa et al. 2001), cette protéine membranaire est hautement inductible par des cytokines, en particulier l'IL-2, l'IL-15 ou encore l'IFN-gamma (Zamai, Ahmad et al. 1998; Kashii, Giorda et al. 1999; Kayagaki, Yamaguchi et al. 1999). Quant à Fas-L (Suda, Takahashi et al. 1993), il est également exprimé par les cellules T et les NK activés.

La lyse par TRAIL est un mécanisme important dans l'arsenal des cellules NK : en effet, l'inhibition de TRAIL par un anticorps bloquant augmente encore la létalité (évaluée suivant le nombre de métastases) de tumeurs sensibles aux NK dans des souris déficientes en perforine (Smyth, Cretney et al. 2001). Fas-L est également impliqué dans le rejet de tumeur Fas positives, ce phénomène étant amplifié par l'IL-2 (Bradley, Zeytun et al. 1998) ; il faut cependant noter que, bien que de nombreuses cellules soient Fas négatives, les cellules NK peuvent induire l'expression de Fas sur de telles cellules (Screpanti, Wallin et al. 2001), et donc favoriser leur lyse.

Certaines des stratégies utilisant des facteurs solubles ligands de récepteurs de mort ont permis de mettre en évidence que la mort induite par des ligands de DR5 favorisait l'induction d'une réponse immunitaire (Takeda, Yamaguchi et al. 2004), et sensibilise les cellules tumorales à d'autres agents, en ayant par exemple une action synergique avec le trastuzumab (Stagg, Sharkey et al. 2008). L'action anti-tumorale des NK via TRAIL revêt donc un double intérêt : celui de lutter directement contre la tumeur en la rendant vulnérable et celui de favoriser la survenue d'une réponse immunitaire spontanée.

2.5.1.2.3 IFN-gamma

Les lymphocytes NK, une fois activés, produisent de nombreuses cytokines, en particulier l'IFN-gamma. Cette cytokine est capable d'agir sur les autres cellules du système immunitaire, notamment en induisant les cellules dendritiques à produire de l'IL-12, qui permettra à ces dernières d'engager des lymphocytes T dans la voie Th1. De plus, L'IFN-gamma est également capable d'une action directe sur les cellules tumorales.

D’une part, l’IFN-gamma augmente l’expression de molécules des CMH-I et CMH-II (Berman, Duncan et al. 1985; Rubin, Riond et al. 2008), ce qui rend les cellules plus sensibles à la lyse par des cellules T et moins à l’action des cellules NK, en augmentant l’activité de présentation antigénique. De même, l’IFN-gamma modifie la composition des complexes protéasomes (Yang, Waters et al. 1992), en provoquant l’expression de protéases codées dans le locus du CMH (Brown, Driscoll et al. 1991). On parle alors d’immunoprotéasome (Tanaka 1994).

D’autre part, l’IFN-gamma possède un effet antiprolifératif sur la plupart des cellules, y compris les cellules tumorales. Des cellules tumorales rendues insensibles à l’action de l’IFN-gamma (cellules MethA transfectées par un récepteur dominant négatif de l’IFN de type II) prolifèrent plus vite *in vivo* que leurs homologues sauvages (Shankaran, Ikeda et al. 2001). De même, il a été montré dans des modèles de métastase pulmonaire (tumeurs DA3 et RM1) que l’activité antitumorale des NK pouvait se résumer aux actions conjuguées et indépendantes de la perforine et de l’IFN-gamma (Street, Cretney et al. 2001). Enfin, l’IFN-gamma permet l’augmentation de l’expression de TRAIL sur les cellules NK elles-mêmes (Smyth, Cretney et al. 2001; Takeda, Smyth et al. 2001; Takeda, Smyth et al. 2002).

Il faut également signaler que l’IFN-gamma peut entraîne la production d’IP-10 (Interferon-inducible Protein 10) (Luster, Unkeless et al. 1985), qui est une molécule anti-angiogénique (Sgadari, Angiolillo et al. 1996), par de nombreux types cellulaires (Luster and Ravetch 1987). Ceci explique les effets dépendants de l’IFN-gamma de l’administration d’IL-12 dans des modèles de tumeurs murines : l’IL-12 favorise la production de l’IFN-gamma, ce qui permet aux NK de lyser plus efficacement les cibles tumorales via TRAIL (Smyth, Cretney et al. 2001) et induit la sécrétion d’IP-10, qui par son activité anti-angiogénique va contrarier la croissance de la tumeur (Sgadari, Angiolillo et al. 1996).

2.5.1.3 Echappement tumoral à l’action des NK

Il n’est pas rare de constater que des tumeurs sont partiellement ou totalement déficiente en ce qui concerne la présentation antigénique (Restifo, Esquivel et al. 1993). Le fait d’exprimer moins de molécules du CMH-I à leur surface, qui leur permet d’échapper aux lymphocytes T $CD8^+$, les rend en théorie plus sensible à l’action des cellules NK. Malheureusement, malgré l’étendue de leur arsenal, dans le cas des tumeurs « réelles », les NK sont rarement suffisants

pour empêcher la tumeur de tuer son hôte. Il a été en effet constaté que les lymphocytes infiltrant les tumeurs étaient nettement moins actives que leurs alter-ego sanguins (Holmes 1985).

D'une part, le nombre de cellules NK infiltrant la tumeur est faible (Benencia, Courreges et al. 2005). D'autre part, comme le cancer est un phénomène vivant, il va s'adapter aux pressions de sélection qu'il subit.

Ainsi, il existe des tumeurs qui résistent aux NK de différentes manières, dont voici une description rapide et non-exhaustive:

- Production de cytokines immunosuppressives, comme le TGF-beta, qui empêchera l'activation des cellules cytotoxiques (Wallick, Figari et al. 1990; Tada, Ohzeki et al. 1991), ou l'IL-10 qui limitera la production des cytokines de type 1 (IFN-gamma) par les cellules T (Fiorentino, Zlotnik et al. 1991) et par les NK (Hunter, Subauste et al. 1994).

- Production de ligands de NKG2D solubles (Groh, Wu et al. 2002). Ces tumeurs sont capables d'inactiver l'ensemble des récepteurs NKG2D de l'organisme : le sérum des patients devient alors lui-même inhibiteur de NKG2D (Ogasawara, Hamerman et al. 2003).

- Diminution de l'expression de récepteurs de mort Fas et DR5 (récepteur de TRAIL) par la tumeur (Nguyen, Zhang et al. 2001). L'action des perforines et granzymes étant liés à l'apoptose par la voie intrinsèque, à laquelle les cellules tumorales résistent particulièrement bien, la perte d'efficacité des récepteurs de mort prive les NK de leur alternative.

2.5.2 Concept d'AP-NK

Le concept de la cellule tueuse présentant l'antigène (AP-NK) est simple : une cellule immunitaire capable de tuer sa cible puis de présenter les antigènes captés constituerait un lien entre les parties innées et adaptatives du système immunitaire. Ainsi, une cellule capable d'attaquer rapidement, puis d'induire efficacement une réponse spécifique d'antigène minimiserait les intervenants et donc le temps de réaction de l'organisme.

Mais un des problèmes de la lutte anti-tumorale, c'est précisément que les réponses adaptatives sont difficiles à induire. En effet, les cellules tumorales possèdent peu d'antigènes de rejet (TRA, pour Tumor Rejection Antigen), et cela est dû à leur genèse : les cellules tumorales ont initialement les antigènes de différenciation de leur tissu d'origine. Ce n'est qu'avec le temps et l'accumulation de mutations que des protéines aberrantes ou ectopiques seront produites, qui permettraient leur reconnaissance par l'immunité adaptative. Or les cancer qui posent problème sont justement ceux qui parviennent à échapper à cette réponse adaptative, notamment en diminuant leur présentation de l'antigène.

L'existence des cellules NK permet de contrebalancer ce phénomène, précisément en permettant la lyse des cellules possédant peu de molécules de CMH. Même s'ils sont également sensible à d'autres signaux d'inhibition (tels que le TGF-beta), les cellules NK, au contraire des cellules T, demeurent plus facilement activables, car leur activation est indépendante de l'antigène.

Des NK tueurs (K-NK) auraient donc comme rôle de lyser des cellules tumorales et de produire des cytokines capables d'améliorer l'action de l'immunité innée (tel l'IFN-gamma) ; et, devenus des AP-NK, pourraient également présenter l'antigène à des cellules T naïves, potentialisant toute une chaîne d'effecteurs.

2.5.3 Prémisses

Si on se réfère aux propriétés d'une CPA professionnelle, ces AP-NK seraient nécessairement CMH-II positifs. Or, l'existence de NK CMH-II^{+} est avérée dans plusieurs modèles, de même que des cellules dendritiques tueuses (Bonmort, Dalod et al. 2008; Chauvin and Josien 2008).

2.5.3.1 Chez l'humain

Il est connu depuis longtemps que les NK humains peuvent être DR^{+} suivant une activation (Phillips, Le et al. 1984) ; mais leur capacité de présentation d'antigène semble limitée (D'Orazio and Stein-Streilein 1996; Burt, Plitas et al. 2008). Une première mise en évidence des capacités de CPA des cellules NK humaines fut faite en montrant que des NK peuvent présenter un super antigène à des cellules T (D'Orazio and Stein-Streilein 1996). Une population de cellules sanguines humaines dendritiques CD56^{+}DR^{hi} semble être incapable de lyser, mais présente efficacement l'antigène, tandis qu'une population de cellules NK

$CD56^{+}DR^{lo}$ est bien lytique et possède une faible mais réelle capacité de présentation de l'antigène (Burt, Plitas et al. 2008).

2.5.3.2 Chez le rat

En 1997, Josien *et al* décrivent une cellule NK/DC chez le rat (Josien, Heslan et al. 1997). Il s'agit d'une cellule dendritique splénique qui exprime le marqueur de NK, NKRP-1, et est capable de lyser des cibles NK. La méthode de purification des DC de rat étant déjà connue pour produire des CPA, ces cellules sont donc nécessairement fonctionnellement à la fois « DC » (présentatrices d'antigène) et « NK » (lytiques).

Par la suite, la même équipe montra que l'activité cytotoxique des DC splénique chez le rat est contenue dans une population $CD4^{-}$, et que son mécanisme est indépendant du calcium et des récepteurs de la famille du TNFR (i.e. le TNFR lui-même, Fas, et TRAIL) (Trinite, Voisine et al. 2000). Ils montrèrent également que la lyse induite par ces DC $CD4^{-}$ est également indépendante des caspases, de FADD et de Bcl-2, mais ressemblant morphologiquement à l'apoptose. De plus, ces DC sont capables de phagocyter *in vitro* les débris de leurs victimes, contrairement à leurs homologues $CD4^{+}$, de même la lyse et la phagocytose sont très diminuées si les DC étaient maturantes (Trinite, Chauvin et al. 2005). Plus récemment, les mêmes auteurs ont montré que ces Killer DC (KDC) sont capables de tuer *in vitro* des cellules tumorales (ostéosarcome) et d'opérer une présentation croisée des antigènes tumoraux *in vivo* : après vaccination des rats avec des KDC ayant tuée des cibles tumorales, on observe un retard à la croissance des tumeurs sous-cutanées, cet effet étant dépendant des cellules T $CD8^{+}$ (Chauvin, Philippeau et al. 2008). Par ailleurs, ces cellules dendritiques tueuses sont phénotypiquement distinctes des cellules NK de rat, car elles n'expriment pas le marqueur NKP46 (Chauvin, Philippeau et al. 2008), qui est maintenant reconnu comme un marqueur spécifique des cellules NK de mammifères (Walzer, Blery et al. 2007; Walzer, Jaeger et al. 2007).

2.5.3.3 Chez la souris

L'hypothèse selon laquelle les lymphocytes NK pourraient faire un lien entre les immunités innée et adaptative n'est pas nouvelle non plus chez la souris : il a été décrit en 1995 que des souris dont les cellules NK ont été détruites par un anticorps monoclonal ne peuvent plus

former de réponse lymphocytaire primaire en réaction à un vaccin formé de cellules tumorales (mélanome B16-F10) tuées par la mitomycine C (Kurosawa, Harada et al. 1995).

Par ailleurs, il existe également chez la souris des cellules apparentées aux DC et non aux NK, qui, parallèlement aux KDC chez le rat, sont lytiques et présentatrices d'antigène. Il a ainsi été montré qu'une population de DC intratumorale peut réagir au BCG en devenant lytique via TRAIL (Roux, Apetoh et al. 2008).

D'autres démarches plus ciblées ont été publiées sur des AP-NK chez la souris.

2.5.3.3.1 NK/DC

En 2002 fut publiée une étude qui montrait pour la première fois une cellule NK/DC chez la souris (Homann, Jahreis et al. 2002). La mise en évidence passait par l'observation d'une tolérance immunitaire provoquée par le transfert adoptif de cellules $DX5^+CD11c^+$ dans un modèle de diabète viro-induit. Ces cellules étaient capables de lyser des cibles NK et d'effectuer une présentation en croix d'un antigène soluble. Ces auteurs estiment que 30% des cellules $CD11c^+$ co-expriment le DX5 dans une rate de souris naïve.

2.5.3.3.2 NKDC

Suivant l'observation que des cellules $NK1.1^+CD11c^+$ existent (Lian, Okada et al. 2003; Miller, Pillarisetty et al. 2003), l'équipe de Ronald DeMatteo étudia des cellules NKDC de souris (Pillarisetty, Katz et al. 2005; Chaudhry, Katz et al. 2006; Chaudhry, Kingham et al. 2006; Chaudhry, Plitas et al. 2007; Plitas, Chaudhry et al. 2007; Burt, Plitas et al. 2008). Ils démontrèrent que ces cellules sont capables de lyser des cibles NK et de présenter l'antigène à des lymphocytes T naïfs (Pillarisetty, Katz et al. 2005). Ces cellules prolifèrent en réponse au FLT3-L (Chaudhry, Katz et al. 2006) et répondent *in vivo* à un traitement CpG+IL-18 en produisant de l'IFN-gamma et en augmentant leur expression de molécules de co-stimulation (Chaudhry, Kingham et al. 2006). Ces NKDC (Natural Killer Dendritic Cells) sont également capables de freiner l'évolution d'une tumeur en condition inflammatoire, via l'IL-15 produite par les DC (Chaudhry, Kingham et al. 2006; Chaudhry, Plitas et al. 2007), comme on sait depuis que c'est le cas pour les NK (Lucas, Schachterle et al. 2007). D'autres études confirmèrent la capacité des cellules $NK1.1^+CD11c^+$ de produire de grandes quantités d'IFN-gamma dans le cadre d'une infection par Listeria monocytogenes (Chang, Wang et al. 2007; Plitas, Chaudhry et al. 2007).

Enfin, une étude d'une autre équipe (Chen, Calomeni et al. 2007) montra une relation entre les compartiments des monocytes, des NKDC et des DC conventionnelles : suite à des transferts adoptifs, ces auteurs ont été capables de montrer que des monocytes peuvent se différencier *in vivo* en NKDC, et que ces derniers peuvent devenir des cellules dendritiques conventionnelles.

2.5.4 IKDC

Le terme IKDC (Interferon-producing Killer Dendritic Cell) désigne une population de cellules immunitaires définie phénotypiquement comme $NK1.1^{+}CD11c^{int}B220^{+}CD3^{-}CD19^{-}Gr1^{-}$. Leurs propriétés initialement décrites étaient d'être capables de lyser les cibles tumorales en utilisant TRAIL et de présenter l'antigène. Si elles furent décrites et nommées en 2006, les IKDC furent parfois observées auparavant, mais sans que leurs fonctions soient étudiées en détail.

2.5.4.1 Prémisses

D'un point de vue phénotypique, une des particularités des IKDC est de porter à la fois les marqueurs NK1.1 (CD161c) et B220 (CD45R). En effet, ces deux marqueurs furent pendant longtemps présentés comme spécifiques des cellules NK et des cellules B, respectivement. Cependant, la présence de B220 sur des cellules NK avait déjà été observée dès 1990 (Ballas and Rasmussen 1990). En 1993, les mêmes auteurs décrivent comment les lymphokine-activated killer (LAK) produits en IL-2 ou IL-4 expriment le B220 ; ils en concluent que cette protéine est un marqueur d'activation (Ballas and Rasmussen 1993). Ils observent également que, parmi les LAK $B220^{+}$ on peut distinguer différentes populations : les LAK $NK1.1^{+}CD3^{-}$, $NK1.1^{-}CD3^{+}$ et $NK1.1^{+}CD3^{+}$. Cependant, ils remarquent que seuls les LAK $NK1.1^{+}CD3^{-}B220^{+}$ lysent les cibles NK typiques.

Le clonage de l'IL-15 (Grabstein, Eisenman et al. 1994; Tagaya, Bamford et al. 1996) permit d'étudier son action sur les lymphocytes, et en particulier les NK. Ainsi, Puzanov décrit en 1996 comment les cellules NK cultivées en IL-15 peuvent proliférer (Puzanov, Bennett et al. 1996), et indique que ces conditions de culture leur fait exprimer le B220, de manière plus efficace mais néanmoins similaire à l'IL-2. Ces observations de LAK *in vitro* permettent de penser que le B220 est bien un marqueur d'activation par cytokine des NK.

La première observation directe des IKDC date probablement de 1996. Dans une étude, Rolink *et al* étudient les différences entre le B220 et le CD19 pour l'étude des lymphocytes B et de leurs précurseurs dans la moelle osseuse de souris (Rolink, ten Boekel et al. 1996). A leur étonnement, ils isolent par tri cellulaire en cytométrie de flux une population de cellules $B220^{+}CD19^{-}$. Ils constatent que cette population contient environ 60% de progéniteurs de cellules B et 40% de cellules $NK1.1^{+}$ qu'ils parviennent à cultiver en IL-2 : ces cellules produisent alors de la perforine et deviennent lytiques. Cette stratégie de tri cellulaire ($B220^{+}CD19^{-}$) permettra en 2005 une autre observation des IKDC en tant que « sous-population de cellule dendritique plasmacytoïde » $B220^{+}CD19^{-}CD11c^{int}Gr1^{-}NK1.1^{+}DX5^{+}$ (Pelayo, Hirose et al. 2005).

Enfin, en 1997, Evans et al utilisent une combinaison associant l'IL-15 et l'endoxan pour le traitement de tumeurs 76-9 établies (Evans, Fuller et al. 1997). Les auteurs administrent l'endoxan, puis prélèvent des tumeurs des souris (un rhabdomyosarcome) et cultivent les cellules obtenues en présence d'IL-15. Les cellules lymphoïdes qui prolifèrent sont $NK1.1^{+}$ et $B220^{+}$, et capables de lutter contre la croissance tumorale après transfert adoptif.

De ces études, nous pouvons tirer une conclusion certaine : il peut exister des cellules $NK1.1^{+}B220^{+}$, et leur première capacité est d'être lytique. Selon les études, la présence du B220 a été interprétée ou bien comme un marqueur d'activation (activation *in vitro* de NK spléniques ou intra-tumoraux) ou de précocité (présence de cellules $NK1.1^{+}B220^{+}$ dans la moelle). Mais aucune étude précise de cette population n'avait été faite avant 2006.

2.5.4.2 Découverte

2.5.4.2.1 L'IKDC, une cellule tueuse

La mise en évidence de l'IKDC dans un contexte tumoral est indissociable du traitement médicamenteux qui l'a permise.

2.5.4.2.1.1 Imatinib mesylate

L'imatinib mesylate (IM, STI 571, Glivec®) est un inhibiteur de tyrosine kinase. C'est un médicament issu d'une conception moléculaire : cette molécule a été conçue pour inhiber la protéine Abl, une tyrosine kinase qui est la cause principale de certaines formes de leucémie myéloïde chronique (LMC) ou de leucémies aigües lymphoblastiques. En effet, dans ces

maladies, et particulièrement dans la LMC, il existe fréquemment une translocation chromosomique t(9,22) (chromosome de Philadelphie), provoquant la synthèse d'une protéine de fusion BCR-Abl. Cette protéine, produite en quantité importante par la cellule transformée, induit sa prolifération rapide.

L'inhibition de BCR-Abl par l'IM provoque l'arrêt de cycle des cellules tumorales dans la LMC, et rapidement les mécanismes d'apoptose se mettent en place. C'est un paradigme parfait de thérapie ciblée, et le développement de cette molécule est souvent pris en exemple comme l'illustration de la démarche de recherche&développement moderne.

Par ailleurs, l'IM inhibe également d'autres tyrosines kinases, en particuliers celles du PDGF-R (platelet-derived growth factor receptor) et du c-kit (CD117). L'IM fut logiquement testé dans d'autres maladies impliquant ces tyrosines kinases, en particulier les GIST (gastro-intestinal stromal tumors), car une proportion importante des patients souffrant de ce cancer à sombre pronostic possède une mutation d'une de ces protéines (Rubin, Singer et al. 2001; Heinrich, Corless et al. 2003). Cette thérapie est souvent un succès, mais curieusement, des patients atteints de GIST mais sans mutation de c-kit peuvent également bénéficier du traitement par l'IM (Bauer, Corless et al. 2003). Ceci a induit l'idée d'un effet strictement dépendant de l'hôte dans l'efficacité du traitement.

Il a été en effet démontré un effet antitumoral de l'IM via le dialogue entre les NK et les DC (Borg, Terme et al. 2004). Des tumeurs résistantes à l'action intrinsèques de l'IM *in vitro* peuvent ainsi être sensibles à son action *in vivo*, de façon dépendante des cellules NK. Le modèle dégagé de ces résultats est que l'IM agit sur le c-kit des DC et permet à ces dernières d'activer très fortement les cellules NK. Dans un modèle sensible aux NK (mélanome B16F10), cet effet permet de retarder la croissance des métastases pulmonaires, et cet effet est synergique avec la co-administration de FLT3-L (Borg, Terme et al. 2004).

2.5.4.2.1.2 IL-2

L'interleukine-2 (IL-2) a été la première interleukine décrite : en 1965, il est mis en évidence que le surnageant de culture mixte de leucocytes provoque la synthèse d'ADN dans des leucocytes (Gordon and MacLean 1965; Kasakura and Lowenstein 1965). Ce « facteur de croissance des lymphocytes T » (T-cell growth factor) est renommé interleukine-2 en 1979 (1979).

Cette cytokine, très étudiée, possède de très nombreuses actions, sur des cellules variées, en particulier sur les cellules NK. L'IL-2 provoque la prolifération des cellules NK et leur activation cytotoxique (Ballas, Rasmussen et al. 1987), en augmentant la production de perforine (Zychlinsky, Joag et al. 1990), et l'expression de TRAIL (Kayagaki, Yamaguchi et al. 1999). Ainsi, des tumeurs résistantes à la lyse par des NK peuvent être sensibles à l'action des lymphocytes activés par l'IL-2 (Grimm, Mazumder et al. 1982; Mazumder and Rosenberg 1984). De même, des cellules NK activées par l'IL-2 *ex vivo* peuvent permettre l'élimination de métastases pulmonaires (Yang, Hokland et al. 2003).

2.5.4.2.1.3 Imatinib + IL-2

D'après les travaux de Borg *et al*, l'efficacité de l'IM sur la tumeur B16F10 semblait dépendre d'une cellule $NK1.1^+$ (Borg, Terme et al. 2004). Mais lors d'études histologiques, Taïeb *et al* mirent en évidence que la combinaison IM+IL-2 permet l'accumulation de cellules $CD11c^+$ et $B220^+$ dans les poumons métastasés de souris C57BL/6 (Taieb, Chaput et al. 2006). En confrontant ces deux données, il fut possible d'identifier un type cellulaire portant les marqueurs phénotypiques B220, CD11c et NK1.1, tout en n'exprimant pas le marqueur Gr1. Ces cellules semblaient la clef du fonctionnement synergique de la combinaison, puisque chaque médicament utilisé séparément ne permet pas d'obtenir la même accumulation, ni une efficacité clinique comparable. Par ailleurs, la moitié de ces cellules exprimaient les molécules du CMH-II.

Ces cellules, une fois isolées à partir de souris porteuses de tumeurs et traitées par IM+IL-2, étaient capables de lyser efficacement des cibles tumorales *ex vivo*, de manière dépendante de TRAIL. Il était également possible d'inhiber l'effet du traitement IM+IL-2 *in vivo* avec un anticorps bloquant l'activité du TRAIL membranaire. Cette lyse via TRAIL fut reliée à la capacité des IKDC de produire de grandes quantités d'IFN-gamma.

La combinaison de ces deux médicaments permit donc d'identifier clairement cette population comme acteur du système immunitaire. La présence de ces cellules dans des souris alymphoïdes ($Rag^{-/-}$ x IL-2$Rgamma^{-/-}$) permit de les considérer myéloïdes. Mais si sa nature lytique (via l'IFN-gamma et TRAIL) la rapprochait des NK, une autre étude les rapprocha des DC.

2.5.4.2.2 L'IKDC, une présentatrice d'antigène

L'équipe de Frank Housseau, indépendamment de la précédente, observa cette même cellule chez la souris Balb/c (Chan, Crafton et al. 2006). Leur observation initiale était qu'il existe une population de cellules $B220^{+}CD11c^{int}Gr1^{-}CD49b^{+}$ dans les organes lymphoïdes des souris (rate et ganglions lymhatiques). Intrigués par cette population dont le phénotype la place ou parmi les DC ($B220^{+}CD11c^{int}$) ou parmi les NK ($Gr1^{-}CD49b^{+}$), ils l'étudièrent et purent ainsi constater que cette cellule possède des propriétés étonnantes, dont la principale est non seulement d'être lytique spontanément, de manière dépendante de NKG2D, et de produire de l'IFN-gamma, mais également d'être capable après stimulation de perdre cette capacité de lyse tout en devenant plus proche d'une cellule dendritique. En effet, après mise en contact de TLR9-L (CpG-ODN), les IKDC perdent leur expression de NKG2D et régulent en hausse l'expression de CMH-II, et produisent des cytokines spécifiques des cellules dendritiques : l'IFN-alpha et l'IL-12. Ces cellules furent donc nommées IKDC, pour « cellule dendritique tueuse productrice d'interferon » (Interferon-producing Killer Dendritic Cell).

De plus, cette plasticité de fonction se retrouvait *in vivo*, les IKDC spléniques étant fonctionnellement lytique et les IKDC des ganglions lymphatiques étant fonctionnellement proches des DC. De même, les IKDC spléniques triées, marquées puis réinjectées à des souris naïves, migrent dans une proportion non négligeable dans les ganglions lymphatiques et deviennent MHC-II positive. Enfin, des IKDC triées à partir de ganglions de souris infectées avec une bactérie Listeria monocytogenes transgénique exprimant l'ovalbumine permettent d'induire *ex vivo* la prolifération d'un clone lymphocytaire T $CD4^{+}$ spécifique de l'Ova, tout en produisant de grandes quantité d'IFN-gamma, recoupant les données sur les cellules $NK1.1^{+}CD11c^{+}$ (Chang, Wang et al. 2007; Plitas, Chaudhry et al. 2007).

Cette étude permit pour la première fois de montrer à la fois des phénotypes et fonctions NK puis des phénotypes et fonctions DC chez une même population cellulaire purifiée. Cette notion de plasticité de ces cellules IKDC posait déjà la question de l'origine hématopoïétique de ces cellules.

Ces cellules IKDC furent nommées « dendritiques », car d'une part les « cellules dendritiques » sont la référence en ce qui concerne la présentation de l'antigène (le marqueur CD11c étant commun aux DC conventionnelles, DC plasmacytoïdes (pDC) et aux IKDC), et d'autre part parce qu'il est possible d'observer des dendrites sur les IKDC au microscope

électronique à transmission. La production d'IL-12, d'IFN-alpha et les fonctions CPA montrées par cette étude permettait de les différencier nettement des NK, tout comme leurs propriétés lytiques via NKG2D les séparent nettement des DC.

Peu de temps après, les IKDC furent étudiées d'un point de vue ontogénique : l'équipe de Paul Kincade décrivit qu'un progéniteur lymphoïde $Lin^{-}Sca\text{-}1^{+}CD117^{+}Thy1.1^{+}CD62L^{+}$ pouvait produire des IKDC, et qu'il s'agissait d'un progéniteur différent des NK et des pDC, les deux types cellulaires phénotypiquement les plus proches des IKDC (Welner, Pelayo et al. 2007).

Ces trois études permirent donc de présenter un nouveau type cellulaire, caractérisé par son origine hématopoïétique, et sa plasticité fonctionnelle : à la fois lytique et présentatrice d'antigène. Mais ces conclusions furent remises en cause par la suite.

2.5.4.3 Concept défié en 2007

Dans un premier temps, la production d'IFN-alpha, qui rapprochait les IKDC des cellules dendritiques, ne fut pas confirmée par une étude indépendante (Vremec, O'Keeffe et al. 2007). Bien que la production d'IFN-gamma fût quant à elle retrouvée, les interférons de type I ne le furent pas dans des conditions expérimentales comparables. Cette publication se faisait ainsi l'écho de l'étude de 2005 identifiant les cellules $B220^{+}CD19^{-}Gr1^{-}$ comme des pDC ne produisant pas d'IFN-I (Pelayo, Hirose et al. 2005).

Ensuite, trois équipes publièrent côte-à-côte leurs résultats concernant les cellules $NK1.1^{+}B220^{+}$ (Blasius, Barchet et al. 2007; Caminschi, Ahmet et al. 2007; Vosshenrich, Lesjean-Pottier et al. 2007). Les trois études reprenaient les deux articles de 2006 et contredisent certains résultats de ceux-ci, en particulier : les IKDC seraient absentes des souris alymphoïdes, ce qui exclut leur appartenance à la lignée myéloïde, *a fortiori* dendritique ; leurs facteurs de dépendances sont les mêmes que ceux des NK ; aucune capacité de présentation de l'antigène ne peut être retrouvée dans la population IKDC. Enfin, le marqueur B220 peut être induit sur des NK *in vitro* et *in vivo*.

Leurs conclusions se recoupaient pour affirmer que les IKDC n'appartiennent pas à la lignée des cellules dendritiques, mais bien à la lignée des cellules NK. La présence du marqueur B220 se résumerait donc à un marqueur d'activation et/ou de prolifération des NK, mais pas à une cellule différente. Une étude par signature génétique a montré la parenté ontogénique

entre les IKDC et les NK, plaçant les IKDC nettement dans les cellules lymphoïde(Robbins, Walzer et al. 2008).

2.5.4.4 Synthèse

Si on embrasse l'ensemble des informations disponibles, il est possible de tirer quelques impressions et hypothèses intéressantes.

La présence de B220 sur des NK en prolifération peut être interprétée de deux manières différentes : ou bien le B220 est acquis par toute cellule NK en prolifération, ou bien seules les cellules NK $B220^+$ prolifèrent dans les conditions décrites, ce qui conduit à leur enrichissement.

Par ailleurs, d'après les travaux de Hayakawa *et al*, les lymphocytes NK de souris peuvent se répartir selon trois populations en fonction de l'expression des marqueurs CD11b et CD27: $CD11b^-$, Double Positifs et $CD27^-$. D'après Hayakawa *et al*, les deux premières populations sont $B220^+$, or ce sont les populations les plus immatures (Hayakawa and Smyth 2006) : celles qui se différencient en premier à partir de la moelle. De même, l'expression de CD117, en tant que marqueur d'immaturité, reflète cette conclusion.

D'autre part, Colucci *et al* avaient déjà publié en 2000 que dans les souris chimère W/W, dont les cellules hématopoïétiques portent une mutation perte de fonction de c-kit, le marqueur B220 était absent des cellules NK mais l'IL-2 ou l'IL-15 peut le leur faire exprimer (Colucci and Di Santo 2000).

Il a été proposé que les IKDC puissent être un équivalent murin des cellules NK humaines $CD56^{hi}$ (Blasius, Barchet et al. 2007). Or, une étude récente a annoncé que cette population de lymphocytes NK humains contient une sous-population $HLA\text{-}DR^+$ douée d'une capacité de présentation de l'antigène, certes faible, mais réelle (Burt, Plitas et al. 2008).

Pour résumer, on peut affirmer que le phénomène AP-NK, bien que controversé, existe probablement chez l'être humain et chez la souris. Plusieurs équipes ont mis en évidence des cellules NK capables de présenter l'antigène ; cette capacité se concentrant chez la souris dans des cellules NK exprimant le CD11c et le B220 (Homann, Jahreis et al. 2002; Pillarisetty, Katz et al. 2005; Chan, Crafton et al. 2006).

3 Objectifs de la thèse

Mon travail a consisté en l'étude des cellules IKDC, dans une optique d'étude des effets immunitaires de médicaments de chimiothérapie antitumorale. Dans le détail, les objectifs étaient donc :

- Etude des dépendances immunologiques de l'efficacité de la combinaison IM+IL-2.
- Mise au point d'une méthode de culture des IKDC spléniques.
- Recherche d'une capacité de présentation de l'antigène par les IKDC et étude de ses modalités en contexte tumoral.

J'ai également eu la chance durant ma thèse de pouvoir participer à un autre projet, consistant à explorer l'immunogénicité de la mort tumorale induite par les traitements antitumoraux conventionnels, et qui a produit deux publications (annexes 1 et 2). Ma participation dans ce projet m'a également permis d'écrire une revue (annexe 3).

4 Résultats

4.1 Les IKDC, agent du traitement IM + IL-2

4.1.1 Rappels

Les travaux précédents sur l'action de l'IM sur le compartiment NK1.1$^+$ des souris porteuses métastases pulmonaire de mélanome avaient permis un certain nombre d'hypothèses. Notamment :

- L'IM agit contre le développement *in vivo* d'une tumeur pourtant résistante *in vitro*. Cette action passe par les cellules NK1.1$^+$. L'IM favorise une activation des cellules NK via une interaction avec les cellules dendritiques. L'inhibition de c-kit dans les DC les prive donc d'une fonction régulatrice/inhibitrice.

- Le traitement combiné IM+IL-2 dans les mêmes conditions met en évidence une synergie entre les deux traitements (Taieb, Chaput et al. 2006). Cette efficacité est corrélée avec la présence de cellules B220$^+$CD11c$^+$ dans les sites tumoraux. Ces cellules peuvent être isolées et étudiées *in vitro* : elles sont capables de lyser les cibles tumorales via TRAIL. Ces mêmes cellules sont suffisantes pour induire un retard à la pousse dans des modèles de tumeurs implantés dans des souris alymphoïdes. Enfin ces cellules expriment le CMH-II au site tumoral, suggérant une capacité de CPA.

4.1.2 Le rôle critique de l'IL-15 dans l'effet antitumoral du traitement associant l'imatinib mesylate et l'interleukine-2 (ARTICLE 1)

4.1.2.1 Résumé

D'après Borg *et al* (Borg, Terme et al. 2004), l'action dépendante de l'hôte de l'IM dépend des NK, via leur activation par les DC. Mais il a été également décrit que c-Kit était une protéine primordiale dans le développement et la fonction des NK (Colucci and Di Santo 2000). Nous avons donc voulu comparer différents activateurs de NK : le cyclophosphamide, qui en éliminant les Treg (Ghiringhelli, Larmonier et al. 2004; Ghiringhelli, Menard et al. 2007), permet d'augmenter l'activation des NK via les DC (Terme, Chaput et al. 2008) ; le CpG qui induit l'activation des NK via l'IL-12 produite *in vivo* (Ballas, Rasmussen et al.

1996) ; et l'IL-2, qui les active directement (Hefeneider, Conlon et al. 1983). La combinaison IM+IL-2 nous a paru être la plus efficace.

Comme il avait déjà été décrit que l'efficacité de la combinaison IM+IL-2 reposait sur les IKDC (Taieb, Chaput et al. 2006), nous avons examiné le comportement de ces cellules dans différentes souches de souris. Nous avons testé l'efficacité de ce traitement dans des souris IL-15$^{-/-}$ (facteur de dépendance des IKDC) et IFN-alpha$^{-/-}$ (qui participe à la sécrétion de l'IL-15 par les DC) et dans un deuxième temps CCL2$^{-/-}$. Nous avons constaté que l'efficacité du traitement dépendait de la présence de ces trois facteurs solubles. Ceci nous a amené à la conclusion que la prolifération des IKDC pendant le traitement nécessite la présence d'IL-15 ; l'IFN-alpha, par ailleurs, agit en synergie avec l'IL-15 pour la production de CCL2 par les IKDC, et cette même IL-15 permet à l'IKDC d'exprimer le récepteur de CCL2, CCR2. Enfin, CCL2 est nécessaire à l'accumulation des IKDC dans les sites tumoraux pendant le traitement.

4.1.2.2 Discussion

Cette étude (Mignot, Ullrich et al. 2008) a permis de faire un lien clair entre l'efficacité du traitement IM+IL-2 et l'IL-15. Ainsi, l'axe IL-15/IFN-alpha/CCL2 nous parait-il primordial. De plus, la baisse d'efficacité du traitement dans des souris KO nous donne l'indice supplémentaire de l'importance du recrutement au site tumoral des IKDC via CCL2.

Cette dépendance à l'IL-15 de l'efficacité du traitement IM+IL-2, sachant que l'efficacité de l'IM passe par l'activation des NK par les DC (Borg, Terme et al. 2004), et que cette cytokine est cruciale pour le dialogue DC-NK (Lucas, Schachterle et al. 2007; Hochweller, Striegler et al. 2008), nous donne l'indice que le dialogue DC-IKDC existe. Par ailleurs, la quasi-absence de cellules NK1.1^{+} dans les souris IL-15$^{-/-}$ nous permet d'émettre deux hypothèses concernant cette dépendance : l'efficacité du traitement IM+IL-2 nécessite la présence de l'IL-15 car elle permet la présence des effecteurs NK, et parce qu'elle permet l'activation des effecteurs par les DC.

Concernant le marqueur B220, plusieurs hypothèses s'offrent à nous. D'une part, si le B220 est un marqueur de cellules NK en prolifération, l'IL-2 induit la production d'un grand nombre de cellules NK B220^{+}. L'IM permet de son coté leur interaction fructueuse avec les

DC de l'hôte, en inhibant un signal inhibiteur, ce qui permet de produire une grande quantité de NK pleinement activés, qui peuvent lutter contre la tumeur.

D'autre part, si le B220 est un marqueur de NK immatures, alors l'IM possède deux actions : il inhibe une tyrosine kinase inhibitrice dans les cellules dendritiques, et il inhibe l'activation des NK immatures $CD117^{+}$ (Colucci and Di Santo 2000). Le rôle de l'IL-2 dans ce cas serait de permettre aux NK d'outrepasser cette anergie.

The Critical Role of IL-15 in the Antitumor Effects Mediated by the Combination Therapy Imatinib and IL-2[1]

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The synergistic antitumor effects of the combination therapy imatinib mesylate (IM) and IL-2 depended upon NK1.1-expressing cells and were associated with the accumulation of CD11c^{int}B220$^+$NK1.1$^+$ IFN-producing killer dendritic cells (IKDC) into tumor beds. In this study, we show that the antitumor efficacy of the combination therapy was compromised in IL-15 and IFN-type 1R loss-of-function mice. IL-15Rα was required for the proliferation of IKDC during IM plus IL-2 therapy. *Trans*-presentation of IL-15/IL-15Rα activated IKDC to express CCR2 and to respond to type 1 IFN by producing CCL2. Moreover, the antitumor effects of the combination therapy correlated with a CCL2-dependent recruitment of IKDC, but not B220$^-$ NK cells, into tumor beds. Altogether, the IL-15-driven peripheral expansion and the CCL-2-dependent intratumoral chemoattraction of IKDC are two critical parameters dictating the antitumor efficacy of IM plus IL-2 in mice. ***The Journal of Immunology,*** **2008, 180: 6477–6483.**

Accumulating evidence indicates that the innate and adaptive immune systems make a crucial contribution to the antitumor effects of conventional chemotherapy and radiotherapy-based cancer treatments (1, 2). The molecular and cellular bases of the immunogenicity of cell death induced by cytotoxic agents are being progressively unraveled (3–6). Along these lines, we highlighted the off-target immunological effects of a tyrosine kinase inhibitor imatinib mesylate (IM;[4] STI571/Gleevec) (7, 8). The 2-phenylaminopyrimidine compound has been initially designed to specifically block the ATP binding site of BCR/ABL tyrosine kinase and found to inhibit the kinase activity of the three related kinases BCR-ABL, platelet-derived growth factor receptor, and KIT (8, 9). IM is a clinically approved drug commonly used for the treatment of gastrointestinal stromal tumors because it directly targets the pathogenomic c-kit mutation responsible for the deregulated proliferation of tumor precursors (10). However, IM could mediate antitumor effects using an alternate mode of action than the direct effect on tumoral c-kit mutations. Borg et al. (7) selected mouse tumor models that resisted the antiproliferative effects of IM in vitro, yet responded to long-term exposure to IM in vivo. They showed that IM acted on host dendritic cells (DC) to promote NK cell activation and NK cell-dependent antitumor effects in mice and humans (7). c-kit triggering was required for the DC-mediated NK cell activation induced by IM.

To potentiate the NK cell activation induced by c-kit tyrosine kinase inhibition in vivo, IM was combined with IL-2 (11). Flow cytometry studies of single-cell suspensions obtained from regressing lung metastases highlighted that the CD11c$^+$B220$^+$NK1.1$^+$ cells were increased by 4-fold during the combination therapy with IM plus IL-2 compared with each treatment modality alone (IM or IL-2). The CD11c$^+$B220$^+$NK1.1$^+$ cell population expressed other NK cell markers such as the integrin VLA-2 recognized by anti-CD49b/Dx5 mAb, CD122, NKG2D, CD11b, but failed to express CD3, CD4, CD8α, CD25, PDCA-1, and costimulatory molecules (CD40, CD80, CD86). Because up to 50% of the CD11c$^+$B220$^+$NK1.1$^+$ cells coexpressed I-A^b in tumors (11) and because they could stimulate naive OVA -specific TCR-transgenic T cells when isolated from lymph nodes (12) and produce IFN-γ in contact with tumor cells (11), these CD11c$^+$ cells were named IFN-producing killer DC (IKDC).

In an attempt to delineate the mechanisms by which IKDC could proliferate and/or be recruited into tumor beds, we investigated the role of key regulatory cytokines and chemokines in the antitumor effects mediated by IM plus IL-2 and found the critical involvement of the type 1 IFN/IL-15/CCL2 axis in the proliferation and recruitment of IKDC into tumors.

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[4] Abbreviations used in this paper: IM, imatinib mesylate; DC, dendritic cell; IKDC, IFN-producing killer DC; pDC, plasmacytoid DC; rh, recombinant human; bid, twice a day; CTX, cyclophosphamide; ODN, oligodeoxynucleotide; WT, wild type; cDC, conventional DC.

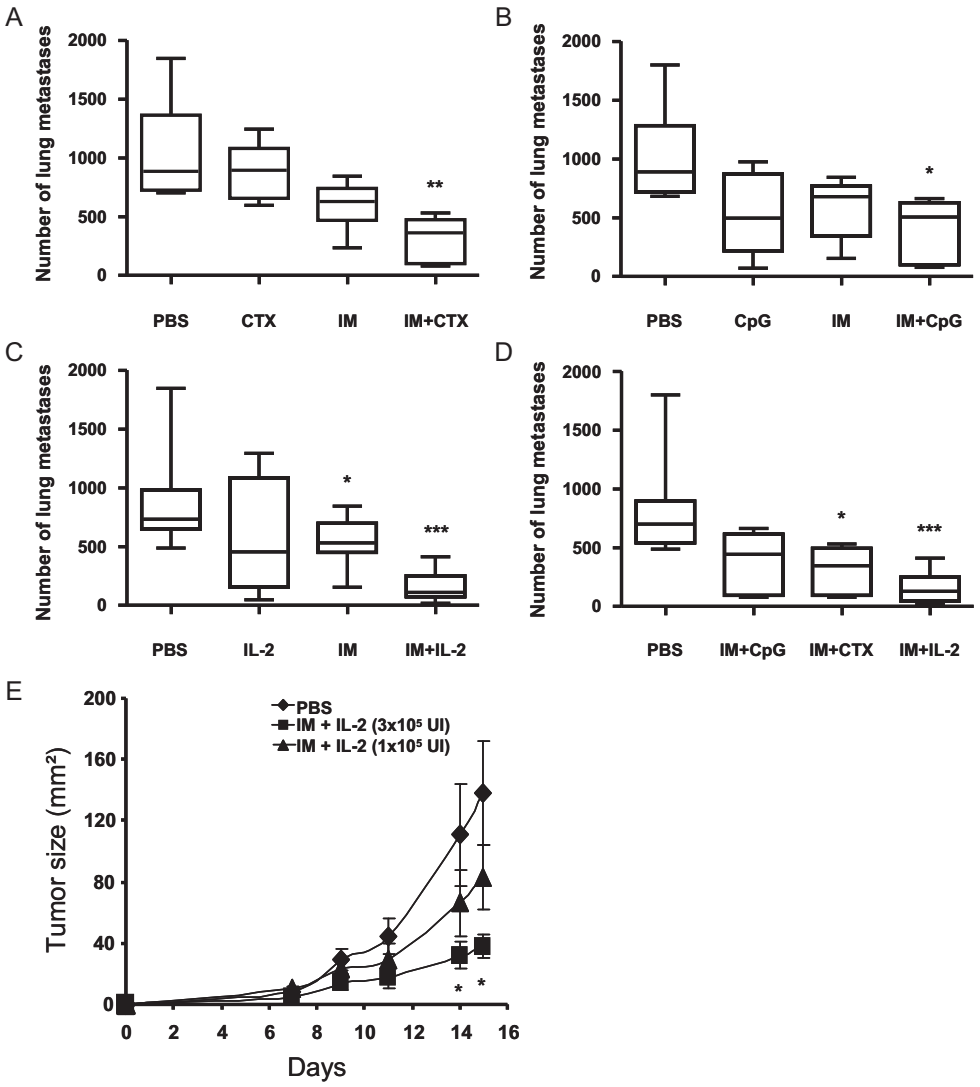

FIGURE 1. Combining IM with NK cell adjuvants. Established B16F10 lung metastases model in C57BL/6 mice. B16F10 tumor cells (5×10^5) were inoculated in the tail vein at day 0. *A,* Mice were injected i.p. with PBS or low-dose CTX (100 mg/kg) and were fed with H_2O or IM bid at 150 mg/kg per day from days 0 to 10. *B,* Mice were fed with H_2O or IM as in *A*, but were treated with s.c. administration of PBS or CpG ODN (10 μg/day) from days 7 to 10. *C,* Mice were fed with H_2O or IM as in *A*, but were treated bid with i.p. administration of PBS or 10^5 IU of rhIL-2 from days 7 to 10. *D,* Mice were fed and treated systemically with the above-mentioned immunotherapies (*A–C*) in the same experiment. Graphs represent the number of lung metastases at day 11. *E*, s.c. B16F10 tumor model in C57BL/6 mice. Same as in *C* but 3×10^5 B16F10 cells were inoculated in the skin of flanks and two doses of IL-2 (10^5 IU or 3×10^5 IU bid) were used. The tumor size was monitored twice a week. A representative experiment of two to five including five mice per group is depicted. Statistical analyses were performed using the Kruskal-Wallis test. Values of p inferior to 0.05, 0.01, or 0.001 are indicated with *, **, or ***, respectively.

Materials and Methods

Mice and cell lines

Female C57BL/6 wild-type (WT) mice were obtained from the Centre d' Elevage Janvier and used at 6–10 wk of age. IFN-type $1R^{-/-}$ and CCL2/MCP-$1^{-/-}$ mice backcrossed on a C57BL/6 background were provided by Centre d'Elevage d'Orléans. IL-15R$\alpha^{-/-}$ and IL-$15^{-/-}$ mice were backcrossed on a C57BL/6 6–8 times and maintained at the animal facility of EB (Research Center Borstel, Borstel, Germany). Animals were all maintained according to the Animal Experimental Ethics Committee Guidelines. B16F10 is a melanoma cell line syngeneic of C57BL/6 (provided by M. T. Lotze, University of Pittsburgh, Pittsburgh, PA) that was cultured in RPMI 1640 (Life Technologies 31870) with 10% heat-inactivated FBS enriched with 5% L-glutamine, non essential amino acids, sodium pyruvate, and antibiotics. MS5 feeder cell lines (provided by W. Vainchenker, Institut Gustave Roussy, Villejuif, France) were cultured in IMDM (Sigma 13390) containing 10% heat-inactivated FBS, 5% L-glutamine, sodium pyruvate, and antibiotics.

Tumor model

Lung metastases model. B16F10 cells (3×10^5) were injected at day 0 into the tail vein and mice were sacrificed at day 11. Two hundred microliters of H_2O or 150 mg/kg IM (Gleevec; Novartis) was given orally in mice twice a day (bid) from days 0 to 10 after tumor inoculation alone or combined with other therapies. Control groups (H_2O plus PBS named PBS), IM alone (IM plus PBS named IM), IL-2 alone (H_2O plus IL-2 named IL-2), cyclophosphamide (CTX, Endoxan; Baxter) alone (H_2O plus CTX named CTX), CpG oligodeoxynucleotide (ODN) alone (H_2O plus CpG named CpG), and combination therapies IM plus CTX, IM plus CpG, or IM plus IL-2 were preformed as stated. rhIL-2 (1×10^5 IU; Chiron) were injected bid by the i.p. route from days 7 to 10 or 100 mg/kg CTX was administered i.p. at day 0, while 10 μg of CpG ODN (5′-TGACTGT GAACGTTCGAGATGA, given by A. Carpentier (Paris, France), AP-HP Pitié Salpétrière) were injected s.c. from days 7 to 10. PBS was injected i.p. or s.c. in control groups.

Skin model. B16F10 cells (3×10^5) were injected s.c., and mice were treated with combination therapy IM plus IL-2 as described above, but with two different doses of IL-2 (10^5 or 3×10^5 bid).

Depletion of plasmacytoid DC (pDC). Mice were depleted by injection of 300 μg of anti-PDCA1-depleting mAb (clone 120G8; Miltenyi Biotec) at day −3 before tumor inoculation and day 0, day +3, and day +9 after tumor inoculation. Effective depletion was monitored by FACS using the pDC-specific staining ($CD3^-CD19^-NK1.1^-CD11c^{int}B220^+Gr1^+$).

Abs and flow cytometry analyses

FACS analyses were performed using allophycocyanin-conjugated anti-CD11c mAb (HL3), PE-Cy7-conjugated anti-NK1.1 mAb (PK136), allophycocyanin-Cy7-conjugated anti-B220 mAb (RA3-6B2), FITC-conjugated anti-CD3 mAb (17A2), FITC-conjugated CD19 (1D3), and PE-conjugated Gr1. IKDC are defined as $CD3^-CD19^-NK1.1^+B220^+CD11c^{int}GR1^-$, NK as $CD3^-CD19^-NK1.1^+B220^-CD11c^{+/-}GR1^-$, and pDC as $CD3^-CD19^-NK1.1^-B220^-CD11c^{int}GR1^+$. Abs were purchased from BD Pharmingen or eBioscience. Anti-CCR2 mAb (MC21, rat IgG2a) has been provided by M. Mack (Universitätsklinikum Regensburg, Abteilung für Nephrologie, Regensburg, Germany) (13). Cells were preincubated with Fc block for 20 min (CD16/CD32, 2.4G2; BD Pharmingen, diluted in PBS with 2% mice serum and 2% FBS) and afterward stained for 20 min at 4°C with the different Abs at 1/200 (anti-CCR2 at 1/20). Alexa

Fluor 488 goat anti-rat IgG has been used as secondary Ab at 1/400 (Invitrogen). Immediately before FACS analysis, 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) was added. FACS analysis was performed by an LSRII (BD Biosciences) using FACSDiva software (BD Biosciences), CellQuestPro software (BD Biosciences), or Flow Jo (Tree Star).

Sorting of NK and IKDC cells and in vitro expansion of IKDC15 is described in our unpublished data. Sorting of NK and IKDC was realized on a Mo-Flo instrument (DakoCytomation), $CD3^-CD10^-C11c^{int}B220^+NK1.1^+$ cells defined "IKDC" and $CD3^-CD19^-CD11c^{+/-}B220^-NK1.1^+$ cells defined $B220^-$NK cells. In vitro expansion of IKDC15 was realized as follows: 10^4 freshly cell sorted IKDC were cultured in the presence of murine stromal cells MS-5 in DMEM (GIBCO 41966) culture medium containing 4500 mg/l of glucose, 5% L-glutamin, pyruvate, and enriched with Abs, 10% BGS (Lot ANB 18298, HyClone), and 20 ng/ml rmIL-15/ml (R&D Systems). While $B220^-$ NK cells could not proliferate ex vivo in similar conditions as IKDC, we could maintain NK cells at higher concentrations (5×10^5/ml) for 7 days on MS-5 feeders and rIL-15 to allow fair comparisons with IKDC.

Cytokine profiling of IKDC and NK cells

In brief, 10^5 freshly sorted NK cells and IKDC or IKDC15 (obtained at day 7 of ex vivo expansion) or NK and IKDC stimulated with recombinant murine IL-15 (20 ng/ml; R&D Systems) for 24 h in the presence of MS-5 were further incubated with recombinant human (rh) IL-2 (10^5 IU/ml) or type 1 IFN at 2500 IU/ml (14) (provided by M. Ferrantini, Instituto di Sanita, Rome, Italy). These in vitro cultures were performed in 200 μl of complete medium in 96-round-bottom well plates. After 24–36 h, cell supernatants were collected and commercial LUMINEX kits, (Linco and BioSource International) were used to determine chemokine and cytokine profiles (used according to the manufacturer's conditions).

In vivo proliferation assays

WT C57BL/6 and IL-15R$\alpha^{-/-}$ mice were treated with IL-2 combined with IM according to a protocol already reported (11). Mice received an i.p. injection of 100 μg of BrdU/100 μl PBS) 1 day before sacrifice. Spleen cells were harvested and processed according to the manufacturer's protocol (BrdU Flow Kit; BD Biosciences). Briefly, cells were stained for surface Ags, fixed, and permeabilized. DNase digestion followed by staining with anti-BrdU mAb was performed before flow cytometry analyses.

Statistical analyses

Aberrant values were excluded using Dixon's test. Normality of distributions was assessed using the Shapiro-Wilk test. Normal distributions were compared by the Student *t* test; non-normal samplings were compared using the Mann-Whitney *U* test. ANOVAs were performed with the Kruskal-Wallis test. Values of *p* inferior to 0.05 were considered significant. All tests were done using Prism 5 software (GraphPad).

Results

c-kit tyrosine kinase inhibition synergized with NK adjuvants

The natural immunosurveillance against B16F10 melanoma developing into lung metastases is known to rely on innate NK cell effectors (15, 16). We previously showed that IM could induce $NK1.1^+$ cell-dependent tumor regressions (7). To further improve the NK cell triggering effects of IM, we combined IM with various NK adjuvants such as TLR9 ligands (CpG ODN (17, 18)) or metronomic CTX suppressing the regulatory T cell-mediated NK cell inhibitory effects (19–21) or IL-2 (22, 23). We observed significant synergistic effects with CTX (Fig. 1*A*) with CpG ODN (Fig. 1*B*) or rhIL-2 (Fig. 1*C*) but the most remarkable antitumoral effects were achieved with the combination of IM + IL-2 (Fig. 1*D*). Similarly, the growth of B16F10 melanoma inoculated into the skin was also impaired by the combination therapy but higher dosages of IL-2 were required for a significant tumor growth delay (Fig. 1*E*). It is of note that all of these agents did not impact on B16F10 proliferation in vitro (data not shown). We already reported that depleting anti-NK1.1 mAb completely curtailed the tumoricidal activity induced by the combination of IM + IL-2 (11).

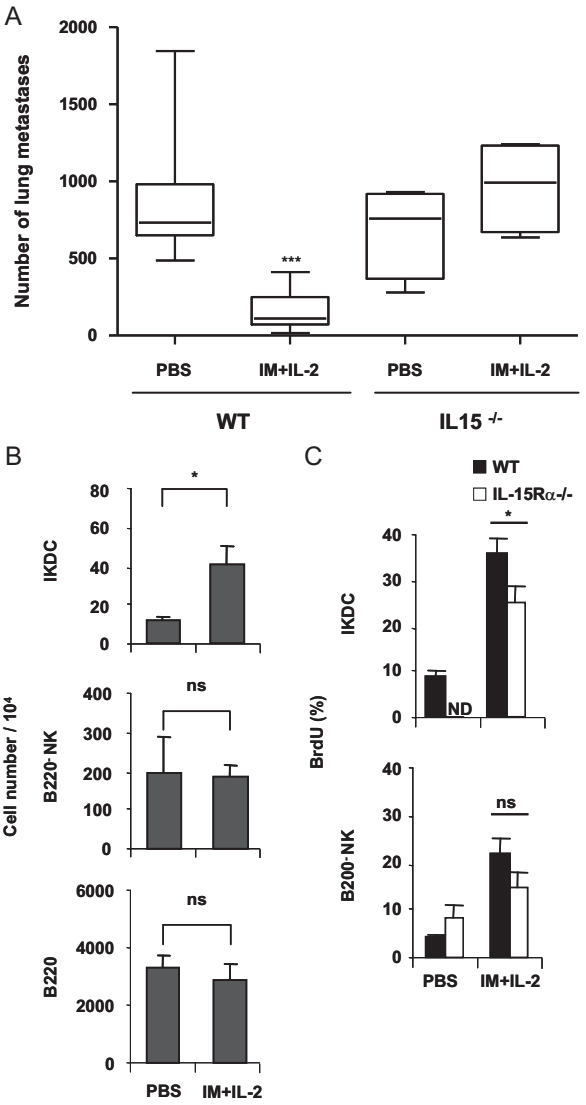

FIGURE 2. IL-15 is required for the proliferation of IKDC and the antitumor effects of IM + IL-2. *A,* Established B16F10 lung metastases model in C57BL/6 mice (WT) and IL-15 knockout (IL-15$^{-/-}$) mice. Same experimental setting as in Fig. 1*C*, but injections were performed in WT vs IL-15$^{-/-}$ mice. Statistical analyses were performed using the Kruskal-Wallis; ***, $p < 0.001$. *B,* Naive C57BL/6 mice (WT) were treated with PBS and combined therapy IM + IL-2. Splenocytes were harvested at day 11 for the enumeration of IKDC ($CD3^-CD19^-CD11c^{int}B220^+NK1.1^+$), $B220^-$ NK cells ($CD3^-CD19^-B220^-NK1.1^+$), and $B220^+$ cells. *C,* Naive C57BL/6 mice (WT) or IL-15R$\alpha^{-/-}$ mice were treated with PBS and combined therapy IM + IL-2 and injected with BrdU before flow cytometry analysis of splenocytes according to protocols described in *Materials and Methods*. Dividing cells that incorporated BrdU are measured as a percentage among the IKDC (*upper panel*) or $B220^-$ NK cell subsets (*lower panel*). *, $p < 0.05$; ND, Not detected; ns, nonsignificant.

Thus, combining IM to IL-2 appeared to be the optimal therapeutic option to induce NK cell-mediated tumor regressions.

Absolute requirement for IL-15 in the antitumor efficacy of IM + IL-2

IKDC were found in tumor beds and were necessary and sufficient to prevent tumor outgrowth after adoptive cell transfer in Rag$^{-/-} \times$ IL-2R$^{-/-}$ hosts (11, 24, 25). Moreover, we just showed that IL-15/IL-15Rα dictates IKDC effector functions (our unpublished data). Therefore, we investigated the role of IL-15 in the efficacy of IM + IL-2 against B16F10 lung metastases by comparing the

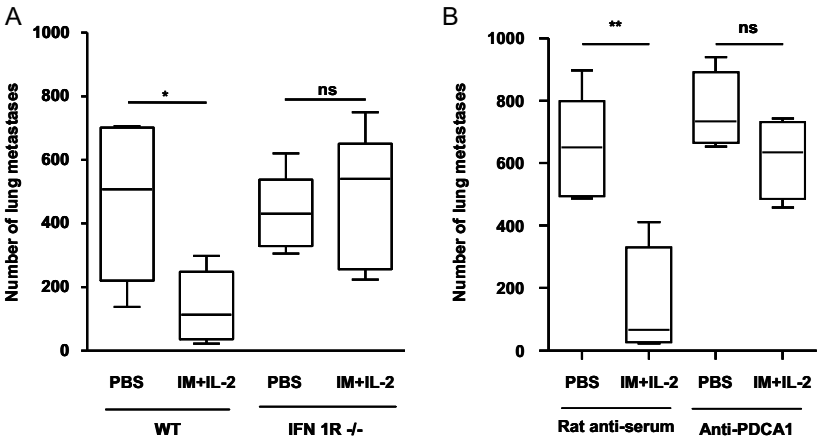

FIGURE 3. Role of type 1 IFN in IM + IL-2-mediated antitumor effects. *A,* Established B16F10 lung metastases model in C57BL/6 mice (WT) and type I IFN receptor-deficient mice. Same experimental setting as in Fig. 1*C*. *B,* Depletion of C57BL/6 mice using the anti-PDCA1 Ab. Same experimental setting as in Fig. 1*C*. A representative experiment of two to three including five mice per group is depicted. Statistical analyses were performed using the Kruskal-Wallis test. Values of p inferior to 0.05 or 0.01 confidence interval are indicated with * or **, respectively. ns, nonsignificant.

numbers of tumor foci in lung parenchyma of WT vs IL-15$^{-/-}$ mice (devoid of IKDC). IL-15 was not only involved in the natural immunosurveillance against B16F10 establishment but also in the IM + IL-2-induced antitumor activity (Fig. 2*A*). Like recombinant murine IL-15, IM + IL-2 could promote the accumulation of IKDC in the spleen by 4-fold (Fig. 2*B* and our unpublished data) while the absolute numbers of B220$^-$ NK and B220$^+$ cells remained unchanged (Fig. 2*B*). Importantly, IM + IL-2 promoted the entry of cells into cycle but IKDC cell proliferation was more efficient than that of B220$^-$NK cells (Fig. 2*C*). The IM + IL-2-driven IKDC proliferation was not entirely abolished in IL-15R$\alpha^{-/-}$ mice (Fig. 2*C*), presumably as a result of a direct effect of IL-2 (our unpublished data). Nevertheless, these data outline the involvement of IL-15/IL-15Rα in the proliferation of IKDC during IM + IL-2 treatment.

In conclusion, the combination of IM + IL-2 promoted the regression of established tumors in an IL-15-dependent manner.

The role of pDC and IFN-type 1R signaling in tumor regression

Trans-presentation of IL-15 is a biological attribute of conventional DC (cDC) stimulated through IFN-type 1R (26, 27) and is essential for the cDC-mediated NK cell triggering during TLR activation in vivo. We addressed whether the combination of IM + IL-2 would also implicate IFN-type 1R signaling. IFN-type 1R played a major role in the tumor growth inhibition provoked through therapy combining IM + IL-2 (Fig. 3*A*). Since the main source of IFN-α remains pDC (28), we addressed the role of pDC in the antitumor effects of the combination therapy. Depletion of pDC using the 120G8 mAb before tumor inoculation significantly curtailed the therapeutic efficacy of IM + IL-2 (Fig. 3*B*).

Because pDC were not found in tumor infiltrates (11), it remains to be investigated at which sites pDC may play a role and whether they could represent the source of IFN-α.

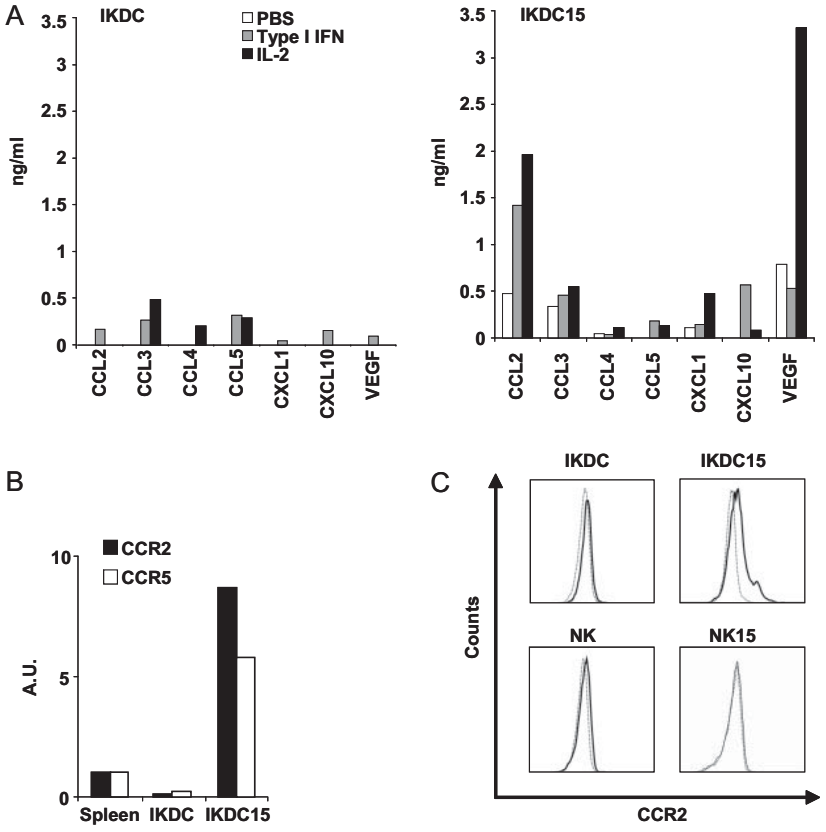

FIGURE 4. IL-15/IL-15Rα stimulates IKDC to respond to type 1 IFN and IL-2 for CCL2 and CCR2 expression. *A,* Cytokine and chemokine release. Resting IKDC or IKDC15 (IL-15Rα/IL-15 expanded IKDC, as described in our unpublished data) were stimulated for 24 h with medium, type 1 IFN, or IL-2. Multiplex analyses of cytokine and chemokine release were performed in the culture supernatants. *B,* Quantitative RT-PCR were performed on freshly sorted IKDC and IKDC15 (at day 7 of expansion). *C,* FACS analyses of CCR2 expression were performed on freshly sorted IKDC or B220$^-$ NK cells or on IL-15/IL15-Rα-stimulated IKDC or NK (for 7 days). Dotted line, Isotype control; black line, anti-CCR2 Ab staining.

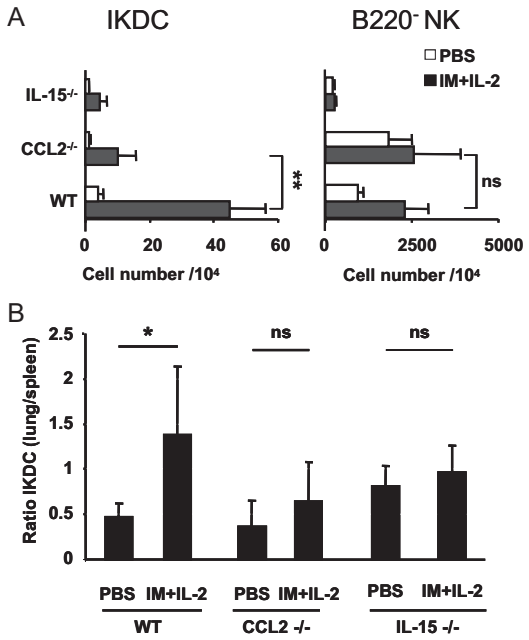

FIGURE 5. CCL2-dependent IKDC homing into tumor tissues. B16F10 tumor cells (5×10^5) were inoculated in the tail vein at day 0 and mice were sacrificed at day 11. *A,* Enumeration of tumor-infiltrating IKDC and B220$^-$ NK cells during PBS or combined therapy with IM + IL-2 in C57BL/6 (WT), CCL2-deficient (CCL2$^{-/-}$), and IL-15-deficient (IL-15$^{-/-}$) mice after lung dissociations. *B,* As in *A*, but calculation of an IKDC ratio defined as: number of IKDC in the lung divided by number of IKDC in the spleen (no. of lung IKDC ÷ no. of spleen IKDC). These data have been obtained from seven animals per group. Statistical analyses were performed using Student's *t* test, *p* inferior to 0.05 are indicated with ∗. ns, nonsignificant.

In summary, IL-15 and IFN-type 1 signaling are markedly involved in the therapeutic effectiveness of the c-kit tyrosine kinase inhibitor combined with IL-2.

The synergistic effects of IFN-type 1 and IL-15 for CCL2 production by IKDC

Because IL-15 and type 1 IFN are major players in IM + IL-2-mediated immunosurveillance against B16F10, we investigated their role in modulating the chemokine/chemokine receptor expression profile in freshly sorted IKDC before and after stimulation by IL-15 *trans*-presentation (so-called "IKDC15" (our unpublished data)). In resting conditions, IKDC cells responded weakly to type 1 IFN and IL-2 for CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL10 (IFN-γ-inducible protein 10), and vesicular endothelial growth factor (Fig. 4*A*). Following *trans*-stimulation with IL-15, IKDC strongly produced CCL2 (MCP-1) and factors involved in tissue remodeling and/or angiogenesis such as vesicular endothelial growth factor and CXCL1 (KC/GROα) in response to type 1 IFN or IL-2 (Fig. 4*A, right panel*). Moreover, following *trans*-stimulation with IL-15, IKDC enhanced their transcription of the inflammatory chemokine receptors CCR2 and CCR5 (Fig. 4*B*). Flow cytometry analyses confirmed the expression of CCR2 and CCR5 on the IKDC15 cell surface (Fig. 4*C* and data not shown). Importantly, IL-15/IL-15Rα-driven B220$^-$ NK cells failed to express CCR2 (Fig. 4*C*).

In conclusion, IL-15 *trans*-presentation licensed IKDC to respond to type 1 IFN and IL-2 and greatly synergized with these stimuli (Fig. 4*A*) for the production of CCL2 by IKDC. In addition, IL-15/IL-15Rα induced the up-regulation of CCR2 receptors on the surface of IKDC as a prerequisite for an autocrine-positive feedback loop for IKDC recruitment in tumor beds.

CCL2-dependent trafficking of IKDC into tumor beds

We next addressed the role of CCL2 in the accumulation of IKDC into B16F10 lung metastases. Because our in vitro data support the view that CCL2 production by IKDC is stimulated by the synergistic effects between IL-15/IL-15Rα and type 1 IFN (Fig. 4), we determined the impact of IM + IL-2 therapy on the proportion of NK1.1$^+$ innate effectors (B220$^-$NK vs IKDC) accumulating in tumor beds in WT, IL-15$^{-/-}$, and CCL2$^{-/-}$. IM + IL-2 significantly increased (up to 10-fold) the numbers of IKDC in WT mice in a CCL2 and IL-15-dependent manner (Fig. 5*A, left panel*). In contrast, B220$^-$NK cell numbers barely increased in a CCL2-independent fashion (Fig. 5*A, right panel*). Accumulation of IKDC into lung metastases could result from IKDC recruitment and/or local proliferation. The ratio between spleen and tumor-infiltrating IKDC augmented (by up to 3-fold) during therapy in WT but not CCL2-deficient hosts, supporting the contention that both recruitment and proliferation occurred during IM + IL-2 (Fig. 5*B*).

Therefore, IL-15 and CCL2 are mandatory for proliferation and recruitment of IKDC into tumor beds during therapy with IM + IL-2.

Discussion

These findings underscore the antitumoral efficacy of associating two NK cell adjuvants, one blocking the c-kit tyrosine kinase of cDC (7) and the IL-2, a major lymphokine-inducing proliferation and activation of T and NK cells (22). This study addresses the mechanisms by which IKDC, recently described as novel innate effectors involved in antitumor immunosurveillance, could accumulate in tumor beds and be associated with tumor regression (11). In this study, we unravel the key role of the IFN type 1R/IL-15/CCL2 axis in the antitumor effects mediated by IM + IL-2 and underscore the potential of IL-15 to license IKDC to proliferate, kill (our unpublished data) and respond to IFN type 1 (and IL-2) by producing CCL2 and expressing CCR2. Finally, we highlight the relevance of the IL-15-driven CCL2/CCR2 selective expression on IKDC for their preferential homing to tumor tissues.

Before the description of IKDC cells, the functional interaction between conventional DC and bona fide NK cells (29, 30) was described to play a key role in the interplay between innate and cognate immunity (31, 32). IL-15/IL-15Rα is crucial for the DC-mediated NK cell priming in vitro (26, 33) and in vivo (26). TLR ligands or agonistic anti-CD40 mAb could both induce the expression of IL-15Rα on DC in vivo (26). Moreover, IFN-α has been identified as a key inducer of IL-15/IL-15Rα (26, 27, 34). The precise mechanism by which IM + IL-2 could induce IL-15/IL-15Rα in our model system remains unclear. Our unpublished data indicated that IM alone could not mediate antitumor efficacy in TRIF$^{-/-}$ mice, indicating that IFN type 1 could be necessary in the immunostimulatory effects of IM. Supporting this contention, we now show that the combination of IM + IL-2 required IFN type IR signaling (Fig. 3*A*). A previous work reported that IKDC derived from BALB/c mice and stimulated through TLR9 ligation could secrete IFN-α (12), but these data remain controversial (35). Since pDC constitute a main source of IFN-α (28), at least during viral infection or TLR7 and TLR9-dependent insults, we could hypothesize that pDC may also be implicated in the efficacy of IM + IL-2. Depleting pDC resulted in an abrogation of the IM +IL-2-mediated antitumor effects in vivo (Fig. 3*B*).

We could postulate that IM + IL-2 might stimulate IFN-α production by pDC in vivo, leading to the up-regulation of IL-15/IL-15Rα on cDC, thereby promoting the DC/NK cell cross-talk. Alternatively, a direct pDC-NK cell interaction could occur whereby IL-2 and/or IM could play a regulatory role. Indeed, we and others reported that IL-2 could trigger TRAIL expression on both pDC and NK cells during a pDC/NK cell cross-talk in vitro (36) and that IL-2-activated NK cells could stimulate IFN-α and IL-6 production by pDC (37). Interestingly, IM could synergize with a concomitant viral stimulation to enhance IFN-α production by pDC in leukemic patients (38). It remains to be determined whether IM could increase IFN-α secretion by pDC when associated with IL-2 in our model. In these two above-mentioned hypotheses (cDC/NK or pDC/NK dialogs), bona fide NK cells could be activated either through *trans*-presentation of IL-15 and/or by IFN-α, accounting for the efficacy of IM + IL-2. However, several lines of evidence argue against those hypotheses. First, a significant proliferation of NK cells could not be documented during IM + IL-2 therapy (Fig. 2), neither in a tumor-free (Fig. 2) nor in tumor-bearing mice (Ref. 11 and not shown). Second, IM + IL-2 could not only prevent the establishment of B16F10 lung metastases (that are notoriously NK cell dependent) but also the s.c. expansion of the same tumor (Fig. 1), supporting the notion that effectors other than NK cells might be involved. Third, in synergy with IL-15/IL-15Rα, IFN type 1 and IL-2 could directly stimulate B220$^-$ NK cells for CCL3 (MIP-1α) secretion (data not shown). However, CCR5 was not critical for the efficacy of IM + IL-2 (our unpublished data).

To date, it remains quite complex to directly ascribe the tumoricidal activity of IM + IL-2 to IKDC. Since adaptive transfer of IM + IL-2-activated IKDC into Rag$^{-/-}$ $\times$ IL-2R$\gamma^{-/-}$ mice could abrogate B16F10 growth while NK cells failed to do so (11), we proposed that IKDC could represent the most potent final effector leading to an efficient antitumor activity of IM + IL-2. Supporting this view, we demonstrate here that IKDC are significantly enriched compared with B220$^-$ NK cells in spleens of mice treated with IM + IL-2 (Fig. 2*B*). Indeed, the IKDC:B220$^-$ NK cell ratio was increased by 4-fold during therapy (0.2 vs 0.05, in IM + IL-2 and PBS-treated mice, respectively, $p < 0.001$). Moreover, we showed the crucial role of IL-15/IL-15 Rα in the proliferation and in the CCR2/CCL2-dependent trafficking of IKDC cells into tumor beds during the therapy (Figs. 2, *B* and *C* and 5*A*). Finally, in our unpublished data, we showed that IL-15Ra/IL-15 licensed IKDC (and not bona fide NK cells) with TRAIL-dependent killing, which was critical in the efficacy of IM + IL-2 (11). However, the final demonstration of the key role of IKDC in tumor immunosurveillance, as an effector or conceivably as a professional APC (12), will await further investigations and the delineation of specific markers assigned to IKDC and not to other subsets of NK cells (39).

Because our data suggested that the combination therapy with IM + IL-2 could be useful to boost the natural immunosurveillance against tumors sensitive to TRAIL-dependent apoptosis (11) and was more potent than other combination therapies (Fig. 1), we launched a phase I trial "IMAIL-2" at the Institut Gustave Roussy aimed at targeting IM-resistant or stabilized gastrointestinal sarcomas and TRAIL-sensitive cancers. It is conceivable that the monitoring of innate effectors in patients treated with high dosages of IL-2 combined with Gleevec might allow the identification of the human counterpart of mouse IKDC.

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Disclosures

The authors have no financial conflict of interest.

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4.1.3 La trans-présentation de l'IL-15 détermine les fonctions effectrices des IKDC (ARTICLE 2)

4.1.3.1 Résumé

En parallèle de ces travaux *in vivo* sur la dépendance à l'IL-15 des IKDC et de l'efficacité anti-tumorale du traitement IM+IL-2, nous avons procédé à d'autres expérimentations. Nous avons précisé les facteurs de dépendance de l'IKDC : la quantité d'IKDC diminue fortement dans des souris IL-15$^{-/-}$, IL-15Ralpha$^{-/-}$, IL-2$^{-/-}$ ou IL-15$^{-/-}$xIL-2$^{-/-}$, de même que dans des IL-2Rgamma$^{-/-}$ xRAG1$^{-/-}$. Ceci nous confirme que l'IKDC dépend pour son développement homéostatique de la présence de l'IL-15/IL-15Ralpha. Nous avons ensuite testé cette dépendance des IKDC lors d'une inflammation : en traitant des souris sauvages ou IL-15Ralpha$^{-/-}$ avec de l'IL-2, de l'IL-15 ou du CpG, nous avons pu constater que les IKDC des souris KO étaient devenues incapables de proliférer en réponse au CpG et à l'IL-15 (confirmant l'importance de la présence de l'IL-15Ralpha dans un contexte infectieux) mais conservaient une réponse prolifération normale à l'IL-2.

Nous avons par la suite établi que l'IL-15/IL-15Ralpha était nécessaire et suffisante pour permettre l'expansion *ex vivo* des IKDC. Cette culture d'IKDC en IL-15 sur cellule nourricière MS5 IL-15Ralpha^{+} permet d'obtenir des quantités importantes de cellules après une semaine de culture.

Cette exposition à l'IL-15 modifie cependant les fonctions des IKDC : elles deviennent incapables de présenter l'antigène, elles voient leur capacités lytiques augmenter (via perforine, granzyme B et TRAIL), et deviennent capable de répondre à la présence de TLR-3L et -4L en produisant CCL5 et CCL2, respectivement.

Plus étonnant, les IKDC-15 cultivées avec des cellules tumorales permettent d'induire après tranfert *in vivo* un retard à la pousse sous-cutanée de ces mêmes tumeurs, phénomène qui n'est pas retrouvé avec des cellules NK cultivées dans les mêmes conditions. Cette faculté résiste à l'action immunosuppressive du TGF-beta.

4.1.3.2 Discussion

Les effets de l'IL-15 sur les cellules NK1.1^{+} sont connus depuis plus de dix ans (Puzanov, Bennett et al. 1996), et l'importance de la trans-présentation de l'IL-15 est étudiée, notamment dans le contexte tumoral (Stoklasek, Schluns et al. 2006).

Cette étude (Ullrich, Bonmort et al. 2008) a donc permis de séparer deux phénoménologies distinctes : les IKDC et les NK différent en certains points majeurs. D'une part, les IKDC peuvent bien exprimer du CMH-II et présenter l'antigène à un clone CD4, tandis que les NK en sont incapables ; cette propriété est perdue en présence d'IL-15. D'autre part, les IKDC traitées par IL-15 sont capables de lyser des cibles tumorales via TRAIL, contrairement aux NK qui utilisent principalement la voie perforine/granzyme B dans les mêmes conditions. Ceci a été confirmé depuis par une autre équipe qui, utilisant la technique du transfert de gène hydrodynamique, a montré que la production d'IL-15 *in vivo* augmente le nombre d'IKDC et leur fonctions lytiques via TRAIL (Arina, Murillo et al. 2008).

Ensuite, l'IL-15 permet aux IKDC de réagir à des stimuli bactériens (TLR-3L et -4L) et viraux (IFN-alpha) via CCL2 et CCL5. Enfin, l'IL-15 permet à l'IKDC de modifier son comportement chimiotactique en exprimant CCR2. Ces différentes observations ne permettent cependant pas d'affirmer que ces deux phénotypes appartiennent à deux populations très différentes ou à deux aspects de la même entité.

Il est à noter que dans cette étude nous avons été incapables d'induire l'expression du B220 sur des NK. Concernant cette expression du B220 sur les NK, il est possible de raisonner pour expliquer la différence avec les résultats de Vosshenrich *et al*. Comme indiqué par ces auteurs, la distribution du B220 sur le total des cellules NK1.1^{+}/CD3^{-}/CD19^{-} n'est pas bimodale. Il est donc possible que nos stratégies de tri cellulaire aient différé : si nous trions les B220$^{hi/int}$ et d'autres auteurs les B220^{hi}, la fraction notée B220^{-} peut sensiblement différer. Ainsi, si la fraction « NK » contient des cellules B220^{int}, qui sont en fait des IKDC, celles-ci vont proliférer notablement plus vite que des NK B220^{-}, et vont envahir la fraction NK.

Par ailleurs, les expériences de prolifération *in vivo* (incorporation de bromodeoxyuridine (BrdU)) ont montré que le B220 n'est pas un marqueur spécifique de NK en prolifération, car le BrdU ne co-localise pas avec le B220. Par contraste, les cellules BrdU^{+} étaient intégralement CD11c^{+} dans toutes les conditions d'activation testées (Figure 2).

a) b)

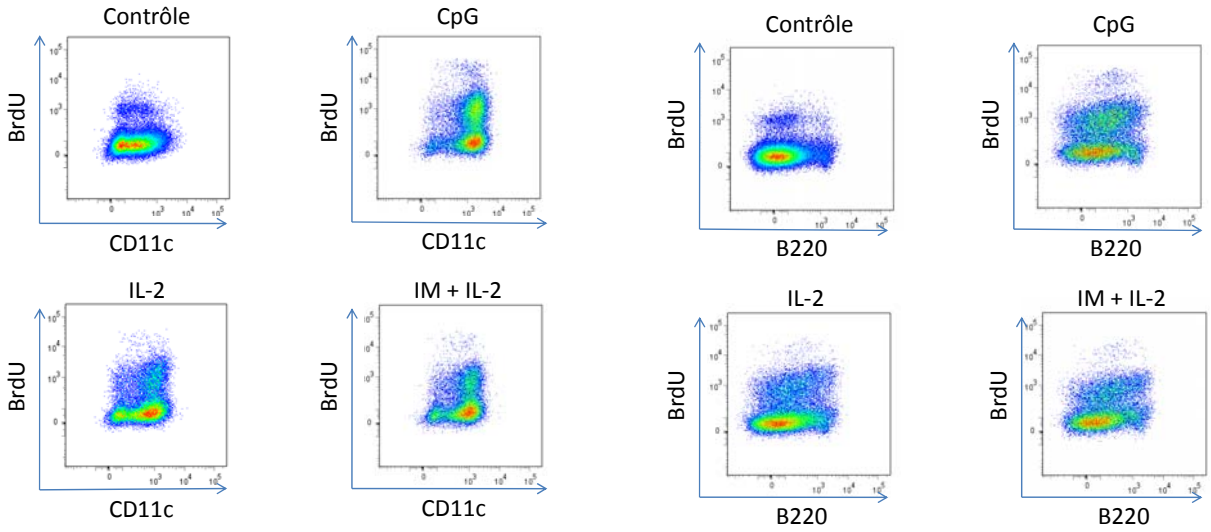

Figure 2 : Incorporation du BrdU dans des lymphocytes NK activés *in vivo*. Il s'agit de splénocytes CD3$^-$ CD19$^-$NK1.1$^+$DX5$^+$ de souris contrôles (en haut à gauche), traitées avec du CpG (en haut à droite), de l'IL-2 (en bas à gauche) ou la combinaison IM+IL-2 (en bas à droite). En a) l'incorporation du BrdU est montrée en fonction du CD11c et en b) en fonction du B220. (Voir matériel et méthodes de l'article 2.)

Enfin, il nous a été impossible d'induire l'expression du CMH-II sur les IKDC uniquement avec des cytokines recombinantes. La seule condition qui permet d'observer du CMH-II sur les IKDC est la co-culture avec des DC dérivée de la moelle osseuse. Ceci nous permet donc d'affirmer qu'un contact cellulaire est nécessaire pour cette expression de CMH-II, mais implique une interaction spécifique, puisque le contact des DC le provoque, mais pas celui des MS5.

Trans-Presentation of IL-15 Dictates IFN-Producing Killer Dendritic Cells Effector Functions[1]

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IFN-producing killer dendritic cells (IKDC) were initially described as B220$^+$CD11c$^+$CD3$^-$NK1.1$^+$ tumor-infiltrating cells that mediated part of the antitumor effects of the combination therapy with imatinib mesylate and IL-2. In this study, we show their functional dependency on IL-15 during homeostasis and inflammatory processes. *Trans*-presentation of IL-15 by IL-15Rα allows dramatic expansion of IKDC in vitro and in vivo, licenses IKDC for TRAIL-dependent killing and endows IKDC with immunizing potential, all three biological attributes not shared by B220$^-$NK cells. However, IL-15 down-regulates the capacity of IKDC to induce MHC class I- or II-restricted T cell activation in vitro. *Trans*-presentation of IL-15 by IL-15Rα allows IKDC to respond to TLR3 and TLR4 ligands for the production of CCL2, a chemokine that is critical for IKDC trafficking into tumor beds (as described recently). We conclude that IKDC represent a unique subset of innate effectors functionally distinguishable from conventional NK cells in their ability to promptly respond to IL-15-driven inflammatory processes. *The Journal of Immunology,* 2008, 180: 7887–7897.

Interferon-producing killer dendritic cells (IKDC)[4] represent a rare but unique entity sharing hybrid features in-between dendritic and NK cells (1–7). They could be found in trace amounts in all lymphoid organs at the steady-state but accumulated during certain inflammatory processes, such as tumor regression under the influence of imatinib mesylate (IM) + IL-2 (2) or *Listeria* infection (1). In such circumstances, IKDC expressed high levels of MHC class II molecules and maintained CD11c expression while lacking CD19 and CD40 molecules, thereby diverging from bona fide B cells. Chan et al. (1) could demonstrate that IKDC derived from lymph nodes of BALB/c mice behaved as conventional myeloid DC in their capacity to traffic to secondary lymphoid organs and to present Ag to MHC class II-restricted CD4$^+$ TCR transgenic (Tg) T cells. Nevertheless, IKDC resembled NK cells in that they exhibited potent killing activity during tumor regression (2, 3, 8–10). Thus, NK cell experts may consider IKDC as a subset of immature NK cells (11–14), that could display molecules such as B220, CD11c (15), or even MHC class II upon activation. In an attempt to delineate a functional hierarchy between IKDC and NK cells, we sought to determine the regulatory cytokine dictating homeostasis and activation of IKDC in vitro and in vivo. Although IL-15 is crucial for the homeostasis of both innate effectors at the steady state, *trans*-presentation of IL-15 endowed IKDC with unique biological functions that are not shared by conventional B220$^-$NK cells. Indeed, *trans*-presentation of IL-15 by IL-15Rα licensed IKDC for proliferation in vitro and in vivo, for killing in a TRAIL-dependent manner, and, finally, for induction of antitumor immunity. Therefore, these data underscore the fundamental role of IL-15 in IKDC biology, suggesting a potential involvement of IKDC not only in the control of tumor growth, but also in various inflammatory processes.

Materials and Methods

Mice and cell lines

Female C57BL/6 wild-type (WT) mice were obtained from the Centre d' Elevage Janvier (Le Genest St. Isle, France) and used at 6–10 wk of age. IFN type 1R$^{-/-}$, CCL2/MCP-1$^{-/-}$, and CD45.1$^+$ mice backcrossed on a C57BL/6 background were provided by Centre d'Elevage d'Orléans (Centre de distribution, typage et archivage animal Orléans, France). IL-15R$\alpha^{-/-}$, IL-15$^{-/-}$, IL-2/IL-15$^{-/-}$, IL-2$^{-/-}$, and IL-15 Tg mice were backcrossed on a C57BL/6 six to eight times and maintained at the animal facility of S. Bulfone-Paus (Research Center Borstel, Borstel, Germany). IL-2R$\gamma^{-/-}$ × Rag2$^{-/-}$ were kindly provided by E. Vivier, Centre d'Immunologie de Marseille, France. Tg OTI and OTII mice were a kind gift by O. Lantz (Institut Curie, Paris, France). Animals were all maintained according to the Animal Experimental Ethics Committee Guidelines. B16F10 is a melanoma cell line syngeneic of C57BL/6 (provided by M. T. Lotze,

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[4] Abbreviations used in this paper: IKDC, interferon-producing killer dendritic cells; BMDC, bone marrow-derived DC; IM, imatinib mesylate; rm, recombinant murine; ODN, oligodeoxynucleotide; rh, recombinant human.

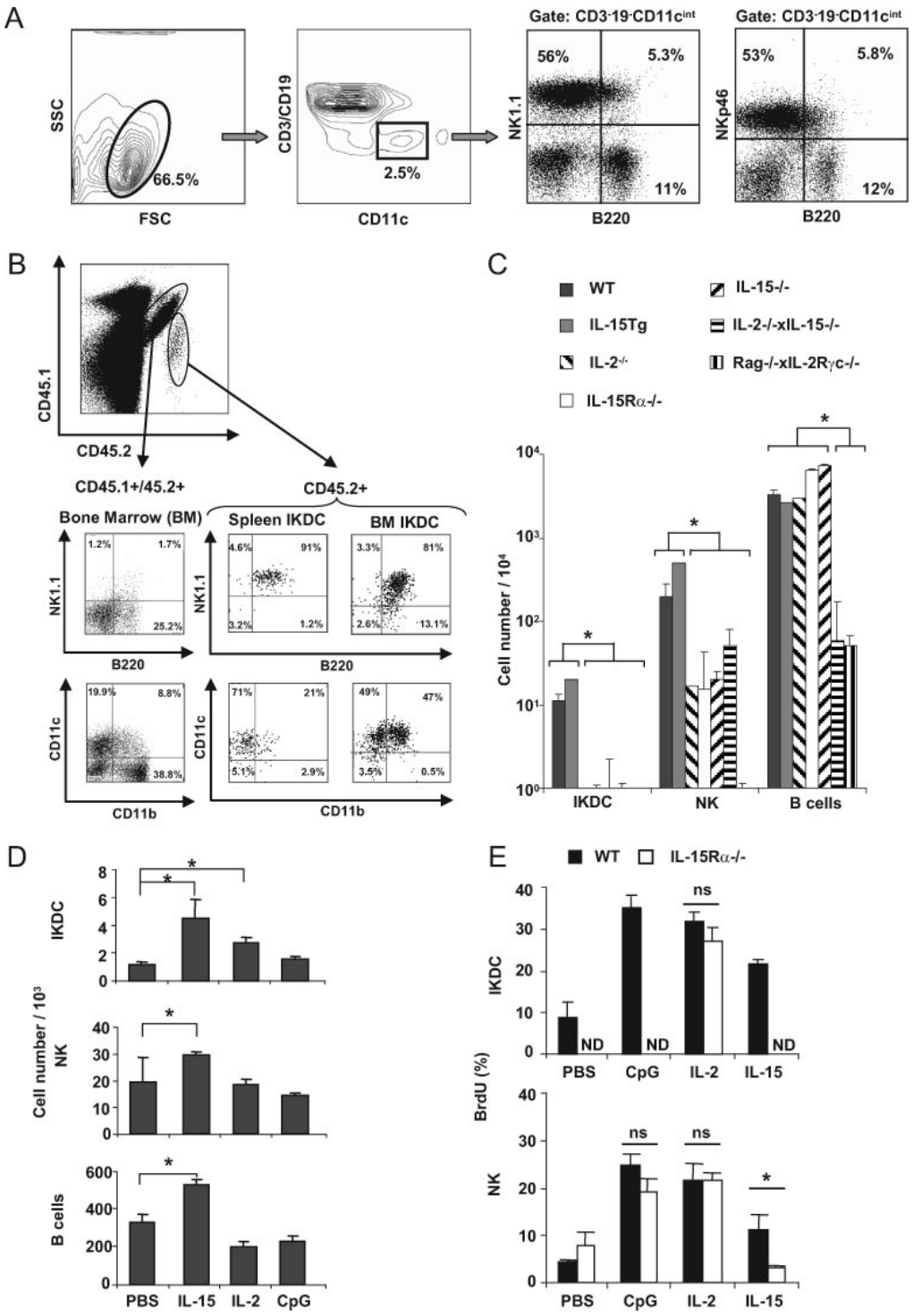

FIGURE 1. IL-15 drives homeostasis and inflammation-induced proliferation of IKDC. *A*, Phenotypic characterization of IKDC. Flow cytometry analyses were performed after staining splenocytes with FITC anti-CD3/CD19, PE anti-CD11c, PerCP anti-B220, PE-Cy7 anti-NK1.1, and Alexa 647 anti-NKp46 mAb. *B*, Adoptive transfer of IKDC in vivo. IKDC were purified from spleen and bone marrow of CD45.2$^+$ mice and transferred into lethally irradiated CD45.1$^+$ mice, which were rescued with 1×10^5 CD45.1$^+$×CD45.2$^+$ bone marrow cells. Flow cytometry was performed on day 12 on splenocytes using FITC anti-CD45.1, PE anti-CD45.2, PerCP anti-B220, allophycocyanin anti-CD11b, PE-Cy7 anti-NK1.1, and PB anti-CD11c mAb. *C*, Enumeration of IKDC and NK cells in mice deficient in γ common chain-dependent cytokines. Flow cytometry analyses were performed on splenocytes derived from different mouse genetic backgrounds after staining as shown in *A*. IKDC were defined as CD3$^-$CD11c$^+$B220$^+$NK1.1$^+$, NK cells as CD3$^-$CD11c$^{+/-}$B220$^-$NK1.1$^+$ and B cells as the whole B220$^+$ population. *D*, Enumeration of IKDC and NK cells in C57BL/6 mice after different stimuli in vivo. Flow cytometry was performed as described above. *E*, BrdU incorporation in IKDC and NK cells following stimulation in vivo. WT or IL-15Rα loss of function mice were treated with different stimuli and injected with BrdU before flow cytometry analysis of splenocytes according to protocols described in *Materials and Methods*. The mean percentages ± SEM of BrdU incorporation in 7–10 mice are presented; (*) indicates statistically significant differences at 95% confidence between IL-15Rα$^{-/-}$ compared with WT C57BL/6 mice. ND, not determinable.

University of Pittsburgh, PA) and was cultured in RPMI 1640 (Invitrogen) with 10% heat-inactivated FBS enriched with 5% L-glutamine, non-essential amino acids, sodium pyruvate, and antibiotics. MS-5-feeder cell lines (provided by W. Vainchenker, IGR, Villejuif, France) were cultured in IMDM (Sigma-Aldrich) containing 10% heat-inactivated FBS, 5% L-glutamine, sodium pyruvate, and antibiotics.

Table I. *Flow cytometric analyses of cytokine-stimulated B220$^-$NK and IKDC cells*[a]

		24-h Duration				48-h Duration			
		B220 (%)		Class II (%)		B220 (%)		Class II (%)	
Cytokine 1	Cytokine 2	NK	IKDC	NK	IKDC	NK	IKDC	NK	IKDC
IL-2		0.25	96, 39	0.43	1.88	0.72	94, 80	1.29	3, 63
	IL-12	0.79	97, 38	1.56	1.24	0.82	96, 79	0.85	2, 99
	IL-18	0.23	96, 46	0.43	2.35	1.67	92, 23	1.61	5, 22
	IFN-α	0.51	97, 08	0.27	1.05	0.54	95, 65	0.63	3, 2
IL-15		0.34	96, 32	1.33	3.08	0.24	97, 64	0.93	5, 12
	IL-12	0.52	96, 83	1.34	2.06	0.46	95, 93	1.28	2, 59
	IL-18	0.78	97, 41	1.15	2.27	1.35	94, 55	0.64	6, 09
	IFN-α	1.11	97, 55	1.26	1.17	0.61	95, 52	0.98	3, 47
BMDC	IL-15	0.89	97, 18	1.02	11, 03	1.32	95, 84	0.87	7, 35

[a] B220$^-$NK (CD3$^-$CD19$^-$B220$^-$NK1.1$^+$) and IKDC (CD3$^-$CD19$^-$B220$^+$NK1.1$^+$) were cell-sorted and incubated in vitro in the presence of different cytokines or with BMDC (1:1). After 24 or 48 h, cells were harvested and analyzed for B220 and MHC class II expression. This table indicates the percentage of B220$^+$ or I-A/I-E$^+$ cells amongst total NK1.1$^+$ cells. IL-2 is used at 50,000 IU/ml, IL-15 at 20 ng/ml, IL-12 at 10 ng/ml, IL-18 at 2.5 ng/ml, and IFN-α at 2,500 IU/ml. Cells were plated at 100,000 cells per well in 96-well, round-bottomed plates in 100 μl of complete medium. These experiments have been performed three times with similar results.

Abs and flow cytometry analyses

FACS analyses of IKDC, IKDC15, and NK cells were performed using allophycocyanin-conjugated anti-CD11c (HL3), PE-Cy7-conjugated anti-NK1.1 mAb (PK136), allophycocyanin-Cy7-conjugated anti-B220 mAb (RA3–6B2), and PerCP-conjugated anti-CD3 mAb (17A2). We further stained with PE- or FITC-conjugated mAb to examine the following molecules: MHC class II (AF6-120.1), CD40 (3/23), CD80 (16.10A1), CD86 (GL1), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD122 (TM-β), CD49b (Dx5), NKG2D (CX5), ckit (ACK2), CD69 (H1.2F3), CD19 (1D3), or CD27 (LG.3A10). Abs were purchased from BD Pharmingen or eBioscience. The anti-NKp46 mAb was kindly provided by E. Vivier, CIML, France. Cells were preincubated with Fc block for 20 min (CD16/CD32, 2.4G2; BD Pharmingen) in 2% FBS and 2% mouse serum and afterward stained for 20 min at 4°C with the different Abs at 1/200. Immediately before FACS analysis, 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) was added. FACS analysis was performed by LSRII (BD Biosciences) using FACSDiva software (BD Biosciences) and CellQuestPro software (BD Biosciences) or FlowJo (Treestar).

Sorting of NK and IKDC cells

NK and IKDC cells from C57BL/6 mice were sorted on a Mo-Flo instrument (DAKO) in two steps. First, we enriched NK1.1$^+$ cells. Second, we sorted CD3$^-$CD19$^-$CD11c^{int}B220$^+$NK1.1$^+$ cells (defined as "IKDC") and CD3$^-$CD19$^-$CD11c$^{+/-}$B220$^-$NK1.1$^+$ cells (defined as B220$^-$NK cells henceforth). Cells were stained with FITC-conjugated anti-CD3 and CD19 mAb, PE-conjugated anti-CD11c mAb, PE-Cy7-conjugated anti-NK1.1 mAb, and PB-conjugated anti-B220 mAb. The purity of cell separation exceeded 97%. Purified NK cells and IKDC were then used for functional experiments.

In vitro expansion of IKDC15

Freshly cell sorted IKDC were cultured in the presence of murine stromal cells MS-5 (16). One or 2 days before coculture with IKDC, MS-5 cells were plated in round-bottom 96-well plates (7500 cells per well). Cultures of IKDC were initiated by seeding 10^4 freshly sorted IKDC in MS-5 precoated 96-well plates in DMEM (Invitrogen) culture medium containing 4500 mg/l of glucose, 5% L-glutamin, pyruvate, and enriched with antibiotics, 10% Bovine Growth serum (Lot no. ANB 18298, HyClone), and 20 ng recombinant murine (rm) IL-15/ml (R&D Systems). Upon expansion of IKDC, stromal cells and culture medium were replaced twice a week. Inhibition of IL-15 *trans*-presentation was performed using anti-mIL-15Rα Ab (AF551, R&D Systems) at a concentration of 20–30 μg/ml in the presence of 20 ng/ml IL-15. Limiting dilution assays were also initiated in 96-well plates (1 cell per well) using the automated cell device unit. Although B220$^-$NK cells could not proliferate ex vivo in similar conditions as IKDC, we could maintain NK cells at high concentrations (5×10^5/ml) for 7 days on MS-5 feeders and rIL-15 to allow fair comparisons with IKDC.

Cytokine profiling of IKDC and NK cells

A total of 10^5 freshly sorted NK cells and IKDC or IKDC15 (obtained at day 7 of ex vivo expansion) or NK and IKDC stimulated with rmIL-15 (20 ng/ml; R&D Systems) for 24 h were further incubated with LPS at 100 ng/ml (InvivoGen) or CpG oligodeoxynucleotide (ODN) 1668 (MWG Biotech) at 5 μg/ml. These in vitro cultures were performed in 200 μl RPMI (Invitrogen) 10% FBS (Invitrogen) in 96 round-bottom well plates. After 24–36 h, cell supernatants were collected and commercial LUMINEX kits were used to determine chemokine and cytokine profiles (used according to the manufacturer's conditions, Linco Research/BioSource International).

Activation of OTII Tg T cells in vitro

FACS sorted 10^5 CD4$^+$ resting OTII lymphocytes purified from naive OTII Tg mice were incubated at various effector/T cell ratios (1:1, 1:5, 1:20, and 1:100) with different effector cells (such as resting IKDC, B220$^-$NK, bone marrow-derived DC (BMDC), IKDC15, or NK15 cells) after a 24-h coculture of effector cells with B16 tumor cells in the presence of 1 mg/ml OVA protein followed by extensive washing (three times in PBS 1× to remove resting traces of OVA protein). After a 20-h incubation period, T cells were stained with anti-CD3, anti-CD4, anti-Vα2, and anti-CD69 Ab and analyzed by FACS.

Cytotoxicity assay

^{51}Cr release killing assay was performed according to standard protocols using 2×10^3 $Na_2{}^{51}CrO_4$-labeled B16F10 tumor cells (T) incubated with various E:T ratios (1:1, 5:1, 10:1, 15:1, and 30:1) of effector (E) cells (NK vs IKDC stimulated or not with rmIL-15 in *trans*-presentation) for 4, 8, or 12 h. Supernatants were harvested for the measurement of chromium release (E) using γ emission counting (Topcount NXT, Packard Instrument). Spontaneous ^{51}Cr release (S) was counted in target cells alone, maximal ^{51}Cr release (M) from target cells treated with 5% alkyltrimethylammonium bromide and specific lysis was calculated according to the following: % lysis = (E-S)/(M-S)×100. As an additional method, crystal violet assay was used. Effector and target cells were mixed at different ratios for 24 or 48 h. Live tumor cells were revealed using a crystal violet staining as previously reported (17). Cocultures of E:T were performed in the presence of neutralizing anti-TRAIL Ab (N2B2, provided by H. Yagita, Juntendo University School of Medicine, Tokyo, Japan), commercial anti-FasL mAb (CD95L; eBioscience) at 10 μg/ml, or EGTA (Sigma-Aldrich) at 1 mM or Concanamycin A (Sigma-Aldrich) at 20 nM.

In vivo proliferation assays

WT C57BL/6, IL-15R$\alpha^{-/-}$ mice were treated with IL-2 alone (100,000 IU i.p. twice a day for 4 days), or rmIL-15 (R&D Systems at 0.5 μg i.p. daily for 4 days) or CpG ODN 1668 (MWG Biotec at 5 μg i.p. daily for 4 days). Mice received an i.p. injection of BrdU (100 μg/100 μl PBS) 1 day before sacrifice. Spleen cells were harvested and processed according to the manufacturer's protocol (BD Biosciences BrdU Flow kit). Briefly, cells were stained for surface Ags, fixed, and permeabilized. DNase digestion followed by staining with anti-BrdU mAb were performed before flow cytometry analyses.

Confocal microscopy

Cultured IKDC15 were resuspended in RPMI 1640, washed, and 5×10^4 cells were gently spread onto a slide coated with poly-L-lysine (Sigma-Aldrich). Slides were incubated for 45 min at 37°C. Cells were fixed in 4%

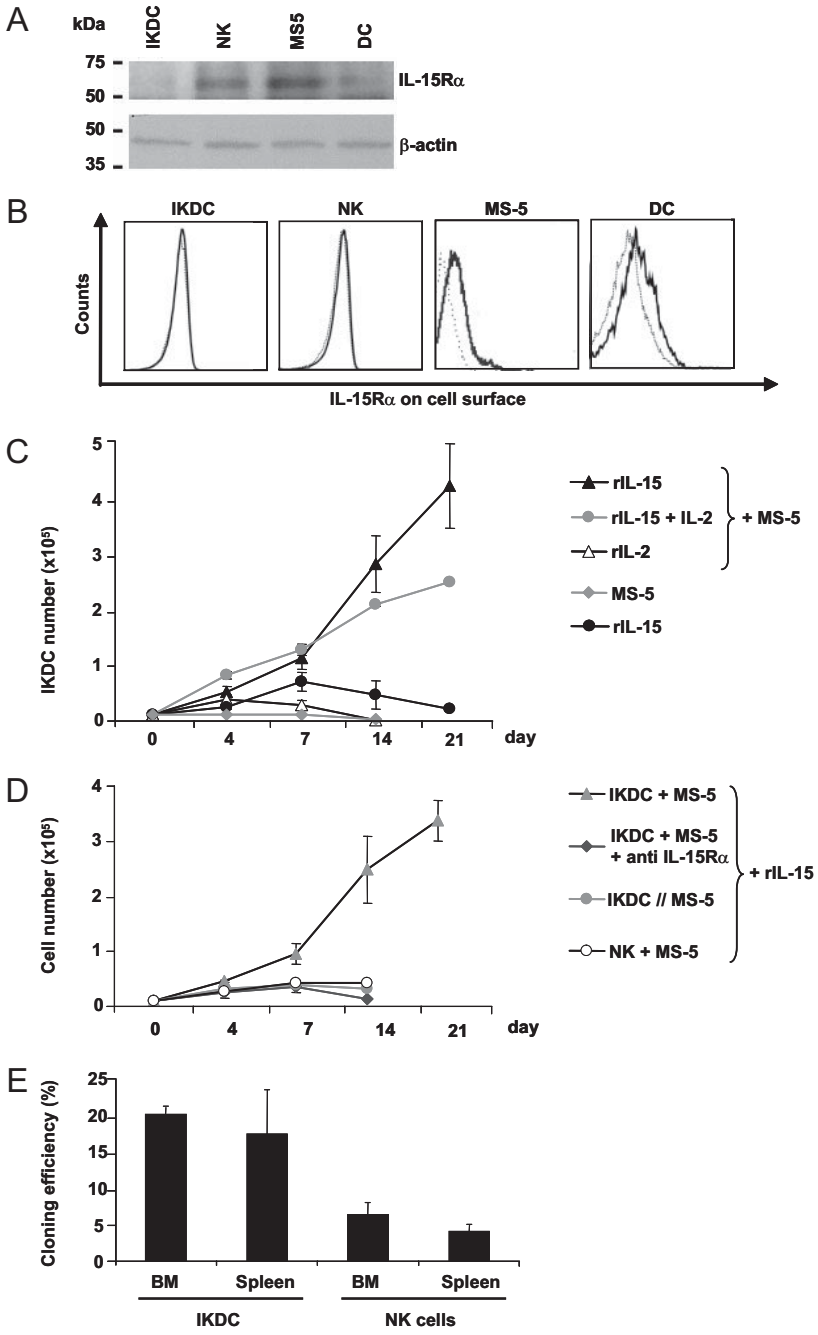

FIGURE 2. *Trans*-presentation of IL-15 allows ex vivo expansion of IKDC. IKDC fail to express IL-15Rα. *A*, Immunoblot analysis of IKDC, NK, conventional BMDC (grown in GM-CSF + IL-4), and MS-5 lysates for IL-15Rα expression (anti-IL15Rα at a dilution of 1/200, N-19; Santa Cruz Technology). *B*, Flow cytometry analyses of IKDC, NK, BMDC, and MS-5 using biotinylated anti-IL-15Rα Ab (plain line) and the isotype control Ab (dotted line). Ex vivo expansion of spleen IKDC. *C*, A total of 10^4 freshly sorted IKDC were cultured with or without MS-5 feeder cells in the presence of rmIL-15 (20 ng/ml) or rhIL-2 (50,000 U/ml) or both. *D*, IKDC and B220$^-$NK were cultured under the above mentioned conditions (rmIL-15 + MS-5). Proliferation of IKDC has further been tested in the presence or absence of transwells (//) physically separating MS-5 and by adding control mAb or neutralizing anti-IL-15Rα mAb. The graphs show cell numbers enumerated using trypan blue exclusion assays followed by FACS analysis to verify the surface expression of CD11c/B220/NK1.1 by IKDC. One representative experiment is shown as means + SEM. The experiments were reproduced >3 times with identical results. *E*, Histogramms of cloning efficiency of IKDC and B220$^-$NK cells after single cell sorting cultured on MS5 + rmIL-15 for 7–10 days. The percentage of positive wells is indicated for each culture condition. The experiment has been performed three times with similar results.

paraformaldehyde and permeabilized with 0.1% SDS. After 20 min of blocking in 10% FBS and washing, cells were stained with the appropriate anti-MHC class II (NIMR-4; Southern Biotechnology Associates), anti-Perforin, and anti-Granzyme B mAbs (BD Pharmingen) in PBS containing 1% BSA for 1 h. Next, slides were extensively washed and incubated with the appropriate secondary Ab (Alexa Fluor 488 goat anti-rat IgG) for 1 h and, after an additional washing step, with DNA-labeling Topro3 (Invitrogen) for 10 min. Finally, 0.17-mm cover glasses were mounted on the slides. Stacks of confocal images were collected with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) using a × 63 1.4 NA apochromat plan objective. Z-projection of slices and image analyses were performed using Zeiss LSM Image examiner software.

Isolation of RNA and RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Low cell number samples were precipitated in the presence of 10 μg/sample GlycoBlue (Ambion). After RNA purification, samples were treated with DNase to remove contaminating genomic DNA (DNaseI Amplification grade, 18068; Invitrogen) and Superscript II Reverse transcriptase (Invitrogen). Gene specific primers were purchased from NBS BIOTECH Scrl; sequences and detailed amplification protocols are available upon request. The iQ SYBR Green Supermix (Bio-Rad) was used to run relative quantitative real-time PCR of the samples according to the manufacturer's instructions. Reactions were run in triplicate on an iCycler (Bio-Rad) and generated products analyzed with the iCycler iQ Optical System software (Version 3.0a; Bio-Rad). Gene expression was routinely normalized both based on β-Actin mRNA and 18S rRNA contents with overlapping results. The amounts of target mRNAs are expressed in arbitrary units calculated as the relative change compared with spleen samples. Data are displayed as 2^{-ddCt} values and are representative of at least three independent experiments.

Statistical analyses

Aberrant values were excluded using Dixon's test. Normality of distributions was assessed using the Shapiro-Wilk's test. Normal distributions were compared by the Student's *t* test; non-normal samplings were compared using the Mann-Whitney test. Statistical analyses of survival curves were performed using Log-rank (Mantel Cox) test. Values of *p* inferior to 0.05 were considered significant. All tests were done using Prism 5 software (GraphPad).

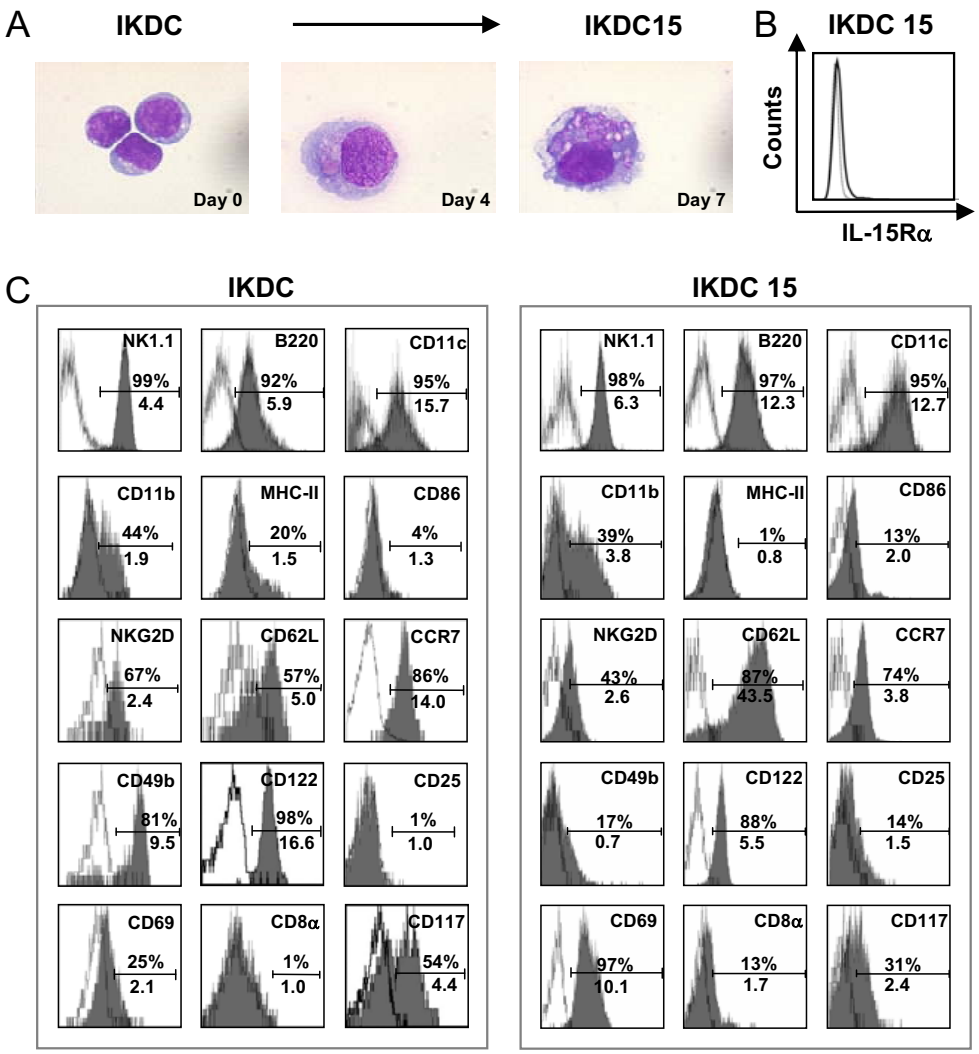

FIGURE 3. Morphological and phenotypical analysis of expanded IKDC15. *A*, Morphological changes of cultured IKDC15. Cytospins of freshly sorted cells or expanding IKDC were stained with May-Grünwald-Giemsa (Sigma-Aldrich) and analyzed on a Leica Microsystems DMLB microscope with ×800 magnification, using a Camera SONY 3CCD and TRIBVN ICS Version 1.4 software. Representative photographs are depicted. *B*, IKDC15 do not express IL-15Rα. FACS analysis on IKDC15 as described in Fig. 2*B*. Anti-IL-15Rα Ab (plain line) and isotype control Ab (dotted line). *C*, Phenotype of IKDC15 compared with freshly sorted IKDC. FACS analyses were performed on IKDC15 at day 7 of expansion compared with freshly sorted IKDC using the Abs listed in *Materials and Methods*. Mean fluorescence intensity fold increase and percentages of positive cells compared with the isotype matched control Ab are shown for each staining. A representative staining is shown. The experiments have been performed three times with identical results.

Results

Phenotypic definition and isolation of IKDC

IKDC were previously described as $CD11c^{int}B220^{+}NK1.1^{+}$ cells mediating the antitumor effects of the combination therapy with IM and IL-2. IKDC represent ~2% of bone marrow $CD11c^{+}$ cells and 1–2% of spleen derived-$CD11c^{+}$ in resting C57BL/6 mice and increased by 4-fold during the combination therapy with IM + IL-2 (2). Phenotype wise, IKDC are a specific cell population coexpressing CD11c, B220, NK1.1, and NKp46 (a recently described NK cell marker) (18) (Fig. 1*A*). To avoid possible contamination with plasmacytoid DC, conventional DC. B lymphocytes, or NK cells, we sorted IKDC in two steps. First, we performed a preselection of $CD3^{-}CD19^{-}NK1.1^{+}$ cells. Second, we gated on $CD11c^{int}$ $B220^{+}NK1.1^{+}$ cells (defined as "IKDC") and $CD11c^{+/-}B220^{-}$ $NK1.1^{+}$ cells (defined as $B220^{-}$NK cells henceforth).

Importantly, the expression of B220 molecules on bona fide $B220^{-}$NK cells did not appear to represent a marker of activation (12, 13, 19) because a 24–48 h stimulation of NK cells with cytokines (such as IL-2, IL-12, IL-15, and IL-18) and/or maturing DC were not sufficient to convert $B220^{-}$NK cells into $B220^{+}$IKDC counterparts (Table I). Conversely, IKDC did not convert or differentiate into $B220^{-}$NK cells neither in vitro (Table I) nor in vivo (Fig. 1*B*). Indeed, we performed an adoptive transfer of FACS sorted IKDC from *CD45.2* donor mice into irradiated congenic *CD45.1* C57BL/6 recipient mice. To rescue recipient hosts, coinjection of bone marrow derived from *CD45.1*×*CD45.2* chimera was performed in parallel. IKDC were cell sorted either from spleen or bone marrow. A similar number of $CD45.2^{+}$IKDC could be recovered in the spleen at day 12, whether originating from spleen or bone marrow (Fig. 1*B*). IKDC did not lose CD11c nor B220 cell surface markers and did not acquire LyC6/Gr1, CD4, or CD8α molecules (Fig. 1*B* and our unpublished data). At later time points (3 wk), $CD45.2^{+}$IKDC were almost undetectable (not shown). Therefore, these data support the notion that IKDC are terminally differentiated cells.

IKDC might be considered as a subpopulation of the so-called "NKDC" (containing all $CD11c^{+}NK1.1^{+}$ cells) (20, 21). However, it is noteworthy that the $B220^{-}$ fraction of NKDC was not significantly different from bona fide NK cells for all the hallmark criteria that the manuscript will describe (our unpublished data).

Absolute requirements for IL-15 in IKDC homeostasis

IL-2Rγ-chain-dependent cytokines, such as IL-15, are critical to promote lymphoid homeostasis and, more specifically, to maintain survival and proliferation of NK cells. We have previously reported that $CD11c^{+}CD49b^{+}B220^{+}$ cells (described as such by Chan et al. in BALB/c littermates) (1) could be found in old (>3 mo) $Rag^{-/-}$×IL-2Rγ-$chain^{-/-}$ mice. However, those cells did not express NK1.1 molecules (not shown). An almost complete deprivation in IKDC (as defined in Fig. 1*A*) was found in IL-$15R\alpha^{-/-}$ and IL-$15^{-/-}$ animals, supporting that IL-15 is a requirement for the differentiation of not only $B220^{-}$NK cells

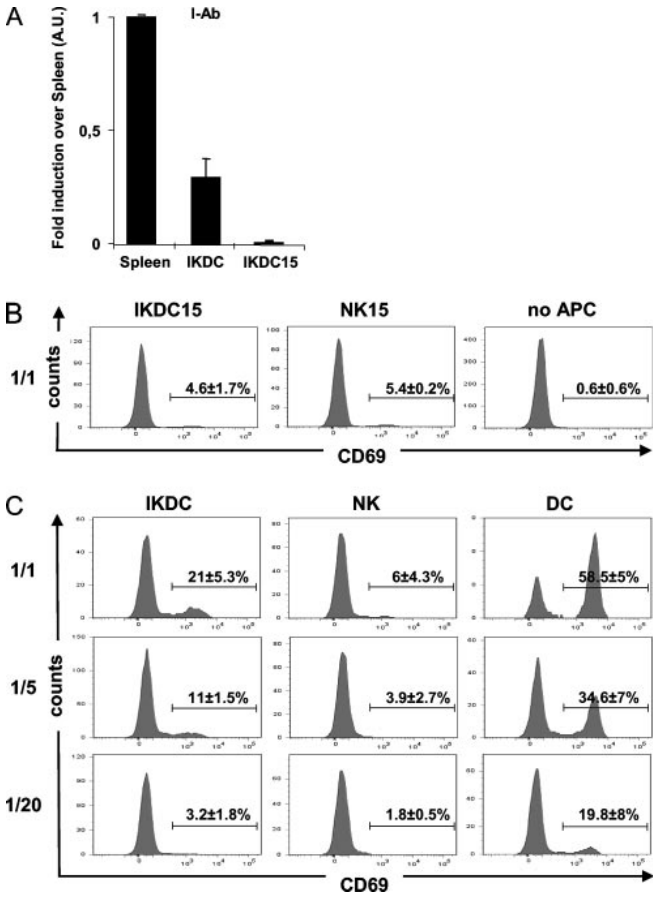

FIGURE 4. IKDC15 lose their MHC class II –restricted Ag presenting capacities in vitro. *A*, Down-regulation of MHC class II mRNA in IKDC15. Quantitative RT-PCR was performed on freshly sorted IKDC and on IKDC15 (at day 7 of expansion). *B* and *C*, Activation of OTII Tg T cells in contact with IKDC but not IKDC15 in vitro. FACS sorted 10^5 $CD4^+$ resting OTII lymphocytes purified from naive OTII Tg mice were added at various effector/T cell ratios (as indicated) to different effector cells (either resting IKDC, $B220^-$NK, immature BMDC (*C*), or *trans*-IL-15-activated IKDC or NK cells (*B*)) or to a control without APC (*B*) after a 24-h coculture with B16 tumor cells in the presence of 1 mg/ml OVA protein. After a 20-h incubation period, cocultures were stained with anti-CD3, anti-CD4, anti-Vα2, and anti-CD69 Ab and analyzed by FACS. A representative experiment is depicted of three yielding identical results. The mean + SEM of the % of CD69 OTII cells is indicated on the graphs.

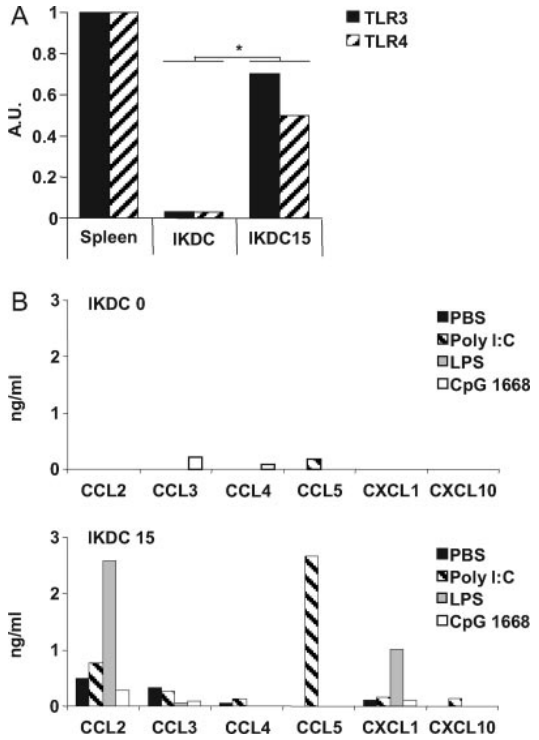

FIGURE 5. *Trans*-presentation of IL-15 licenses IKDC to respond to TLR3 and TLR4 ligands. *A*, IL-15 *trans*-presentation induced TLR3 and TLR4 expression in IKDC. Quantitative RT-PCR was performed on freshly sorted IKDC and on IKDC15 (at day 7 of expansion). *B*, Chemokine release by IKDC and IKDC15. After 24-h stimulation with medium, TLR3L (poly (I:C)), TLR4L (LPS), or TLR9L (CpG) multiplex analysis of chemokine release were performed on IKDC stimulated or not with *trans*-IL-15 presentation. IKDC0 meant freshly sorted cells without ex vivo stimulation. The experiments were performed at least twice with identical results.

(which decreased by 10-fold in IL-$15^{-/-}$ or IL-$15R\alpha^{-/-}$ mice) but also of IKDC in vivo (Fig. 1*C*). Moreover, IL-2 plays a redundant role in both the $B220^-$NK and IKDC developmental pathways (Fig. 1*C*). Surprisingly, IL-15 Tg animals did not contain enhanced numbers of IKDC at the steady state (Fig. 1*C*). In contrast, administration of rIL-15 was associated with the accumulation of IKDC in the spleen (Fig. 1*D*) resulting from their proliferation, as assessed by incorporation of BrdU in IKDC more than in $B220^-$NK cells (Fig. 1*E*). IL-15 but also IL-2 could promote a 2–4-fold accumulation of IKDC in the spleen (Fig. 1*D*). About one third of IKDC underwent cell division during exogenous administration of IL-15 or IL-2 in WT mice while <20% did in the $B220^-$NK cell fraction (Fig. 1*E*). Importantly, IKDC proliferation induced by TLR9 ligands (CpG 1668) was IL-15Rα-dependent in contrast to $B220^-$NK cells (as assessed by comparing BrdU incorporation in WT vs IL-$15R\alpha^{-/-}$ mice, Fig. 1*E*). However, although TLR9L induced IKDC proliferation, IKDC did not appear to accumulate in the spleen following administration of CpG ODN (Fig. 1*D*).

Hence, IKDC critically depended upon IL-15/IL-15Rα for their homeostasis and CpG driven-proliferation in vivo.

Ex vivo expansion of IKDC required IL-15/IL-15Rα

There are trace numbers of IKDC in lymphoid organs of naive animals (~50,000/spleen). Based on the IL-15/IL-15Rα requirement for IKDC differentiation in vivo, we set up culture conditions allowing ex vivo IKDC proliferation and/or differentiation. Immunoblot analysis indicated that IKDC do not harbor IL-15Rα in contrast to $B220^-$NK cells or DC (Fig. 2*A*). However, cell surface expression of IL-15Rα was detectable only on MS-5 stromal cells and DC using FACS analyses (Fig. 2*B*). We used IL-15Rα expressing MS-5 to test the hypothesis of the role of *trans*-presentation of IL-15 by IL-15Rα in the biology of IKDC. "*Trans*-presentation" of IL-15 defines a phenomenon by which IL-15 is presented by IL-15Rα on a bystander cell to neighboring cells lacking IL-15Rα and responding through the IL-2/15Rβ and γ-chains (22).

Accordingly, *trans*-presentation of rIL-15 by MS-5 to IKDC was successful and mandatory to promote ex vivo expansion (up to 10–30-fold) of spleen or bone marrow derived-IKDC within 7–12 days (Fig. 2, *C* and *D*). Indeed, IKDC proliferated only in the condition of IL-15 *trans*-presentation, neither on MS-5 alone, nor in rIL-15 alone (Fig. 2*C*). IKDC cultured in rIL-15 in the absence of MS-5 feeder cells expanded by 5-fold by day 7 but lost their proliferative potential afterward (Fig. 2*C*). IL-15 could not be substituted by rIL-2 and there was no additive effect with the combination of rIL-2 or rIL-18 + rIL-15 (Fig. 2*C* and unpublished data).

IKDC proliferation on the MS-5 stromal cells in the presence of rIL-15 was dependent on cell to cell contact, as shown by transwell

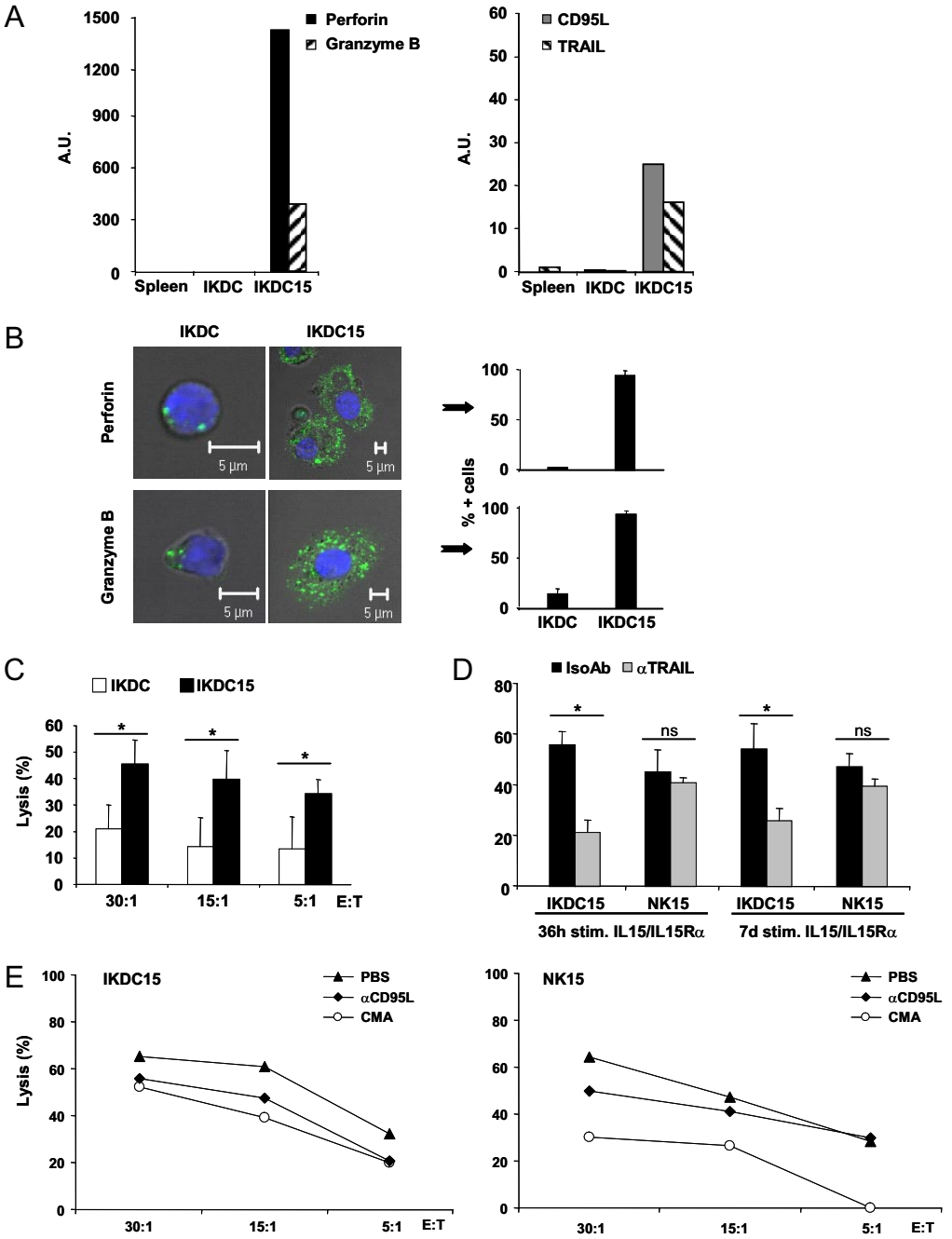

FIGURE 6. IL-15 triggers the TRAIL-dependent killing machinery selectively in IKDC. *A*, Transcription profile of IKDC and IKDC15 for perforin, granzyme B, TRAIL, and FasL (CD95L) encoding genes. Quantitative RT-PCR was performed on freshly sorted IKDC and on IKDC15 (at day 7 of expansion). *B*, Perforin, granzyme B protein expression as assessed by confocal microscopy. Confocal microscopy was performed on the same cells after intracellular staining using anti-mouse perforin, granzyme B primary Ab, Alexa 488 secondary Ab and DNA staining with Topro. Isotype control Abs were used in parallel. Background staining was not significant. A representative cell is shown in each condition and the percentage of cells containing positive granules is depicted on the *right panel*. *C*, IKDC15 exerted a more potent lysis of B16F10 compared with freshly sorted IKDC. ^{51}Cr release assays were performed 12 h at different ratios of IKDC or IKDC15 effector cells on ^{51}Cr-labeled B16F10 cells allowing calculation of the percentages of B16F10 lysis as described in *Materials and Methods*. *D* and *E*, Study of the TRAIL and perforin/granzyme B-dependent lytic pathway in NK15 and IKDC15. Cytotoxicity assays were performed at 36 h (*D*) or 7 days (*D* and *E*) after IL15/IL15Rα-stimulation of IKDC or B220$^-$NK cells. Crystal Violet assays were performed in 24 h at a ratio of 30:1, 15:1, and 5:1 of effector:B16OVA cells. Lysis of B16OVA was blocked by treatment with anti-CD95L Ab, anti-TRAIL (N2B2) Ab, or concanamycin A (CMA). The experiments were at least performed three times.

experiments (Fig. 2*D*), and could be abrogated by anti-IL-15Rα neutralizing Abs (Fig. 2*D*). Interestingly, this culture procedure did not allow the expansion of B220$^-$NK cells (Fig. 2*D*), although NK cell proliferation also depended on *trans*-presentation of IL-15 in vivo (Fig. 1*E*). The cloning efficiency of IKDC on MS-5 + rIL-15 as determined by limiting dilution analysis after single-cell sorting was ~20% (Fig. 2*E*). Clones derived from CD117$^+$ or CD117$^-$ bone marrow or spleen IKDC expanded exponentially in culture to colonies of 3×10^3 cells by 7 days (not shown). Under these culture conditions, cloning efficiency of NK cells was not significant (Fig. 2*E*).

Ex vivo expanded IKDC (referred to as "IKDC15" henceforth) acquired a large blastic cytoplasm and contained numerous granules and vacuoles (Fig. 3*A*). At days 7–10 after expansion, IKDC15 still failed to harbor membrane expression of IL-15Rα

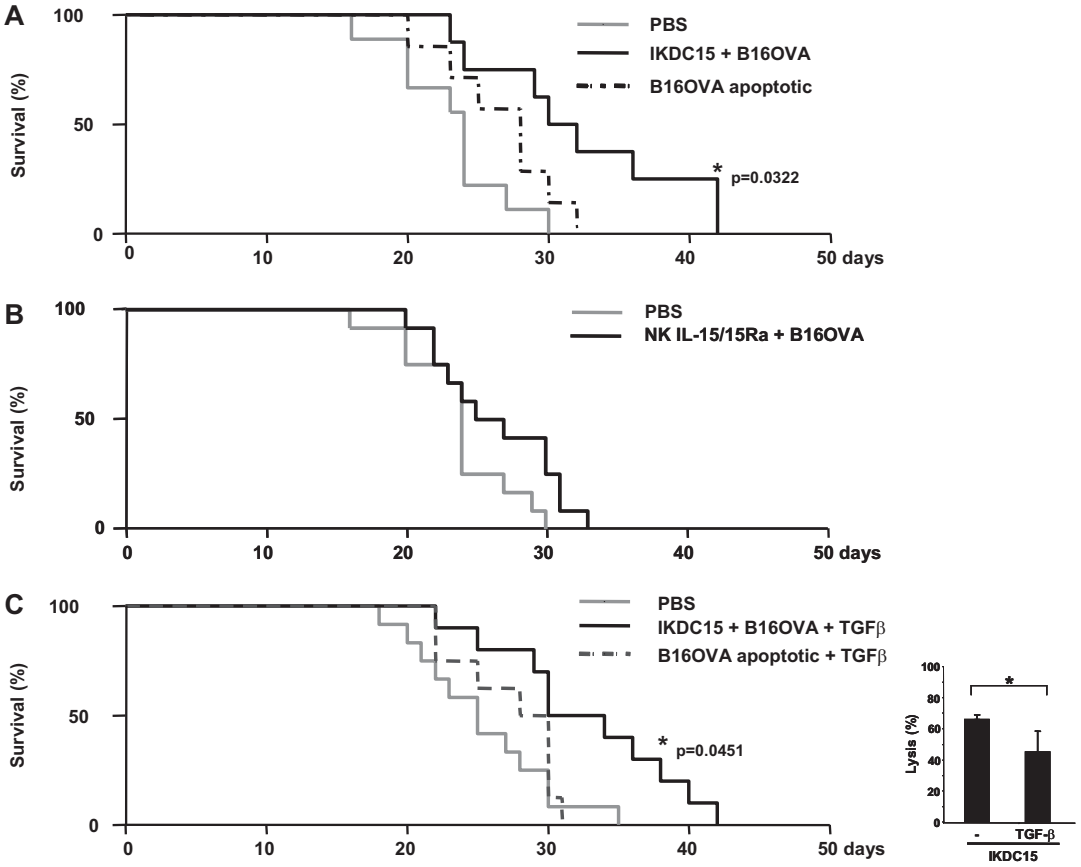

FIGURE 7. IKDC15 have immunizing potential and resist to TGF-β-induced immunosuppression. A total of 10^6 IKDC15 or NK15 cells (maintained 7 days in MS-5 + IL-15) were incubated with B16OVA at a 10:1 E:T ratio for 16 h before inoculation into the footpad of C57BL/6 mice. Rechallenge was performed 10 days later with a lethal tumorigenic dose of B16OVA (3×10^5 cells) s.c. in the flank of mice (*A* and *B*). Controls included untreated mice (PBS) or immunization with 10^5 B16OVA tumor cells incubated 15 h with 5 μM doxorubicin (23) (apoptotic B16OVA). The same experiments were performed adding TGF-β to IKDC15 or apoptotic tumor cells for 6 h before washing and injection into the footpads (*C*). Graphs show survival curves of animals ($n = 8$–12 per group) from at least four independent experiments. Statistical analyses were performed using Mantel Cox test. The *inset* of *C* depicts the TGF-β reduced killing of IKDC15 in vitro. IKDC15 incubated for 24 h with B16OVA in medium alone or with TGF-β (2 ng/ml) were subjected to a 24-h crystal violet assay against B16OVA. Results of a representative experiment of three are depicted as means $\pm$ SEM.

(Fig. 3*B*) but maintained the hallmark criteria of IKDC such as the expression of CD11c, B220, and NK1.1 (as well as CD11b, CCR7, and CD62L, Fig. 3*C*). Interestingly, IKDC lost the expression of MHC class II molecules, both at the mRNA and protein levels (Figs. 4*A* and 3*C*). Accordingly, IKDC15 lost their capacity to activate MHC class II-restricted OTII cells in vitro. Following pulsing with soluble OVA protein, neither IKDC15 nor NK15 could induce CD69 expression on naive OVA-specific I-A^b-restricted Tg OTII cells (Fig. 4*B*), while IKDC (but not NKB220$^-$ cells) and BMDC could do so (Fig. 4*C*). Similarly, IKDC15 do not have the capacity to activate MHC class I-restricted OTI cells in vitro (not shown).

Altogether, IL-15 *trans*-presentation allowed the selective ex vivo expansion of IKDC. However, IKDC15 seem to be poor APCs in both classical MHC pathways.

IL-15 trans-*presentation is a prerequisite for IKDC responsiveness to TLR3 and TLR4 ligands*

Resting IKDC did not express basal levels of mRNA encoding any of the 11 mouse TLR and could not respond to TLR stimuli (Fig. 5*A* and our unpublished data). However, *trans*-presentation of IL-15 significantly up-regulated the transcription levels of TLR3 and TLR4 in IKDC (Fig. 5*A*). Thus, IKDC15 acquired the capacity to respond to TLR4 ligands (LPS) by producing high levels of CCL2 (MCP-1) and CXCL1 (KC-GROα) (Fig. 5*B*). It is important to note that this responsiveness of IKDC required *trans*-presentation of IL-15 (not shown). Importantly, IKDC15 acquired the capacity to produce CCL2 even after 2–3 days culturing in IL-15/MS-5 (not shown). In addition, IKDC15 responded to TLR3 ligands (poly(I:C)) for the production of CCL5 (RANTES, Fig. 5*B*). Moreover, *trans*-presentation of IL-15 lead to responsiveness of IKDC to IL-2 and IFNα for the secretion of high amounts of CCL2 and CCL5 (23).

Trans-presentation of IL-15 allowed IKDC to respond to TLR3 and TLR4 ligation.

IL-15 trans-*presentation promoted IKDC effector functions*

The basal transcription level of the killing machinery (perforin/granzyme B/FasL), which was detectable in resting NK cells (not shown), was absent in resting IKDC (Fig. 6*A*). However, upon IL-15 *trans*-presentation, the transcription of perforin, granzyme B, CD95L, and TRAIL was dramatically induced in IKDC (Fig. 6, *A* and *B*). Interestingly, the transcription levels of perforin increased by 1000-fold in ex vivo expanded IKDC15 compared with freshly sorted IKDC. At the protein level, similar conclusions could be drawn in that IKDC15 contained high amounts of granules of perforin and granzyme B compared with resting IKDC as observed in confocal microscopy (Fig. 6*B*) or flow cytometry (not shown). Accordingly, the lytic activity of IKDC against B16F10 was markedly enhanced by *trans*-presentation of IL-15 (Fig. 6*C*).

The lytic activity of IKDC15 was mainly dependent on TRAIL molecules (Fig. 6*E*). The side by side comparisons between IKDC and NK cells both stimulated for short (36 h) or long (7 days) periods of time with MS-5/IL-15 revealed qualitative but not quantitative differences. Although NK cell-mediated killing was dependent on granule exocytosis, IKDC lytic functions mostly rely on TRAIL molecules (Fig. 6, *D* and *E*).

Therefore, *trans*-presentation of IL-15 endowed IKDC with TRAIL-dependent killing capacities, a biological attribute not shared by conventional NK cells.

Ex vivo expanded IKDC mediated a TGF-β resistant-protective immunity against tumor cells

We previously reported that IKDC invade tumor beds and were necessary and sufficient to prevent tumor outgrowth after adoptive cell transfer in $Rag^{-/-}\times$IL-2R$\gamma^{-/-}$-deficient hosts (2). However, the immunizing potential of IKDC in nonimmunocompromized animals remained to be assessed. In as much as NK and IKDC diverge in their mechanisms of killing tumor cells, we addressed the differential immunizing potential of both innate effectors. We used B16OVA as target cells incubated with IKDC15 or $B220^-$NK cells (equally activated in MS-5/IL-15) for 16 h before s.c. inoculation as immunization protocols. The ex vivo killing of B16OVA was comparable to that of B16F10 (Figs. 6 and 7*C*, *inset*). When mice were rechallenged 10 days later with a lethal dose of B16OVA, only those vaccinated with IKDC15, but not with 24 h or 7 day IL-15/IL-15Rα stimulated $B220^-$NK cells or 1×10^5 dying tumor cells (24–26), exhibited delayed tumor outgrowth associated with a significantly prolonged survival compared with untreated animals (Fig. 7, *A* and *B*). It is noteworthy that inoculation of an increased number of at least 3×10^6 doxorubicin-treated B16OVA tumor cells could confer a significant protection after rechallenge (24). Because IKDC invade tumor beds and could theoretically be subjected to TGF-β-induced immunosuppression, we analyzed the effects of recombinant human (rh) TGF-β on their killing potential and their immunogenicity in vivo. TGF-β could substantially reduce the killing potential of IKDC against B16OVA in vitro (*Inset*, Fig. 7*C*), but did not abrogate their protective activity against tumor challenge in vivo (Fig. 7*C*). The prophylactic effects of IKDC15 treated with TGF-β were not observed in Nude counterparts, suggesting that IKDC15/TGF-β mediated T cell-based antitumor immunity (not shown).

In this study, we demonstrate that ex vivo expanded IKDC15 not only gained lytic capacity in vitro (Fig. 6), but also protective antitumor function in vivo (Fig. 7*A*) that even resists to immunosuppressive TGF-β. These data support the hypothesis that IKDC15 could link innate and cognate immunity and, therefore, would be capable of inducing an antitumor immune response resistant to tumor-induced tolerance.

Discussion

This manuscript describes for the first time the pivotal role of IL-15 *trans*-presentation in the biology of IKDC, a novel subset of innate effectors sharing markers of both NK cells and conventional DC (1, 2). We initially reported that IKDC were $B220^+CD11c^+$ $NK1.1^+$ cells expressing MHC class II molecules during treatment with IM + IL-2 and invading tumor beds to kill in a TRAIL-dependent fashion, whereas Chan et al. (1) described that lymph node IKDC in BALB/c mice were endowed with MHC class II-restricted Ag presenting function in vitro. Therefore, IKDC may be considered as a MHC class II expressing NK cell subset or alternatively as a DC endowed with TRAIL-dependent killing capacities. This view has been recently challenged by several authors supporting the notion that IKDC, rather, represent an activated state of conventional NK cells (12, 13, 27). This manuscript aimed at clarifying the functional differences between IKDC and NK cells.

First, IKDC exhibited marked proliferative potential in vitro and in vivo following IL-15/IL-15Rα-driven stimulation (Figs. 1 and 2). Indeed, we showed that CpG ODN, rIL-15 (Fig. 1), and even IM + IL-2 (23) all drove IKDC proliferation in vivo in an IL-15Rα-dependent manner. Interestingly, despite their cloning expansion capacity, IKDC appeared to represent fully differentiated cells because they did not convert into bona fide $B220^-CD11c^-$NK cells after adoptive transfer into congenic animals (Fig. 1*B*). In sharp contrast, $B220^-$NK cells failed to proliferate in vitro during stimulation with IL-15/IL-15Rα and their CpG driven-proliferation in vivo was IL-15Rα-independent. Although harboring intracytosolic IL-15Rα (Fig. 2*A*), why did $B220^-$NK cells fail to respond to IL-15 for ex vivo proliferation? Several hypotheses can be drawn to account for the IL-15-driven proliferation of IKDC and not NK cells. There are several isoforms of IL-15Rα. The full-length sIL-15Rα ectodomain resulting from the proteolytic degradation of IL-15Rα is inhibitory when binding to IL-15. In contrast, some isoforms, such as the sushi sIL-15Rα resulting from an alternative splicing of the mRNA of IL-15Rα, are agonists (28). Moreover, there is a reciprocal activation of IL-15Rα with a tyrosine kinase receptor Axl leading to the phosphorylation of both receptors upon binding of IL-15 or Gas6 (the ligand for Axl) and survival effects of the transduced cell type (29). Hence, it is plausible that NK cells might secrete the antagonist form of IL-15Rα and would not be able to benefit from IL-15 and/or that IKDC do secrete a sushi-like isoform of IL-15Rα. Likewise, it is unlikely that Axl plays a dominant role because Axl was not found in Western blot analyses, neither in IKDC nor NK cells (not shown).

Second, following *trans*-presentation of IL-15, IKDC acquired high lytic capacities (against B16F10 (Fig. 6*C*) and B16OVA (Fig. 7)) that were fully abrogated in the presence of anti-TRAIL neutralizing Ab (N2B2, Fig. 6). In contrast, $B220^-$NK cells exhibited high basal transcription levels of perforine (in contrast to IKDC, not shown) and killed target cells using secretory granules and not TRAIL molecules (Fig. 6, *D* and *E*). TRAIL-dependent cytotoxicity was shown to play a dominant role in the prevention and treatment of neoplasia (30, 31). IKDC15 became capable of sensing and killing tumor cells mainly through TRAIL molecules while also up-regulating their levels of perforine and granzyme B (Fig. 6). Although previous observations tend to demonstrate that IL-15 can up-regulate TRAIL and boost TRAIL-dependent cytotoxicity of murine NK cells in vitro, it remains to be determined whether the IKDC component of the mouse NK cell pool was in fact mediating these TRAIL-dependent effects (32).

Third, following *trans*-presentation of IL-15, B16OVA-lysing IKDC mediated T cell-dependent protective effects in vivo, even in the presence of TGF-β. Such prophylactic immunization properties were not found with IL-15/IL-15Rα-stimulated NK cells (displaying equivalent quantitative killing capacities as IKDC). One of the main issues remains whether IKDC could not only play a scavenger role by mediating tissue destruction but also a role in T cell priming. Because we have shown that IL-15 *trans*-presentation skews IKDC toward cytotoxic effector cells rather than APC, we suggest that footpad inoculation of IKDC15 encountering B16OVA may indirectly promote recruitment and activation of conventional DC that will prime naive T lymphocytes. Given that IKDC15 differ from NK15 in their TRAIL-dependent killing of targets, we anticipate that programmed cell death triggered by the extrinsic (membrane bound, TRAIL-mediated) as opposed to the intrinsic (mitochondrial perforine/granzyme-mediated) cell death

pathways could matter in the outcome of the prophylactic potential of both effectors. Indeed, our group has reported that apoptosis mediated by anthracyclines, oxaliplatinum, or X Rays was immunogenic, whereas other cytotoxic agents failed to promote an immunogenic cell demise. This was due to the ability of some cytotoxic compounds to induce ecto-calreticulin (CRT) at the plasma membrane of dying cells (26) and to release HMGB1 alarmins to interact with TLR4 harbored on DC (33). Although ecto-calreticulin was required for phagocytosis by DC of dying tumor cells, HMGB1 was involved in the processing of apoptotic material by DC. Therefore, whether the immunogenicity of IKDC15-mediated cell death is TRAIL-, HMGB1-, and/or CRT-dependent needs to be addressed.

Fourth, as recently demonstrated *trans*-presentation of IL-15 allowed CCR2 expression on IKDC but not on B220$^-$NK cells, likely contributing to their CCL2-dependent intratumoral trafficking (23).

It is interesting to note that B220 and CD11c molecules were not acquired by conventional NK cells after 24–48 h of stimulation with a variety of cytokines or DC (Table I), presumably because such NK cells do not enter cell cycle in vitro. Moreover, IL-15 or IL-2 down-regulated MHC class II transcription levels on IKDC, supporting the notion that B220, CD11c, and MHC class II unlikely correspond to activation markers because they were differentially modulated by these activating cytokines.

IL-15 is a pivotal cytokine for the development and function of innate immune cells such as NK, NKT, and TCR$\gamma\delta$ intestinal intraepithelial lymphocytes and DC (34). IL-15 also affects acquired immunity by stimulating the proliferation and survival of naive and memory CD8$^+$ T cells (35). T cell-dependent delayed type hypersensitivity responses are impaired in IL-15$^{-/-}$ mice but restored by injection of IL-15 producing WT DC in vivo (36). Furthermore, IL-15 could mediate deleterious effects and has been involved in the exacerbation of numerous inflammatory processes, such as rheumatoid arthritis (37, 38), inflammatory bowel disease (39, 44, 45), type C chronic hepatitis (40), sarcoidosis (41), multiple sclerosis (42), and celiac disease (43). Elevated IL-15 production and IL-15 producing cells were identified as potential initiators of the inflammation. IL-15 can be produced by DC, macrophages, monocytes, and endothelial cells (34). Recently, Ohteki et al. (46) could identify DC derived-IL-15 as the initiator for the development of liver inflammatory diseases. The authors showed that DC-derived IL-15 could stimulate an autocrine loop leading to IL-12 and IFN-γ production and a cascade of inflammatory processes involving CCL2, CCL3, and CCL4 culminating in granuloma formation and liver injury (46). Although their results suggested that asialo-GM1 expressing cells (which include IKDC, our unpublished data) were not involved in the inflammatory cascade, the role of IKDC as a master regulator of the initial steps of granuloma formation and/or at later stages during hepatic injury has yet to be defined. This hypothesis is also driven by other results suggesting the crucial role of CCL2 in granuloma formation promoted by *Propionibacterium acnes,* zymosan, or *Mycobacterium tuberculosis* (47). Therefore, one of the major challenges will be to delineate the relevance of IKDC in the sequential events where IL-15 is beneficial or deleterious.

Another unsolved question remains the identification of the human counterpart for IKDC. Some authors have discussed the possibility that IKDC represent the mouse ortholog of human CD56^{bright}NK cells (4, 13). Human CD56^{bright}NK cells are mostly localized in lymph nodes and were considered as potential precursors of more mature CD56^{dim}NK (48). Although indeed IKDC preferentially home and accumulate in lymph nodes (1), they do not appear to convert into B220$^-$NK cells in vitro (Table I) nor in vivo upon adoptive transfer in irradiated hosts (Fig. 1*B*). It is clear that the identification of more specific IKDC markers will allow not only to characterize the human IKDC ortholog but also to delineate the biological significance of IKDC in pathophysiology.

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Disclosures

The authors have no financial conflict of interest.

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4.2 Antigen Presenting IKDC

4.2.1 Rappels

La première description des propriétés AP-IKDC vient de Chan *et al* (Chan, Crafton et al. 2006), qui avaient montré :

- Les IKDC spléniques ont une activité K-IKDC, tandis que les IKDC ganglionnaires sont AP-IKDC.
- L'infection par listéria ou la stimulation par TLR-9L transforme les K-IKDC en AP-IKDC.
- Les IKDC de ganglion peuvent présenter l'antigène en CMH-II

Ces notions semblaient valables pour un modèle infectieux. Mais dans notre modèle tumoral, il était également possible d'observer du CMH-II pour les IKDC au site tumoral. Nous avons donc axé notre travail sur les propriétés CPA des IKDC.

4.2.2 Les fonctions présentatrices d'antigènes des IKDC sont dans leur fraction CD11b$^+$ et sont déclenchées par les cellules tumorales (ARTICLE 3)

En partant du constat que les IKDC acquièrent les molécules de CMH-II membranaires dans les tissus cancéreux, la régulation des fonctions de présentation de l'antigène par la présence de la tumeur a été testée *in vitro*. Nous avons pu constater que seule la combinaison IKDC+Tumeur B16 (Tum) + antigène soluble ovalbumine (Ova) permet, après lavage de l'antigène soluble, de faire réagir les OT-II naïfs. Cette propriété est meilleure dans la fraction CD11b$^+$ des IKDC.

Sachant les objections opposables à cette interprétation, nous avons mené une série d'expérience *in vivo* destinés à tester l'existence d'une présentation de l'antigène lié à la présence des IKDC. Nous avons donc mis en évidence :

- L'incubation *in vitro* des IKDC avec l'antigène soluble Ova seul permet une activation *in vivo* des OT-II via les IKDC et les NK, mais seules les IKDC induisent une prolifération, sans aller jusqu'à la sécrétion.

- L'incubation *in vitro* des IKDC avec Tum+Ova permet d'induire *in vivo* une réponse T CD4 primaire jusqu'à la sécrétion d'IFN-gamma.

- Cette prolifération se retrouve dans des souris CD11c-DTR, dépourvues de cDC, pour la présentation en CMH-II aux cellules T $CD4^+$, et dans des souris beta2-microglobuline$^{-/-}$ pour la présentation en CMH-I aux cellules T $CD8^+$.

- Il est possible d'augmenter l'effet vaccinal en traitant les IKDC avec un anti-B7H1 : en bloquant sur la cellule vaccinale une molécule de costimulation inhibitrice, nous mettons en évidence la réalité des fonctions APC des IKDCs.

Ce dernier point peut être interprété de deux manières différentes : il existe des molécules B7H1 sur les IKDC et sur les tumeurs utilisées (B16RAE). L'ajout de l'anticorps bloquant permet donc potentiellement le blocage de l'inhibition des IKDC par le B7H1 de la tumeur ou le blocage de l'inhibition des T par le B7H1 des IKDC.

Dans les deux cas, si nous observons une augmentation de l'effet vaccinal de la préparation, c'est bien que l'effet vaccinal est lié aux cellules injectées.

Nous avons donc expérimentalement l'indication selon laquelle l'IKDC est capable de capter un antigène soluble et de le présenter en CMH-II. Nous savons que la prise en charge de l'Ova dans les DC est liée au CD206 (récepteur du mannose). Nous avons donc testé la présence de CD206 sur les populations de cellules $NK1.1^+$: la sous-population $CD11b^+B220^+$ est 30% $CD206^+$, tandis que les autres le sont <10%. Fort de cette notion, nous avons avec succès inhibé la capture de l'antigène soluble Ova avec un anticorps inhibiteur dirigé contre le CD206.

Ensuite, nous avons testé les capacités des IKDC à présenter en croix l'antigène en CMH-I. Nous avons donc testé notre protocole avec des $CD8^+$OT-I, et constaté qu'ils s'activent en présence des IKDC incubées dans les mêmes conditions que celles faisant réagir les OT-II. Mais encore une fois, cette interprétation pouvait être mise en doute : ne s'agit-il pas d'une contamination de nos préparations par un peptide issu de la dégradation spontanée *in vitro* de la protéine ?

Pour éliminer cette objection, nous avons utilisés un produit de génie protéique : une protéine de fusion entre la toxine de Bordetella pertussis et le peptide immunodominant de l'Ova. Cette protéine CyaA-Ova, peut se fixer au CD11b membranaire et être présenté en croix en CMH-I des DC (Guermonprez, Khelef et al. 2001; Schlecht, Loucka et al. 2004). Nous l'avons injecté en intraveineuse à des souris dont nous avons ensuite trié les IKDC spléniques. Une fois encore, les IKDC $CD11b^+$ sont bien capable de faire réagir les OT-I, démontrant une capacité de présentation de l'antigène *in vivo*.

4.2.3 Discussion

Ce travail permet de montrer qu'il existe bien une capacité de présentation de l'antigène dans les cellules $NK1.1^+$, et qu'elle se concentre dans la population $CD11b^+B220^+$. D'après nos résultats, il est possible que l'ensemble des cellules $NK1.1^+CD27^+CD11b^+$ soient capables de présenter l'antigène, tandis que les cellules $CD11b^-$ ne le seraient pas.

Par ailleurs, nous confirmons quelques-uns des résultats concernant les faibles capacités APC des IKDC, car dans les mêmes conditions (tumeur seule, antigène soluble seul), nous ne trouvons pas non plus de réactivité des cellules T (Caminschi, Ahmet et al. 2007).

Ceci permettrait de concilier les rapports concernant les NKDC ($NK1.1^+CD11c^+$) et les IKDC ($NK1.1^+B220^+CD11c^{int}$), qui se comportent de manière similaire d'après la littérature. Nous pourrions alors proposer un modèle dans lequel le phénomène IKDC décrit en fait la capacité d'une sous-population de NK à présenter l'antigène. La population $B220^+$ serait alors un heureux mélange entre des cellules $CD27^+CD11b^-$, très immatures et cytotoxiques via TRAIL et des cellules $CD27^+CD11b^-$, immatures et capables de présentation de l'antigène. Les IKDC tueuses ($CD11b^-$) proliférant plus vite que les $CD11b^+$, ce sont elles qui envahissent les cultures en présence d'IL-15, ce qui explique la perte des capacités de présentation de l'antigène dans les IKDC-15 (Ullrich, Bonmort et al. 2008).

Le fait est que nous avons été capables d'observer une faculté de prise en charge de l'antigène *in vivo*. La stratégie utilisée (le ciblage du CD11b) est à comparer à une étude récente utilisant des produits de fusion f(ab')2 –Ova (Castro, Tutt et al. 2008). Cette étude comparait des f(ab')2 anti-CD11c, anti-CD205 et anti-CMH-II, et conclue à la nette supériorité du ciblage du CD11c. Cet élément ne doit pas être négligé : les cellules exprimant le CD11c sont plus nombreuses que celles exprimant le CD205. Un rôle pour les cellules $NK1.1^{+}CD11c^{+}$ (parmi lesquelles les cellules IKDC $B220^{+}$) dans ce phénomène, c'est-à-dire la prise en charge de l'antigène soluble, n'est pas à exclure.

The Dendritic Cell-Like Functions of Interferon-Producing Killer Dendritic Cells Reside in the CD11b$^+$ Subset and Are Licensed by Tumor Cells

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Abbrevations: APC: antigen presenting cells, **BM-DC:** bone marrow-derived DC, **cDC**: conventional dendritic cells, **CTL**: cytotoxic T lymphocyte, **CyaA**: Bordetella pertussis adenylate cyclase toxin, **DLN**: draining lymph node, **DTR**: diphteria toxin receptor, **IKDC:** interferon producing killer dendritic cells, **ND**: not done, **NK**: natural killer cells, **MMR**: mannose receptor, **TCR**: T cell receptor.

Abstract

Interferon producing Killer Dendritic Cells (IKDC) were originally defined as $CD11c^{int}$ $B220^{+}NK1.1^{+}$ (or $CD49b^{+}$) cells that exert a potent tumoricidal activity in animals lacking B, T and conventional NK effectors. MHC class II expression on tumor infiltrating IKDC prompted us to investigate their putative antigen presenting function. Here we show that tumor cells license IKDC to acquire the properties of antigen presenting cells, i.e expression of MHC class II and costimulatory CD86 molecules, priming of naïve $CD4^{+}$ T cells and cross-priming of naïve $CD8^{+}$ T lymphocytes. Licensing of IKDC by tumor cells involved IFNγR and was mandatory for the full differentiation of T cells into polarized effectors. IKDC could engulf soluble proteins and process soluble ovalbumine in a CD206-dependent manner. Finally, we demonstrate that the $CD11b^{+}$ subset of IKDC is selectively endowed with CTLA4Ig-inhibitable antigen presenting capacities and that targeting this subset with the detoxified adenylate cyclase toxin of *Bordetella pertussis* fused to antigen resulted in efficient cross-presentation of antigen by IKDC to specific TCR transgenic $CD8^{+}$T cells *in vivo*. Collectively, our data indicate that upon exposure to tumor cells, IKDC subserve DC-like functions.

Introduction

Tumors can regress as a result of autonomous cell death pathways and/or invading myeloid and lymphoid effectors that act in concert to kill cancer cells. While myeloid cells are widely recognized as antigen presenting cells and lymphoid cells as classical effectors, the functional concept of "natural killer myeloid DC"[1,2] is emerging in murine[3,4] and human[5-7] *in vitro* studies but lacks in vivo relevance. Conversely, a subset of non T, non B cells called "IKDC" was recently identified as potentially endowed with the dual potential of killing and antigen presentation[8-10].

Interferon-producing Killer Dendritic Cells (IKDC) were originally defined as $CD11c^{int}B220^{+}NK1.1^{+}$ (or $CD49b^{+}$) cells lacking the expression of CD3, CD19 and Gr-1 molecules[8,10]. The nomenclature "IKDC" relied upon the presence of functional MHC class II (MHCII) molecules harboured on IKDC in lymph nodes[8] and tumor beds[9,10] together with their capacity to produce IFNα (after viral infection or TLR9 triggering)[8] or IFNγ (after exposure to tumor cells or IL-12+IL-18) and to kill a variety of cancer cell types[9,10]. However, several observations suggested that IKDC could represent a subset of NK cells. First, IKDC express receptors that are typically present on NK cells such as CD122, NKG2D and Ly49 molecules[8-10]. Second, IKDC rely on the trans-presentation of IL-15 by IL-15Rα for their homeostatic[9,11] and inflammation-driven proliferation[9,12]. Based on the facts that IKDC express the NK-specific marker NKp46[11], that B220 and CD11c molecules can be acquired upon NK cell proliferation[11], and the poor antigen presenting capabilities of IKDC[13,14], three independent teams of investigators have challenged the view that IKDC belong to a separate DC lineage.

Considering that MHC II expression by IKDC was upregulated in tumor beds[10] and downregulated by trans-presentation of IL-15 by IL-15Rα *in vitro*[9], we studied the antigen presenting capacities of resting IKDC freshly isolated from splenocytes. Our data indicate that the $CD11b^{+}$ subset of IKDC selectively exerts DC-like functions i.e priming of naïve $CD4^{+}$ and $CD8^{+}$T lymphocytes to a soluble antigen in a CTLA4Ig-repressable fashion *in vitro and in vivo*. Furthermore, IKDC required prior exposure to tumor cells to become fully competent at polarizing Th1 and Tc1 lymphocytes *in vivo*. Finally, cross-presentation to $CD8^{+}$T cells of antigens fused to the detoxified adenylate cyclase of *Bordetella pertussis* toxin capable of binding to the $\alpha_M\beta2$ integrin was mediated by $CD11b^{+}$ IKDC *in vivo*. Collectively, these data support the

concept that a subset of "activated NK cells" can acquire DC-like capacities in a tumor environment.

Material and methods

Cell lines and mouse strains. C57Bl/6 wild type and H-2K^b/H-2D^b/β2 microglobulin knock-out mice were obtained from the Centre d'Elevage Janvier (Le Genest-St-Isle, France) and from Charles River Laboratories (L'Arbresle, France) and used at 7-10 weeks of age. C57Bl/6*Rag*$^{-/-}$ TCR transgenic OT-II mice (specific for the Ova$_{257-264}$ peptide) were kindly provided by Dr. O.Lantz (Institut Curie, Paris, France), C57Bl/6*Rag*2$^{-/-}$TCR (Vα2, Vβ5) transgenic mice (OT-I) by Dr A.Boissonas (Institut Curie), CD11c-GFP/DTR mice[15] by P.Aucouturier (INSERM, St. Antoine Hospital, Paris, France), CD83$^{-/-}$mice[16] by T. Tedder (Duke University, USA). Animals were all maintained according to the Animal Experimental Ethics Committee Guidelines.
B16-Rae1 and B16-Ova are stably transfected with Rae1 or Ova encoding cDNA, respectively (kindly provided by E. Tomasello, CIML, Marseille, France and C. Théry, Institut Curie, Paris, respectively).

Antibodies and flow cytometry analyses. FACS analyses were performed using mAb against CD11c (HL3), NK1.1 (PK136), B220 (RA3-6B2), CD3 (17A2), CD19 (1D3), MHCII (AF6-120.1), IA/IE (NIMR-4), CD40 (3/23), CD80 (16.10A1), CD86 (GL1), NKG2D (CX5), CD69 (H1.2F3), or CD27 (LG.3A10). Antibodies were purchased from Pharmingen (San Diego, CA) or eBioscience (San Diego, CA). Immediately before FACS analysis, DAPI was added. FACS analysis was performed by LSRII using FACS Diva Software and CellQuestPro Software (Becton Dickinson, Mountain View, CA) or Flowjo (Treestar, Ashland, OR). Neutralizing antibodies were used in some experiments : neutralizing anti-IFNγ (Pharmingen), anti-TRAIL Ab (kindly provided by H.Yagita, Japan), anti-CD206 mAb targeting the mannose receptors (MMR, clone 310301, R&DSystems), CTLA4Ig (Roche, Milan, Italy) or anti-CD40L antibody (Clone MR-1, Pharmingen).

Sorting and purification of IKDC, B220$^-$NK and cDC. The procedures were previously described[9]. Briefly, IKDC and B220$^-$NK cells were sorted from spleens of naïve C57Bl/6 mice after enrichment of NK cells (negative selection, Miltenyi Biotec, Germany). IKDC were sorted as CD3$^-$CD19$^-$CD11c^{int}B220$^+$NK1.1$^+$ cells, B220$^-$NK cells as CD3$^-$CD19$^-$CD11c$^-$B220$^-$NK1.1$^+$ cells. We sorted separately CD11b$^+$IKDC and CD11b$^-$IKDC. cDC were sorted as CD11c$^+$I-Ab$^+$ cells. Cell separation was performed on a Mo-Flo instrument (DAKO, Glostrup, Denmark). The purity of cell separation exceeded 98%.

Licensing experiments and *in vitro* T cell priming. BM-DC were generated as previously described[17]. For licensing experiments, 1.5x10^5 FACS-sorted IKDC, NK or cDC from spleens of naïve C57Bl/6 mice were cocultured with B16 tumor cells at the 10:1 ratio in presence of 1 mg/ml of Ova protein for 12hrs. Then, either FACS was performed to analyse MHCII or co-stimulatory molecules expression, or co-cultures were washed extensively (to remove resting traces of Ova protein) to proceed to test T cell priming *in vitro*. Resting T lymphocytes purified from naïve OT-I or OT-II mice (negative selection, Miltenyi Biotec) were incubated at the 1/1 effector/T cell ratio with different APCs (see Fig. Legend). After a 24 hr-incubation period, IFNγ and IL-2 secretion were measured by ELISA (Pharmingen) and T cells were stained with anti-CD3, anti-CD4 or anti-CD8, anti-Vα2, anti-CD69 Ab.

Confocal microscopy. 5x10^{4}cells were spread onto a slide coated with poly-L-lysine (Sigma). Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% sodium dodecyl sulfate (SDS). After blocking in 10% FBS and washing, cells were stained with the appropriate anti-MHCII (NIMR-4, Southern Biotech, Birmingham, AL) or isotype control in PBS containing 1% BSA. Slides were extensively washed and incubated with the secondary antibody (Alexa fluor 488 goat anti-rat IgG) and with DNA-labelling Topro3 (Invitrogen Carlsbad, CA). Finally, 0.17mm cover glasses were mounted. Stacks of confocal images were collected with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) using a x 63 1.4 NA apochromat plan objective.

Uptake of FITC-Dextran by effector cells. cDC, IKDC, B220$^-$NK cells were incubated or not with FITC-Dextran at 37°C versus 0°C for 4 hrs. Cells were analyzed for FITC staining in flow

cytometry and in confocal microscopy after membrane labeling with WGA A647 fluorescent dye (Wheat Germ Agglutinin-Alexa647, Invitrogen, Carlsbad, CA).

***In vivo* priming studies.** 10^6 resting CFSE-labelled OT-I, OT-II or GFP/OT-I lymphocytes purified from naïve OT-I or OT-II mice were injected i.v. into naïve C57Bl/6 mice 18 hrs prior to footpad inoculation of the IKDC, NK or DC (see experimental settings in figure insets). $1.5x10^5$ FACS-sorted IKDC or NK cells or $3x10^5$ DC were pre-treated for 12 hrs as follows: i) unpulsed or pulsed with 1mg/ml of Ova protein, ii) cocultured with B16 (B16-Rae1 or B16-Ova) tumor cells at a 10:1 E:T ratio in the presence (or not) of 1 mg/ml of Ova holoprotein. In Fig.4C, After an extensive washing, IKDC, NK or DC were injected into the footpad of mice. After 3-5 days, popliteal lymph node cells were harvested and examined to define the activation, proliferation and polarization status of adoptively transferred transgenic T cells. i) DLN cells were stained with anti-CD69 Ab or proliferation of CFSE-labeled T cells was analyzed by FACS by gating on OT-I or OT-II cells. ii) DLN cells (10^5 cells per well) were restimulated or not with 1mg/ml of Ova protein or SIINFEKL peptide *in vitro*. Supernatants were harvested 72 hours later, IFN-γ secretion was assessed by ELISA. iii) Alternatively, DLN cells were restimulated with 1mg/ml of Ova protein for 12h (with addition of Golgi-Stop, Pharmingen) and stimulated for 2 additional hours with PMA (50 ng/ml) and ionomycin (1 µg/ml), followed by staining with Abs specific for Vα2, CD3, CD4 or CD8, anti-IL-2 and anti-IFN-γ mAbs.

CyaA-based vaccine studies. The detoxified adenylate cyclase toxin of *Bordetella pertussis* used as carrier of the immunodominant Ova peptide SIINFEKL (CyaA-Ova) or of a negative control (CyaA-HPV16-E7), kindly provided by Daniel Ladant (Institut Pasteur), was injected i.v. (50µg/mouse) in the absence or presence of CpG 1668 oligodeoxynucleotide (0.5 µg i.v.). Twelve hours later, cDC and IKDC were sorted according to CD11b expression and incubated with resting OT-I cells for 24 hrs. CD69 expression on OT-I was examined in flow cytometry and mouse IL-2 and/or IFNγ secretion was monitored in the supernatants.

Statistical analysis. All results are expressed as means ± standard error of the mean (SEM) or as ranges when appropriate. Aberrant values were excluded using Dixon's test. Normality of distributions was assessed using the Shapiro-Wilk's test. Normal distributions were compared by

the Student's t test; non-normal samplings were compared using the Mann-Whitney's test. Analyses of variances were performed with the Kruskal-Wallis test. Statistical analyses were performed using Prism 5 software (GraphPad, San Diego, CA).

Results

Licensing of IKDC by tumor cells for $CD4^+$ T cell priming *in vitro*

We previously reported that a high proportion of $CD11c^{int}B220^+NK1.1^+$ IKDC infiltrated regressing (but not progressive) tumors following therapy of established lung B16F10 metastases with imatinib mesylate and IL-2[10,12]. IKDC possessed TRAIL-dependent killing activity and controlled tumor progression in the absence of other lymphoid effectors[10]. IKDC produced IFNγ in contact with tumor cells[10,18] and expressed MHC class II molecules in regressing tumor beds[10]. Here, we addressed whether IFNγ could dictate the DC-like phenotype of IKDC. Splenic IKDC were obtained from C57Bl/6 mice by a combination of positive and negative selection processes, as $CD3^-CD19^-CD11c^{int}B220^+NK1.1^+$ cells. Splenic IKDC freshly isolated from naïve mice did not express MHC class II antigen. However, upon contact with a variety of distinct tumor cell lines (B16F10 melanoma, CT26 colon cancer, MCA205 sarcoma), IKDC acquired MHC class II molecules on the cell surface, as shown by flow cytometry (Fig.1A) or confocal microscopy (Fig. 1B). In identical conditions, freshly isolated $B220^-$NK cells did not acquire MHC class II in response to transformed cells (Fig.1A,B). The expression of MHC class II molecules by IKDC depended upon the autocrine production of IFNγ because IKDC sorted from $IFN\gamma R^{-/-}$ mice failed to express MHC class II in coculture with B16F10, even though they produced IFNγ (Fig.1C and not shown). Similar results were achieved using neutralizing anti-IFNγ Ab (not shown).

Beyond the induction of MHC class II molecules, tumor cells stimulated the expression of the costimulatory molecule CD86 on IKDC but not on $B220^-$NK cells (whether $CD11c^+$ or $CD11c^-$) (Fig.1A and not shown). Although TRAIL expression reportedly can be upregulated by IFNγ[19], blocking TRAIL failed to inhibit the upregulation of CD86 molecules on IKDC (Fig.1D).

In conclusion, IKDC (but not conventional NK cells) can be licensed by cancer cells to exhibit a DC-like phenotype *in vitro*.

IKDC elicit the differentiation of Th1 cells *in vivo*

$CD11c^{int}B220^{+}NK1.1^{+}$ cells could be subdivided into $CD11b^{+}$ and $CD11b^{-}$ subsets (Suppl. Fig.1). After co-culture with B16F10 tumor cells (or B16-Ova and B16-Rae1, not shown), both subsets of IKDC indistinguishably acquired MHC class II expression, a phenomenon that we refer to as "licensing". We assessed the functionality of MHC class II molecules expressed by tumor-licensed $CD11b^{+}$ *versus* $CD11b^{-}$IKDC *in vitro*. Freshly isolated splenic IKDC were cocultured for 12 hrs with B16F10 cells overexpressing the NKG2D ligand Rae1 (B16-Rae1) in the presence of highly purified ovalbumine (Ova) protein (licensing incubation), the coculture was then washed and IKDC exposed to naive TCR-transgenic OT-II cells purified from spleens of $Rag2^{-/-}$xOT-II mice. Only licenced $CD11b^{+}$ IKDC triggered the IL-2 production from naïve OT-II cells. Thus, in contrast to cDC, IKDC required licensing by tumor cells for their action as antigen presenting cells (APC) (Fig.2A). Recognition and/or killing of B16-Rae1 through NKG2D receptors play an important role in the IKDC-mediated activation of OTII cells *in vitro* (Fig.2B).

Next, we addressed which minimal signals are required for IKDC to trigger naïve Ova-specific TCR-transgenic $CD4^{+}$T cells to become activated, to proliferate and to differentiate into Th1 cells *in vivo.* We pulsed freshly purified splenic IKDC with soluble Ova in the presence or absence of licensing by B16-Ova tumor cells. It is noteworthy that B16-Ova cells were poorly killed by resting IKDC but IFNγ was produced during the licensing period (data not shown). 18 hours after adoptive transfer of CFSE-labeled naïve OT-II lymphocytes into C57Bl/6 mice, IKDC were injected in the footpad and compared with cDC (bone marrow-derived DC) and $B220^{-}$NK cells that were subjected to a similar pre-conditionning with Ova and/or B16-Ova cells. 4-6 days post-injection, CD69 expression, division and IFNγ production were analyzed among the OT-II cells contained in the draining lymph nodes (DLN). Pulsing of IKDC with soluble Ova was necessary and sufficient to induce the CD69 expression and proliferation of naive OT-II cells (Fig.2C). However, for promoting the differentiation of OT-II cells into IFNγ-producing Th1 cells, it was required that IKDC would be exposed to Ova and, in addition, would be licenced by tumor cells (Fig.2D). In all of the conditions tested here (pulsing with Ova alone or in combination with tumor cells), $B220^{-}$NK cells failed to stimulate T cell proliferation and polarization (Fig.2C, D). As a control, tumor cells alone or admixed with soluble Ova could not prime naïve T cells in this model (not shown and Fig.2H).

To exclude the possibility that tumor cells (rather than IKDC) became immunogenic after encountering IKDC, we used B16-Rae1 cells (which in contrast to B16-Ova cells lack the model antigen Ova) to license IKDC *in vitro*, then we inoculated these licensed IKDC into the footpad of C57Bl/6 mice and monitored IL-2 production by adoptively transferred OT-II cells. $CD11b^+$-licensed IKDC and cDC pulsed with soluble Ova promoted the priming and differentiation of Ova-specific $CD4^+$T lymphocytes (Fig.2E). However, without prior licensing by B16-Rae1 tumor cells, Ova-pulsed $CD11b^+$ IKDC failed to promote T cell polarization to IL-2 production *in vivo* (Fig.2E). In this setting, Ova-pulsed B16-Rae1 failed to stimulate cognate T cell responses *in vitro* or *in vivo* (not shown). Of note, $B220^-$NK cells were unable to activate OT-II cells in DLN in similar conditions (Fig.2E), underscoring the general incapacity of such cells to act as APC.

To exclude the possibility that host-derived-cDC presented antigens released by IKDC and/or B16-Ova tumor cells, we inoculated Ova-pulsed IKDC (that were licensed by coculture with B16-Ova) into two different models i.e in mice expressing the diphtheria toxin (DT) receptor under the control of the CD11c promoter and in $CD83^{-/-}$ mice expressing reduced MHC class II expression on antigen presenting cells[16,20]. In CD11c-DTR mice, injection of DT causes a conditional and transient depletion of conventional myeloid $CD11c^{high}$DC[15]. We observed that the OT-II proliferation driven by IKDC pulsed with Ova and licensed by tumor cells was as efficient in WT as in CD11c-DTR mice treated with DT (Fig.2F). In $CD83^{-/-}$mice, which lack MHC class II expression in LN (Fig.2G, inset), $CD11b^+$IKDC pulsed with Ova and licensed by tumor cells maintained their ability to induce CD69 expression on OT-II-transferred T cells (Fig.2G). Hence, IKDC are likewise the cells that present Ova-derived, class II-restricted peptides *in vivo*.

To further explore antigen presentation by IKDC, we exploited the fact that IKDC express B7-H1/CD274, an immunosuppressive molecule involved in the downregulation of T cell responses[21]. Neutralization of B7-H1/CD274 on IKDC with specific mAb during the licensing period markedly augmented the priming capacity of $CD11b^+$IKDC, as determined on OT-II cells (Fig.2H). As an internal control, however, B7-H1/CD274 blockade could not convert OVA-pulsed $CD11b^-$IKDC or B16-Rae1 cells into functional APC *in vivo* (Fig.2H).

Altogether, these data illustrate the potential of $CD11b^+$IKDC to behave as DC-like cells that display functional MHCII/peptide complexes after contact with cancer cells and soluble antigen.

CTLA4Ig-repressable priming of naïve CD8$^+$ T lymphocytes by CD11b$^+$ IKDC

The principal hallmark that distinguishes cDC from other cells endowed with APC-like functions is "cross-presentation", a phenomenon whereby captured extracellular antigens are presented on MHC class I molecules for the activation of CD8$^+$T cells[22,23]. We investigated *in vivo* cross-presentation by incubating IKDC with soluble Ova in the presence or absence of B16-Rae1 tumor cells (at a 10:1 E:T ratio), and followed by adoptive transfer of these IKDC (or cDC or NK cells as controls) with freshly isolated naive SIINFEKL-specific TCR transgenic OT-I lymphocytes. Loading of IKDC (but not cDC) with soluble Ova was not sufficient to promote the proliferation and differentiation of OT-I cells in DLN (Fig.3A). IKDC (but not cDC) required licensing by tumor cells to become cDC-like APC endowed with the capacity to present soluble Ova (Fig.3A). Of note, CD11b$^+$IKDC (but not CD11b$^-$IKDC or NK cells) were electively competent in cross-presenting exogenous antigens *in vivo* (Fig. 3A). As little as 3x10^{4}IKDC could trigger OT-I activation *in vivo* (Fig.3B). To reinforce that IKDC themselves (rather than host-derived DC) could function as APC, we analyzed OT-I activation triggered by WT IKDC injected into H-2D^b/H2-K^b/β2-microglobuline$^{-/-}$ mice. The APC function of CD11b$^+$IKDC was intact in hosts compromised for MHC class I presentation (Fig.3C), indicating that CD11b$^+$IKDC themselves act as APC.

Priming of naïve OT-I by CD11b$^+$IKDC required B7 molecules because a CTLA4Ig fusion protein abrogated the activation of OT-I by IKDC (as well as by cDC) *in vitro* (Fig.3D) and *in vivo* (Fig.3E), in a setting in which neutralizing CD40L with a specific antibody had no inhibitory effect (Fig.3F).

Altogether, these data support the notion that CD11b$^+$IKDC can cross-present class I-restricted antigen, but only when they are licensed by tumor cells.

IKDC engulf soluble antigen: a role for mannose receptors

It is well established that cDC can take up soluble Ova through mannose receptors[24] and that cDC can macropinocytose soluble extracellular molecules such as dextran[25,26]. Since IKDC apparently could process soluble Ova for antigen presentation (Fig.2), we investigated their

endocytic capacity, by incubating freshly isolated splenic IKDC (including $CD11b^+$ and $CD11b^-$) with the fluid phase marker dextran conjugated with fluoresceine isothiocyanate (FITC-DX). A significant fraction of IKDC was indeed labeled with FITC-DX at 37°C (but not at 0°C, not shown), as demonstrated by flow cytometry (Fig.4A, left panels) and corroborated by confocal fluorescence microcopy (Fig.4A, right panels). Moreover, IKDC were capable of binding and/or engulfing FITC-Ova (not shown). Importantly, $CD11b^+$IKDC contained higher levels of mannose receptors (CD206, MMR) than any other NK cell subset present in resting spleens (Fig.4B). In the next step, we added a neutralizing anti-MMR Ab to IKDC (or other APC) when they were licensed by tumor cells and loaded with Ova. MMR neutralization abolished the activation of OT-I cells by cDC and $CD11b^+$IKDC *in vivo* (Fig.4C).

These data indicate that $CD11b^+$IKDC have the potential to internalize fluid phase proteins and/or to take up antigens via mannose receptors.

Targeting of IKDC *in vivo*

To further explore targeting and antigen delivery to IKDC *in vivo*, we used a new vaccine vector that takes advantage of the adenylate cyclase (CyaA) of *Bordetella pertussis.* CyaA binds specifically to the $\alpha_M\beta_2$ integrin (CD11b/CD18[27]) and delivers its catalytic domain into the cytosol of $CD11b^+$ cells[28,29]. Therefore, $CD8^+$T cell epitopes fused with the catalytic site of CyaA are processed and presented by MHC class I molecules at the surface of $CD11b^{high}$cDC[30]. We injected the CyaA-Ova protein[31] intravenously and monitored the capacity of FACS-sorted $CD11b^+$ or $CD11b^-$IKDC or cDC to activate OT-I lymphocytes *ex vivo*. In these experimental conditions, $CD11b^+$IKDC (but not $CD11b^-$ IKDC), as well as $CD11b^+$cDC, mediated OT-I activation (CD69 expression and IFNγ secretion), provided that the animals had been injected with a CyaA-Ova vaccine (but not with an irrelevant CyaA-E7 fusion protein as control) (Fig. 5A, B). In the presence of TLR9 agonists coinoculated with CyaA, $CD11b^+$IKDC (but not $CD11b^-$IKDC), as well as $CD11b^+$cDC, promoted the production of IL-2 by OT-I cells *ex vivo* (Fig. 5C).

These results underscore the antigen-presenting capacity of $CD11b^+$IKDC, as evaluated in an *in vivo/ex vivo* system.

Discussion

DC represent a unique CD11c^{high}MHCII^{+} leukocyte subset, defined using functional criteria, that is specialized in antigen presentation and cross-presentation of endogenous and exogenous antigens for the priming of naïve T lymphocytes[32]. When first identifying the CD11c^{int}CD49b^{+}MHCII^{+} cell detectable at low frequency in RagxIL-2R$\gamma^{-/-}$ mice, we and others decided to name this new cell type "dendritic", admittedly without comprehensively exploring their antigen presenting capacities[8,10,33]. Subsequently, several groups challenged this view and proposed to classify the population that we had apostrophed as IKDC among the pool of "activated NK cells" that undergo cell divisions yet lack APC functions[11,13,14]. The aim of the present study was to revisit this notion and to address the regulation of APC functions in IKDC harvested from naïve spleens. Here, we produce evidence in favor of an unexpected DC-like function of IKDC. Indeed, our data indicate that i) IKDC can prime naïve CD4^{+} and CD8^{+}T cells *in vivo*, in the Ova model system, in a CD206 and B7-dependent manner (Fig.2, 3, 4); ii) the DC-like functions of IKDC mostly reside in the CD11b^{+} subset (Fig.2, 3, 4); iii) the DC-like functions of IKDC are dictated by their contact with tumor cells (Fig.1, 2, 3); (iv) CD11b^{+}IKDC targeted by the *Bordetella pertussis* CyaA *in vivo* were able of cross-presenting exogenous antigens to CD8^{+}T cells (Fig.5). In all experimental conditions explored in this work, B220^{-}NK cells failed to function as APC, in conditions in which IKDC (and in particular CD11b^{+}IKDC) did present MHC class I or class II-restricted antigens. Antigen transfer to endogeneous cDC is unlikely since CD11b^{+}IKDC remained capable of stimulating CD4^{+}T cells in CD11c^{hi}DC-depleted mice (CD11c-DTR, Fig.2F) or in mice lacking MHC class II expression (CD83$^{-/-}$, Fig.2G), and CD8^{+}T cells in H2D$^{-/-}$K$^{-/-}\beta$2m$^{-/-}$ mice (Fig.3C). Thus, IKDC possess a unique APC activity that is not shared by other NK cell populations.

Like cDC, IKDC subserve different functions at various stages of their differentiation. When isolated from mice treated with rIL-15 or CpG or imatinib mesylate plus IL-2, IKDC proliferated and accumulated in lymphoid organs in an IL-15Rα/IL-15-dependent manner[9,12]. In these circumstances, IKDC downregulated MHC class II molecules, both at the mRNA and protein levels and failed to activate naïve OT-II *in vitro*[9] or tumor-specific T cells *in vivo* (Mignot, unpublished data). Following trans-presentation of IL-15, IKDC acquired an enhanced

killing potential[9]. Thus, upon signaling through the IL-2Rβ/γ chain, IKDC lost their DC-like functions and became NK-like effectors. IKDC remained distinguishable from the other B220$^-$ NK cells by a set of characteristics that were all inducible by IL-15Rα/IL-15, namely a high proliferative potential, CCR2 expression, and TRAIL-dependent killing[9,12].

After encountering a variety of distinct tumor cell types (B16F10, B16-Ova, B16-Rae1, MCA205, CT26), spleen derived-IKDC acquired the cell surface expression of CD69, MHC class II and CD86 molecules (Fig.1 and not shown). Thus, tumor driven-licensing of IKDC stimulated their phenotypic conversion into DC-like cells capable of migrating to DLN and of triggering the activation and differentiation of naïve TCR transgenic OT-I and OT-II into polarized T cells (Fig.2, 3). Pletneva and coll. could corroborate that licensing of IKDC by a by-stander cell was required to switch on the DC-like properties of IKDC. Indeed, MCMV-infected fibroblasts but not resting fibroblasts could license IKDC to activate OT-1 and OT-II *in vitro* and *in vivo* (see companion paper). Killing of virally infected fibroblasts was mandatory since perforine$^{-/-}$IKDC failed to activate OT-II in this model system. Accordingly, our data indicate that killing target cells is critical for the efficient priming of naïve T cells since anti-NKG2D mAb abrogated the IKDC-mediated triggering of OTII cells (Fig.2B) and in as much as B16-Ova, yet expressing the candidate antigens, was not sufficient to induce the IKDC-mediated T cell priming in the absence of exogenous proteins (Fig.2 and Fig.3). Indeed, killing of B16-Ova target cells by resting IKDC (<10% at 10:1 E:T ratio) was not sufficient to promote the release of sufficient soluble Ova (or the cell-based transfer of Ova from tumor cells to IKDC) for its processing and presentation by IKDC. At least at this effector/target ratio, IKDC only presented soluble Ova antigen that was supplied to the cultures.

Licensing of IKDC by B16-Rae1 for T cell priming involved an autocrine loop of IFNγ production because anti-IFNγ neutralizing Ab abolished this process and because IFN type IIR$^{-/-}$ IKDC failed to acquire MHC class II and CD86 molecules in contact with tumor cells (Fig.1C and not shown). Although less efficient than B16-Rae1, B16-Ova could license IKDC for OT-I and OT-II activation (Fig.2 and not shown). This reduced activity of B16-Ova in licensing IKDC may be explained by the lack of NKG2D ligands expression and hence a reduced induction of IFNγ secretion by IKDC (Fig. 1C). However, signaling through NKG2D (or NKp46) activating receptors (by *ex vivo* cross-linking) in IKDC was not sufficient to induce their MHCII expression

(Chaput, unpublished data), indicating that other signals may be required for this process. Blockade of TRAIL did not compromise IKDC licensing by tumor cells (Fig.1D).

Why do IKDC diverge so much from B220$^-$NK cells with regard to their APC function? Several arguments pertaining to antigen uptake and "maturation" or "licensing" can be enumerated.

First, genome-wide transcriptome analysis of resting IKDC and B220$^-$NK cells sorted from the spleens of C57Bl/6 mice revealed few but important differences. For instance, IKDC overexpressed CCR7 and CD83, as compared with B220$^-$NK cells (Ullrich and Schultze, unpublished data), results that were corroborated by Pletneva and coll. (companion article). CCR7 is involved in the trafficking of naïve T cells and DC to the lymph nodes[34,35], explaining why IKDC may possess a better migratory potential to LN than conventional NK cells. CD83 molecules participate in the traffic of MHC class II molecules to the cell surface and act as costimulatory molecules[20,36]. Our preliminary data indicate that, indeed, CD83$^{-/-}$IKDC were compromised in their ability to activate naïve OT-II cells (Ullrich, unpublished data).

Secondly, licensing of IKDC, and more specifically of CD11b$^+$IKDC by tumor cells is a mandatory requirement to switch NK-like IKDC into DC-like APC (Fig.2, 3, Suppl.Fig.1). In response to coculture with tumor cells (but not immortalized fibroblasts, not shown), CD11b$^+$ and CD11b$^-$IKDC produced equivalent amounts of IFNγ and exhibited similar levels of MHCII or CD86 expression (Ullrich, not shown). Therefore, more work is required to identify which molecular patterns expressed by transformed cells specifically stimulate IKDC (but not B220$^-$NK cells).

Thirdly, the difference between IKDC and other NK subsets might also rely on their distinct abilities to capture antigens. Significant internalization of soluble proteins or fluid phase markers was observed for IKDC (Fig.4A and not shown). Accordingly, IKDC expressed higher levels of the C-type lectin mannose receptor (CD206, MMR) (Fig.4B) than B220$^-$NK cells. MMR has been involved in the recognition and clearance of microorganisms and serum glycoproteins, mostly by macrophages[37,38]. A role in antigen presentation has been reported based on its expression on cDC and its role in the uptake of mannosylated structures such as dextrans into MHCII-enriched cellular compartments[39],[24,40]. Here, we reveal a functional role for MMR in the DC-like functions of IKDC. Thus, the Ova-specific OT-I activation triggered by the adoptive

transfer of CD11b$^+$IKDC loaded with Ova proteins was reduced in the presence of anti-CD206 blocking antibodies (Fig.4C).

It is important to note that the DC-like function of IKDC was mainly confined to the CD11b (CD11b/CD18, CR3, Mac1)-expressing fraction of IKDC[41]. DC-like IKDC indeed represent about 40% of the CD27$^+$CD11b$^+$NK cell population in the LN, lungs, and spleen[42]. Potential ligands of CD11b/CD18 are fibrinogen, iC3b, ICAM1, and β-glucans, which dock to the lectin-like binding site of CD11b/CD18. CD11b/CD18 can efficiently be internalized by endocytosis[43-46]. The role of CD11b in the DC-like functions of IKDC remains unclear. Triggering and/or binding of CD11b could represent a "licensing" signal delivered by tumor cells or infectious agents or the complement cascade. Alternatively, CD11b could represent a route of uptake of β-glucan-containing particles[47]. The selective binding of CyaA toxin from *Bordetella pertussis* to CD11b-expressing cells has been exploited to target myeloid DC *in vivo*. Detoxified mutants of CyaA (that do not elevate cAMP) can be utilized to pharmacologically deliver tumor[31] or viral[48] antigens to the cytosol of myeloid DC. After proteasome-dependent intracellular processing, peptide epitopes are presented by MHC I molecules to CD8$^+$T lymphocytes, leading to protective CTL responses against viral or tumor challenges[29,31,48]. Here, we show that CyaA also targeted CD11b$^+$IKDC and allowed efficient cross-priming of naïve CD8$^+$T cells *in vivo* (Fig.5). In this *in vivo* system, the CD11b$^+$IKDC licensing (switch to APC like function) remains unknown.

It is unclear whether IKDC exert their DC-like functions in peripheral tissues and/or in the draining LN like more classical APC. Our preliminary data indicated that during the course of an efficient therapy of B16-Ova lung metastases with imatinib mesylate and IL-2, tumor-infiltrating IKDC failed to activate OT-I or OT-II *ex vivo* after sorting from the lungs (Bonmort, unpublished data). Since we showed that the tumoricidal activity of the combination therapy depended upon IL-15Rα/IL-15[12], this result might reflect the NK-like functions displayed by IKDC at this time point. In contrast, Anderson and coll. could demonstrate the capacity of IKDC recruited in tumor beds en route to LN to exhibit DC-like functions on antigen-experienced T cells (companion paper).

Altogether our data (as well as those contained in the two co-submited papers) indicate that bifunctional cells that encompass characteristics of DC and NK cells exist. As little as 30x10^{3}IKDC could prime naïve OT-I (Fig.3B) and OT-II cells (Fig.2) *in vivo*. Interestingly, both

IKDC and cDC functions are controlled by the B7 subfamily members. Indeed, they both stimulate T cells in a CTLA4Ig-inhibitable fashion (Fig.3D-E-F) and B7-H1 molecules appeared to represent a negative regulator, both of cDC[49,50] and of the DC-like functions of IKDC (Fig.2G). Thus, PD-1-expressing tumor infiltrating T lymphocytes might operate as a retro-control on all potential DC-like functions in tumor beds. However, cDC and IKDC may differ in their capacity to phagocytose particulate antigens. Indeed, our preliminary data indicate that 200 nm beads can be phagocytosed by cDC but not by IKDC (Bonmort, not shown). Hence, it is plausible that IKDC may be specialized in handling circulating mannosylated particulate antigens that could be captured right after entry through high endothelial venules within LN. It is likely that non transformed cells residing in this location might license IKDC.

The results presented in this work underscore the potential of IKDC to exhibit DC-like functions. However, as for natural killer myeloid DC or DC-like NK cells, future studies will be required to identify their pathophysiological relevance *in vivo*.

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Authorship:

Conceived and designed the experiments: MT, GM, EU, MB, NC and LZ. Performed the experiments: MT, GM, EU, MB and JLS. Analyzed the data: MT, GM, EU, MB, NC and LZ. Provided reagents: VMC, AJ and CL. Wrote the paper: LZ, NC, MT and GK.

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Figure Legends

Figure 1. Tumor cells license IKDC to express MHC class II and CD86 molecules.

A-B. *Tumor cells license IKDC for MHC class II and CD86 expression*. B16F10 tumor cells were incubated for 24 hrs with spleen-derived IKDC or B220$^-$NK cells ("NK") sorted from naïve C57Bl/6 animals, at a 10:1 E:T ratio. Flow cytometry analyses (A) of effector cells were performed gating on IKDC or B220$^-$NK cells respectively, after staining with anti-I-A^b/I-E^b Ab or anti-CD86 or anti-CCR7 mAb. A representative experiment out of three is depicted. Confocal microscopy allowed to visualize MHC class II expression and cell-cell contacts between a variety of tumor cells and IKDC or B220$^-$NK cells (B, left panel) and to enumerate the MHC class II expressing effectors (B, right panel). The photographs depict typical cocultures and the results are presented as means$\pm$SEM of fluorescent cells examined in 100 cells. **C-D.** *IFNγR-dependent tumor licensing of IKDC*. Identical setting as in A, but IKDC were sorted from spleens of naïve WT and IFNγR$^{-/-}$ mice (C) or incubated in the presence of N2B2 (anti-TRAIL neutralizing Ab) (D). The experiment was performed twice with identical results.

Figure 2. CD11b$^+$IKDC can prime naïve OT-II cells after tumor licensing.

A. *CD11b$^+$IKDC can prime naïve OTII cells after tumor licensing in vitro.* IKDC (CD11b$^+$ or CD11b$^-$) or B220$^-$NK cells or cDC (DC) were first incubated for 12 hrs in the presence of B16-Rae1 tumor cells ("Tum") at a 10:1 E:T ratio and/or soluble Ova. The coculture was washed before addition of naïve OT-II cells at a 1:1 APC:OT-II ratio for 18 hours. Supernatants were analyzed by commercial ELISA detecting the specific production of mIL-2. **B.** Id. as in A. but anti-NKG2D neutralizing or isotype control antibodies were added to the licensing period. OTII cells were next analyzed in flow cytometry for CD69 expression**. C**. *CD11b$^+$IKDC can prime naïve OT-II cells after tumor licensing in vivo.* 10^6 CFSE labeled-OT-II were inoculated i.v., 150-250x10^3 live IKDC (or B220$^-$NK cells) pulsed with soluble Ova and licensed (or not) with tumor cells were inoculated in the footpad of C57Bl/6 mice 18 hours later. Positive controls included footpad inoculation of 300x10^3 live BM-DC (DC) cultured in similar conditions or increasing dosages of Ova protein (see experimental setting in the inset of panel 2C). After 6 days, DLN were harvested, stained with anti-CD69 mAb and analyzed in flow cytometry analysis.

Representative dot plots of CD69 and CFSE dilution of CFSE-labeled OT-II cells are shown (C, upper panels). The means ± SEM of percentages of CD69 expressing $CFSE^+$ cells and the percentages of dividing cells (C, lower panels) in three independent experiments containing 2 mice /group. APC could be licensed by B16-Ova tumor cells (as in Fig. 1) during the pulsing period with Ova prior to inoculation (D). In another group of mice, day 6 DLN were restimulated with PBS or Ova protein and the production of IFNγ was measured by commercial ELISA (D) or intracellular staining of IL-2 after Ova restimulation (as described in Material and Methods) gating on CFSE-labeled OTII cells (upper panel) or all Vα2 unlabeled $CD4^+$ T cells (lower panel) (E). **F.** *Ablation of host cDC did not compromise IKDC-mediated OT-II stimulation.* WT or CD11c- DTR transgenic mice were treated with DT one day prior to OT-II injection. At day 2, IKDC pulsed with Ova were injected. The graph outlines a representative staining of CD69 expressing $CFSE^+$ cells of two independent experiments containing 2 mice /group. Flow cytometry analyses of $CD11c^{high}$/MHC class II expressing cDC revealed their ablation in control animals (not shown). **G.** *IKDC are competent APC in CD83-deficient mice.* MHC Class II expression was checked by flow cytometry on gangliocytes obtained from $CD83^{-/-}$ and WT mice (upper panel). $CD11b^+$ IKDC, $B220^-$ $CD11c^-$ NK cells, cDC licensed by tumor cells and pulsed with Ova protein were injected into $CD83^{-/-}$ mice, one day after OT-II cells transfer. 18 hours later, CD69 expression was monitored on OT-II cells defined as $CD3^+CD4^+V\alpha2^+$ cells (lower panel). **H.** *B7-H1 molecules severely impaired the DC-like functions of resting $CD11b^+$IKDC.* Same setting as in D. but neutralizing anti-B7-H1 mAb was added during the licensing period prior to washing and footpad inoculation. Negative controls included footpad inoculation of Tum+Ova in the presence of anti-B7-H1 mAb. A representative experiment out of two containing two mice /group is shown depicting the mean IFNγ productions ± SEM after restimulation of lymph node cells with PBS versus Ova. * $p<0.05$; *** $p<0.001$; ND means not done.

Figure 3. Licensed $CD11b^+$IKDC cross-present soluble antigens to naïve $CD8^+$T cells.

A-B. *IKDC prime naïve OT-I cells after tumor-driven licensing.* Identical settings as in Fig. 2D but adoptive transfer of naïve OT-I-GFP lymphocytes was performed and the intracellular staining identified IFNγ producing OT-I. (A) depicts a representative experiment out of two yielding similar conclusions. showing the proportion (left panel) and absolute number per lymph node (right panel) of IFNγ producing OT-I. Escalation dose of IKDC or cell-sorted cDC injected

in the footpad, at day 6 after injection restimulation of DLN with the H-2^b-restricted Ova peptide (SIINFEKL 10µg/ml) to monitor IFNγ secretion (B). **C.** *IKDC are competent APC in H-$2D^b$/H-$2K^b$/β2 microglobuline loss-of-function mice.* OT-I cells defined as $CD3^+$ $CD8^+$ $V\alpha2^+$ T lymphocytes were monitored for CD69 expression in the DLN of wild type (WT) and β2 microglobuline knock out host after transfer (18 hrs later) of WT DC or IKDC licensed with tumor cells and Ova protein. A representative experiment containing 3 mice /group is shown. **D-F.** *Licensed $CD11b^+$IKDC can prime naïve $CD8^+$T cells in a B7-dependent manner.* The IKDC subsets and cDC were cocultured with B16-Rae1 and Ova protein for 18 hrs prior to incubation with OT-I cells in the presence or absence of control or CTLA4Ig fusion proteins. Cytokine production was assessed at 48 hrs using commercial ELISA. (D) depicts the means ± SEM of triplicate wells and represents one experiment out of two yielding similar results. E-F. 10^6 OT-I CD8+ T cells were inoculated iv. 18 hours later, IKDC or DC were injected in footpads of C57Bl/6 mice after licensing wih B16-Rae and Ova protein. Neutralizing CTLA4Ig fusion proteins or anti-CD40L Ab were coinjected together with the APC. At day 6, DLN were restimulated with the SIINFEKL peptide (10µg/ml). Cytokine production was assessed at 48 hrs using IFNγ ELISA. A representative experiment containing 3 mice/group is depicted. *$p<0.05$, ND means not done.

Figure 4. CD206-dependent T cell priming by IKDC.

A-B. *IKDC can internalize fluid phase markers*. IKDC and $B220^-$NK cells (A) were incubated or not with FITC-Dextran at 37°C. At 0°C, the specific staining was not observed (not shown). Cells were analyzed at 4-6 hours for FITC staining in flow cytometry and confocal microscopy after membrane labeling with WGA A647 fluorescent dye. A representative picture is shown (A, right panels). The uptake was performed twice with identical results. **B.** *Mannose receptor expression on NK or IKDC*. Naïve spleens were stained intracellularly with anti-CD206 mAb in addition to the classical six colors staining allowing the identification of IKDC and $B220^-$NK cells in flow cytometry analyses. The dot plots (left panel) is a representative staining of IKDC. The right panel represents the data pooled of three independent stainings. **C**. *Mannose receptor-dependent activation of OT-I by IKDC in vivo.* Same experimental setting as in Fig. 3E-F but using anti-MMR neutralizing or isotype control Ab during the licensing phase. DLN were restimulated with the H-2^b-restricted Ova peptides (SIINFEKL at 10 µg/ml) for 24 hrs and IFNγ levels were

measured using ELISA. The mean values of triplicate wells are depicted in a representative experiment containing 3 mice per group.*$p<0.05$.

Figure 5. Targeted antigen delivery via CD11b/CD18 revealed IKDC cross-priming capacities.

In vivo targeting of CD11b/CD18 expressing cells using systemic inoculation of CyaA-Ova (or CyaA-E7 as negative controls) in the absence (**A, B**) or presence (**C**) of CpG ODN inoculated iv. Conventional DC and IKDC were sorted according to CD11b expression and incubated with resting OT-I cells for 24 hrs. CD69 expression on OT-I was examined in flow cytometry (A) and mouse IFNγ (B) and/or IL-2 (C) secretion was monitored in the supernatants of APC:OT-I cocultures at 24 hrs. Each experiment contained 2-3 mice /group and was performed two to three times yielding identical results.*$p<0.05$.

Supplemental Figure

Supplemental Figure 1. CD11b expression on splenic IKDC.

Flow cytometry analyses of $CD3^{-}CD19^{-}CD11c^{int}B220^{+}NK1.1^{+}$ cells using anti-CD11b Ab. IKDC sorted from naïve spleens can be subdivided into two subsets based on CD11b expression. One representative graph is shown out of three independent experiments.

Figure 1

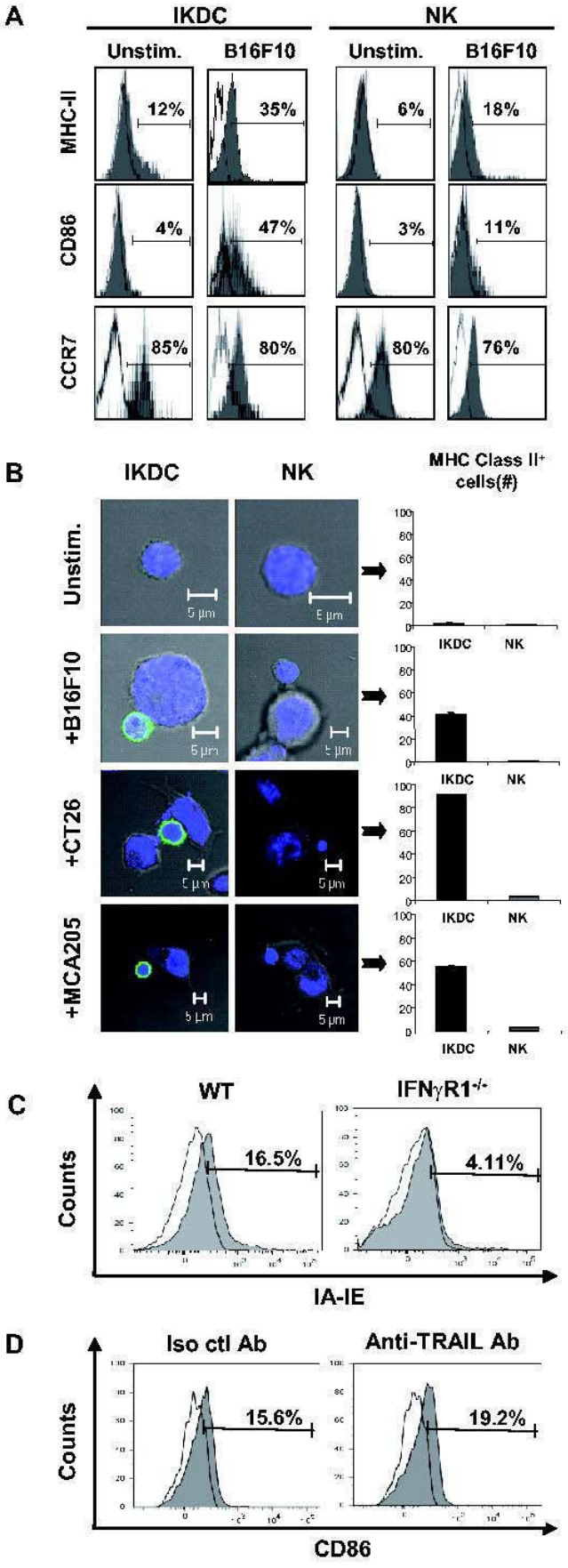
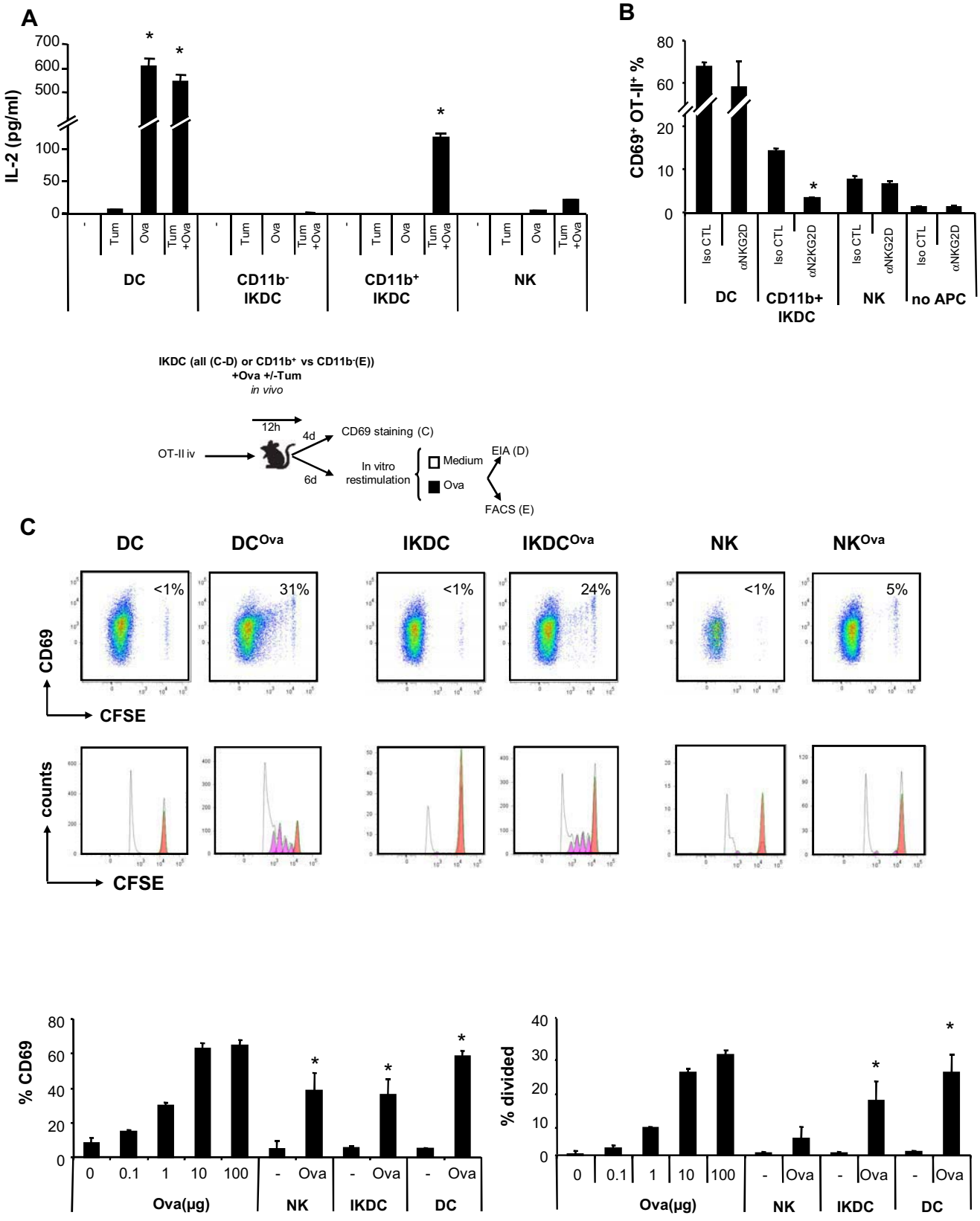

A
IKDC
NK
Unstim.
B16F10
Unstim.
B16F10
MHC-II
12%
35%
6%
18%
CD86
4%
47%
3%
11%
CCR7
85%
80%
80%
76%
B
IKDC
NK
MHC Class II+ cells(#)
Unstim.
+B16F10
+CT26
+MCA205
5 μm
IKDC
NK
C
WT
IFNγR1-/-
Counts
16.5%
4.11%
IA-IE
D
Iso ctl Ab
Anti-TRAIL Ab
Counts
15.6%
19.2%
CD86

Figure 2


A
IL-2 (pg/ml)
700
600
500
100
50
0
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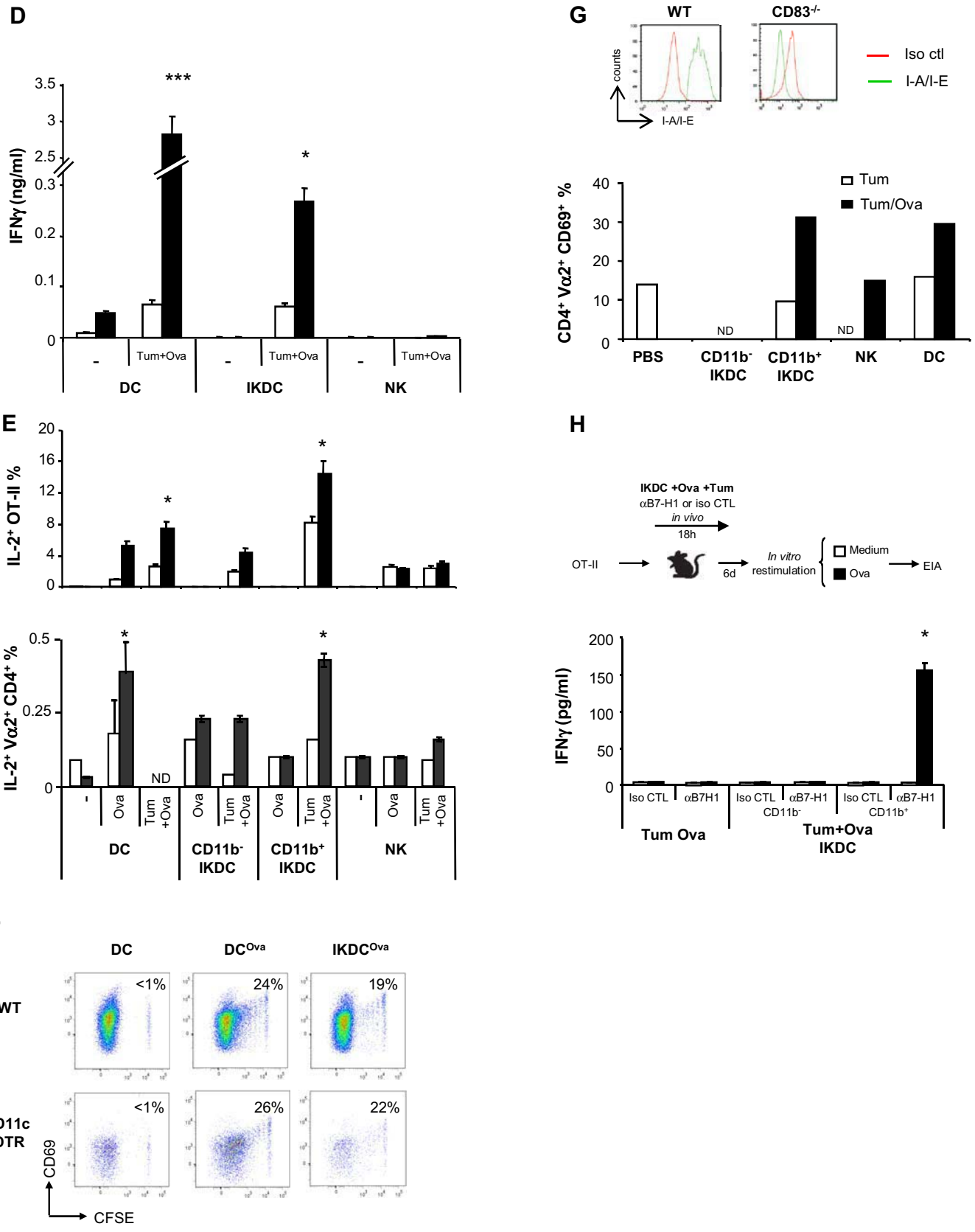
*
-
Tum
Ova
Tum +Ova
DC
CD11b- IKDC
CD11b+ IKDC
NK
B
CD69+ OT-II+ %
80
60
20
10
0
*
Iso CTL
αNKG2D
αN2KG2D
DC
CD11b+ IKDC
NK
no APC
IKDC (all (C-D) or CD11b+ vs CD11b-(E))
+Ova +/-Tum
in vivo
OT-II iv
12h
4d
6d
CD69 staining (C)
In vitro restimulation
Medium
Ova
EIA (D)
FACS (E)
C
DC
DCOva
IKDC
IKDCOva
NK
NKOva
<1%
31%
<1%
24%
<1%
5%
CD69
CFSE
counts
CFSE
% CD69
80
60
40
20
0
0
0.1
1
10
100
Ova(µg)
-
Ova
NK
IKDC
DC
% divided
40
30
20
10
0
0
0.1
1
10
100
Ova(µg)
NK
IKDC
DC

Figure 2


D
IFNγ (ng/ml)
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0.3
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Tum+Ova
DC
IKDC
NK
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WT
CD83-/-
counts
I-A/I-E
Iso ctl
I-A/I-E
CD4+ Vα2+ CD69+ %
40
30
20
10
0
Tum
Tum/Ova
ND
PBS
CD11b- IKDC
CD11b+ IKDC
NK
DC
E
IL-2+ OT-II %
20
16
12
8
4
0
IL-2+ Vα2+ CD4+ %
0.5
0.25
ND
-
Ova
Tum +Ova
DC
CD11b- IKDC
CD11b+ IKDC
NK
H
IKDC +Ova +Tum
αB7-H1 or iso CTL
in vivo
18h
OT-II
6d
In vitro restimulation
Medium
Ova
EIA
IFNγ (pg/ml)
200
150
100
50
0
Iso CTL
αB7H1
CD11b-
CD11b+
Tum Ova
Tum+Ova IKDC
F
DC
DCOva
IKDCOva
WT
CD11c -DTR
<1%
24%
19%
<1%
26%
22%
CD69
CFSE

Figure 3

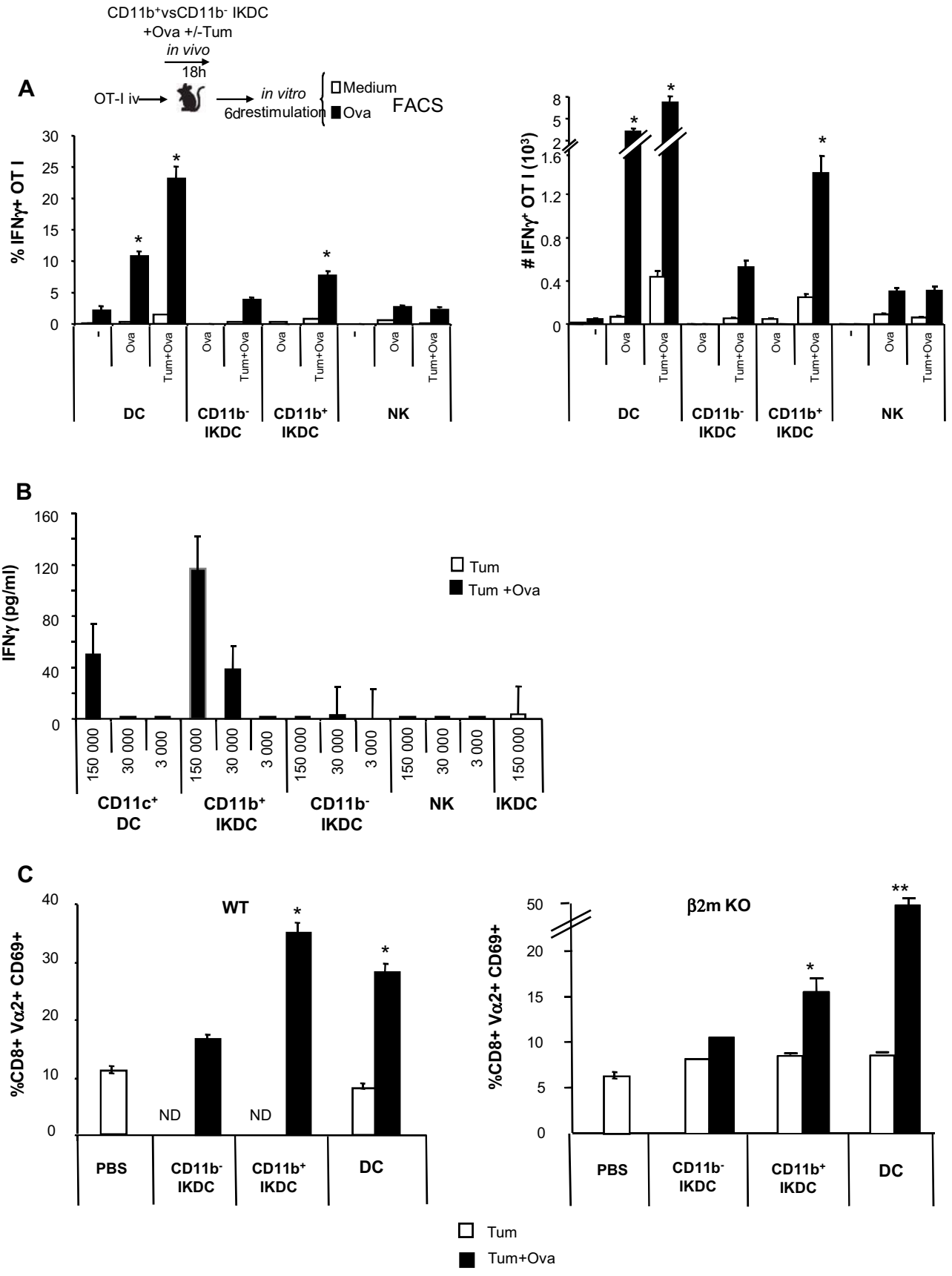
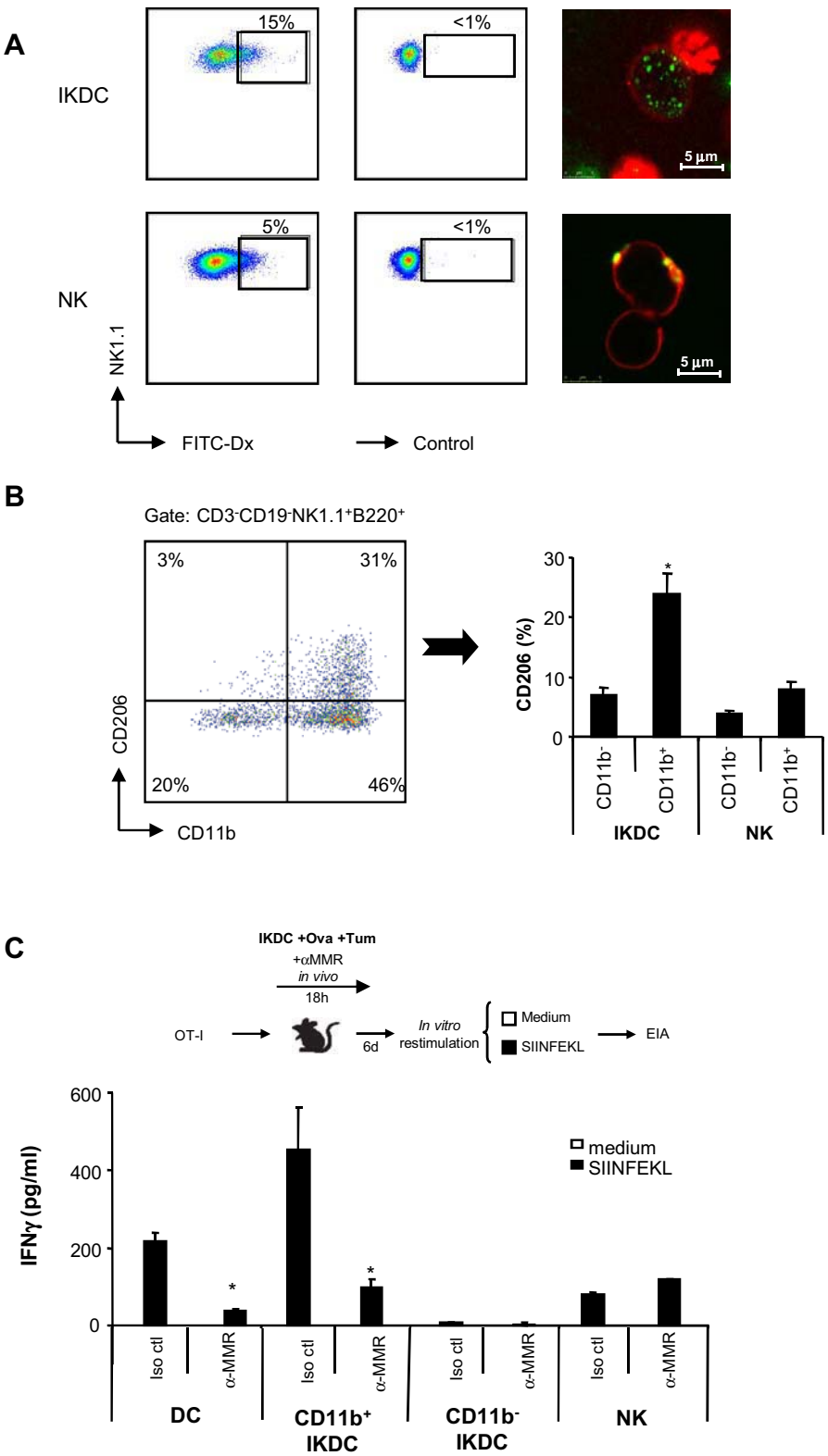

CD11b+vsCD11b- IKDC
+Ova +/-Tum
in vivo
18h
A
OT-I iv
in vitro
6d restimulation
Medium
Ova
FACS
% IFNγ+ OT I
# IFNγ+ OT I (10³)
Ova
Tum+Ova
DC
CD11b- IKDC
CD11b+ IKDC
NK
B
IFNγ (pg/ml)
Tum
Tum +Ova
150 000
30 000
3 000
CD11c+ DC
CD11b+ IKDC
CD11b- IKDC
NK
IKDC
C
WT
β2m KO
%CD8+ Vα2+ CD69+
ND
PBS
CD11b- IKDC
CD11b+ IKDC
DC
Tum
Tum+Ova

Figure 4


A
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15%
<1%
5 µm
NK
5%
<1%
5 µm
NK1.1
FITC-Dx
Control
B
Gate: CD3-CD19-NK1.1+B220+
3%
31%
20%
46%
CD206
CD11b
CD206 (%)
30
20
10
0
CD11b-
CD11b+
CD11b-
CD11b+
IKDC
NK
C
IKDC +Ova +Tum
+αMMR
in vivo
18h
OT-I
6d
In vitro
restimulation
Medium
SIINFEKL
EIA
IFNγ (pg/ml)
600
400
200
0
medium
SIINFEKL
Iso ctl
α-MMR
Iso ctl
α-MMR
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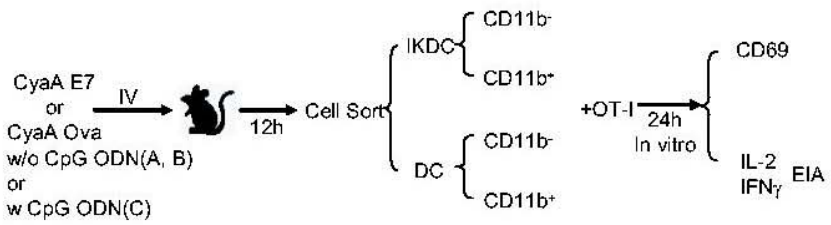
α-MMR
Iso ctl
α-MMR
DC
CD11b+ IKDC
CD11b- IKDC
NK

Figure 5


CyaA E7
or
CyaA Ova
w/o CpG ODN(A, B)
or
w CpG ODN(C)
IV
12h
Cell Sort
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CD11b+
DC
CD11b-
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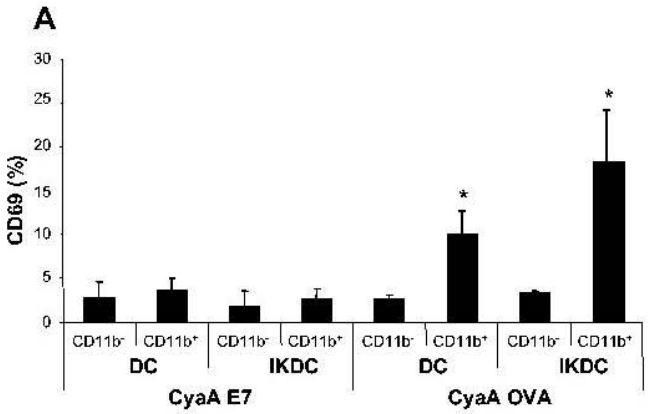
+OT-I
24h
In vitro
CD69
IL-2
IFNγ
EIA


A
CD69 (%)
30
25
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10
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0
*
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CD11b- CD11b+ CD11b- CD11b+ CD11b- CD11b+ CD11b- CD11b+
DC
IKDC
DC
IKDC
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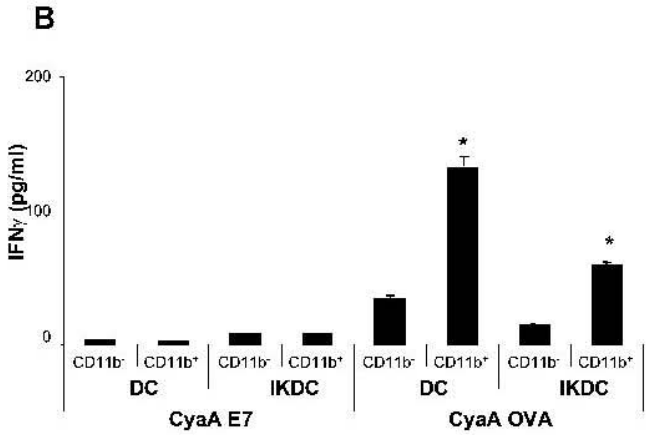
CyaA OVA


B
IFNγ (pg/ml)
200
100
0
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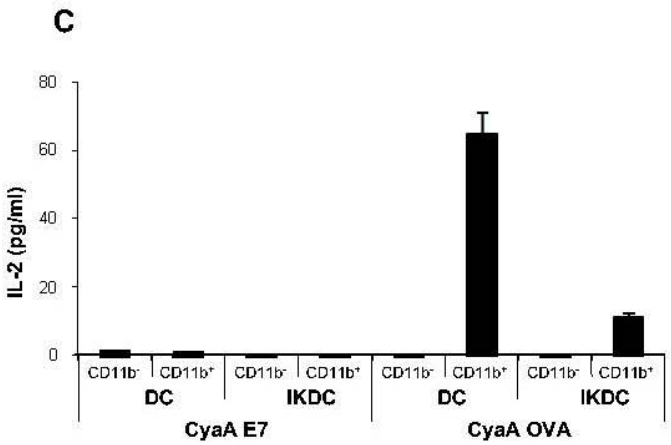
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5 Discussion

Suite à la mise en évidence du phénomène IKDC, une polémique est née entre experts. Les IKDC sont-elles justes des NK CMH-II+ ? Ou bien les IKDC sont-elles vraiment des DC tueuses ? Les données accumulées permettent de donner des éléments de réponse.

Comme publié par Vosshenrich *et al* (Vosshenrich, Lesjean-Pottier et al. 2007), Blasius *et al* (Blasius, Barchet et al. 2007) et Caminshi *et al* (Caminschi, Ahmet et al. 2007), et confirmé par notre propre étude (Ullrich, Bonmort et al. 2008), les IKDC semblent plus proche du lignage lymphoïde que myéloïde. Leur dépendance à l'IL-15 les fait ressembler aux NK. Dans ces conditions, comment les classer parmi les NK ?

Les IKDC définies sur le B220 sont une population hétérogène. En effet, il est possible d'observer une distribution bimodale du B220 sur les NK CD11b$^-$, mais jamais sur les CD11b$^+$. Or, la fraction des NK exprimant spontanément TRAIL dans une rate de souris naïve est plutôt CD11b$^-$ (Figure 3). Par ailleurs, nous avons mis en évidence une supériorité des fractions CD11b$^+$NK1.1$^+$ sur les CD11b$^-$ en ce qui concerne la présentation de l'antigène.

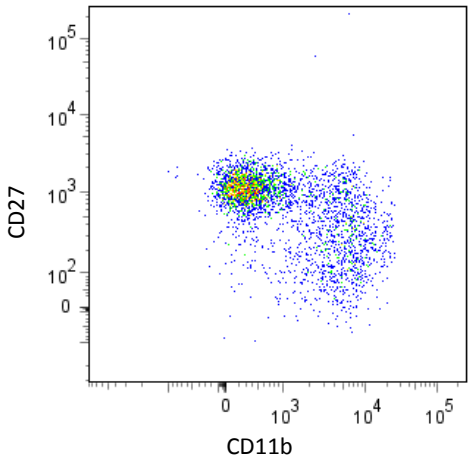

Figure 3 : Expression des marqueurs CD27 et CD11b sur des splénocytes NK1.1$^+$CD3$^-$CD19$^-$TRAIL$^+$. Voir matériel et méthode de l'article 2.

Nous avons donc devant nous une évidence qui nous avait échappée : les IKDC définies sur la seule expression du B220 sont un mélange de deux populations : d'une part une population facilement définissable CD11b$^-$B220$^+$, qui prolifère très bien en IL-15/IL-15Ralpha, et qui

exprime TRAIL ; et d'autre part une population $CD11b^{+}B220^{+}$, qui prolifère plus mal en IL-15/IL-15Ralpha, et qui possède un potentiel de CPA. Etant donné que 100% des $B220^{+}$ NK sont $CD27^{+}$, nous pouvons faire une hypothèse en nous appuyant sur la littérature.

Selon le travail de Hayakawa *et al* (Hayakawa and Smyth 2006), les NK de souris se répartissent en trois populations : les $CD11b^{-}CD27^{+}$, jugées immatures, les doubles positives $CD11b^{+}CD27^{+}$ et les $CD11b^{+}CD27^{-}$, jugées matures et cytotoxiques. Nous pouvons introduire dans ces populations le « facteur B220 » : les $CD11b^{-}CD27^{+}B220^{+}$ sont capables de proliférer en IL-15/IL-15Ralpha et sont lytiques via TRAIL, ce qui valide leur statut immature. Par ailleurs les $CD11b^{+}CD27^{+}$ contiennent une population, semble-t-il mal définie par le B220, qui est capable de présenter l'antigène. De manière intéressante, cette population $CD11b^{+}CD27^{+}$ est aussi celle qui migre le plus facilement aux ganglions lymphatiques après injection de DC, comme montré récemment (Watt, Andrews et al. 2008).

Le marqueur B220 sur les NK est-il donc un marqueur d'activation ou bien un marqueur de NK immature ? Car dans le premier cas, comment expliquer que les populations de NK matures ($CD27^{-}$) soient les moins activées ($B220^{-}$) ? Et dans le second cas, pourquoi serait-ce les NK immatures ($B220^{+}$) qui seraient les plus actifs ?

Le plus probable pourtant est bien que le B220 est un marqueur de NK immatures. Il existe plusieurs indices à ce propos :

- Co-expression de TRAIL (Takeda, Cretney et al. 2005; Taieb, Chaput et al. 2006)
- Co-expression de CD27 (Hayakawa and Smyth 2006; Blasius, Barchet et al. 2007)
- Co-expression de CD117 (Colucci and Di Santo 2000; Ullrich, Bonmort et al. 2008)
- Fort pouvoir prolifératif (Vosshenrich, Lesjean-Pottier et al. 2007; Ullrich, Bonmort et al. 2008)

Ce qui peut alors expliquer la présence de B220 dans une culture de NK triée $B220^{-}$ est le fait que les cellules NK $CD27^{-}$ prolifèrent peu par rapport aux autres (Hayakawa and Smyth 2006), qui sont eux-mêmes $B220^{int}$ ou $B220^{hi}$ (Hayakawa and Smyth 2006). Ainsi, suivant la « sévérité » du tri sur le B220, les cellules $CD27^{+}$ $B220^{+}$ restantes envahiront la culture, qui paraîtra être devenue $B220^{+}$.

5.1 Note sur les chausse-trappes de l'étude des NK de souris

Il existe une fraction des cellules B qui est capable de fixer le NK1.1. Il est relativement facile de les discriminer avec un marquage CD19, mais on ne saurait se contenter d'un CD3 pour l'étude phénotypique des cellules NK en cytométrie en flux. Ainsi des cellules $NK1.1^{+}CMH\text{-}II^{+}B220^{+}$ peuvent-elles exprimer le CD11c ou le CD19 (Figure 4). Ces deux marqueurs sont mutuellement exclusifs en condition normale, mais qu'en est-il en condition inflammatoire ? Or, une étude a montré, dans un modèle humain de leucémie lymphoïde chronique à cellules B, que des cellules B leucémiques peuvent dans certains cas (exposition à la bryostatine, un modulateur de protéines kinases C) exprimer le CD11c (Thomas, Pepper et al. 2004).

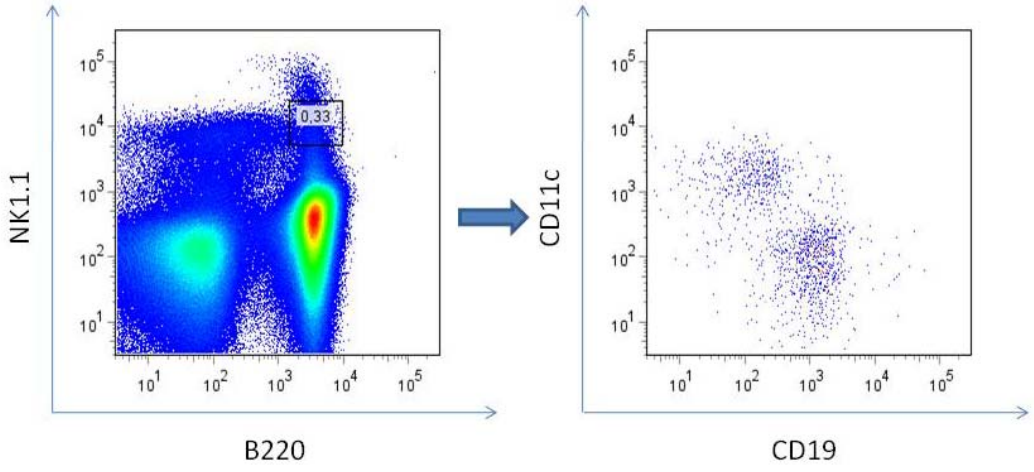

Figure 4 : Expression du CD11c et CD19 sur splénocytes vivants sélectionnés $NK1.1^{hi}B220^{+}$. (Voir matériel et méthodes de l'article 2)

Par ailleurs, il existe dans certaines conditions inflammatoires une population de cellules $CD11b^{hi}$ qui fixe le NK1.1 mais pas le DX5 ni le NKP46. Cette population est $CMH\text{-}II^{+}$ et $CD40^{+}$, est probablement d'origine myéloïde, tel un monocyte ou une cellule dendritique. Il est parfois très difficile de s'en affranchir, notamment en conditions inflammatoires, où plus de la moitié des cellules $NK1.1^{+}$ sont $CD11b^{hi}$.

6 Perspectives

6.1 Rôle physiologique des IKDC

Nos travaux et ceux d'autres équipes nous permettent de dire que les IKDC sont capables de présenter l'antigène, mais dans des conditions plus restrictives que les DC. L'hypothèse selon laquelle les NK $B220^{+}$ seraient capables de prendre en charge l'antigène soluble dans un contexte tumoral ou infectieux est à approfondir : quels sont le(s) signal(-aux) présent(s) chez les cellules tumorales qui permet aux IKDC de présenter l'antigène ? Si ce signal est identifié, il conviendrait de tester sa présence dans différentes lignées tumorales, d'autres cibles NK (cellules infectées par un virus), et de chercher un équivalent humain.

6.2 IKDC humaine

Il a été proposé que les IKDC soient l'équivalent murin des cellules NK $CD56^{hi}$ humaines. Il est alors possible d'étudier les similitudes entre les espèces de ces sous-populations de cellules NK, afin de mieux comprendre comment les phénomènes étudiés chez la souris pourraient être exploités en clinique.

6.3 Applications possibles

Le fait de disposer d'une cellule lytique et/puis présentatrice d'antigène ouvre des perspectives d'immunothérapie du cancer. Auparavant, les injections de cellules produites *in vitro* visaient des buts distincts : ou bien la lyse des cellules tumorales, avec l'injection de cellules cytotoxiques, ou bien la vaccination des patients contre leur tumeur, avec les injections de DC ou de cellules transgéniques.

Mais si une cellule tueuse et/puis présentatrice d'antigène peut être produite et contrôlée, alors les deux stratégies pourront être mises en place en même temps. L'immunothérapie active pourrait être envisagée, induisant dans le corps du patient la production et/ou l'activation des IKDC : c'est dans cette optique qu'un essai IM+IL-2 est actuellement en cours à l'Institut Gustave Roussy dans le traitement des cancers TRAIL-sensibles et des sarcomes gastrointestinaux résistants à l'IM seul.

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8 ANNEXES

8.1 Annexe 1 : Identification d'un signal pro-phagocytaire impliqué dans l'immunogénicité de la mort tumorale induite par les anthracyclines : la calreticuline

8.1.1 Résumé

En se basant sur les résultats de Casares *et al* (Casares, Pequignot et al. 2005), nous avons poursuivit l'étude des mécanismes rendant la mort par exposition aux anthracyclines immunogène. La première question était de savoir si un signal membranaire particulier, dont dépendait la phagocytose, était acquis par les corps apoptotiques des cellules tuées par anthracyclines. Il fut établit que la phagocytose des corps apoptotiques et des cellules mourantes par les cellules dendritiques était un phénomène rapide, excluant donc la participation d'une protéine produite *de novo* par la cellule mourante. L'hypothèse d'une translocation d'une protéine intracellulaire vers la surface -ou à l'inverse, une internalisation d'un signal inhibiteur- fut envisagée. En étudiant par électrophorèse bidimensionnelle les protéines des membranes des cellules tumorales CT26 avant et après chimiothérapie, et en les comparant avec les protéines membranaires de ces mêmes cellules traitées conjointement avec une anthracycline et un inhibiteur de capsase, il fut possible d'identifier une protéine qui apparaissait sur les membranes uniquement dans les préparation de cellules immunogènes : la calréticuline (CRT) (Obeid, Tesniere et al. 2007).

La présence de cette protéine sur les cellules tumorales fut testée dans différents cas d'exposition à divers cytotoxiques, et les cellules traitées furent testées dans deux contextes : d'une part leur phagocytose par des DC *in vitro*, d'autre par leur capacité à induire une réponse immunitaire antitumorale protectrice *in vivo*. Dans les deux cas, il fut possible d'établir une corrélation entre la présence de CRT à la membrane des cellules mourante, leur phagocytose par les DC et l'efficacité de la vaccination induite.

Par ailleurs, dans des conditions où les cellules mourantes sont immunogènes, l'invalidation de l'expression de la CRT par interférence d'ARN abolit cette immunogénicité, et ce phénomène est réversible par l'ajout de CRT recombinante. De même, des cellules mourantes non-immunogènes peuvent le devenir si on leur adjoint de la CRT, dans un modèle de vaccination préventive anti-tumorale.

Enfin, l'utilisation d'une drogue (inhibiteurs de PP1/GADD34) favorisant l'exposition membranaire de CRT permet d'observer un effet anti-tumoral synergique avec un traitement cytotoxique chez des animaux immunocompétents porteurs de tumeurs, mais pas chez des animaux immunodéficients (Obeid, Tesniere et al. 2007).

8.1.2 Discussion

Cette étude (Obeid, Tesniere et al. 2007) a été la première à mettre en évidence un marqueur membranaire associé à l'immunogénicité des corps apoptotiques. La CRT est une protéine abondante dans le reticulum endoplasmique, et est une protéine chaperone, capable de fixer des peptides immunogènes et donc de posséder des propriétés d'adjuvants immunitaire. Cependant l'utilisation de CRT recombinante sur des cellules tuées permet l'induction de la réponse immunitaire : le rôle de chaperon n'est donc pas à priori impliqué dans celui qui nous intéresse ici.

Favoriser l'expression de la CRT par des inhibiteurs de PP1/GADD34 permet de rendre immunogène un traitement chimiothérapeutique qui ne l'est pas. Cette possibilité permettrait d'étudier les effets immunologiques potentiels d'autres médicaments anticancéreux.

L'intérêt de l'exposition membranaire de la CRT ne se trouve cependant que dans le cas d'une cellule tumorale mourante : en effet, des cellules vivantes exprimant la CRT ne provoquent pas de réponse immunitaire.

Le récepteur cellulaire impliqué dans la détection de la CRT membranaire reste à identifier ; mais il semble établit dans ce modèle de tumeur (CT26), il y a une nette corrélation entre expression membranaire de CRT et immunogénicité des cellules mourantes.

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Calreticulin exposure dictates the immunogenicity of cancer cell death

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Anthracyclin-treated tumor cells are particularly effective in eliciting an anticancer immune response, whereas other DNA-damaging agents such as etoposide and mitomycin C do not induce immunogenic cell death. Here we show that anthracyclins induce the rapid, preapoptotic translocation of calreticulin (CRT) to the cell surface. Blockade or knockdown of CRT suppressed the phagocytosis of anthracyclin-treated tumor cells by dendritic cells and abolished their immunogenicity in mice. The anthracyclin-induced CRT translocation was mimicked by inhibition of the protein phosphatase 1/GADD34 complex. Administration of recombinant CRT or inhibitors of protein phosphatase 1/GADD34 restored the immunogenicity of cell death elicited by etoposide and mitomycin C, and enhanced their antitumor effects *in vivo*. These data identify CRT as a key feature determining anticancer immune responses and delineate a possible strategy for immunogenic chemotherapy.

Induction of immunogenic cancer cell death should be one of the aims of anticancer chemotherapy, because it would allow the immune system to contribute through a 'bystander effect' to the eradication of chemotherapy-resistant cancer cells and cancer stem cells[1–3]. Despite a growing body of research, the circumstances that trigger an immune response against dying tumor cells remain unclear[4].

It has long been hypothesized that apoptotic cell death is poorly immunogenic (or even tolerogenic) whereas necrotic cell death is truly immunogenic[5–7]. This difference was thought to result from the intrinsic capacity of cells dying a nonapoptotic cell death to reduce an immune response—for example, by stimulating local inflammatory responses ('danger signals'), triggering the maturation of dendritic cells (DCs) or both[8,9]. In contrast to necrosis (which is defined by brisk plasma membrane rupture), apoptosis is associated with a series of subtle alterations in the plasma membrane that render the dying cells palatable to phagocytic cells[10]. Such 'eat me' signals, which include the adsorption of soluble proteins (such as C1q and thrombospondin) from outside the cell and the translocation of molecules (such as phosphatidylserine and calreticulin (CRT)) from inside the cell to the surface, as well as the suppression of 'don't eat me' signals (such as surface CD47)[11–15], elicit the recognition and removal of apoptotic cells by professional and nonprofessional phagocytes. Suboptimal clearance of apoptotic cells can trigger unwarranted immune reactions and lead to autoimmune disease[16,17].

Nonetheless, it seems that the dichotomy between immunogenic necrosis versus tolerogenic apoptosis is an oversimplification. Thus, unscheduled (necrotic) tumor cell death may induce local immunosuppression[18]. Moreover, the capacity of apoptotic tumor cells to trigger the immune response was found to depend on the apoptosis inducer, leading to the identification of two morphologically indistinguishable subcategories of apoptosis—namely, immunogenic versus nonimmunogenic apoptosis[19,20]. Here we identified one particular alteration in the plasma membrane of dying cells, the surface exposure of CRT, as an event that occurs only in immunogenic cancer cell death. Exogenous CRT or an external supply of signals that induce CRT exposure confers immunogenicity to otherwise nonimmunogenic cell death, allowing for an optimal anticancer chemotherapy.

RESULTS

CRT exposure defines immunogenic cell death

By double staining with the vital dye 4,6-diamidino-2-phenylindole (DAPI) and the phosphatidylserine-binding dye annexin V, we identified ~20 distinct inducers of apoptosis (all of which induced ~70 ± 10% cell death; **Fig. 1a**). We exposed dying CT26 mouse colon cancer cells to these inducers and then injected the cells into one flank of immunocompetent BALB/c mice. The mice were then rechallenged with live tumor cells injected into the opposite flank 8 d later. Protection against tumor growth was interpreted as a sign of antitumor vaccination (**Fig. 1b**), because such protection was not observed in

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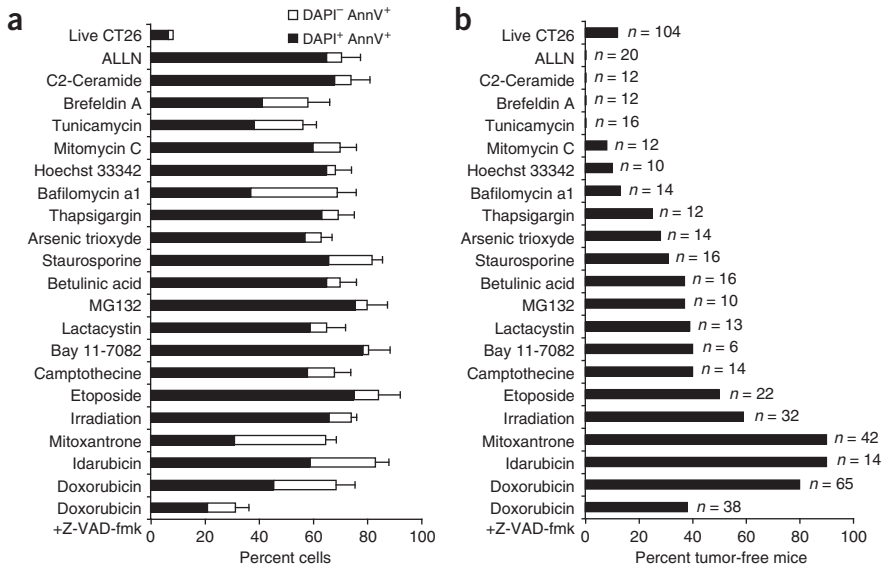

Figure 1 Immunogenic cell death induced by anthracyclins. (**a**) Percentage of dead and dying cells after treatment with distinct chemotherapeutic agents. CT26 cells were cultured in the presence of the indicated agents for 24–48 h and then stained with annexin V–FITC and the vital dye 4,6-diamidino-2-phenylindole (DAPI). (**b**) Identification of inducers of immunogenic cell death. CT26 cells cultured as described in **a** were injected into the left flank; this was followed by injection of live tumor cells into the right flank 8 d later. The percentage of tumor-free mice was determined 120 d later (*n* represents the absolute number of mice used in all experiments).

athymic (*nu/nu*) BALB/c mice (ref. 19 and data not shown). Most cytotoxic compounds, including agents that target the endoplasmic reticulum (ER) (such as thapsigargin, tunicamycin and brefeldin), mitochondria (arsenite, betulinic acid and C2 ceramide) or DNA (Hoechst 33342, camptothecin, etoposide and mitomycin C) did not induce immunogenic cell death, whereas anthracyclins (doxorubicin, idarubicin and mitoxantrone) did (**Fig. 1b** and **Supplementary Fig. 1** online). We found no correlation between immunogenicity and the ratio of *bona fide* apoptotic (annexin V^+DAPI^-) versus necrotic (annexin V^+DAPI^+) cells (**Fig. 1a**,**b**).

To identify changes in the plasma membrane proteome, we affinity-purified biotinylated surface proteins from cells that were either untreated or treated for 4 h with doxorubicin alone or doxorubicin

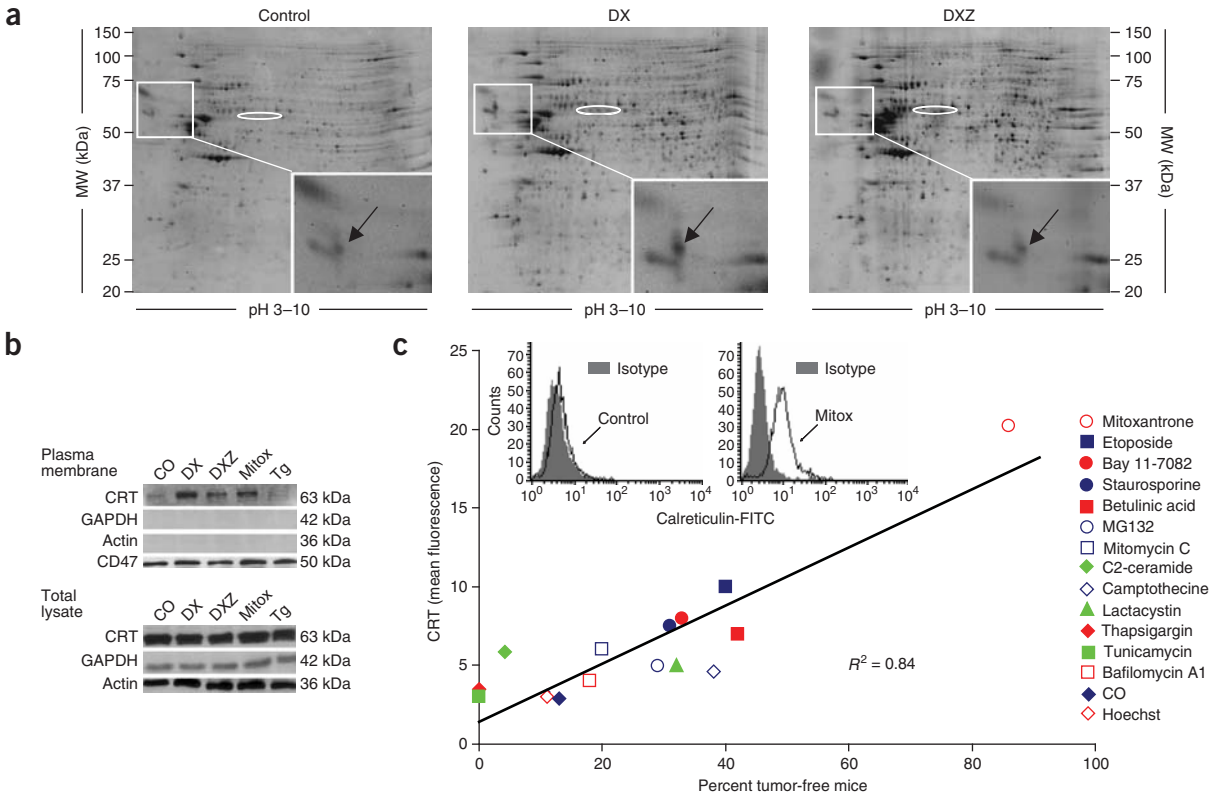

Figure 2 Surface exposure of CRT in immunogenic cell death. (**a**,**b**) Identification of CRT as a protein that is exposed on the cell surface upon treatment with anthracyclins. Cells were treated for 4 h with doxorubicin (DX), alone or in combination with Z-VAD-fmk (DXZ). This was followed by purification of the biotinylated plasma membrane proteins and either 2D gel electrophoresis (**a**) or immunoblot detection of CRT in the plasma membrane protein fraction or the total cell lysate (**b**). In **a**, circles indicate the position of ERP57, insets show gels at higher magnification, and arrows indicate one DX-induced spot (identified as CRT). (**c**) Correlation between CRT exposure and immunogenicity. The surface exposure of CRT was determined by immunofluorescence cytometry among viable (propidium iodine–negative) cells (exemplified in insets for untreated control or mitoxanthrone-treated cells stained with an isotype control or an anti-CRT antibody) 24 h after treatment and was correlated with the immunogenicity of cell death (as determined in **Fig. 1**). Note that idarubicin and doxorubicin (not included in **c**) emit strong autofluorescence, rendering FACS determinations of CRT exposure inaccurate. CO, control; Mitox, mitoxantrone.

and Z-VAD-fmk, a pan-caspase inhibitor that reduces the immunogenicity of doxorubicin-elicited cell death (ref. 19 and **Fig. 1b**). Comparison of two-dimensional (2D) electrophoreses (**Fig. 2a**), followed by mass spectroscopic analyses, led to the identification of CRT (**Supplementary Fig. 2** online) as a protein that was strongly (by a factor of 6) induced by doxorubicin, but less so (by a factor of 1.8) by doxorubicin combined with Z-VAD-fmk. Another protein whose surface exposure was specifically induced by doxorubicin was identified as ERP57 (**Fig. 2a**), a CRT-interacting chaperone[21]. Immunoblot analyses of 2D gels (data not shown) and conventional electrophoreses of purified plasma membrane surface proteins (**Fig. 2b**) confirmed the surface exposure of CRT after treatment with anthracyclins. This CRT surface exposure was also detectable by immunofluorescence staining of anthracyclin-treated live cells (**Supplementary Fig. 2**) and was not accompanied by a general increase in the abundance of intracellular CRT (**Fig. 2b**). The induction of CRT exposure by anthracyclins was a rapid process, detectable as soon as 1 h after treatment, and hence preceded the apoptosis-associated phosphatidylserine exposure (**Supplementary Fig. 3** online). CRT exposure did not correlate with alterations in CD47 expression (**Fig. 2b**). Of note, there was a strong, positive linear correlation ($P < 0.001$) between the appearance of CRT at the cell surface (measured at 24 h among viable cells) and the immunogenicity elicited by the 20 distinct apoptosis inducers (**Fig. 2c**). Although additional parameters may determine the immunological characteristics of cell death (note the discrepancy between low CRT exposure and low versus medium immunogenicity for mitomycin C and etoposide, respectively, in **Fig. 2c**), these data suggest that CRT exposure is a major determinant of immunogenicity.

CRT on dying tumor cells is required for phagocytosis by DC

In view of the established role of CRT as an 'eat me' signal[14,22], we decided to further investigate the possible implication of CRT in the phagocytosis of anthracyclin-treated tumor cells by DC, a cell type that is stringently required for mounting an immune response against apoptotic tumor cells[8,19,20]. Anthracyclin-treated tumor cells were phagocytosed by DC quickly, well before the manifestation of apoptotic changes and within a few hours after treatment with doxorubicin or mitoxantrone (**Fig. 3a**). This correlated with the rapid induction of CRT (**Fig. 3b**, **Supplementary Fig. 3**) and the acquisition of immunogenicity (**Supplementary Fig. 4** online). The presence of CRT on the surface of tumor cells treated with the distinct cell-death inducers strongly correlated with their DC-mediated phagocytosis, suggesting that CRT is important in mediating the uptake of tumor cells by DC (**Fig. 3b**).

Accordingly, blockade of the CRT present on the surface of mitoxantrone-treated cancer cells by means of a specific antibody

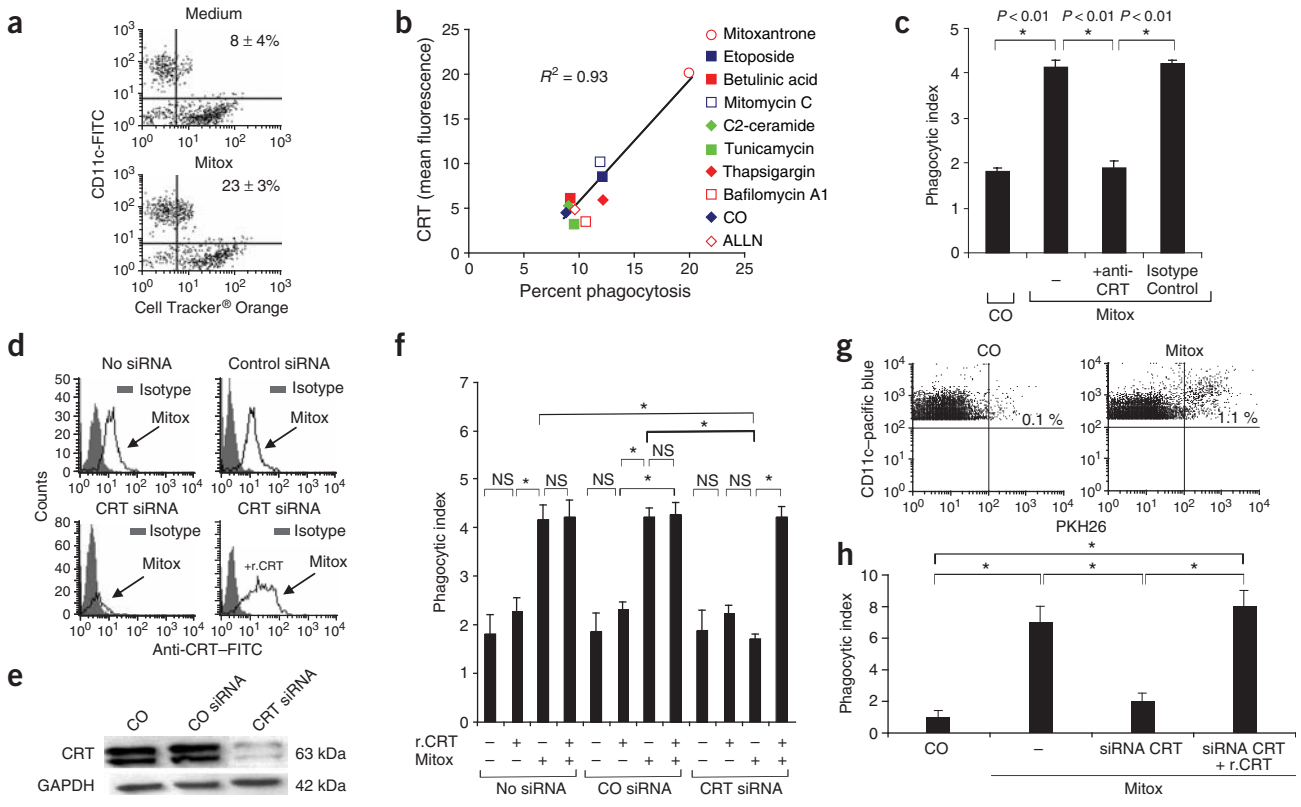

Figure 3 Requirement of surface CRT for phagocytosis of tumor cells by DC. (**a**,**b**) Correlation between tumor cell phagocytosis and CRT exposure. Tumor cells were treated for 4 h with different cell-death inducers, then labeled with Cell Tracker Orange and cultured with CD11c-expressing DC. The percentage of DC taking up tumor cells was determined (**a**) and correlated with CRT surface exposure (**b**), measured as in **Figure 2e**. (**c**) Blockade of CRT inhibits DC-mediated phagocytosis. Mitoxantrone-treated or control cells were incubated with a blocking chicken antibody to CRT. This was followed by detection of phagocytosis by DC. (**d**–**f**) Knockdown of CRT inhibits DC-mediated phagocytosis, and rCRT restores phagocytosis *in vitro*. Cells were transfected with the indicated siRNAs and optionally treated with rCRT. This was followed by detection of surface CRT (**d**), immunoblotting (**e**) and measurement of phagocytosis by DC (**f**). (**g**,**h**) Surface CRT determines the phagocytosis of tumor cells by DC *in vivo*. Representative pictograms are shown in **g**. The phagocytotic index is the ratio between values obtained with control cells and those obtained after mitoxantrone treatment (with or without siRNA CRT, and with or without rCRT). Results are triplicates from one experiment (mean ± s.d.) and representative of three independent experiments. *$P < 0.001$ (Student's *t*-test).

of avian origin (which cannot interact with mouse Fc receptors) inhibited their phagocytosis by DC (**Fig. 3c**). Similarly, knockdown of CRT with a specific siRNA (**Fig. 3d**,**e**) suppressed the phagocytosis of anthracyclin-treated tumor cells by DC, both *in vitro* (**Fig. 3f**) and *in vivo* (**Fig. 3g**,**h**). Addition of recombinant CRT protein (rCRT), which binds to the surface of the tumor cells, reversed the defect induced by the CRT-specific siRNA, in terms of both CRT surface exposure (**Fig. 3d**) and phagocytosis by DC *in vitro* (**Fig. 3f**) and *in vivo* (**Fig. 3g**,**h**). Of note, rCRT alone could not promote DC maturation *ex vivo* over a large range of concentrations (10 ng/ml to 100 μg/ml, data not shown). Hence, surface CRT specifically elicits phagocytosis by DC.

Requirement of CRT for immunogenicity of dying tumor cells

The knockdown of CRT compromised the immunogenicity of mitoxantrone-treated CT26 cells, and this defect was restored when rCRT was used to complement the CRT defect induced by the CRT-specific siRNA. Additional knockdown of ERP57 did not further reduce the immunogenicity of the cells (data not shown). CRT exposure was found to be critical for immunogenicity in two distinct experimental systems: (i) when CT26 tumor cells were injected into the flank of BALB/c mice (or MCA205 cells were injected into C57Bl/6 mice— data not shown) to assess the efficacy of antitumor vaccination (**Fig. 4a**), and (ii) when the tumor cells were injected into the foot pad to measure interferon-γ production by popliteal lymph node cells (**Fig. 4b**). In the latter system, absorption of rCTR to the plasma membrane surface greatly enhanced the immunogenicity of cells that usually do not induce an immune response, such as mitomycin-treated cells (**Fig. 4c**).

To determine the impact of $CD11c^+$ DC on the CRT-dependent immune response *in vivo*, we injected diphteria toxin into transgenic mice expressing the diphteria toxin receptor specifically in DC (under the control of the CD11c promoter)[23]. Lymph node cells from vehicle-injected control mice readily produced interferon-γ after the injection with CT26 cells treated with mitomycin C and rCTR followed by *in vitro* rechallenge with CT26 lysates. However, lymph node cells from DC-depleted mice did not produce interferon-γ in response to the same stimuli (**Fig. 4d**). Etoposide-treated rCTR-coated cells exhibited a vigorous antitumor immune response *in vivo*, under conditions in which etoposide-treated sham-coated cells were poorly

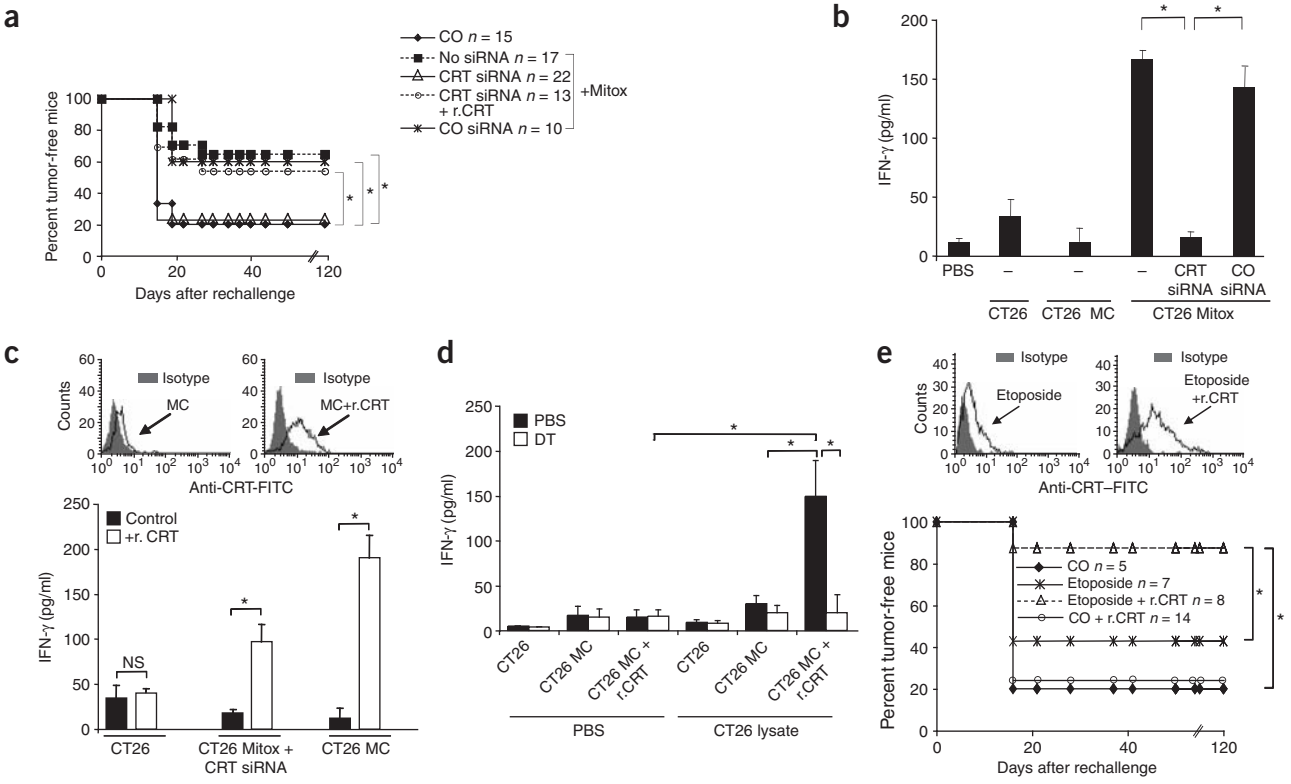

Figure 4 CRT is required for the immune response against dying tumor cells. (**a**) *In vivo* anticancer vaccination depends on CRT. CT26 colon cancer cells were transfected with the indicated siRNAs, then treated with rCRT and/or mitoxantrone (as in **Fig. 3d**). The antitumor response was measured by challenging BALB/c mice simultaneously with mitoxantrone-treated tumor cells in one flank and untreated, live tumor cells in the opposite flank. (**b**) Priming of T-cell responses depends on CRT. CT26 tumor cells were left untransfected or were transfected with the indicated siRNAs, then treated with medium alone, mitomycin C or mitoxantrone, and injected into the right foot pad of BALB/c mice to determine the capacity of draining lymph node cells to produce IFN-γ in response to CT26 lysates. (**c**) Exogenous supply of CRT enhances the immunogenicity of CRT-negative dying cells. CT26 cells lacking CRT expression after depletion of CRT with an siRNA and mitoxantrone treatment or after mitomycin treatment were coated with r.CRT (insets) and then injected into the foot pad, followed by assessment of the IFN-γ secretion by cells from the draining lymph nodes as in **b**. (**d**) DC is required for the CRT-dependent immune response. Transgenic mice specifically expressing the diphteria toxin (DT) receptor in DC were pretreated with PBS alone or a dose of DT that depletes DC. The mice were then challenged with CT26 tumor cells treated with mitomycin C plus rCRT into the food pad and IFN-γ secretion by lymph node cells was measured as in **b**. (**e**) CRT-mediated amelioration of the immune response against etoposide-treated tumor cells. CT26 cells were treated for 24 h with etoposide (or PBS) and rCRT was optionally absorbed to the cell surface (insets), followed by simultaneous injection of live tumor cells into opposite flanks and monitoring of tumor growth (mean ± s.e.m.) $^*P < 0.001$ (Student's *t*-test).

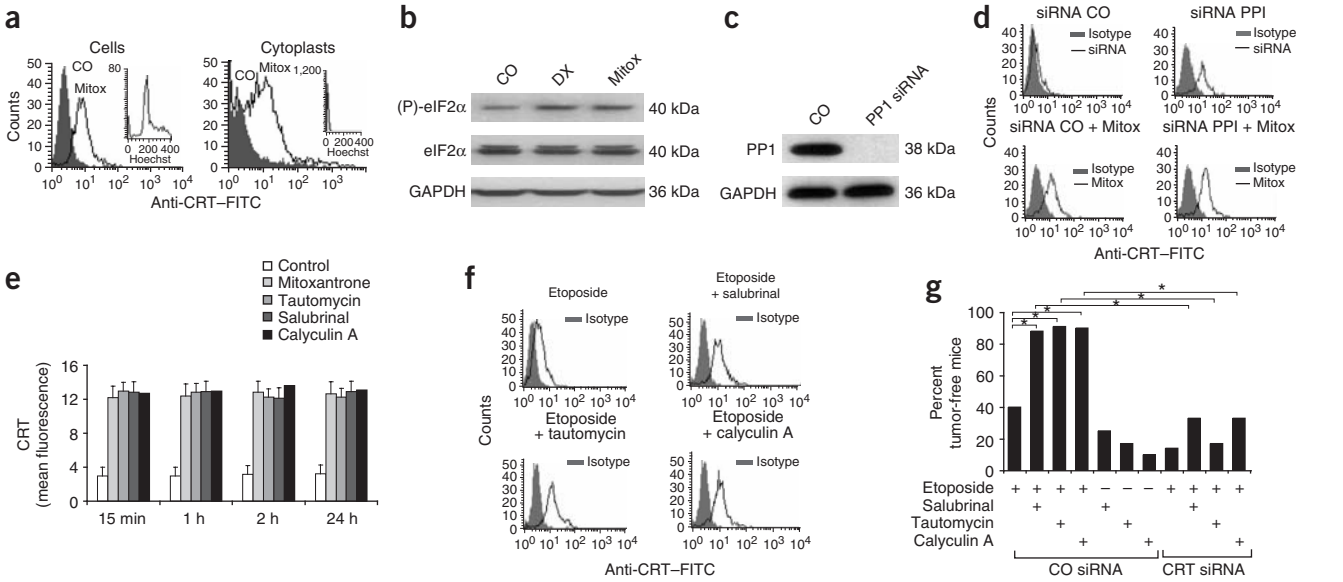

Figure 5 Induction of CRT exposure and immunogenic cell death by inhibition of the PP1/GADD34 complex. (**a**) CRT exposure after anthracyclin treatment in the absence of a nucleus. Intact cells or enucleated cells (cytoplasts) were treated for 2 h with mitoxantrone, followed by immunofluorescence detection of CRT exposure. Insets show the effective removal of Hoechst 33342-stainable nuclei from the cytoplasts. (**b**) Phosphorylation of eIF2α after treatment with anthracyclins. Cells were treated for 4 h with mitoxantrone or doxorubicine, followed by immunoblot detection of phosphorylated eIF2α, eIF2α irrespective of its phosphorylation state and GAPDH as a loading control. (**c**,**d**) Induction of CRT exposure by knockdown of PP1. Cells were transfected with siRNAs specific for PP1 and were treated with mitoxantrone for 2 h before immunoblot (**c**) and cell surface staining (**d**). (**e**) Kinetics of CRT exposure determined by FACS analysis after incubation of cells with the indicated agents. (**f**,**g**) PP1/GADD34 inhibitors render cells immunogenic via CRT. Tumor cells were first transfected with a control siRNA or a CRT-specific siRNA and then treated *in vitro* with etoposide, alone or in combination with PP1/GADD34 inhibitors. Two hours later, the surface CRT was detected (**f**), and later, cells were injected as in **Fig. 1a** to determine their capacity to inhibit the growth of live tumor cells inoculated one week later (**g**). The results represent the % of tumor free mice (comprising a total of 12 to 18 mice per group).

immunogenic (**Fig. 4e**). Coating with rCTR also enhanced the immunogenicity of cells killed with the proteasome inhibitor MG123. However, one single cycle of freeze-thawing (which induces necrosis) destroyed the immunogenicity of etoposide- or mitomycin C–treated, rCRT-coated tumor cells, indicating that the apoptotic nature of cell death is important in determining immunogenicity (**Supplementary Fig. 5** online). Similarly, absorption of rCTR to the cell surface without prior treatment with cell-death inducers did not elicit an anticancer immune response, and live rCTR-pretreated cells injected into mice formed tumors, both in immunocompetent mice (**Fig. 4e**) and in immunodeficient mice (data not shown). Thus, CRT critically determines the immunogenicity of cell death *in vivo* but does not determine cell death *per se*.

Inhibitors of PP1/GADD34 induce CRT exposure and immunogenicity

Because anthracyclin-induced CRT exposure was a rather rapid process (it occurred within 1 h, **Supplementary Fig. 3**), we suspected that anthracyclins might exert effects that are not mediated by genotoxic stress. In response to mitoxantrone, enucleated cells (cytoplasts) exposed CRT readily (within 1 h) (**Fig. 5a**) and were phagocytosed by DCs (data not shown) as efficiently as intact cells (**Fig. 3a**), indicating the existence of a cytoplasmic (non-nuclear) anthracyclin target. Anthracyclins did not induce immediate mitochondrial stress (data not shown), yet caused the rapid phosphorylation of eIF2α (**Fig. 5b**), a protein that is typically hyperphosphorylated in ER stress due to the activation of stress kinases[24]. Knockdown of either GADD34 (data not shown) or the catalytic subunit of protein phosphatase 1 (PP1) (**Fig. 5c**), which together form the PP1/GADD34 complex involved in the dephosphorylation of eIF2α, was sufficient to induce CRT exposure (**Fig. 5d**). The CRT exposure triggered by PP1 or GADD34 depletion was not further enhanced by mitoxantrone (**Fig. 5d**), suggesting that PP1/GADD34 and anthracyclins act on the same pathway to elicit CRT translocation to the cell surface. CRT exposure was efficiently induced by the chemical PP1/GADD34 inhibitors tautomycin, calyculin A (both inhibit the catalytic subunit of PP1)[25] and salubrinal (which inhibits the PP1/GADD34 complex)[26] (**Fig. 5e**). All these PP1/GADD34 inhibitors induced CRT exposure with rapid kinetics, similar to the anthracyclins, both in cells (**Fig. 5e**) and in cytoplasts (data not shown).

Mitoxantrone and salubrinal induced CRT exposure in several tumor cell lines of mouse (MCA205, B16F10 and J558) and human (LNCAP, A549 and HCT116) origin. CRT exposure induced by anthracyclins and PP1/GADD34 inhibitors was not affected by inhibitors of transcription, translation or microtubule polymerization (data not shown), yet was abolished by latrunculin A, an inhibitor of the actin cytoskeleton and exocytosis (**Supplementary Fig. 6** online). Inhibition of the PP1/GADD34 complex with salubrinal, calyculin A or tautomycin was not sufficient to induce immunogenic cell death (**Fig. 5f**,**g**); moreover, these treated cells, which did not die, formed lethal tumors when injected into mice. However, these inhibitors greatly enhanced CRT exposure (**Fig. 5f**) and the immunogenic potential of cells succumbing to etoposide (**Fig. 5g**) or mitomycin C (data not shown), and this immunostimulatory effect was abrogated by knocking down CRT (**Fig. 5g**). Altogether, these results demonstrate that PP1/GADD34 inhibition induces CRT exposure, which in turn can stimulate the antitumor immune response.

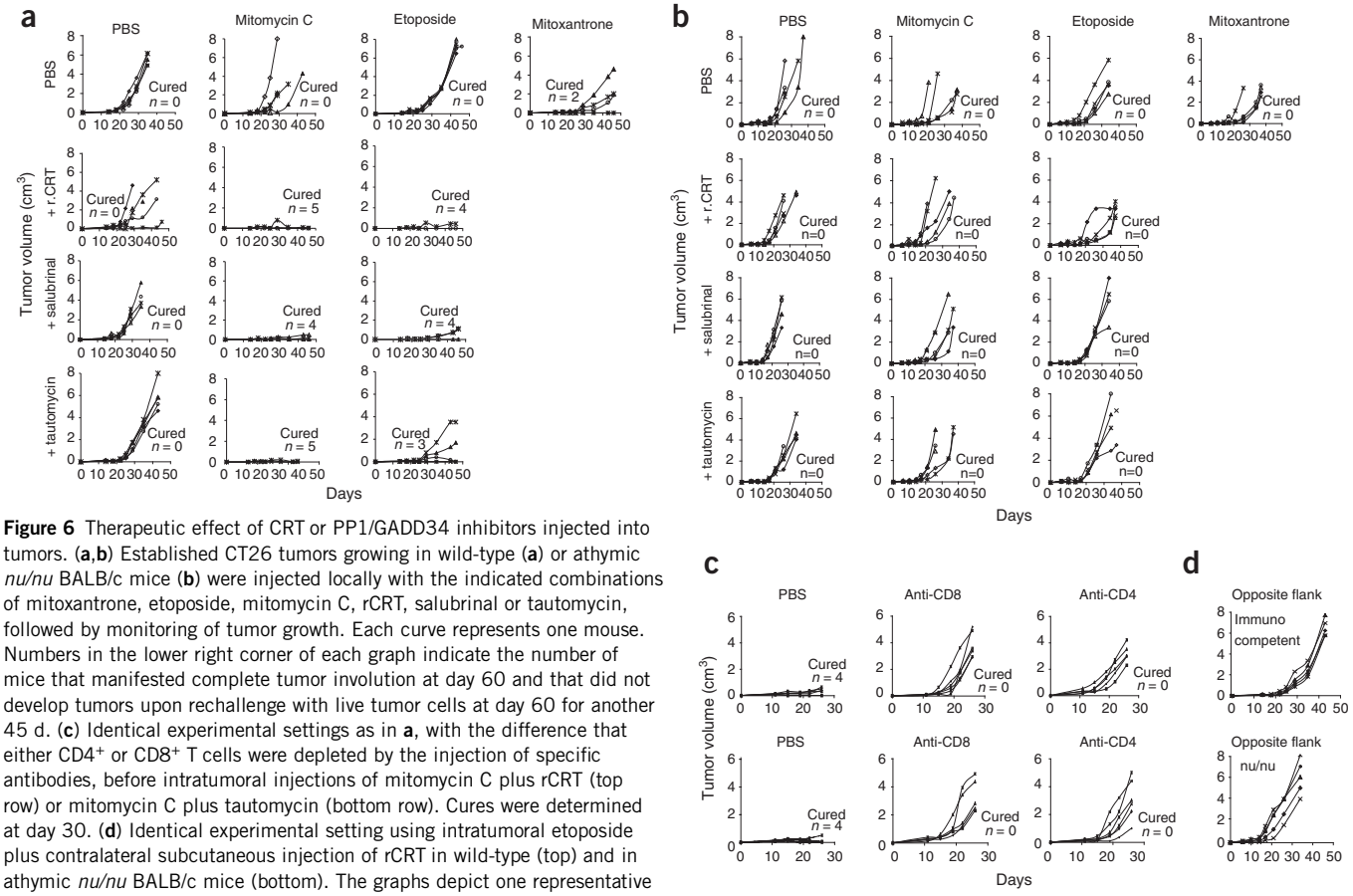

Figure 6 Therapeutic effect of CRT or PP1/GADD34 inhibitors injected into tumors. (**a**,**b**) Established CT26 tumors growing in wild-type (**a**) or athymic *nu/nu* BALB/c mice (**b**) were injected locally with the indicated combinations of mitoxantrone, etoposide, mitomycin C, rCRT, salubrinal or tautomycin, followed by monitoring of tumor growth. Each curve represents one mouse. Numbers in the lower right corner of each graph indicate the number of mice that manifested complete tumor involution at day 60 and that did not develop tumors upon rechallenge with live tumor cells at day 60 for another 45 d. (**c**) Identical experimental settings as in **a**, with the difference that either $CD4^+$ or $CD8^+$ T cells were depleted by the injection of specific antibodies, before intratumoral injections of mitomycin C plus rCRT (top row) or mitomycin C plus tautomycin (bottom row). Cures were determined at day 30. (**d**) Identical experimental setting using intratumoral etoposide plus contralateral subcutaneous injection of rCRT in wild-type (top) and in athymic *nu/nu* BALB/c mice (bottom). The graphs depict one representative experiment out of two, comprising five mice per group.

Injection of rCRT or PP1/GADD34 inhibitors for cancer therapy

A single intratumoral injection of mitoxantrone into established 14-d-old CT26 tumors was able to cause their permanent regression in some, but not all, cases, if the tumors were established in immunocompetent BALB/c mice (**Fig. 6a**). However, mitoxantrone did not result in regression if the tumors were carried by immunodeficient *nu/nu* mice (**Fig. 6b**). The intratumoral injection of rCTR, salubrinal, tautomycin, etoposide or mitomycin C had no major therapeutic effect, in either immuncompetent or *nu/nu* mice. However, the combined intratumoral injection of a cell-death inducer (etoposide or mitomycin C) plus rCRT was able to cause tumor regression in immunocompetent (but not immunodeficient *nu/nu*) mice.

The depletion of either $CD4^+$ or $CD8^+$ T cells abrogated the antitumoral effects of intratumoral injections of mitomycin C plus rCRT (**Fig. 6c**). To obtain a therapeutic effect, rCRT had to be injected into the tumor, as rCRT injected into a distant site did not ameliorate the antitumoral effects of intratumorally injected etoposide (**Fig. 6d**). We found that the combination of etoposide or mitomycin C with drugs that induce CRT exposure (salubrinal or tautomycin) led to stable disease or complete tumor regression in immunocompetent (but not immunodeficient) hosts (**Fig. 6a–c**). Live CT26 cells did not grow in mice that had been cured of CT26 tumors, indicating the establishment of a permanent antitumor immune response. Similar results were obtained when established MCA205 sarcomas (in C57Bl/6 mice) were treated by local injections of weakly immunogenic cell-death inducers plus rCRT or PP1/GADD34 inhibitors (data not shown). These results delineate a strategy of immunogenic chemotherapy for the cure of established cancer.

DISCUSSION

It has been a mystery as to which particular biochemical changes determine the distinction between immunogenic and nonimmunogenic cell death. Here we provide evidence that CRT is exposed on cells that succumb to immunogenic cell death but is lacking on the surface of cells that undergo nonimmunogenic cell death (**Figs. 1**,**2**). CRT exposure is known to be induced by ultraviolet-C (UVC) light[14]. Here we report that CRT exposure is triggered by anthracyclins (**Fig. 2**) and PP1/GADD34 inhibitors (**Fig. 5**), by a process that involves the translocation of intracellular CRT to the cell surface. This translocation occurs by means of a molecular mechanism that is not fully understood and that likewise involves the presence of saturable CRT receptors[15] on the cell surface that can bind exogenous CRT as well as endogenous, preformed CRT. Accordingly, inhibition of mRNA synthesis (by addition of actinomycin D or by enucleation) and inhibition of protein synthesis did not inhibit CRT exposure (**Fig. 5a** and **Supplementary Fig. 6**). We were able to dissociate CRT exposure, as induced by anthracyclins or PP1/GADD34 inhibitors, from phosphatidylserine exposure, which occurred much later after anthracyclin treatment (**Supplementary Fig. 3**) and not at all after transient PP1/GADD34 inhibition (data not shown). Thus, CRT exposure occurs upstream of apoptosis or necrosis, as part of a specific danger-signaling system. Doxorubicin-induced CRT exposure was partially reduced by caspase inhibition (**Fig. 2**), a regimen that also reduced the immunogenicity of cell death (**Fig. 1**). However, CRT exposure did not involve caspase cleavage, which would reduce the molecular mass of the protein (**Fig. 2b**). Hence, the involvement of caspases in CRT exposure must be indirect, and it remains to be

elucidated in mechanistic terms. CRT is one of the most abundant proteins in the ER (refs. 27,28), implying that plasma membrane translocation of only a fraction of CRT would be sufficient to cause detectable CRT exposure. However, ER stress does not elicit the appearance of CRT on the cell surface (**Fig. 2c**) and cell death provoked by ER stress is particularly nonimmunogenic (**Fig. 1b**). Rather, inhibitors of ER stress such as salubrinal (which targets PP1/GADD34 and protects cells from lethal ER stress[26]) stimulate CRT exposure (**Fig. 5**), and anthracyclins that induce CRT exposure reportedly attenuate the ER stress response[29].

CRT has adjuvant properties, meaning that chimeric constructs encoding CRT and tumor antigens delivered with a viral vector[30] or a gene gun[31] are more immunogenic than the tumor antigen alone. CRT is also a chaperone capable of binding unglycosylated peptides. Thus CRT purified from tumors can elicit tumor-specific immunity, a finding that has been attributed to the peptides associated with CRT (ref. 32). However, this property of CRT has been linked to necrosis and not to apoptosis, because CRT is usually released only from necrotic (not apoptotic) tumor cells[33]. Moreover, as shown here (**Supplementary Fig. 5**), exogenous rCRT protein enhances the immunogenicity of cells killed in conditions in which the proteasome (and hence the production of antigenic peptides within the apoptotic cells) is inhibited[20], suggesting that complexes formed by CRT and tumor-specific antigenic peptides cannot be solely responsible for the adjuvant effect of CRT. The data presented here suggest that exposure of CRT on the cell surface is a major factor in determining the antitumor immune response.

The inhibition of CRT exposure by blocking antibodies or by knocking down the CRT transcript abolished the phagocytosis of tumor cells by DC (**Fig. 3**) and abrogated the immunogenicity of anthracyclin-induced cell death (**Fig. 4**). In contrast, external supply of CRT or administration of PP1/GADD34 inhibitors (which enforce CRT exposure) converted nonimmunogenic cell death into an immunogenic event (**Figs. 5**,**6**), correlating with enhanced phagocytosis by DC (**Fig. 3**). Although rCRT adsorbed to the surface of live cells did enhance their phagocytosis by DC *in vitro* (**Fig. 3f**) and *in vivo* (**Fig. 3h**), CRT had to be combined with a cell-death inducer to elicit a local (**Fig. 4c**) or systemic immune response *in vivo* (**Fig. 4e**, **Fig. 6**). Thus, whereas the presence or absence of CRT on the cell surface determines the difference between immunogenic and nonimmunogenic cell death, CRT alone, without a genotoxic agent, is not sufficient to elicit antitumor immunity. Accordingly, CRT alone as a soluble protein or when absorbed to live cells did not promote DC maturation or activation (data not shown), which must rely on additional signals emanating from the dying tumor cells.

In conclusion, a nonimmunogenic chemotherapeutic agent became immunogenic when combined with rCRT or PP1/GADD34 inhibitors. The challenge for future investigation is to apply this strategy to the treatment of human cancer and to identify the pattern recognition receptors of the innate immune system that promote the antitumor immune response.

METHODS

Cell lines and induction of cell death. CT26 cells were cultured at 37 °C under 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, 1 mM pyruvate and 10 mM HEPES in the presence of doxorubicin (DX; 24 h, 25 μM, Sigma), mitoxantrone (Mitox; 24 h, 1 μM, Sigma), idarubicin (24 h, 1 μM, Aventis), mitomycin C (48 h, 30 μM, Sanofi-Synthelabo), zVAD-fmk (24 h, 50 μM, Bachem), tunicamycin (24 h, 65 μM), thapsigargin (24 h, 30 μM), brefeldin A (24 h, 50 μM), etoposide (48 h, 25 μM), MG132 (48 h, 10 μM), ALLN (48 h, 45 μM), betulinic acid (24 h, 10 μM), Hoechst 33342 (24 h, 0,2 μM), camptothecine (24 h, 15 μM), lactacystin (48 h, 60 μM), BAY 11-8072 (24 h, 30 μM), staurosporine (24 h, 1.5 μM), bafilomycin A1(48 h, 300 nM), arsenic trioxide (24 h, 30 μM), C2-ceramide (C2-C; 24 h, 60 μM), calyculin A (48 h, 30 nM) or tautomycin (48 h, 150 nM, Sigma) and/or salubrinal (48 h, 20 μM, Calbiochem). Cells were analyzed on a FACS Vantage after staining with DAPI (10 min, 2.5 μM, Molecular Probes) for determination of cell viability, and annexin V conjugated with fluorescein isothiocyanate (Bender Medsystems) for the assessment of phosphatidylserine exposure[34].

siRNAs and manipulation of surface CRT. siRNA heteroduplexes specific for CRT (sense strand: 5′-rCrCrGrCUrGrGrGUrCrGrArAUrCrRrArATT-3′), GADD34 (5′-rCrArGrGrArGrCrArGrAUrCrArGrAUrArGrATT-3′), PPICα (5′-rGrCUrGrGrCrCUrAUrArArGrAUrCrArGrATT-3′) or an unrelated control (5′rGrCrCrGrGUrAUrGrCrCrGrGUUrArArGUTT-3′) were designed in our laboratory and synthesized by Sigma-Proligo. CT26 cells were transfected by siRNAs at a final concentration of 100 nM using HiPerFect (Qiagen). Thirty-six hours after transfection, CT26 cells were assessed for total CRT content by immunoblotting. To restore CRT expression, cells were exposed to rCRT, produced in insect cells[35], at 3 μg/10^6 cells in PBS on ice for 30 min, followed by three washes.

Antitumor vaccination and treatment of established tumors. All mice were maintained in specific pathogen-free conditions and all experiments followed the guidelines of the Federation of European Animal Science Associations. All animal experiments were approved by the Ethical Committee of Institut Gustave Roussy (IGR). 3×10^6 treated CT26 cells were inoculated subcutaneously (s.c.) in 200 ml of PBS into the lower flank of BALB/c six-week-old female mice (Charles River), and 5×10^5 untreated control cells were inoculated into the opposite flank. For the tumorigenicity assay, 3×10^6 treated or untreated CT26 cells were injected s.c. into *nu/nu* mice (IGR Animal Facility). To assess the specificity of the immune response against CT26, we injected either 5×10^5 or 5×10^6 CT26 cells (for the mice immunized in a standard protocol or a vaccination protocol, respectively). Size of tumors was evaluated weekly, using calipers. In a series of experiments, BALB/c (wild-type or *nu/nu*) mice carrying palpable CT26 tumors (implanted 14 d before treatment for wild-type mice or 7 d before treatment for *nu/nu* mice by injection of 10^6 tumor cells) received a single intratumoral injection of 100 μM PBS containing the same concentration of anticancer agents and PP1/GADD34 inhibitors as those used *in vitro*, as well as rec.CTR (15 μg). None of these treatments caused macroscopic necrosis. In some mice, $CD4^+$ and $CD8^+$ cells were depleted by intraperitoneal (i.p.) injection of the purified monoclonal antibodies YTS191 (100 μg) and H35.17.2 (250 μg) (from ATCC), respectively, 3 d before and 7 and 14 d after intratumoral injections of anticancer agents.

Assessment of local immune responses. 5×10^5 CT26 cells were injected into the footpad of Balb/c mice. Five days later, popliteal lymph node cells were recovered by homogenizing and filtering the organ through a sterile cell strainer (70 μm; Becton Dickinson). 1×10^5 lymph node cells were cultured in complete culture medium in the presence or absence of CT26 cell lysates killed by a freeze-thaw cycle in 200 μl medium in round-bottom 96-well plates. Three days later, the supernatants were harvested and IFN-γ secretion was determined by ELISA (BD Biosciences). In one series of experiments, CD11c-GFP DT K^b mice[23] were injected i.p. with 100 ng diphteric toxin (or PBS as a vehicle control) on the same day as tumor cells were injected into the food pad.

Statistical analyses. Data are presented as mean ± s.d., or as percentages. All statistical analyses were performed using JMP software (SAS Institute). Student's *t*-test was used to compare continuous variables (comparison of tumor growth), and the χ^2 test for nonparametrical variables (comparison of animal cohorts). For all tests, the statistical significance level was set at $P < 0.05$.

Supplementary methods. The immunodetection of CRT, purification of plasma membrane proteins, mass spectroscopy, generation of bone marrow–derived DC and cytoplasts and phagocytosis assays are detailed in the **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.O., A.T., F.G., G.M.F., L.A., J.-L. P., M.C., T.P., D.M., N.L. and F.C. performed the *in vivo* and *in vitro* experiments. G.M.F. performed mass spectroscopy. N.C. and P.v.E. provided essential reagents. M.P. conducted data analysis. L.Z. and G.K. conceived the study and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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8.2 Annexe 2 : Implication de TLR-4 dans l'efficacité de la chimio- et radiothérapie anticancéreuse

8.2.1 Résumé

La présence de CRT à la surface de cellules tumorales vivantes ne leur permet pas d'être immunogènes. Nous avons donc supposé qu'un autre signal, propre à la mort cellulaire, servait de signal de danger. Les principaux détecteurs de signaux de danger sont les TLRs. La première étape du travail a donc été de tester un à un des modèles de souris invalidées pour un de leur TLR, dans le cadre d'une vaccination avec cellule tumorale mourante. Il fut donc établi que la présence de TLR4 sur les DC de l'hôte était indispensable à l'immunogénicité des corps apoptotiques, le mécanisme étant que les corps apoptotiques relâchent un facteur soluble, HMGB1 (qui est un ligand de TLR-4), qui permet de ralentir la maturation des phagosomes, et donc de diminuer la protéolyse des antigènes, augmentant le rendement de la présentation croisée.

Il fut prouvé que la présence des acteurs HMGB1/TLR4 et MYD88 était indispensable à la présentation croisée d'antigènes membranaires tumoraux. Il convient de noter que les hôtes TLR-4$^{-/-}$ peuvent toujours présenter en croix les antigènes solubles ; de même la maturation des cellules dendritiques induite par les corps apoptotiques ne dépend pas de TLR-4. Ainsi, il est possible de trouver une différence significative entre les croissances chez des hôtes immunocompétent ou imunodéficients (nudes ou TLR4$^{-/-}$) de tumeurs traitées selon les cas par les rayonnements ionisants, les anthracyclines et un sel de platine.

Une étude clinique rétrospective nous a permis de mettre en évidence un lien entre une mutation de TLR-4 et le temps à la rechute de patientes traitées pré-exérèse par anthracyclines pour un cancer du sein : les patientes porteuses d'une mutation TLR-4 ASP299GLY, qui ont une protéine TLR-4 légèrement déficiente pour la fixation de ses ligands, rechutent plus vite (Apetoh, Ghiringhelli et al. 2007).

8.2.2 Discussion

Cette étude (Apetoh, Ghiringhelli et al. 2007) est la première à montrer de manière claire et large la contribution du système immunitaire à l'efficacité de plusieurs stratégies antitumorales couramment utilisées : les rayonnements ionisants, les anthracyclines et un sel

de platine. Dans différents modèles, il a été constaté une nette différence entre les souris immunocompétentes et nudes, dépourvues de lymphocytes T, et le lien entre la mort des cellules tumorales et l'immunité adaptative est fait par le récepteur TLR-4 et la protéine HMGB1.

Cette étude permet donc d'affirmer qu'un signal de danger endogène intervient dans l'efficacité de certaines chimiothérapies. Le fait qu'une étude rétrospective portant sur des patientes traitées par anthracyclines avant exérèse tumorale montre un désavantage pour celles qui portent une mutation diminuant l'activité de TLR-4 indique que la situation chez les humains semble comparable à ce qui a été observé chez les souris.

De plus, la notion selon laquelle le ralentissement de la dégradation des antigènes captés au sein des endosomes de DC favorise la présentation croisée amène une nouvelle option thérapeutique envisageable : la chloroquine, un médicament dont les effets secondaires sont bien connus, permet de rétablir la présentation croisée dans des DC déficientes en TLR4. Il est séduisant d'envisager de rétablir de cette manière une présentation croisée efficace chez des patients porteurs d'une mutation invalidant le produit du gène TLR-4.

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Toll-like receptor 4–dependent contribution of the immune system to anticancer chemotherapy and radiotherapy

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Conventional cancer treatments rely on radiotherapy and chemotherapy. Such treatments supposedly mediate their effects via the direct elimination of tumor cells. Here we show that the success of some protocols for anticancer therapy depends on innate and adaptive antitumor immune responses. We describe in both mice and humans a previously unrecognized pathway for the activation of tumor antigen–specific T-cell immunity that involves secretion of the high-mobility-group box 1 (HMGB1) alarmin protein by dying tumor cells and the action of HMGB1 on Toll-like receptor 4 (TLR4) expressed by dendritic cells (DCs). During chemotherapy or radiotherapy, DCs require signaling through TLR4 and its adaptor MyD88 for efficient processing and cross-presentation of antigen from dying tumor cells. Patients with breast cancer who carry a *TLR4* loss-of-function allele relapse more quickly after radiotherapy and chemotherapy than those carrying the normal *TLR4* allele. These results delineate a clinically relevant immunoadjuvant pathway triggered by tumor cell death.

Apoptosis has been believed to be a silent cell death modality that does not trigger innate or adaptive immune responses[1,2]. Nonetheless, programmed cell death contributes to the onset of an adaptive immune response either directly[2,3] or indirectly, during bacterial and viral infection[4,5]. It is generally assumed that cell death can elicit an immune response only if dying cells emit 'eat me' and 'danger' signals that mediate their efficient phagocytosis by DCs and the maturation of DCs, respectively. Depending on the cell death inducer, some types of tumor cell death can also induce an antitumor immune response, and this property can be exploited to break tumor-induced immune tolerance[6]. Thus, tumor cell death induced by anthracyclines or X-rays can promote a DC-mediated cytotoxic T-lymphocyte (CTL) response that confers permanent antitumor immunity[6]. The obligate immunogenic 'eat me' signal generated by dying tumor cells consists in the exposure of calreticulin on the cell surface[7]. This particular 'eat me' signal is found only on the surface of cells that succumb to immunogenic death and not on that of cells dying in an immunologically silent fashion, indicating that it constitutes the first checkpoint for the immunoadjuvant effect of tumor cell death[8]. However, calreticulin exposure is not sufficient to elicit an antitumor immune response, because live cells that express ecto-calreticulin are unable to induce DC maturation and antigen presentation and hence are non-immunogenic[8]. This suggests that tumor cells must emit one or several additional signals linked to cell death in order to trigger an efficient immune response.

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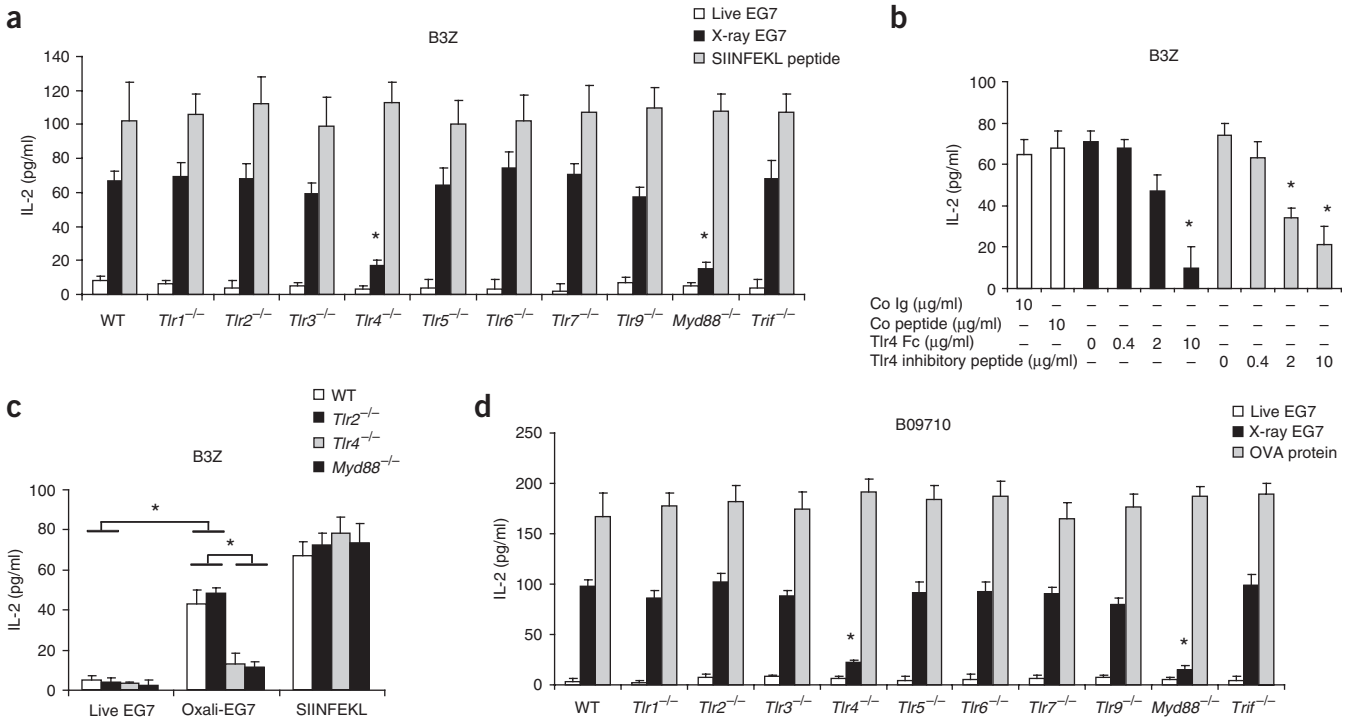

Figure 1 TLR4 controls antigen presentation by DCs engulfing apoptotic bodies *in vitro*. (**a**) TLR4 and MyD88 are required for antigen presentation by DCs loaded with irradiated tumor cells to MHC class I (H-2^b)-restricted T cells. Shown are IL-2 levels in the supernatants from DCs of the indicated genotype (background C57BL/6, H-2^b I-A^b) after they were pulsed with either SIINFEKL peptide or live or irradiated EG7 cells (ratio of EG7 to DCs 1:1) and incubated with the SIINFEKL-specific B3Z hybridoma cells for 48 h. (**b**) Inhibition of TLR4 abrogates antigen presentation. WT DCs were loaded with decreasing amounts of recombinant TLR4-Fc fusion protein (or a control immunoglobulin (Co Ig)) or a TLR4-inhibitory peptide (or a control peptide (Co peptide)), along with irradiated EG7 cells (as in **a**), and assayed for their capacity to elicit IL-2 production by B3Z cells. (**c**) Same setting as in **a**, using live or oxaliplatin-treated tumor cells. (**d**) TLR4 and MyD88 are required for class II-restricted antigen presentation by DC. Same setting as in **a**, using MHC class-II (I-A^b) restricted hybridoma (B09710). Results (means of triplicates ± s.e.m.) are representative of five experiments, and asterisks indicate significant inhibitory effects of TLR4 inhibition or ablation. $^*P < 0.05$.

Toll-like receptors (TLRs) recognize molecules derived from pathogens as well as endogenous danger signals possessing similar chemical structures[9,10]. Upon recognition of their ligands, TLRs transduce signals through two pathways involving distinct adaptors, Toll/IL-1R domain–containing adaptor inducing IFNα (TRIF) and myeloid differentiation primary response protein (MyD88), which is used by all TLRs except TLR3. During microbial infections in which cross-presentation of exogenous antigens is a prerequisite for T-cell activation[11], TLRs present on the surface of DCs or macrophages are triggered by alien components and mediate the activation of antigen-presenting cells (APCs)[5,9]. In addition, TLRs present on internal membranes may control the processing and presentation of peptides derived from internalized cargo[12–14].

While searching for the TLRs[15] that might be involved in the immune response against dying tumor cells, we found that TLR4 expression by DCs is a prerequisite for efficient antigen presentation of tumor antigens furnished by dying cancer cells. This observation led us to the discovery of the 'danger' signal emitted by dying tumor cells: the release of the HMGB1 protein. We demonstrate that both the release of HMGB1 by dying tumor cells and the TLR4–myeloid differentiation primary response protein-88 (MyD88) signaling pathway are required for the immune response against dying tumor cells and also for the efficacy of anticancer chemotherapy and radiotherapy in mice. The clinical relevance of these findings is underscored by the observation that the Asp299Gly *TLR4* mutation, which affects the binding of HMGB1 to the receptor, has a negative prognostic impact on human patients with breast cancer.

RESULTS

TLR4 is required for cross-presentation of dying tumor cells

To determine which TLR might control the immune response against dying tumor cells, we fed dying ovalbumin (OVA)-expressing EG7 mouse thymoma cells to bone marrow–derived DCs (BM-DCs) that were either wild type (WT) or lacking TLRs. We then assessed the antigen-presenting capacity of the DCs by measuring IL-2 production by MHC class I (H-2^b)-restricted $OVA_{257–264}$-specific B3Z and MHC class II (I-A^b)-restricted $OVA_{323–339}$-specific B09710 mouse hybridomas (**Fig. 1**). OVA peptides from irradiated (**Fig. 1a**,**b**,**d**) or oxaliplatin-treated (**Fig. 1c**) EG7 tumor cells, but not from live tumor cells, were efficiently presented by DCs. Although syngeneic WT, *Trif*$^{-/-}$, *Tlr1*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, *Tlr5*$^{-/-}$, *Tlr6*$^{-/-}$, *Tlr7*$^{-/-}$ or *Tlr9*$^{-/-}$ DCs could present antigen from dying tumor cells, *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ DCs were defective in this function (**Fig. 1a**,**c**,**d**). Pulsing of DCs from WT mice with a TLR4 inhibitory peptide[16] or a TLR4-Fc fusion protein also inhibited the MHC class I–restricted OVA-specific response (**Fig. 1b**). In contrast, no such inhibitory effect was observed for an oligonucleotide designed to block both TLR7 and TLR9 (data not shown)[17].

Inoculation of oxaliplatin-treated (but not live) EG7 cells into the footpad primed draining lymph node (DLN) cells for interferon-γ

(IFN-γ) production after *in vitro* re-stimulation with OVA protein. This response was obtained in WT and was intact in all *Tlr*$^{-/-}$ mice except for *Tlr4*$^{-/-}$ mice (**Fig. 2a**). We confirmed this observation for distinct apoptosis inducers and tumor antigens. Mouse CT26 (H-2^{d+} colon cancer) or MCA205 (H-2^{b+} sarcoma) cells treated with doxorubicin efficiently primed tumor-specific T lymphocytes in BALB/c and C57BL/6 mice with, respectively, a WT or a *Tlr2*$^{-/-}$ genetic background (**Fig. 2b**). However, neither tumor cell type elicited efficient T-cell priming in *Tlr4*$^{-/-}$ littermates (**Fig. 2b**). Cross-presentation of OVA from dying EG7 (H-2^{b}) cells was also compromised by the knockout of *Tlr4* in hosts carrying a different MHC class I allele (H-2^{d}) (**Fig. 2c**). Similar results were obtained in C3H/HeJ mice (H-2^{k}), which are defective in TLR4 signaling (data not shown). In contrast, *Tlr4*$^{-/-}$ and C3H/HeJ mice mounted an intact response to soluble OVA protein mixed with TLR9 agonists (CpG oligodeoxynucleotides) (**Fig. 2c** and data not shown). Thus, the absence of TLR4 selectively compromised the immune response against dying cells, not soluble antigen.

T-cell priming by dying tumor cells depended stringently on DCs. Injection of diphtheria toxin into mice expressing a transgenic diphtheria toxin receptor (DTR) in DCs[11] led to DC depletion and abrogated the priming of T lymphocytes elicited by dying tumor cells

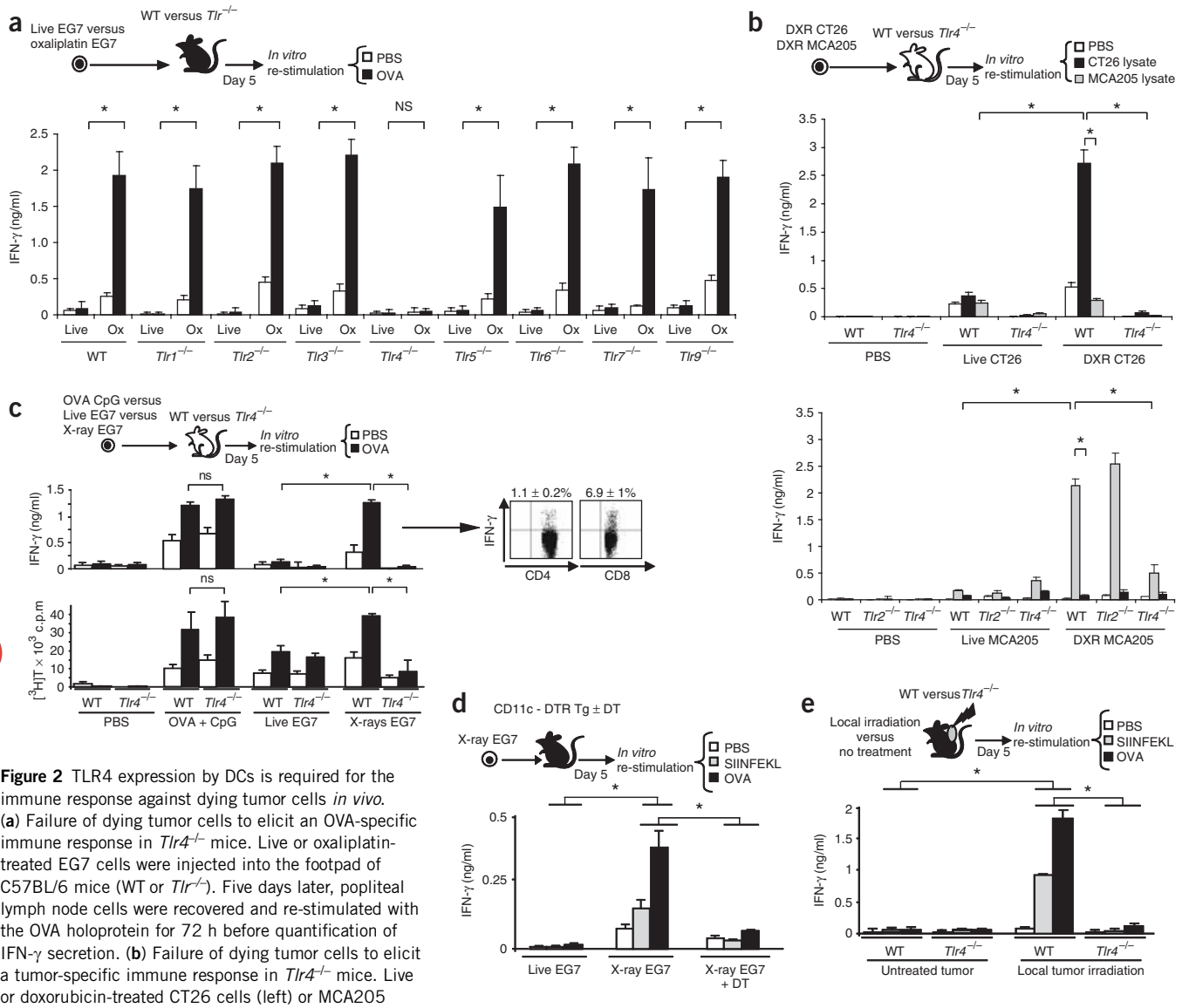

Figure 2 TLR4 expression by DCs is required for the immune response against dying tumor cells *in vivo*. (**a**) Failure of dying tumor cells to elicit an OVA-specific immune response in *Tlr4*$^{-/-}$ mice. Live or oxaliplatin-treated EG7 cells were injected into the footpad of C57BL/6 mice (WT or *Tlr*$^{-/-}$). Five days later, popliteal lymph node cells were recovered and re-stimulated with the OVA holoprotein for 72 h before quantification of IFN-γ secretion. (**b**) Failure of dying tumor cells to elicit a tumor-specific immune response in *Tlr4*$^{-/-}$ mice. Live or doxorubicin-treated CT26 cells (left) or MCA205 (right) were injected into the footpad of BALB/c or C57BL/6 mice (WT or *Tlr*$^{-/-}$), respectively. Lymph node cells were analyzed 5 d later for their capacity to produce IFN-γ upon *in vitro* re-stimulation with tumor lysate. (**c**) Cross-presentation of antigen from dying tumor cells is impaired in *Tlr4*$^{-/-}$ hosts. Live or irradiated EG7 cells (syngeneic to C57Bl/6 mice) were inoculated into the footpad of WT or *Tlr4*$^{-/-}$ BALB/c mice, and the local immune response was measured either as IFN-γ secretion (as in **a**) or as proliferation. As a positive control of antigen presentation, mice were injected with 1 mg of OVA protein plus 10 μg CpG 28 as an adjuvant. The inset illustrates that IFN-γ elicited by cross-presentation is produced by both CD4^{+} and CD8^{+} T cells. (**d**) Requirement for DCs to mount an immune response against dying tumor cells. Irradiated EG7 cells were injected into C57BL/6 mice expressing a transgenic DTR under the control of the CD11c promoter, and the mice were simultaneously injected i.p. with PBS or diphtheria toxin (DT). The local immune response was assessed 5 d later as in **a**. (**e**) TLR4 is required for the immune response promoted by local tumor radiotherapy *in vivo*. EG7 tumors established in the thigh were X-ray irradiated (10 Gy), and inguinal lymph node cells were analyzed 5 d later for their capacity to produce IFN-γ upon *in vitro* re-stimulation. Results (means of triplicates ± s.e.m. $n = 3$) are representative of a typical experiment out of three independent ones. *$P < 0.01$.

(**Fig. 2d**). To confirm that it was the TLR4 present in DCs that determined the immune response, we pulsed WT or *Tlr4*$^{-/-}$ DCs with live or irradiated EG7 cells, transferred them into *Tlr4*$^{-/-}$ hosts and monitored the priming of CD8^{+} T cells in DLN cells. TLR4 deficiency affecting DCs specifically abolished CTL activation (measured as IFN-γ production and proliferation). However, the absence of TLR4

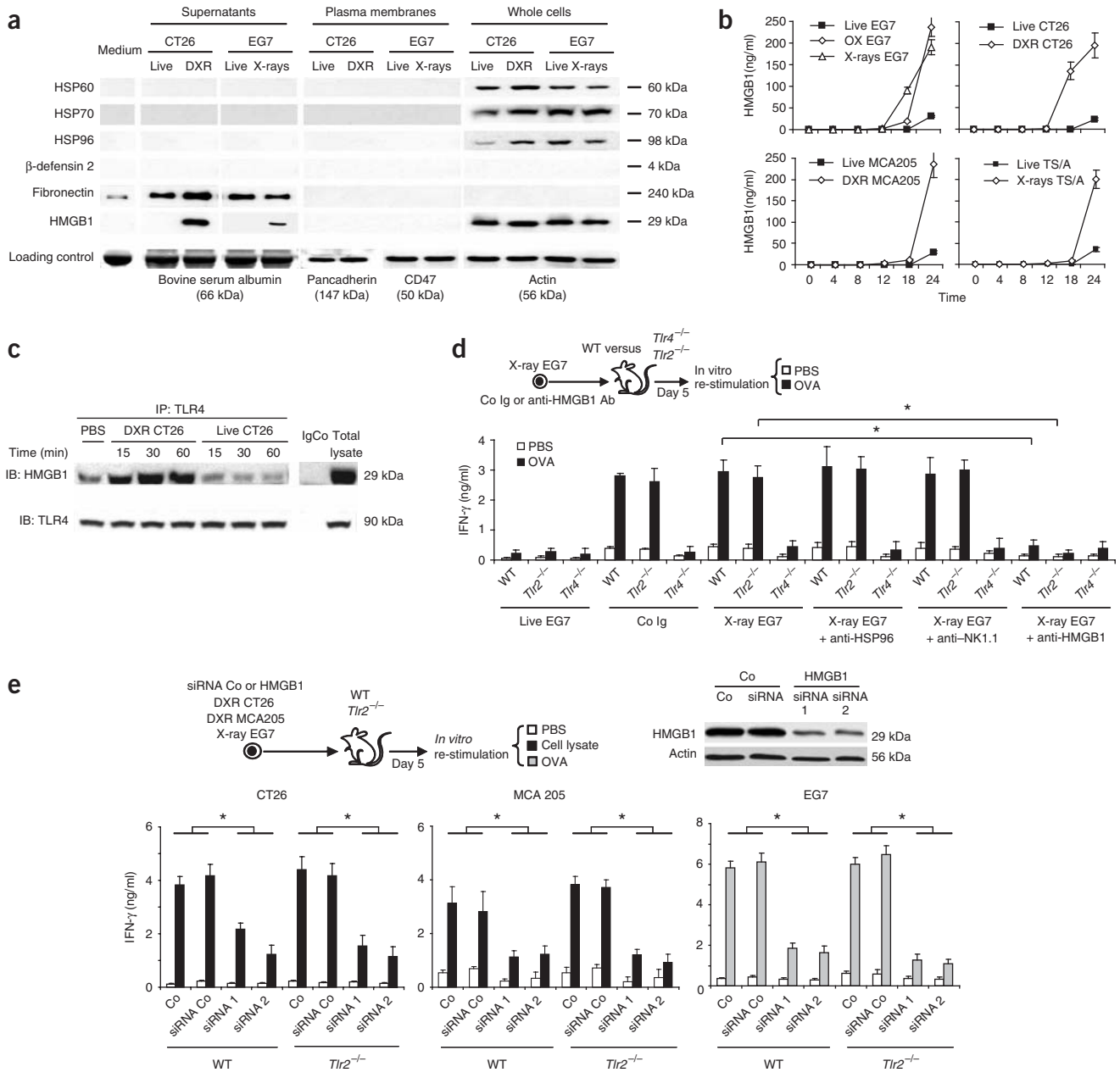

Figure 3 The immunogenicity of dying tumor cells after chemotherapy or radiotherapy depends on the release of the TLR4 ligand HMGB1. (**a**) HMGB1 is selectively released by dying tumor cells. TLR4 ligands were detected by immunoblots in supernatants, plasma membranes and whole-cell lysates of CT26 and EG7 tumors treated (or not treated) with, respectively, doxorubicin and X-rays. (**b**) Kinetic study of HMGB1 release from tumor cell lines. Shown is the accumulation of HMGB1, as measured by ELISA, after the indicated treatment of EG7, CT26, MCA205 or TS/A tumor cell lines (**c**) HMGB1 released from tumor cells binds to TLR4. RAW264.7 cells were incubated with the supernatants of either untreated or doxorubicin-treated CT26 tumor cells for the indicated periods of time. HMGB1 was then detected by western blotting after immunoprecipitation using an antibody against TLR4 (or a control immunoglobulin). The immunoprecipitation assays were performed four times with identical results. (**d**) Cross-presentation of OVA from dying tumor cells is dependent on HMGB1 *in vivo*. Live or X-irradiated EG7 were inoculated into the footpads of WT, *Tlr4*$^{-/-}$ or *Tlr2*$^{-/-}$ BALB/c mice along with an antibody to HMGB1 antibody (or a control immunoglobulin for each individual antibody (Co Ig) and antibodies to HSP96 and NK1.1 (directed against EG7 membrane-associated antigen)), and the local immune response was measured as in **Figure 2a**,**b**. (**e**) Depletion of HMGB1 in dying tumor cells using siRNA abolished antigen presentation. CT26, MCA205 and EG7 tumor cells were transfected with a control siRNA or two different HMGB1-specific siRNA. After 48 h (when immunoblots confirm HMGB1 depletion, top), the cells were injected into mice as in **Figure 2b**. Note that HMGB1 depletion does not alter cell death induction by genotoxic stress (data not shown). All *in vivo* experiments involved three mice per group and were repeated three times yielding identical results. Graphs (means of triplicates ± s.e.m., $n = 3$) are representative of a typical experiment out of three independent ones. *$P < 0.01$.

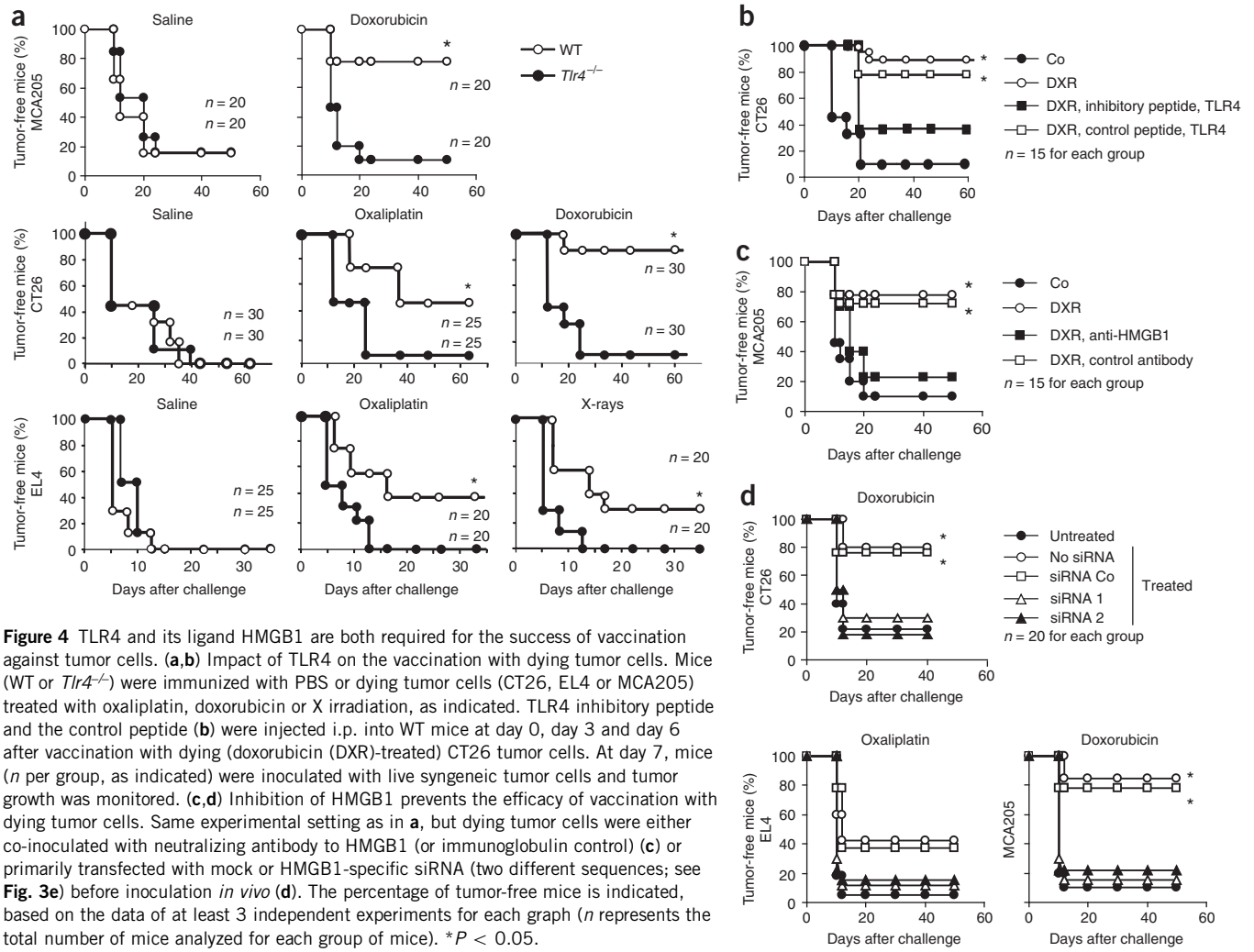

Figure 4 TLR4 and its ligand HMGB1 are both required for the success of vaccination against tumor cells. (**a**,**b**) Impact of TLR4 on the vaccination with dying tumor cells. Mice (WT or *Tlr4*$^{-/-}$) were immunized with PBS or dying tumor cells (CT26, EL4 or MCA205) treated with oxaliplatin, doxorubicin or X irradiation, as indicated. TLR4 inhibitory peptide and the control peptide (**b**) were injected i.p. into WT mice at day 0, day 3 and day 6 after vaccination with dying (doxorubicin (DXR)-treated) CT26 tumor cells. At day 7, mice (*n* per group, as indicated) were inoculated with live syngeneic tumor cells and tumor growth was monitored. (**c**,**d**) Inhibition of HMGB1 prevents the efficacy of vaccination with dying tumor cells. Same experimental setting as in **a**, but dying tumor cells were either co-inoculated with neutralizing antibody to HMGB1 (or immunoglobulin control) (**c**) or primarily transfected with mock or HMGB1-specific siRNA (two different sequences; see **Fig. 3e**) before inoculation *in vivo* (**d**). The percentage of tumor-free mice is indicated, based on the data of at least 3 independent experiments for each graph (*n* represents the total number of mice analyzed for each group of mice). *$P < 0.05$.

did not impair the capacity of DCs to present the soluble peptide SIINFEKL (**Supplementary Fig. 1** online). In addition, EG7 tumors established in the thigh were irradiated (10 Gy), and this local irradiation stimulated OVA-specific T-cell responses in the inguinal lymph node in WT but not *Tlr4*$^{-/-}$ mice (**Fig. 2e**). In conclusion, TLR4 must be present in DCs for the optimal presentation of antigen derived from dying tumor cells.

TLR4 controls tumor antigen processing and presentation

WT and *Tlr4*$^{-/-}$ DCs were equally efficient in engulfing irradiated EG7 thymoma or doxorubicin-treated CT26 colon carcinoma cells (**Supplementary Fig. 2a** online). The acquisition of maturation markers (including MHC class II and the co-stimulatory molecules CD40, CD80, CD86), the production of inflammatory cytokines (IL-6, IL-12p40, TNF-α) and allostimulatory potential by *Tlr4*$^{-/-}$ DCs were deficient in response to bacterial lipopolysaccharide yet intact in response to dying tumor cells (data not shown and **Supplementary Fig. 2b**,**c**). However, TLR4 influenced the kinetics at which DCs express K^{b}-SIINFEKL MHC class I peptide complexes at the plasma membrane surface after loading with dying OVA-transfected TS/A (H-2^{d}) cells. WT and *Tlr4*$^{-/-}$ DCs expressed comparable levels of K^{b} molecules at baseline and acquired K^{b}-SIINFEKL complexes after pulsing with saturable amounts of free SIINFEKL peptides with similar kinetics (data not shown). However, a markedly reduced exposure of K^{b}-SIINFEKL complexes was detected on *Tlr4*$^{-/-}$ DCs, as compared with WT controls, after loading with dying OVA-expressing TS/A cells (**Supplementary Fig. 3a** online).

TLR4 is likely to affect the processing and presentation of antigen. TLR4 has been reported to inhibit the lysosome-dependent degradation of phagosomes[18], meaning that *Tlr4*$^{-/-}$ DCs would degrade dying cells in the lysosomal compartment instead of presenting their antigens[19]. Indeed, the alkalinization of lysosomes with either chloroquine (a lysosomotropic alkaline) or bafilomycin A1 (a specific inhibitor of the vacuolar ATPase responsible for lysosomal acidification), used at subtoxic concentrations, enhanced the capacity of *Tlr4*$^{-/-}$ DCs to present antigen from dying cells yet did not ameliorate antigen presentation by WT DCs (**Supplementary Fig. 3b**). Accordingly, treatment of *Tlr4*$^{-/-}$ DCs with chloroquine restored the ability of DCs to present K^{b}-SIINKEKL complexes to normal levels (**Supplementary Fig. 3a**, right). Next, we directly determined the kinetics of fusion between phagosomes and lysosomes in WT versus *Tlr4*$^{-/-}$ DCs loaded with dying tumor cells. Colocalization of the phagocytic cargo with lysosomes was significantly accelerated in *Tlr4*$^{-/-}$ DCs as compared with WT DCs (**Supplementary Fig. 3c**).

These data confirm that TLR4 regulates the processing and presentation of tumor cell antigens by DCs, presumably by inhibiting the lysosomal destruction of antigens.

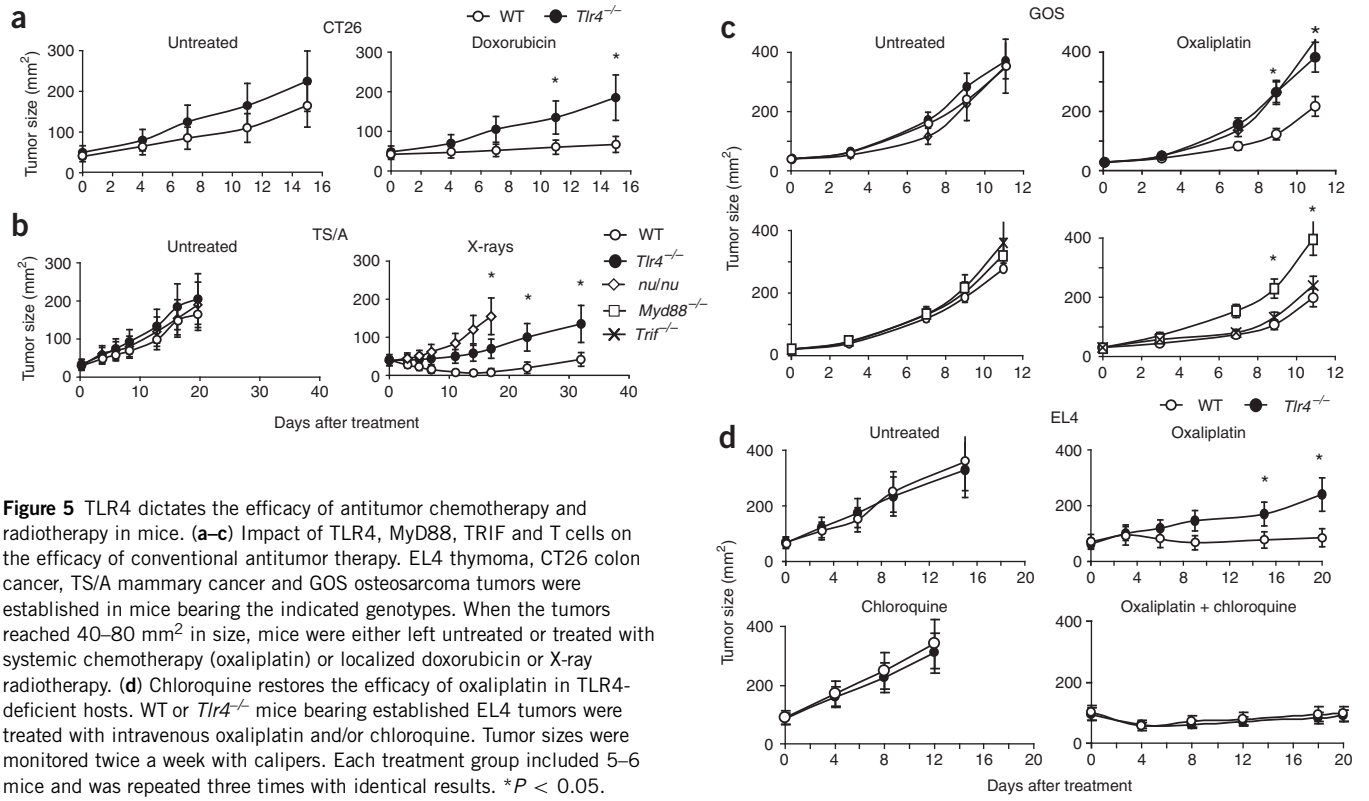

Figure 5 TLR4 dictates the efficacy of antitumor chemotherapy and radiotherapy in mice. (**a–c**) Impact of TLR4, MyD88, TRIF and T cells on the efficacy of conventional antitumor therapy. EL4 thymoma, CT26 colon cancer, TS/A mammary cancer and GOS osteosarcoma tumors were established in mice bearing the indicated genotypes. When the tumors reached 40–80 mm^2 in size, mice were either left untreated or treated with systemic chemotherapy (oxaliplatin) or localized doxorubicin or X-ray radiotherapy. (**d**) Chloroquine restores the efficacy of oxaliplatin in TLR4-deficient hosts. WT or *Tlr4*$^{-/-}$ mice bearing established EL4 tumors were treated with intravenous oxaliplatin and/or chloroquine. Tumor sizes were monitored twice a week with calipers. Each treatment group included 5–6 mice and was repeated three times with identical results. $^*P < 0.05$.

HMGB1 release by dying tumor cells

Reportedly, a number of endogenous proteins bind and stimulate TLR4[20]: heat-shock protein (HSP) 60, HSP70, oxidized LDL, surfactant protein A, hyaluronan breakdown products[21], fibronectin, β-defensin-2 (ref. 22) and the alarmin high-mobility-group box 1 protein (HMGB1)[23,24]. Irradiation of EG7 cells or doxorubicin treatment of CT26 caused the release of HMGB1, yet did not provoke the release or surface exposure of HSPs, β-defensin-2 or fibronectin (**Fig. 3a**). HMGB1 was released 18 h after irradiation of EG7 or TS/A cells or doxorubicin treatment of CT26 or MCA205 cells (**Fig. 3b**), and this release was inhibited by Z-VAD-fmk (data not shown), which suppresses apoptotic caspase activation and delays secondary necrosis. Next we determined whether the HMGB1 contained in the supernatant of dying tumor cells might directly interact with TLR4[23]. Raw264.7 macrophages (which express TLR4) were incubated with supernatants from doxorubicin-treated CT26 cells (containing >200 ng/ml of free HMGB1) or live CT26 cells (containing <20 ng/ml of free HMGB1), washed and then subjected to the immunoprecipitation of HMGB1, and TLR4 was detected in the precipitate (**Fig. 3c**). These results demonstrate that HMGB1 secreted by dying tumor cells binds to TLR4 and hence make it unlikely that another (known or unknown) TLR4 ligand contained in this supernatant would preferentially occupy TLR4 on antigen presenting cells.

Inhibition of HMGB1 secretion by preincubation of the tumor cells with a small interfering RNA (**Supplementary Fig. 4** online) inhibited the capacity of irradiated EG7 cells to stimulate B3Z cells via DCs. Similar data were obtained when DCs were cocultured with dying EG7 tumor cells in the presence of neutralizing antibody to HMGB1 (**Supplementary Fig. 4**). Because HMGB1 is involved in the inflammatory response elicited by dying cells[24–27], we further investigated its contribution to the TLR4-dependent antitumor immune response. Neutralization of all possible TLR4 ligands with recombinant TLR4-Fc fusion protein (**Fig. 1b**) was as efficient in inhibiting the DC-mediated presentation of OVA from irradiated EG7 cells as was neutralization of HMGB1 (**Supplementary Fig. 4**), suggesting that HMGB1 is indeed the principal TLR4-activating agent involved in this system. Local injection of HMGB1-neutralizing antibody (but not an irrelevant antibody to NK1.1, antibody targeting NK1.1 molecules expressed on EG7 or an antibody to HSP96) also inhibited the priming of T cells induced by irradiated EG7 cells (**Fig. 3d**) or doxorubicin-treated CT26 cells (data not shown) *in vivo*. Similarly, doxorubicin-treated CT26 or MCA205 cells and irradiated EG7 cells lost their capacity to prime T cells *in vivo* when they were depleted from HMGB1 by means of specific siRNAs (**Fig. 3e**). In conclusion, HMGB1 represents the principal damage-associated molecular pattern that dictates the TLR4-dependent immune response to dying tumor cells.

HMGB1/TLR4/MyD88 in the efficacy of anticancer drugs

Injection of doxorubicin-treated CT26 colon cancer cells is highly efficient in inducing an immune response that prevents the growth of live CT26 cells inoculated 1 week later[6]. We obtained similar results with doxorubicin-treated MCA205 sarcoma cells, which prevented the growth of MCA205. Although this effective vaccination induced by dying tumor cells applied to WT mice, no tumor vaccination could be achieved with anthracycline- or oxaliplatin-treated cells in *Tlr4*$^{-/-}$ mice (**Fig. 4a**). These data were confirmed for oxaliplatin-treated or irradiated EL4 thymoma cells, which failed to protect *Tlr4*$^{-/-}$ hosts against tumor challenge (**Fig. 4a**). Pharmacological inhibition of TLR4 with a cell-permeable blocking peptide[16] that was co-injected with dying tumor cells also prevented antitumor immunity (**Fig. 4b**). Moreover, the depletion of HMGB1 from doxorubicin- or oxaliplatin-treated tumor cells using neutralizing antibodies (**Fig. 4c**) or HMGB1-specific siRNAs (**Fig. 4d**) compromised the efficacy of antitumor vaccination.

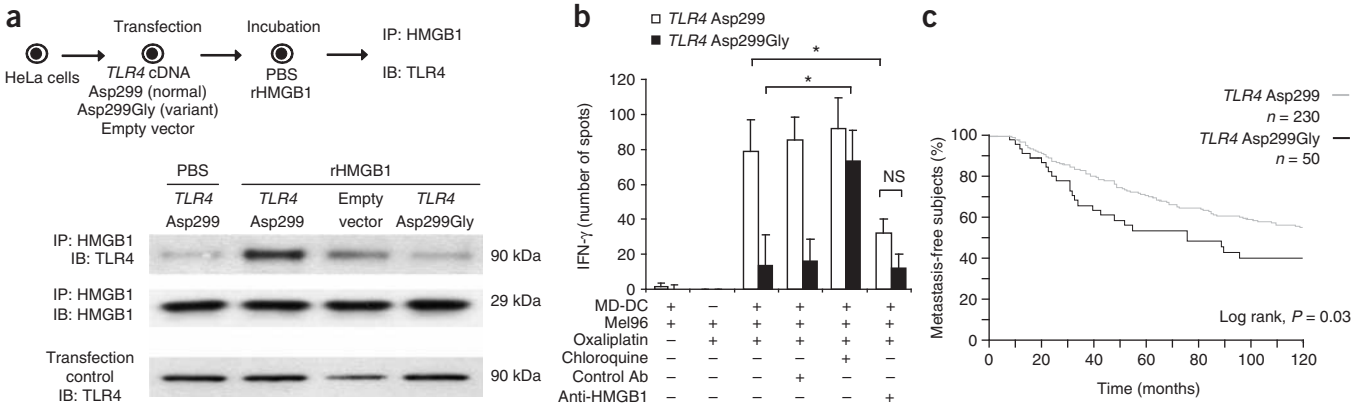

Figure 6 TLR4 dictates the efficacy of antitumor chemotherapy in humans. (**a**) Defective binding of HMGB1 to TLR4 conferred by the Tlr4 Asp299Gly polymorphism. HeLa cells were transfected with a vector containing the *Tlr4* Asp299 (normal) cDNA or the *Tlr4* Asp299Gly mutated cDNA or an empty vector. Transfectants were incubated in the presence of rHMGB1 for 1 h and immunoprecipitation assays were done as indicated. (**b**) Defective capacity of human DCs harboring the TLR4 Asp299Gly mutation to cross-present tumor antigens to CTL clones. HLA-A2–positive MD-DCs were cocultured with dying HLA-A2–negative melanoma cells expressing Mart1 as well as a CTL clone specific for A2/Mart1. IFN-γ ELISPOT assays were conducted to assess CTL activation. The graph represents the mean ± s.e.m. of the number of spots in each condition in a representative experiment (out of two) in a single donor for each genotype. The results obtained with a second pair of individuals are depicted in **Supplementary Figure 5**. (**c**) Kaplan-Meier estimates of time to metastasis between two groups of patients bearing the normal or mutated *Tlr4* alleles. The time to progression was analyzed in 280 women with non-metastatic breast cancer with lymph node involvement who were treated by surgery followed by anthracycline-based chemotherapy and local irradiation.

In the treatment of established tumors with systemic chemotherapy or local radiotherapy, the presence of TLR4 dictated the therapeutic outcome. CT26 colon cancers (**Fig. 5a**), TS/A breast carcinomas (**Fig. 5b**), heterotransplanted GOS osteosarcomas (**Fig. 5c**) and EL4 thymomas (**Fig. 5d**) progressed with similar kinetics in immunocompetent WT, *Tlr4*$^{-/-}$ and *nu/nu* athymic mice. Chemotherapy with appropriate cytotoxic agents or local radiotherapy reduced tumor growth and prolonged the survival of tumor-bearing mice in immunocompetent WT mice yet was less effective in *Tlr4*$^{-/-}$ (**Fig. 5a–d**) and *nu/nu* mice (**Fig. 5b** and data not shown). In accordance with the *in vitro* data (**Fig. 1a**), *Trif*$^{-/-}$ mice mounted a similar chemotherapeutic response as WT mice, whereas *Myd88*$^{-/-}$ mice behaved like *Tlr4*$^{-/-}$ mice (**Fig. 5c**). Moreover, systemic administration of chloroquine enhanced the efficacy of chemotherapy in *Tlr4*$^{-/-}$ mice but not in WT mice (**Fig. 5d**), in agreement with data presented in **Supplementary Figure 3**. These results point to a hitherto unrecognized contribution of TLR4/MyD88-dependent immunity to chemotherapeutic regimens.

Relevance of a TLR4 mutant for chemotherapy efficacy

A sequence polymorphism in *Tlr4* (896A/G, Asp299Gly, rs4986790) affecting the extracellular domain of TLR4 is associated with reduced endotoxin responses and with a reduced susceptibility to cardiovascular disease in humans[28,29]. Although it is a matter of debate whether the *TLR4* Asp299Gly mutation results in deficient LPS signaling[28,30,31], we addressed the possibility that the *TLR4* Asp299Gly polymorphism could affect the response to HMGB1. Immunoprecipitation experiments were performed after adding HMGB1 to HeLa cells that were transfected with normal and mutated human *TLR4*. The binding of HMGB1 to the mutant (Asp299Gly) TLR4 was reduced as compared to its binding to the normal (Asp299) TLR4 (**Fig. 6a**), although both transfectants expressed similar numbers of TLR4 molecules. This defective binding of HMGB1 to the mutated TLR4 might account for the severely impaired capacity of monocyte-derived DCs (MD-DCs) to cross-present melanoma antigens to CTLs. MD-DCs from normal (Asp299) individuals cross-presented Mart1 derived from dying melanoma cells to CTL clones in an HMGB1-dependent manner (**Fig. 6b** and **Supplementary Fig. 5** online). In contrast, MD-DCs from individuals bearing an Asp299Gly *TLR4* allele did not cross-present (**Fig. 6b** and **Supplementary Fig. 5**). This defect was restored by addition of chloroquine (**Fig. 6b** and **Supplementary Fig. 5**). Thus, *TLR4* Asp299Gly inhibits the HMGB1 response.

The breast cancer patients who benefit the most from adjuvant systemic administration of anthracyclines are those presenting with lymph node involvement. Therefore, we analyzed the time to metastasis in a cohort of 280 patients with non-metastatic breast cancer who were treated with anthracyclines after local surgery revealing lymph node involvement. The frequencies of heterozygous and homozygous germline polymorphisms encoding Asp299Gly were 17.1% and 0.7%, respectively (the two will be referred to collectively as 'mutated *TLR4*' hereafter). Patients carrying the mutated *TLR4* allele did not differ from patients with the normal *TLR4* allele with regard to any classical prognostic factors (see **Supplementary Table 1** online). The frequency of metastasis by 5 years after surgery was statistically higher in the group carrying a mutated *TLR4* (40%, versus 26.5% in patients without the mutation; $P < 0.05$, relative risk 1.53, 95% confidence interval 1.1–3.58). Moreover, the Kaplan-Meier estimate of metastasis-free survival showed an overall significantly lower percentage of metastasis-free patients in the group with mutated *TLR4* (log-rank test, $P = 0.03$) (**Fig. 6c**). In contrast, single-nucleotide polymorphisms affecting the *TLR4* intron or 5′ untranslated region (*TLR4* mutations rs1927911 and rs10759932 (ref. 32), respectively) had no correlation with the metastasis-free survival of the same cohort of patients (data not shown).

Hence, a specific mutation of *TLR4* with functional relevance may influence the immunological component of anthracycline-based chemotherapy in human cancer.

DISCUSSION

Cancer patients and their physicians who receive and apply chemotherapy, respectively, do so in the genuine belief that the prime goal of therapy is to destroy tumor cells. Here, we show for the first time that anticancer chemotherapy has an additional, decisive effect. Dying tumor cells elicit an immune response that is required

for the success of therapy. This immune response mediates the suppression of tumor growth and determines the long-term survival of animals and patients. We have defined (one of) the molecular mechanism(s) that dictates the chemotherapy-elicited antitumor immune response, namely the functional interaction between one compound released from dying tumor cells (HMGB1) and one particular receptor that is important for the function of the immune system (TLR4).

Injured tissue can trigger acute and transient immune responses against self antigens[1,33], presumably because dying cells release adjuvant factors that amplify and sustain DC- and T cell–dependent immune responses[34–38]. Recent studies have described the roles of IFN type 1 and the *N*-ethyl-*N*-nitrosourea–induced germline mutation 3d in the T cell–dependent immunogenicity of Fas- and UV-induced apoptotic splenic cells expressing a membrane-associated form of ovalbumin[35,36]. Several damage-associated molecular patterns, including hyaluronans, HSPs and fibronectin, have been identified as TLR4 ligands[39]. However, endogenous 'danger' signals have thus far not been implicated in antitumor immune responses. Here, we show that dying tumor cells produced by cancer therapies trigger a cognate immune response in a TLR4-dependent fashion (**Figs. 1** and **2**). TLR4 has previously been reported to play a part in lung tumorigenesis induced through chemically induced pulmonary inflammation[40]. Nonetheless, this observation did not link TLR4 expression to the induction of specific antitumor immune responses.

In the present study, we were able to identify one particular TLR4 ligand, HMGB1, as indispensable for the death-driven immunoadjuvant effects of chemotherapy. HMGB1 is a nonhistone chromatin-binding nuclear constituent that is passively released by dying cells and actively secreted by inflammatory APCs[25,41]. HMGB1 is a mediator of inflammation in the extracellular environment that exerts an important pathogenic role in late sepsis[26] as well as in hepatic ischemia-reperfusion; TLR4 is known to have a role in the latter process[24]. HMGB1 released by dead cells is a potent adjuvant *in vivo*[27]. Moreover, HMGB1 binding to its receptor, the receptor for advanced glycation end products (RAGE), promoted DC activation and elicited immune responses[42]. A recent report underscored the capacity of HMGB1 to trigger DC migration[43]. Here we provide evidence of a physical and functional interaction between HMGB1 released by tumor cells and TLR4 (**Figs. 3**, **4** and **5**). We were able to exclude the possibility that HMGB1 is required for the maturation of mouse and human DCs (**Supplementary Fig. 2b**,**c** and data not shown). Rather, our results suggest that TLR4 prevents the accelerated degradation of the phagocytic cargo within DCs, thereby allowing for optimal antigen presentation (**Supplementary Fig. 3**). Cross-presentation of tumor antigens derived from dying tumor cells by mouse or human DCs to T-cell hybridoma or CTL clones was selectively impaired in TLR4-deficient DCs and was HMGB1 dependent (**Supplementary Figs. 4** and **5** and **Fig. 6b**). The exact mechanism by which HMGB1-TLR4 interactions influence the processing and presentation of tumor antigens has yet to be deciphered.

Our data suggest that the *TLR4* polymorphism Asp299Gly (and the cosegregating missense mutation Thr399Ile; data not shown), which is found in 8–10% of Caucasians, compromises the efficacy of anticancer chemotherapy, at least in breast cancer. This polymorphism can affect the response of epithelial bronchial cells and alveolar macrophages to inhaled LPS[28]. Our experiments revealed that the *TLR4* Asp299Gly single-nucleotide polymorphism (SNP) reduces the interaction between TLR4 and HMGB1 (**Fig. 6a**) and abolishes the capacity of MD-DCs to cross-present dying melanoma cells to Mart1-specific HLA-A2–restricted-CTLs, a biological property that depends on HMGB1 in WT MD-DCs (**Fig. 6b**). However, the altered cross-presentation ability conferred by the *TLR4* Asp299Gly SNP was restored by culturing MD-DCs with chloroquine (**Fig. 6b**), a treatment that also overcame the defect of antigen presentation by *TLR4*$^{-/-}$ mouse DCs *in vitro* (**Supplementary Fig. 3a**,**b**) and *in vivo* (**Fig. 5d**).

Notably, 17% of the group of breast cancer patients presenting with lymph node involvement carried the *TLR4* Asp299Gly allelic variant, and these *TLR4* Asp299Gly carriers showed a shorter time to progression (**Fig. 6c**). To the best of our knowledge, this is the first report revealing that immunogenetic factors might affect clinical outcome in breast cancer.

Altogether, the immunoadjuvant effect of radiotherapy and chemotherapy relies upon two major checkpoints, calreticulin exposure (the 'eat me' signal) and HMGB1 release (the 'danger' signal) by dying tumor cells, thus licensing DCs for antigen uptake[8] and TLR4-dependent antigen processing, respectively. Only when both the 'eat me' and the 'danger' signals are correctly emitted by dying tumor cells and perceived by DCs will an immune response ensue. This knowledge may be clinically exploited to enhance the immunogenicity of current chemotherapeutic regimens. A major challenge will be to determine whether the immune defect induced by deficient TLR4 signaling can be alleviated by combining chemotherapy with alternate TLR agonists or with lysosomal inhibitors such as chloroquine.

METHODS

Mouse strains. All animals were bred and maintained according to both the FELASA and the Animal Experimental Ethics Committee Guidelines (Val de Marne, France). Animals were used at between 6 and 20 weeks of age. C57BL/6 *Tlr1*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$ (ref. 44), *Tlr5*$^{-/-}$, *Tlr6*$^{-/-}$, *Tlr7*$^{-/-}$, *Tlr9*$^{-/-}$, *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ mice were gifts (see Acknowledgments). Genetic background and origin of other mice are detailed in the **Supplementary Methods** online.

Tumor cell lines and transplantable tumors. CT26 colon cancer cells (syngenic from BALB/c mice), TS/A breast cancer cells (syngenic from BALB/c mice), TS/A-OVA breast cancer cells (syngenic from BALB/c mice), EL4 thymoma cells (syngenic from C57BL/6 mice), EG7 cells (OVA-transfected EL4 cells) and MCA205 fibrosarcoma cells (syngenic from C57BL/6 mice) were cultured at 37 °C under 5% CO_2 in endotoxin-free RPMI 1640 medium supplemented with 10% FCS, penicillin and streptomycin[6], 1 mM pyruvate and 10 mM HEPES. The Glasgow osteosarcoma (GOS) tumor was maintained in C57BL/6 mice over 6 weeks of age and transplanted every 2 weeks as s.c. implants[45].

Bone marrow–derived DCs and T-cell hybridoma assays. We propagated bone marrow–derived DCs as already described[46]. Further information is provided in the **Supplementary Methods**.

Immunoblot analysis. For immunoblot analysis, cells were lysed in lysis buffer. Whole-cell lysates, purified plasma membranes or supernatants were resolved by SDS-PAGE and then transferred onto nitrocellulose membrane and probed with appropriate primary and secondary antibodies. Further details are described in the **Supplementary Methods**.

RNA interference knockdown of HMGB1. We transfected CT26, MCA205 and EG7 cells using HiPerFect (CT26, MCA205) (Qiagen) or nucleofection using Nucleofector Kit L (EG7) (Amaxa) with either PBS, irrelevant siRNA, HMGB1 siRNA 1 or HMGB1 siRNA 2 (siRNA sequences are detailed in the **Supplementary Methods**).

Detection of peptide–MHC class I complexes at the surface of DCs using specific antibody to 25D1.16 (ref. 47). We performed detection of peptide–MHC class I complexes at the surface of DCs as previously described. Further details are provided in the **Supplementary Methods**.

Anticancer vaccination. CT26, EL4 and MCA205 cells were cultured with either PBS, doxorubicin (20 μM for CT26 and 1 μM for MCA205) or

oxaliplatin (5 μg/ml) (Sanofi-Aventis) for 24 h. Alternatively, EL4 cells were subjected to 10 Gy of X-ray irradiation (RT250, Phillips). In some experiments cells were transfected with HMGB1 siRNA or irrelevant siRNA 48 h before *in vitro* treatment. All these treatments resulted in a population containing ~30% annexin V^+ $DAPI^+$ double-positive cells, as assessed by FACS analysis at 24 h. 3×10^6 dying CT26 cells or 5×10^6 dying EL4 cells were injected s.c. into the left flanks of mice. Seven days later, mice were re-challenged in the right flank with 5×10^5 live CT26 or EL4 cells. Tumor growth was then monitored weekly using calipers.

Chemotherapy and radiotherapy of established tumors in mice. WT or loss-of-function mice were injected in the flank with 10^5 EL4 CT26 TS/A cells. Mice were then randomly assigned into treatment groups of 4–6 mice each. Tumor surface was monitored using calipers. When tumor size reached 40–80 mm^2, mice were treated with oxaliplatin (5 mg per kg body weight i.p for EL4), doxorubicin (2 μM injected intratumorally in 100 μl PBS, for CT26) or local X-ray irradiation (for TS/A). For local radiotherapy, mice were briefly anesthetized using isoflurane, placed into plastic constrainers and locally irradiated (10 Gy). The whole body was protected by lead shielding, except for the area of the tumor to be irradiated. For Glasgow osteosarcoma (GOS), oxaliplatin (5 mg/kg intraperitoneally (i.p.)) was administrated at day 5 when tumors became palpable[45].

Genotyping of TLR4 Asp299Gly, Thr399Ile and related SNPs. DNA was isolated from frozen blood leukocytes from subjects. PCR primers (Applied Biosystems) were used to amplify a 101-bp fragment containing the *TLR4* Asp299Gly mutation (rs4986790) site. After PCR amplification, genotypes were assigned to each subject, by comparing the signals from the two fluorescent probes, FAM and VIC, and calculating the –log(FAM/VIC) ratio for each data point[48]. Other *TLR4* polymorphisms and CD14-260 C/T SNPs were also analyzed in parallel using predesigned PCR primers from Applied Biosystems.

Statistical analyses. For the analysis of experimental data, comparison of continuous data was achieved by the Mann-Whitney *U* test and comparison of categorical data by χ^2 or Fisher's exact test, as appropriate. The log-rank test was used for analysis of Kaplan-Meier survival curves. All statistical analyses were performed with JMP 5.1 (SAS Institute). All *P* values are two tailed. A *P* value <0.05 was considered statistically significant for all experiments.

Additional information about reagents and materials, anticancer vaccination, priming experiments, immunoprecipitation and clinical study design are detailed in the **Supplementary Methods**.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

L.A., F.G., A.T., M.O., G.M., M.C.M. and E.U. performed the *in vivo* and *in vitro* experiments. C.O. performed *in vitro* experiments. A.C. performed immunoprecipitations. B.R. provided transgenic mice. F.J.B., H.Y. and F.L. provided essential reagents. R.L., C.N., J.-P.M., A.C., V.J., F.C.-C., S.D. and T.T. recorded and provided the patients' data. L.A. and P. Saulnier performed patients' genotyping. A.T., F.A. and F.G. conducted data analysis. S.A. offered scientific advice and gave technical hints on the direction of the study. L.Z. and G.K. conceived the study and wrote the manuscript. P. Saftig provided the *LAMP2*$^{-/-}$ mice. J.B. set up the radiotherapy protocols *in vivo*.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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8.3 Annexe 3: Immunogénicité des anthracyclines.

Review

Immunogenicity of anthracyclines: moving towards more personalized medicine

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The current method of cancer management takes into account tumor-related factors to predict therapeutic outcome. However, recent evidence indicates that the host immune system also contributes to therapeutic outcome. Here, we highlight anthracyclines, which have been used to treat a broad range of cancers since the 1960s, as an example of an anticancer treatment that can boost the host's immune system to improve the efficacy of chemotherapy. It has recently been revealed that the translocation of calreticulin to the plasma membrane in tumor cells and the release of high-mobility-group box 1 (HMGB1) by tumor cells are two key post-transcriptional events required for the immunogenicity of anthracyclines. These discoveries represent a conceptual advance in the understanding of the mechanisms underlying the immunogenicity of anthracyclines. We review the effects of anthracyclines on the host immune system and discuss how this knowledge can be exploited for anticancer therapy.

Introduction

The clinical use of anthracyclines can be viewed as a double-edged sword. On the one hand, anthracyclines have – for >40 years – had a major role in the management of pediatric, sarcoma, leukemia, lymphoma, Kaposi, uterine, ovarian and breast malignancies (Table 1). On the other hand, chronic administration of anthracyclines induces and/or contributes to cardiomyopathy, congestive heart failure and myelodysplasia [1]. Moreover, observed drug-resistance mechanisms in tumor cells have also fueled the debate that surrounds these compounds. Despite their janus face, anthracyclines continue to raise interest regarding their mechanism of action, the pathophysiology of their cardiotoxic effects, the development of third-generation pharmaceutical formulations and their effects on the host immune system.

Initially described in 1939 as antibiotics recognized for their anti-bacterial properties, anthracyclines became of therapeutic value in the treatment of cancer when the first member of the family, daunorubicin, was isolated in 1963. *In vitro* studies demonstrated that daunorubicin exhibits antiproliferative effects on leukemia cell lines [2], raising oncologists' interest to further investigate this family of cytotoxic compounds. Doxorubicin (previously called adriamycin) was later introduced (Box 1) and became one of the most widely used anthracyclines because of its lower toxicity and potent anti-tumor activity against solid tumors compared with daunorubicin. The first *in vivo* studies performed in 1973, which compared the actions of anthracyclines in immuno-competent and immuno-compromised mice, indicated that part of the anti-tumor activity of anthracyclines could be attributable to the immune system of the host [3]. These findings were later corroborated in various experimental models (Table 2), and anthracyclines were shown to enhance innate and cognate immune functions *in vivo* (Table 3). These data prompted investigators to combine anthracyclines with immunomodulators [4–6] and to analyze the cellular and molecular events that account for the immunogenicity of anthracyclines [7–10].

To comprehensively study the contribution of the immune system of the host to the tumoricidal activity of a drug, the following premises should be taken into account. Briefly, following a spontaneous or drug-induced insult (i.e. stress, damage or distress), tumor cells induce local recruitment and activation of early effectors (such as neutrophils, eosinophils and mastocytes) to establish an inflammatory microenvironment, and myeloid precursors of macrophages and dendritic cells (DC) will also be recruited, which uptake and process dying tumor cells. DC loaded with their phagocytic cargo can respond to a variety of inflammatory signals and will migrate to secondary lymphoid organs (i.e. lymph nodes, LN) to prime naïve T lymphocytes that subsequently differentiate into effector and memory tumor-specific T-cells. Effector T-cells might have acquired the chemokine receptor pattern to return to the inflammatory tumor bed and eliminate tumor cells in a major histocompatibility (MHC) class I-dependent manner (for $CD8^+$ T-cells) or to modulate the tumor microenvironment through cytokine release (for $CD4^+$ T-cells). Moreover, DC will also activate LN natural killer (NK) cells that either participate in the polarization of T-cell responses or might leave the LN to reach the peripheral damaged tissue (tumor), where they have a scavenging role through recognition of stress molecules that are overexpressed in the tumor. It is therefore conceivable that T- and NK (cytotoxic lymphocyte) cells act in concert with early players of inflammation to destroy the residual living

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Table 1. Current clinical uses of anthracyclines

Compound +/− regimen or carrier and conjugate	Investigational or approved cancer indication	Refs
Epirubicin (CEF versus CMF) or CMF +/− epirubicin	Premenopausal N^+ breast cancer (adjuvant)	[89,90]
Epirubicin and taxanes sequentially	N^+ $HR^{+/-}$ *HER2*$^{+/-}$ breast cancer (adjuvant)	[91]
Epirubicin (CEF regimen)	*HER2*$^{+++}$ breast cancer (adjuvant)	[92]
Epirubicin (and Herceptin®)	*HER2*$^{+++}$ and *TOPO IIa* gene amplified breast cancer (adjuvant)	[93]
Epirubicin	High-throughput gene expression profiling in advanced breast cancer (pre-operative)	[94]
Epirubicin	Metastatic breast cancer	[95]
Uncoated citrate-containing liposomal doxorubicin	Metastatic breast cancer	[96,97]
Daunorubicin, idarubicin (and Ara-C) or mitoxantrone	Acute myeloid leukemia	[98,99,100]
Idarubicin, daunorubicin	Acute lymphoblastic leukemia	[101]
Doxorubicin (vincristine and dexamethasone)	Multiple myeloma	[102]
Doxorubicin-based regimen	Advanced non Hodgkin lymphoma (Follicular, ATL)	[103,104]
Doxorubicin-based regimen	Hodgkin lymphoma	[105]
PEG-conjugated liposomal doxorubicin, liposomal daunorubicin	Kaposi's sarcoma	[106,107]
Doxorubicin (platinum and cyclophosphamide)	Non-small-cell lung cancer	[108]
Amrubicin	Refractory small cell lung cancer	[109]
PEG liposomal doxorubicin (platinum and taxanes)	Ovarian cancer	[110]
Single agent doxorubicin	Advanced soft tissue sarcoma	[111]
PEG liposomal doxorubicin and carboplatin	Endometrial cancer	[112]
Doxorubicin-based regimen	Neuroblastoma	[113]
Mitoxantrone	Advanced prostate cancer	[114]
Nemorubicin, morpholinyldoxorubicin	Hepatocellular carcinoma	[115,116]
PEG liposomal doxorubicin	Glioblastomas	[117]
PEG liposomal doxorubicin	Head and neck tumors	[118]

Abbreviations: ARA-c, cytarabine; ATL, adult T cell lymphoma; CEF, cyclophosphamide, epirubicin plus 5-fluorouracil; CMF, cyclophosphamide, methotrexate plus fluorouracil; PEG, poly-ethylene glycol.

cells that are spared by chemotherapy or cytotoxic agents. To test this hypothesis, investigators aimed to compare the anti-tumor efficacy of a given compound in mice bearing either an intact ('immunocompetent') or a compromised immune system [at the level of, for example, NK, T-cells, macrophages and toll-like receptors (TLR) using depleting or neutralizing antibodies or mice carrying gene defects].

Indeed, the relevance of the type of cell demise triggered by cytotoxic drugs [initially classified as 'necrosis' versus 'apoptosis' (see Box 2)] in the reactivity of the host immune system has been addressed since the seminal work of Albert *et al.* [11,12] highlighting the remarkable capacity of DC to cross-present antigens derived from apoptotic cells but not necrotic counterparts.

Here, we review the historical and recent findings that support the possibility that anthracyclines should be

Box 1. Anthracyclines: a family of anticancer agents

Anthracyclines were initially described in 1963 as antibiotics derived from *Streptomyces* sp. [73]. Daunorubicin was the first anthracycline isolated and was quickly introduced as a treatment against leukemia [74]. Doxorubicin (first called adriamycin) was later produced in 1969 and was shown to act against solid tumors [75]. Doxorubicin exhibits stronger efficacy against sarcomas and carcinomas and decreased toxicity compared with daunorubicin. The family of anthracyclines rapidly expanded with the discoveries of idarubicin in 1979 [76] and epirubicin in 1980 [77] (the chemical structures of these anthracyclines are shown in Figure I and Table I). Mitoxantrone, the structure of which is closely compound to anthracyclines, was also synthesized in 1980 [78].

The mechanism of action of the anthracyclines is not completely understood. However, characterization of their binding sites in tumor cells provided several clues as to their enormous tumoricidal potential. Nuclear DNA is the primary target for these drugs, as anthracyclines are intercalators that bind to nuclear DNA by inserting their planar chromophores (Figure I, Table I) between DNA bases. They also interact with chromatin, creating unfolding transitions, leading to chromatin aggregation. Binding of anthracyclines to chromosomal proteins (such as histone H1, topoisomerases I and II, and HMGB1) accounts for their interference with both RNA synthesis and DNA repair. Covalent modification of the DNA by daunorubicin (involving the ion complex of the drug), generation of reactive oxygen species (resulting from the chelation of ions by daunorubicin), the inhibition of topoisomerase II (caused by distortion of the DNA conformation induced by the intercalation process) and the drug-induced intercalation itself, all function in concert to promote chromatin aggregation and fragmentation, leading to apoptosis [79]. However, such cytotoxic mechanisms and targeted modes of action occur in both tumor cells and healthy tissues.

The major limitation to the clinical implementation of anthracyclines is a cumulative cardiac toxicity, leading to discontinuation of treatment even when therapy is successful. At present, liposomal formulations (whether pegylated or uncoated) remain the best-known alternatives that have been found for improving the therapeutic index and spectrum of activity daunorubicin in clinical trials [80,81]. Combination therapies with anthracyclines and other chemotherapeutic agents have been tested and are still employed in the daily management of malignancies. Treatment with anthracyclines in association with platinium was tested as early as 1976 [82], and is still the reference therapy for uterine [83] and ovarian cancer [84]. In addition, anthracycline in combination with paclitaxel or cyclophosphamide is now approved for the treatment of breast cancer [85] (Table 1 of main text). Multiple combination therapies including anthracyclines are also used to treat cancer, such as 'CHOP' (cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone) for lymphoma [86], ECF (epirubicin, cisplatinum, 5-fluorouracil) for gastric carcinoma [87] and FEC (5-fluorouracil, epirubicin, cyclophosphamide) for breast cancer [85].

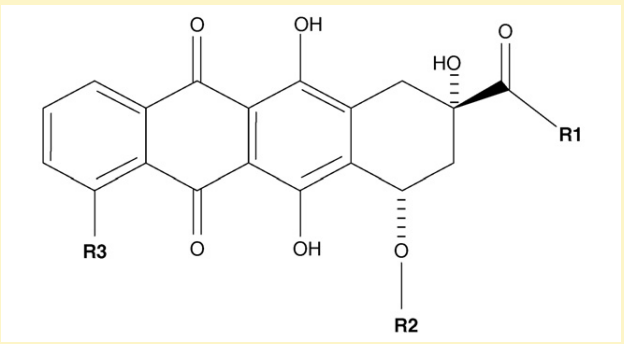
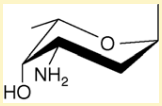
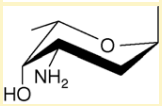
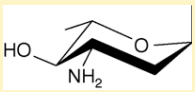
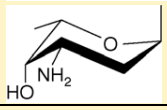


Figure I. Basic molecular structure of the anthracyclines [88].

Table I. Molecular structures of different anthracyclines

Molecule	R1	R2	R3
Daunorubicin	-H		-OCH3
Doxorubicin	-OH		-OCH3
Epirubicin	-OH		-OCH3
Idarubicin	-H		-H

considered not only as direct cytotoxic agents but also as potent immunomodulators that harness the host immune system. The story of an old drug, doxorubicin, pioneers a novel view of modern oncology, supporting the contention that cancer should not be considered as simply a disease of a tissue but, rather, a disease of the entire host.

Immune-mediated action of anthracyclines: the premises

The anti-proliferative activity of anthracyclines on tumor cells was characterized in the 1960s [2]. It was well known at that time that most cytotoxic drugs also exert deleterious side effects on non-cancerous host cells, including hematopoietic cells of the immune system [13]. However, a counterintuitive finding came from a report published in the late 1970s showing that the cytotoxic activity of splenocytes against tumor cells was greater in adriamycin- than in saline-treated mice after mixed lymphocyte tumor cultures [14]. The pioneering studies from Schwartz and Gringley [3] revealed the first evidence of the immune-system contribution to doxorubicin-mediated anti-tumor effects. The researchers aimed to compare the tumoricidal activity of two anthracycline compounds, doxorubicin (DX) and daunorubicin (DN), against the transplantable murine lymphocytic leukemia P-288 in immunocompetent DBA/2 mice [3]. Although biological and pharmacological analyses indicated that DN is the most effective anti-tumor compound *in vitro*, DX featured the strongest activity *in vivo* [3]. Importantly, when the host immune defenses were compromised by whole-body irradiation, or by administration of high doses of cyclophosphamide or methotrexate (both of which ablate lymphocytes), the tumoricidal activity of DX decreased. These data led to the thought-provoking hypothesis that the anti-tumor effects of DX are critically dependent on an intact immune system. Some studies also pointed to a role of DX in enhancing the growth inhibitory potential of macrophages against tumor lines *in vitro* [15,16]. Twenty years later, such a phenomenology was corroborated in a variety experimental settings (Table 2). Maccubbin *et al.* [17] first showed that DX is an effective immunomodulator capable of boosting cytotoxic T lymphocyte (CTL) responses, the tumoricidal activity of macrophages and the Fc-dependent phagocytosis in spleens while depressing NK and lymphokine activated killer (LAK) effector functions [17]. Subsequently, the authors isolated from the EL-4 parental thymoma cell line an anthracycline-resistant EL-4 variant cell line that could not be killed by adriamycin *in vitro*, yet was sensitive to the same drug in immunocompetent C57BL/6 mice [18]. Furthermore, pharmacokinetic parameters indicated that, although DX is taken up by $CD3^+$ T cells and accumulates in tumor-infiltrating lymphocytes (TIL), mature T cells are insensitive to DX-mediated cell death and, instead, are boosted in their cytolytic functions against tumors [19]. Interestingly, DX-resistant tumor cells exhibit enhanced MHC class I expression, rendering tumor cells more sensitive to CTL attack [20]. Other findings corroborated the direct effects of DX on immune cells [21,22].

The notion that, in addition to direct cytotoxic effects, anthracyclines could also exert a host-dependent anti-tumor activity participating in tumor shrinkage prevailed

Table 2. Mouse studies that investigated the immunological actions of doxorubicin

Model	Cellular target	Description	Refs
BALB/c x DBA/2F1 mice, MBL-2	Macrophages	DX-activated macrophages can inhibit tumor growth	[15]
C57BL/6 x A Ft, HeLa	Macrophages	Macrophages, having phagocytosed DX-treated debris, can inhibit tumor growth *in vitro*	[16]
RDM4	CTL and LAK	DX can enhance lysability of tumor cells by CTL and LAK	[119]
BALB/c, RENCA	LAK	Chemoimmunotherapy using DX + LAK can cure advanced renal cell carcinoma	[23]
C57BL/6, P815	Macrophages and NK	DX can induce augmentation of macrophage lytic activity *ex vivo*, and transiently decrease NK activity	[22]
C57BL/6, EL-4	NK and LAK	DX can induce diminution of NK activity *ex vivo*, depending on PGE2 production	[17]
C57BL/6, EL-4	CTL, LAK and macrophages	DX can function via the host immune system *in vivo* because *in vitro* DX-resistant tumors can be DX-sensitive *in vivo*	[18]
C57BL/6, EL-4	CTL	DX + IL-2 combined treatment efficacy relies mainly on CD8+ T cells	[4]
C57BL/6, EL-4	TIL	DX enhances cytotoxicity of TIL *ex vivo*, but TIL are not sensitive to the drug *in vivo*	[19]
C57BL/6, EL-4	CTL	DX and TNF-α can function synergistically; survival correlates with CTL activity.	[5,6]
C57BL/6	Macrophages and NK	DX enhances cytokines (IFNγ, IL-1, TNF-α) production by macrophages and NK	[21]
BALB/c, CT26	APC and CTL	Phagocytosis of DX-treated tumor cells can promote an immune response	[8]
BALB/c, CT26	APC	Phagocytosis of DX-treated tumor cells is dependent on calreticulin exposure by dying cells	[9]
BALB/c, CT26	APC and CTL	Processing of DX-induced apoptotic bodies is dependent on HMGB1 release	[10]

Abbreviations: APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; DX, doxorubicin; HMGB1, high-mobility-group box 1 protein; IFNγ, interferon-γ; IL, interleukin; LAK, lymphokine-activated killer cells; NK, natural killer; PGE2, prostaglandin E2; TIL, tumor-infiltrating lymphocytes; TNF-α, tumor necrosis factor-α.

Table 3. Anthracyclines as immunogenic drugs, from premises to demonstration

Model	Treatment		Effect on tumor	Refs
P288 (leukemia)	DX		Shrinkage	[3]
		+ Cyclophosphamide	Growth	[3]
		+ TBI	Growth	[3]
RENCA (kidney cancer)	DX		Growth	[23]
	LAK		Growth	[23]
	DX	+ LAK	Shrinkage	[23]
EL-4 (thymoma)	DX		Shrinkage	[4]
		+ Anti-CD8	Growth	[4]
		+ Anti-CD4	Moderate growth	[4]
		+ Anti-NK1.1	Shrinkage	[4]
CT26 (colon cancer) in wild type	DX		Shrinkage	[8]
		+ z-VAD-fmk	Growth	[8]
CT26 in nude	DX		Growth	[8]
CT26 in CD11c-DTR	DX		Shrinkage	[8]
		+ DT	Growth	[8]
CT26	DX		Shrinkage	[9]
		+ siRNA CRT	Growth	[9]
	Mitomycin C		Growth	[9]
		+ CRT	Shrinkage	[9]
		+ inh PP1/GADD34	Shrinkage	[9]
CT26 in *tlr4*$^{-/-}$	DX		Growth	[10]
CT26 in wild-type MCA-205 (fibrosarcoma)	DX		Shrinkage	[10]
		+ siRNA HMGB1	Growth	[10]
		+ anti-HMGB1	Growth	[10]

Abbreviations: CRT, calreticulin; DT, diphtheria toxin; DTR, DT receptor; DX, doxorubicin; HMGB1, high-mobility-group box 1; inh PP1/GADD34, inhibitors of protein phosphatase 1/growth arrest and DNA-damage inducible protein 34; LAK, lymphokine-activated killer; siRNA, small interfering ribonucleic acid; TBI, total body irradiation; TLR4, Toll-like receptor 4; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

in the 1990s (Table 2). This concept paved the way for combination therapies whereby DX was administered with adoptive cell transfer of LAK [23] or recombinant interleukin (IL)-2 [4,24,25], tumor necrosis factor (TNF)-α [5,6] or IL-12 [26]. In these experiments, a role for $CD8^+$ T cells (but not NK cells) and/or a correlation between anti-tumor CTL activities and tumor regression or long-term survival was demonstrated. Interestingly, tumor cells had to be sensitive to DX-induced cell death but not necessarily to TNF-α-induced cell death *in vitro* for an immune response to take place [5].

Some clinical trials also reported encouraging results against soft-tissue sarcoma and non-Hodgkin's lymphoma when DX was used in combination with IL-2 and interferon-α (IFNα), respectively [27–29]. Epirubicin combined with IFNα2b or bacille Calmette-Guérin (BCG; a mycobacterium with anti-tumor effects) could be valuable in the treatment of transitional cell carcinoma of the urinary bladder [30–32]. However, these treatments have often been limited by the toxicity of these immunomodulators [27] and/or the poor therapeutic index [33]. These conclusions prompted a search for the mechanisms underlying the immunogenicity of anthracyclines to ameliorate the design of combinatorial immunochemotherapies of cancer.

The cellular and molecular components of anthracycline immunogenicity

Immunogenic versus non-immunogenic cell death

Millions of cells die every day within our body through apoptosis without eliciting an immune response, indicating that apoptosis is a non-immunogenic cell-death process. By contrast, necrosis, an unprogrammed form of cell death, leads to the release of cellular content into the extracellular space, which probably elicits an immune response [34–37]. This sharp dichotomy regarding the immunogenicity of apoptosis and necrosis was questioned by some studies that pointed out that, rather than the type of cell death, the tumor cells themselves or the nature of the death-inducing stimulus influences the immunogenicity of dying cells [38,39] (Box 2).

The knowledge that some cytotoxic agents can selectively exert an immunomodulatory effect [34,40] led to the hypothesis that the type of cellular demise induced by chemotherapeutic agents could account for their immunogenicity. Thus, Casares and colleagues [8] undertook a comparison of the immunogenicity of different classes of cell-death inducers using a vaccination setting in the mouse colon cancer CT26 model. Various compounds were tested such as those targeting endoplasmic reticulum (ER) (i.e. thapsigargin, tunicamycin and brefeldin), lysosomes (i.e. bafilomycin A1), mitochondria (i.e. arsenite, betulinic acid and C2 ceramide), proteasome (i.e. MG132, lactacystin and ALLN), nuclear factor-κB (i.e. Bay 11–7082), or those causing DNA damage (Hoechst 33342). None of these drugs was found to elicit an immunogenic cell death in this model. However, anthracyclines such as DX or idarubicin (but not mitomycin C or etoposide, which are as cytotoxic as DX or idarubicin *in vitro*) could elicit immunogenic cell death *in vivo* [8].

The mechanisms underlying the immunogenicity of anthracyclines in CT26 bearing hosts were then investigated. First, using athymic nude mice (devoid of T lymphocytes) and diphtheria toxin receptor (DTR) transgenic animals expressing DTR under the control of the CD11c promoter (which permits DT-induced ablation of DC), the crucial role of T cells and DCs in the immunogenicity of DX-treated CT26 [41] was demonstrated. Second, by pre-treating CT26 with the pan-caspase inhibitor Z-VAD-fmk during the exposure to DX, it was reported that caspases are crucial for an anti-tumor immune response to take place. Surprisingly, caspases are required not for the maturation of DC following contact with DX-treated CT26 tumor cells but for the uptake of the apoptotic bodies by DC. Indeed, DX-treated CT26, but not CT26 exposed to mitomycin C or etoposide, could be efficiently phagocytosed by myeloid DC both *in vitro* and *in vivo*. These data indicated that phagocytosis of dying tumor cells by DCs constitutes a first checkpoint that accounts for the immunogenicity of anthracyclines.

Calreticulin exposure discriminates immunogenic from non-immunogenic cell death

Phagocytosis of DX-treated CT26 cells occurs within one hour following anthracycline exposure [9], indicating that the molecular events involved in the immunogenicity of DX are unlikely to result from transcription regulatory

Box 2. Immunogenic versus non-immunogenic cell death

Cancer cells react to cytotoxic agents either by differentiation (leading to proliferative arrest and apoptotic removal), proliferative arrest (which, in the case of senescence, is indefinite) or cell death. Tumor-cell demise often occurs through apoptosis, a stereotyped pattern of morphological changes that involves chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), shrinkage of the cytoplasm, blebbing of the plasma membrane and final disintegration of the cell into membrane-surrounded apoptotic bodies. These alterations cannot be monitored easily *in vivo* because dying tumor cells are engulfed by scavengers before they enter the late stages of the apoptotic process. In some cases, tumors can also die by necrosis (characterized by swelling of the cell and the cytoplasmic organelles before the plasma membrane ruptures and the cellular content is spilled extracellularly), by autophagy (sequestration of large portions of the cytoplasm in autophagic vacuoles, often before the cells undergo apoptosis) or as a result of mitotic catastrophe (defined as a multi- or micro-nucleation and/or mitotic arrest before apoptosis).

Although there are teleological arguments to consider that apoptosis must be non-immunogenic or even tolerogenic (to prevent autoimmunity) and although necrosis (a pathological feature) has been condemned as pro-inflammatory, there is no experimental verification that apoptosis = non-immunogenic, and necrosis = immunogenic. Conversely, it seems that apoptosis is non-uniform in biochemical terms. The apoptotic execution phase can involve a variable degree of caspase-dependent and caspase-independent catabolic reactions, meaning that similar morphologies might have been acquired through distinct biochemical routes. Various pathways can induce stimulus-specific changes (eventually leading to cell death) that can be identified by the composition of the proteome associated with the plasma membrane. Therefore, we anticipate that the classification of cell-death modalities will move from the mere description of morphological changes to biochemical and/or metabolic criteria. These parameters are awaited before unambiguously correlating immunogenicity with distinct cell-death pathways.

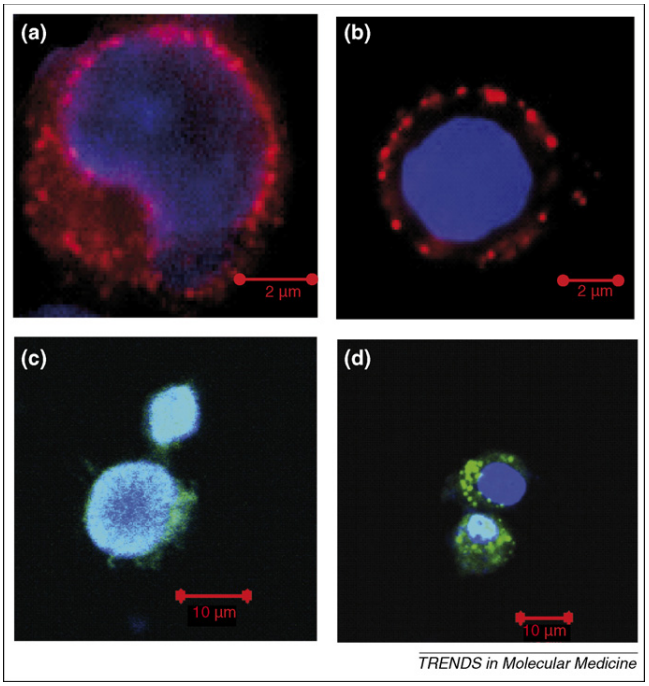


Figure 1. Visualizing calreticulin (CRT) and HMGB1 molecules before and after exposure to an anthracycline. (**a**) Confocal microscopy showing endoplasmic reticulum (ER) localization of CRT (red) in permeabilized Jurkat cells prior to mitoxantrone (MTX) treatment. (**b**) CRT translocation to cell surface. After exposure of this T-cell lymphoma cell line to MTX, CRT translocates from the ER to the plasma membrane in small patches. Chromatin appears in blue (DAPI) staining the nucleus (CRT is visualized with mouse monoclonal, clone fmc75, ab22683, AbCam, and AlexaFluor 568, Molecular Probes, Invitrogen, www.invitrogen.com). (**c**) Nuclear localization of HMGB1 (green) in permeabilized EL4 cells prior to MTX treatment. Nuclei are stained with DAPI (blue). Co-localization of DAPI staining and MHGB1 is represented by lighter-blue staining. (**d**) Nuclear exodus of HMGB1 after MTX treatment. (HMGB1 is visualized with ab18256, AbCam and Alexa Fluor 488, Molecular Probes, Invitrogen).

processes. Therefore, Obeid and colleagues studied the modulations of protein expression on plasma membranes of tumor cells that were untreated or treated with DX, with or without Z-VAD-fmk, using 2-dimensional gel electrophoresis coupled to mass spectrometry analyses. Calreticulin (CRT) was identified as the major protein component that is selectively translocated to the plasma-membrane surface within hours following exposure of a variety of different tumor cells to DX [9] (Figure 1). CRT is a Ca^{2+}-binding lectin chaperone that is mostly present in the ER lumen. Indeed, CRT is frequently used as an ER-specific marker in subcellular localization studies [42,43]. However, CRT can also appear on the surface of dying cells (Figure 1a,b), serving as an 'eat me' signal for adjacent phagocytes [44]. In contrast to the standard kinetics of CRT translocation to the plasma membrane, which parallels that of phosphatidylserine present on the cell surface [44], Obeid *et al.* [9] found that anthracyclines elicit CRT exposure with very rapid kinetics. Indeed, CRT exposure occurred within minutes after addition of anthracyclines, whereas phosphatidylserine exposure occurred only after several hours of treatment.

This result was validated using functional analyses. First, Obeid *et al.* [9] correlated the surface expression of CRT (as assessed by flow cytometry or confocal microscopy) to the capacity of myeloid DC to uptake dying tumor cells *in vitro*, and subsequently to the ability of DX (or other drugs)-induced CT26 to mediate a protective anti-tumor immune response. Second, tumor cells transfected with a small interfering RNA (siRNA) targeting CRT and exposed to DX failed to elicit T-cell priming *in vivo*, but still died. Finally, tumor cells killed by a non-immunogenic cell-death inducer (such as mitomycin C or etoposide; i.e. an agent that fails to induce CRT translocation to the tumor plasma membrane) were rendered immunogenic via the adsorption of recombinant CRT onto the tumor-cell surface [9].

Further experiments revealed that enucleated cells (cytoplasts) treated with anthracyclines translocated CRT to the cell surface as well as intact cells, indicating that the target of anthracyclines is cytoplasmic [9]. Anthracyclines were then shown to induce the phosphorylation of the eukaryotic translation initiation factor eIF2α [9]. Inhibition of the phosphatase that dephosphorylates eIF2α [which is composed of a catalytic subunit, protein phosphatase 1 (PP1) and the adaptor protein GADD34] led to the hyperphosphorylation of eIF2α and efficiently induced the surface exposure of CRT. Chemical inhibitors of PP1 (such as tautomycin or calyculin A) or of the PP1–GADD34 complex (such as salubrinal [9]) induced CRT exposure on the cell surface without any major cytotoxic effects [9].

Because increased CRT expression on the plasma membrane might elicit phagocytosis of tumor cells by DC and thus render tumor-cell death immunogenic, inhibitors of the PP1–GADD34 complex have been implemented in the

treatment of established tumors. When these inhibitors are injected into day six established CT26 together with a non-immunogenic cell-death inducer (i.e. etoposide or mitomycin C) administered intravenously, synergistic anti-tumor effects are observed only in immunocompetent animals (not in nude littermates) [9]. Similar results have also been reported in the fibrosarcoma model MCA205 [45]. CRT must be presented on the dying tumor cells and not on nearby living tumor cells to mediate its immunogenicity [45,46]. Therefore, CRT expressed on dying tumor cells is the first checkpoint of anthracycline-mediated immunogenicity *in vivo*, through elicitation of efficient phagocytosis of dying tumor cells by host DC (Figure 2). Similarly, it has been discovered that bortezomib-induced heat shock protein HSP90 on myeloma cells is a prerequisite for DC-mediated T-cell activation [47].

HMGB1–TLR4 interaction: a second checkpoint to yield immunogenic cell death

In the aforementioned CT26 tumor model, the addition of recombinant CRT fails to enhance the immunogenicity of live tumor cells *in vivo* [46]. Because exposure to anthracyclines induces tumor-cell death, it was assumed that the release of signals from dying tumor cells might account for their immunogenicity [48]. Although widely recognized for their role in sensing pathogen-associated molecular patterns [49,50], TLR might also be involved in the early detection of endogenous signals released by stressed or damaged cells [51]. Therefore, the hypothesis that dying tumor cells might trigger TLR-dependent activating pathways was proposed [10]. A wide and systematic screening of the TLRs implicated in the capacity of CT26 dying tumor cells (colon carcinoma cells treated with DX) to elicit the priming of naïve T cells *in vivo*, led to the conclusion that the immunogenicity of anthracyclines is exclusively dependent on an intact TLR4–MyD88 signaling pathway. Indeed, using TLR4-deficient hosts and an inhibitory peptide that interferes with TLR4 function, TLR4 was shown to be required for efficient cross-presentation of apoptotic tumors by DCs to T-cells *in vitro* and *in vivo* [10,52]. However, TLR4-deficient DCs are still capable of processing and presenting soluble antigens, indicating

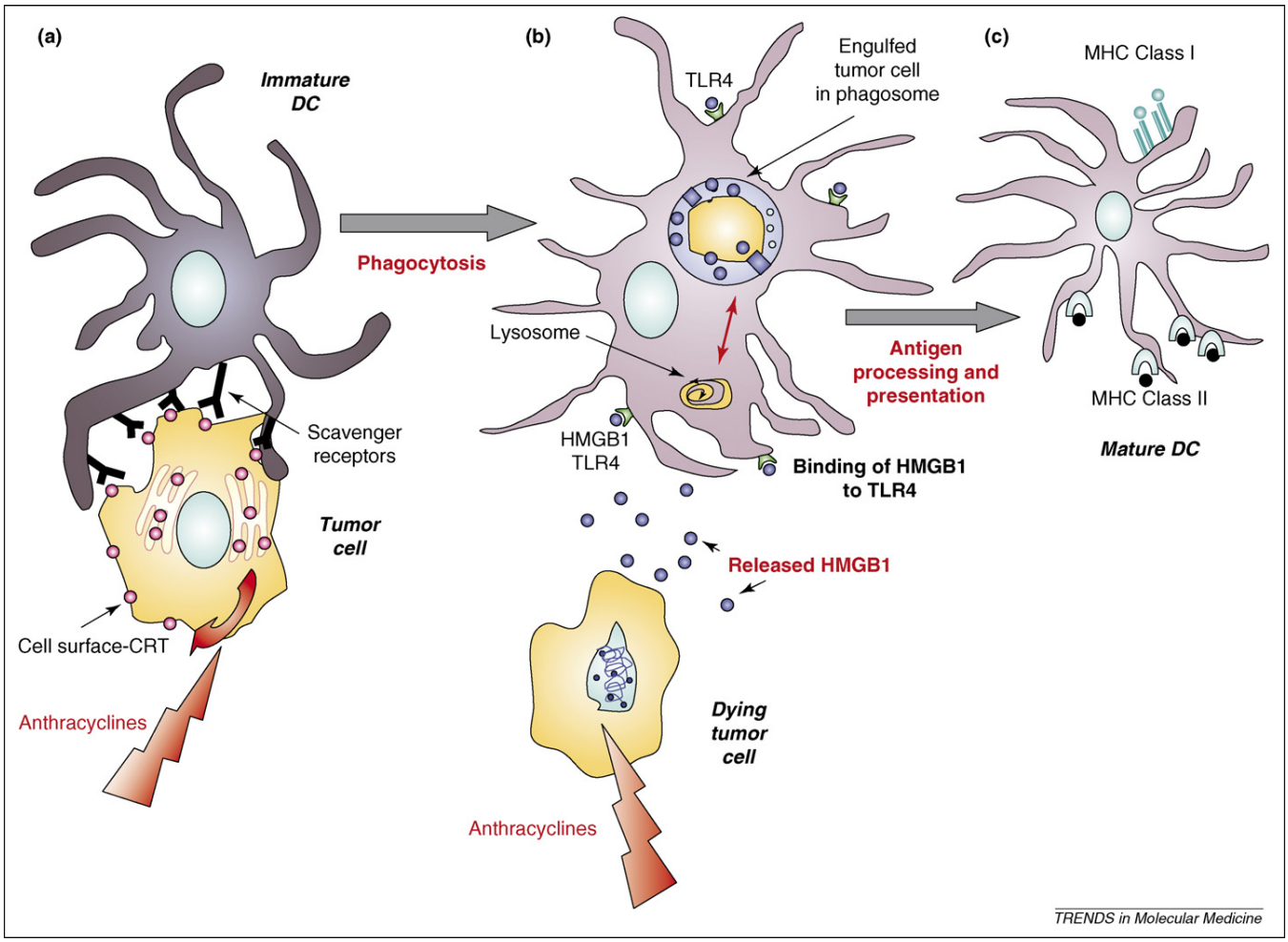


Figure 2. Progression of DC-recognizing anthracycline-treated tumor cells. (**a**) Anthracycline-treated mouse or human tumor cells can readily translocate ER-resident calreticulin (CRT) to the cell surface within 30 min to 1 h after exposure to the drug. The recognition of CRT on the tumor-cell surface by conventional immature DC through scavenger receptors (as yet uncharacterized) is a mandatory step for the engulfment (most likely phagocytosis) of dying tumor cells and the elicitation of an anti-tumor immune response [9,46]. However, DC loaded with their phagocytic cargo require additional signals to process the antigens and to undergo final maturation, which is necessary for productive T-cell priming. (**b**) The alarmin HMGB1, a chromatin-binding non-histone protein with the dual function of generating inflammatory signals once located in the extracellular milieu, is passively (and/or actively) released during anthracycline-mediated tumor-cell stress or death (after 18 h of exposure to the drug *in vitro*). HMGB1 binds to TLR4 expressed on DC, modulating the dynamics of the fusion between phagosomes and lysosomes resulting in (**c**) the effective processing and presentation of antigens in MHC molecules and maturation of DC.

that TLR4 is involved in the processing machinery of membrane-associated antigens [10].

Based on a previous report claiming that TLR4 could inhibit the lysosome-dependent degradation of phagosomes in macrophages [53], Apetoh *et al.* compared the kinetics of fusion between phagosomes and endo-lysosomes in wild-type DC with that in TLR4-deficient DC after addition of dying tumor cells [10]. Under these conditions, TLR4 seems to prevent the accelerated routing of the phagocytic cargo to the lysosomes. In accordance with these data, antigen presentation by TLR4-deficient DCs *in vitro* could be fully restored using either chloroquine (a lysosomotropic alkaline) or bafilomycin A1 (a specific inhibitor of the vacuolar ATPase responsible for lysosomal acidification). Moreover, the concomitant treatment of tumor-bearing $Tlr4^{-/-}$ mice with a cytotoxic compound along with chloroquine could ameliorate the anti-tumor effects of the chemotherapy [10], a result that was not achieved in wild-type littermates.

These findings indicate that an endogenous ligand released from dying tumor cells binds to TLR4 on host DCs and mediates the immunogenicity of DX-exposed tumor cells (Figure 2). Different putative ligands have been reported for TLR4 [54,55], including β defensin 2, heat shock proteins, fibrinogen, fibronectin and high-mobility-group Box 1 (HMGB1). HMGB1 is a non-histone chromatin-binding protein that functions as a transcription factor (but it can also be released upon cellular demise – necrosis rather than apoptosis) and that stimulates antigen-presenting cells [56,57]. Therefore, HMGB1 has been considered to be an alarmin or a damage-associated molecular pattern (DAMP) [58] molecule. In line with this concept, it was shown that treatment of CT26 cells by DX selectively induces the release of HMGB1 (Figure 1c,d) *in vitro* but not that of alternate TLR4 ligands [10]. Similar findings were obtained during the treatment of MCA205 fibrosarcoma with DX. It is noteworthy that HMGB1 release occurred within 18 h following exposure to anthracyclines (or other cytotoxic agents) [10]. The results of immunoprecipitation experiments confirmed that HMGB1 effectively binds to TLR4 exposed on rat mastocyte Raw cells [10].

The role of HMGB1 in the TLR4-dependent immunogenicity of DX-treated CT26 (DX-CT26) has been investigated by reducing HMGB1 levels via specific siRNAs. The results revealed that HMGB1 is mandatory for the priming of naïve T cells by dying DX-CT26 cells and for conferring anti-tumor protective immunity against tumor rechallenge [10]. Moreover, anti-HMGB1 neutralizing antibody completely abrogates both DX-induced immunogenicity of cell death *in vivo* and cross-presentation of dying tumor cells to T-cell hybridoma *in vitro*. Intriguingly, neither CRT nor HMGB1 can account for the DC maturation phenotype induced by apoptotic tumor cells. This conclusion prompts the current search for additional signals emitted or ferried by tumor cells to DCs that could contribute to the polarization of the T-cell priming.

Prospects for clinical use of immunomodulatory properties of anthracyclines

Challenging the notion that chemotherapy or radiotherapy negatively affects the immune system, accumulating evidence (from the 1970s to date) indicates that cell death can induce an immunological cascade that contributes to the anti-tumor effects of conventional cytotoxic agents. The immune system effectors are elicited in three different ways by conventional therapies. Some therapeutic regimens (such as DX, but also oxaliplatin and X-rays [10]) can elicit specific cellular responses that render tumor-cell death immunogenic. Other drugs might have side effects that stimulate the immune system, by transient lymphodepletion (such as high dosing of alkylating agents and fludarabine), by the subversion of immunosuppressive mechanisms (such as metronomic cyclophosphamide, which inhibits regulatory T cells [59], or *all trans* retinoic acid [60] or gemcitabine [61], both of which modulate myeloid suppressor cells), or by direct or indirect stimulatory effects on immune effectors (such as flavonoids, which modify chemokine release [62], or androgen deprivation, which induces B-cell activation and thymopoiesis leading to increased T-cell repertoire, i.e. an augmented number of anti-tumor CTL clones [63]). Moreover, some treatments (such as fluorouracil or X-rays) can sensitize tumor cells to CTL and NK cell attack by modulating the surface expression of tumor-associated antigens [64], Fas [65] and/or NKG2D (natural-killer group 2, member D) ligands [66]. Vaccination against cancer-specific antigens could also sensitize the glioblastoma tumors to subsequent chemotherapeutic treatment [40].

The data described in this review enable formulation of the following predictions for optimization of the clinical use of anthracyclines. First, the potential immunosuppressive effects of some current clinical methods of cancer management should be considered. Systematic ablation of lymph nodes for staging might be discussed when considering that tumor-draining lymph nodes are the primary sites of T-cell education. The dose-intensity scheduling of most chemotherapy is associated with iterative lymphodepletion and might compromise the establishment of T-cell priming. Thus, for instance in breast cancer, low doses of anthracyclines on a weekly basis in the absence of glucocorticoids (used as anti-vomiting agents but notoriously immunosuppressive) could be assessed in randomized trials aimed at monitoring the ensuing immune response.

Second, neoadjuvant chemotherapy (as opposed to adjuvant chemotherapy) of early (non-advanced) cancers would offer the advantage that more tumor antigen would become available for the priming of T cells.

Third, prospective trials should be designed so that serial tumor biopsies, performed before and after chemotherapy, are evaluated for macrophage-, DC-, T- and NK-cell responses as putative prognostic factors. Should the anti-tumor immune responses dictate long-term survival, then local signs of antigen priming (presence of mature DC in a pseudolymphoid architecture) or effector cells with a Th1 or Tc1 phenotype might correlate with favorable responses. Preferably, such data should be matched to gene-expression profiling in tumor beds (either static before therapy or dynamic following one cycle of chemotherapy) that might indicate immune signatures associated with favorable clinical outcome or response to treatment (as shown in follicular lymphoma [67]). Along these lines, a correlation between pathological

complete response to neoadjuvant chemotherapy with therapy-induced high $CD8^+$ effector T lymphocyte and low regulatory T-cell infiltrates has recently been reported in a series of 56 advanced breast cancers [68].

Last, there are direct diagnostic and therapeutic consequences of 'immunogenic cell death'. From a biotechnological point of view, designing of a drug-screening program aimed at selecting products capable of translocating CRT to the plasma membrane of tumor cells could be imagined. Indeed, some, but not all, chemotherapeutic regimens can induce immunogenic cell death with early CRT expression, thus eliciting a therapy-associated anticancer immune response that determines disease outcome. Furthermore, clinicians could perform an early detection of CRT exposure on circulating tumor cells or cytospins from tumor beds one or two days post-chemo- or radio-therapy to predict whether recombinant CRT or peptides inhibiting the PP1–GADD34 phosphatase complex should be combined with the therapeutic regimen to augment therapeutic success. As a result, it might be interesting to monitor not only CRT exposure but also the presence of all factors that are mandatory for the signal-transduction machinery that leads to CRT exposure in tumor cells, with the hope of establishing new prognostic or predictive biomarkers. Therapeutic regimens designed to re-establish CRT exposure (for instance, by inhibiting PP1–GADD34) should enhance the immunogenicity of cell death and, hence, boost the therapeutic efficacy of non-immunogenic regimens *per se*.

In addition, hosts bearing an inherited defect in the *tlr4* gene or an acquired TLR4 dysfunction [69] might not respond to conventional 'immunogenic-prone' regimen. Indeed, a polymorphism in human TLR4 (rs4986790) has been associated with decreased responses to inhaled lipopolysaccharide [70]. This single-nucleotide exchange (A896G) in the *TLR4* gene results in an amino acid substitution (Asp299 → Gly) in the extracellular domain of TLR4. It was also demonstrated that this substitution not only decreases the binding of HMGB1 to TLR4 but also results in weaker activation of the transcription factor nuclear factor-κB (L.A., unpublished) and in a profound alteration of the capacity of monocyte-derived-human DC to cross-present melanoma tumor antigens from dying melanoma cell lines [10]. In a retrospective study, time to metastases was analyzed in a cohort of 280 patients that had been treated for breast cancer presenting with lymph-node involvement following local radiotherapy and an anthracycline-based adjuvant chemotherapy. Patients bearing the *TLR4* Asp299 → Gly allele featured an accelerated disease course compared with patients bearing the normal *TLR4* allele, establishing *TLR4* Asp299 → Gly as an independent predictive factor of early disease progression [10]. These data await further confirmation in various cancer models that have been treated with specific cytotoxic combinations before envisioning treating TLR4-deficient patients with chloroquine in prospective trials.

Taking into account the considerations discussed here, the ideal management of patients with advanced cancer might integrate the following steps: (i) inhibition of tumor-induced immunosuppression [71]; (ii) priming of naïve T cells with vaccines; (iii) induction of tumor-cell death with conventional or more recent therapies (i.e. chemo-, radio- or hormone-antibody therapies, or tyrosine kinase inhibitors); (iv) turning non-immunogenic into immunogenic cell death [i.e. using recombinant calreticulin (rCRT) or inhibition of the PP1–GADD34 complex]; (v) compensation (i.e. using chloroquine treatment) for TLR4 defects or host defects (i.e. polymorphisms in molecular mechanisms that elicit immune responses); (vi) boosting ongoing effector or memory immune responses [e.g. treatment with glycolipids, cytokines, chemokines, anti-KIR (killer inhibitory receptor) antibody, sTRAIL (soluble TNF-related apoptosis inducing ligand), IL-18BP (interleukin-18 binding protein)] [40,72]. This integrated strategy should be evaluated in sizeable randomized trials in the near future.

Concluding remarks

Anthracyclines are thought to mediate their tumoricidal activity by exerting a direct effect on tumor cells, but recent evidence indicates that the host immune system contributes to the anti-tumor activity greatly (Box 3). Indeed, anthracyclines (e.g. daunorubicin, idarubicin and mitoxantrone) promote cross-talk between the dying tumor cells and DC, leading to DC-mediated T-cell priming and the contribution of T cells to long-term survival in experimental tumor models. We have shown that anthracyclines, and also oxaliplatin and X-rays, induce a series of molecular events in tumor cells that can be sensed by DC [10]. First, by inducing the phosphorylation of eIF2α, anthracyclines trigger the rapid translocation of the ER-resident CRT to the plasma membrane of tumor cells, thereby facilitating the uptake of the dying cell by DC. This first step can be elicited by administration of recombinant CRT or by inhibiting the phosphatase complex PP1–GADD34 when tumors and/or treatment fail to promote membrane CRT expression. Second, anthracycline-mediated cell death (apoptosis and necrosis at later stages) induces the release of the chromatin-binding protein HMGB1 18 h after the drug-induced stress *in vitro*. HMGB1 is mandatory to

Box 3. Outstanding questions

- Which of the current drugs used in the clinical armamentarium of oncology can be considered 'immunogenic'? And what are the molecular mechanisms involved in the immunogenicity of cell death induced by such drugs?
- If a drug turns out to mediate immune responses, how should the scheduling and dosing of the current regimen be modified to avoid compromising this immunity? How might immunotherapy synergize (or antagonize) with the ongoing immune response?
- Which tumors lack the molecular machinery leading to CRT exposure and/or HMGB1 secretion? Is there a routine assay that could test these relevant molecular pathways before and during treatment?
- How should the impact of development of an immune response on clinical outcome be demonstrated during the course of conventional cancer therapy?
- How should the prognostic value of host polymorphisms in immune-response genes (i.e. *TLR4*) be shown in the response to anthracyclines, oxaliplatin and X rays?
- Is there any increased risk in developing autoimmune disorders when optimizing the immunogenicity of anthracyclines, specifically in increasing cardiotoxicity through auto-immune reactivities?

mediate the immunogenicity of anthracyclines by binding to host TLR4. DC are required for the response to HMGB1 and, upon TLR4 triggering, they are endowed with antigen-processing capacities by regulating vesicular dynamics of the compartments involved in antigen presentation. We have shown that the lysosomotropic chloroquine can convert a TLR4-deficient DC (mouse and human) into an efficient antigen-presenting cell capable of cross-presentation to CTL. TLR4 loss-of-function animals are unable to mount an efficient anti-tumor immune response following treatment with anthracyclines unless chloroquine is co-administered in the schedule. The relevance of these findings culminates in the validation of a prognostic role of the Asp299 → Gly TLR4 polymorphism in breast cancer patients treated with adjuvant anthracyclines [FEC (5-fluorouracil, epirubicin, cyclophosphamide) protocol; see Box 1]. The Asp299 → Gly TLR4 loss-of-function mutation, which is carried by 12% of all Caucasians, prevents the effective binding of HMGB1 to TLR4, and might account for the shorter disease-free survival observed in this subset of women after adjuvant FEC. Prospective studies will be launched at the Institut Gustave Roussy (www.igr.fr) to (i) show the prognostic value of CRT exposure in the pathological response to neoadjuvant anthracycline-based therapy in breast cancer and (ii) demonstrate the beneficial effects of combining chloroquine with anthracyclines in Asp299 → Gly TLR4 carriers. These findings lead the way towards development of new therapeutics that combine chemo-and immuno-therapy, taking into account the tumor-host-drug relationship to tailor-make optimal therapeutic regimen for cancer treatment.

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