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HIRZ Taghreed

TITRE

Neutrophiles polymorphonucléaires et cancer: L'impact des neutrophiles sur la sensibilité des cellules de lymphome B aux thérapies anti-cancéreuses

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RESUME en français

Alors que le rôle des cellules du système immunitaires innées sur la progression tumorale est l'objet d'une investigation croissante, le rôle des neutrophiles sur la sensibilité à la thérapie n'a pas été précédemment décrit. Jusqu'au présent, nous avons effectué des cocultures de neutrophiles et des différentes lignées cellulaires de lymphome non hodgkinien (LNH) en présence de divers agents cytotoxiques ou des thérapies ciblées. Afin d'évaluer l'effet du traitement sur la prolifération cellulaire et la mort des cellules, des marquages CFSE et DAPI ont été effectués respectivement, en utilisant la cytométrie en flux. Les neutrophiles ainsi que les cellules HL60 différenciées avec des propriétés de neutrophiles, ont atténué la sensibilité de cellules de lymphome à des agents anticancéreux in vitro, à la fois dans les modèles 2D et 3D. L'effet protecteur des neutrophiles a été testée in vivo en injectant des cellules de LNH et des neutrophiles chez des souris SCID/CB17 traités avec vincristine. La coinjection de neutrophiles réduit la sensibilité des cellules LNH à la chimiothérapie. Cet effet protecteur a été validé en utilisant des cellules primaires, purifiée à partir de patients atteints de leucémie lymphoïde chronique, exposés à des agents cytotoxiques ou des agents ciblés en présence de neutrophiles autologues. La protection par les neutrophiles est contact dépendante. Elle est médiée par l'interaction de CD11b et ICAM1, exprimé par les neutrophiles et les lymphocytes B, respectivement, et par la molécule d'adhésion CD44. Elle est également dépendante de Mcl1 et est partiellement abrogée par un composé anti-Mcl1.

TITRE en anglais

Polymorphonuclear neutrophils and cancer: The impact of neutrophils on the sensitivity of lymphoma B cells to cancer therapy

RESUME en anglais

While the role of innate immune cells on tumor progression is the object of increasing scrutiny, the role of neutrophils on sensitivity to therapy has not been previously described. To this end, we performed cocultures of freshly purified human neutrophils and different non-Hodgkin lymphoma (NHL) cell lines in the presence of various cytotoxic and targeted agents. CFSE and DAPI assays were performed to assess the therapeutic effect on cell proliferation and cell death, respectively, using flow cytometry. Neutrophils and differentiated HL60 cells with neutrophil-like properties attenuated the sensitivity of lymphoma cells to anti-cancer agents both in 2D and 3D models in vitro. The protective effect of neutrophils was tested in vivo using SCID/CB17 mice inoculated with NHL cells together with neutrophils, and treated with vincristine. The co-inoculation of neutrophils reduced the sensitivity of NHL cells to chemotherapy. Similar findings were made on primary cells, purified from patients diagnosed with chronic lymphocytic leukemia, exposed to cytotoxic agents or recently approved targeted agents (ibrutinib and idelalisib) in the presence of autologous neutrophils. Neutrophil-induced protection was dependent on cell-cell contact mediated by the interaction of CD11b and ICAM-1, expressed by neutrophils and B cells respectively, and by the adhesion molecule CD44. This protective effect was Mcl-1-dependent and was partially abrogated by an anti-Mcl-1 compound.

DISCIPLINE : Cancérologie

KEYWORDS/MOTS-CLES

Neutrophils, lymphoma, chemotherapy, targeted therapy, cell-cell interactions

Neutrophiles, lymphome, chimiothérapie, thérapie ciblée, interactions cellulaires

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Un résumé substantiel en français:

Chapitre I

1. Introduction

Le système immunitaire est un système complexe, mais bien organisé, qui est composé d'un réseau de cellules, des tissus et des organes qui permet d'identifier et d'éliminer les envahisseurs potentiellement nocifs tels que les bactéries, les virus, les champignons ou des parasites, ainsi que des objets endogènes tels que des complexes immuns ou des cellules transformées. Cette défense est divisée en deux types généraux de réactions: réactions de l'immunité innée et de l'immunité adaptative. En général, l'immunité innée est considérée comme une réponse non spécifique, alors que le système immunitaire adaptatif est considéré comme étant très spécifique. En outre, les récepteurs d'antigène de la réponse immunitaire adaptative sont généralement considérés comme «maître» dont leur engagement dicte la fonction des lymphocytes. Les cellules du système immunitaire sont généralement régulé par des hormones de croissance et la promotion ou la suppression de signaux, mais ils sont tous vulnérables aux erreurs, qui pourraient conduire à la transformation maligne.

Le cancer est une classe de maladies caractérisées par une division incontrôlée des cellules et la capacité des cellules à envahir les tissus normaux et des dommages que ce soit localement ou au site distant du corps. Un lymphome malin survient quand un seul lymphocyte B ou T est arrêté dans une étape spécifique de la différenciation cellulaire conduisant à la transformation maligne et d'expression clonale. Tumeurs malignes de cellules hématopoïétiques ont été divisés en hémopathies myéloïdes malignes, lymphoïdes et. Les lymphomes malignes comprend plus de 67 sous-types des cancers liés qui affectent le système lymphatique; La maladie de Hodgkin compte pour environ 10% de tous les lymphomes et les 90% restants sont désignés comme les lymphomes non hodgkiniens (LNH).

Chapitre II

2. Le lymphome non hodgkinien

2.1. Définition

Lymphome non hodgkinien (LNH) est un terme collectif pour un groupe hétérogène de pathologies malignes lymphoprolifératives avec différents modes de comportement et des réponses au traitement. LNH est le dixième néoplasme le plus fréquemment diagnostiqué dans le monde et occupe le septième rang dans les pays développés. Plus de 65 000 cas de LNH sont diagnostiqués chaque année aux États-Unis, et 85% de ceux-ci se développent à partir des cellules B.

2.2. Biologie des lymphomagenèse

La plupart des tumeurs malignes lymphoïdes sont dérivées de lymphocytes B à divers stades de différenciation. Deux types de tissu lymphoïde existent: central (moelle osseuse et du thymus) et périphérique (sang, la rate, les ganglions lymphatiques, et associé aux muqueuses).

Pour comprendre les mécanismes par lesquels les lymphomes pourraient développer, les événements qui se produisent au cours de la maturation des cellules B normales doivent être considérés.

Au cours du développement des cellules B normales, les cellules proviennent de tissus lymphoïdes centraux où des précurseurs de cellules B subissent un réarrangement de la chaîne lourde et légère des immunoglobulines (Ig) et sont équipés d'un récepteur d'antigène de surface fonctionnelle. Ce réarrangement est activé par des enzymes qui provoquent des cassures de l'ADN double brin. Dans les cellules normales, les processus de réparation d'ADN sont activés, mais de telles ruptures peuvent contribuer à des translocations chromosomiques dans le lymphome qui résultent typiquement de l'activation du proto-oncogène.

Les descendants de ces cellules précurseurs, appelées cellules B naïves, migrent dans les tissus lymphoïdes périphériques. Les cellules B naïves subissent une expansion clonale dans les centres germinatifs (GC), qui se trouvent dans le cortex des ganglions lymphatiques. Dans la GC ganglionnaire, les gènes Ig sont encore modifiés par hypermutation somatique, un processus par lequel les cellules subissent des mutations rapides et une recombinaison de commutation.

2.3. Le traitement de la LNH

Le traitement du NHL est dans la plupart des cas une combinaison de modalités multiples, y compris la chimiothérapie cytotoxique conventionnelle, l'immunothérapie et / ou de petites molécules thérapeutiques ciblées.

2.3.1. Chimiothérapie

La chimiothérapie est un traitement systémique utilisant des médicaments anti-cancéreux qui sont donnés par voie intraveineuse ou par voie orale. C'est le principal traitement de la LNH. La chimiothérapie est administrée de façon répétée (cycles). Le protocole de chimiothérapie utilisé dépend du stade et du type de LNH. La combinaison de la chimiothérapie la plus courante pour le traitement initial est appelée CHOP et contient quatre médicaments: cyclophosphamide, hydroxyle daunomycine (doxorubicine), Oncovin® (vincristine et prednisone). Les patients atteints de lymphome agressif de mauvais pronostic peuvent subir un traitement de chimiothérapie à haute dose suivie de cellules souches autologues ou de la transplantation de moelle osseuse.

2.3.2. Immunothérapie

L'immunothérapie est utilisée pour améliorer la cible, ou de restaurer la fonction du système immunitaire. Les anticorps monoclonaux sont des immunothérapies et l'interféron qui ont été largement utilisés comme traitements pour les différents sous-types de LNH.

2.3.3. Inhibiteurs à petites molécules ciblées

Au cours des dernières années, il y a eu une augmentation substantielle du nombre de petites molécules ciblées entrant dans le développement clinique ou approuvées dans les lymphomes. Des inhibiteurs à petites molécules typiquement interrompent les processus cellulaires en interférant avec la signalisation intracellulaire des enzymes spécifiques. Ces enzymes initient une cascade moléculaire qui peut conduire à la croissance cellulaire, la prolifération, la migration et l'angiogenèse dans les tissus normaux et malins.

Chapitre III

3. Le microenvironnement tumoral de LNH

3.1. Vue d'ensemble

Une avancée considérable dans la compréhension de la pathogenèse ou la progression du lymphome a été réalisée grâce à la dissection moléculaire des cellules malignes, qui a identifié des altérations génétiques et des dérèglements des voies intracellulaires. Cependant, l'importance du microenvironnement où les cellules malignes se posent et résident a été relativement négligé.

Il est connu depuis plus d'un siècle que les tumeurs comprennent non seulement les cellules cancéreuses, mais également une multitude de cellules non malignes et des composants de la matrice extracellulaire (ECM) qui constituent le micro-environnement de la tumeur. Il est devenu plus évident que les éléments du microenvironnement ne sont pas seulement de simples spectateurs de la réponse inflammatoire anti-tumorale de l'hôte, mais ils ont aussi un impact important sur la pathogenèse et la progression tumorale et / ou la suppression tumorale.

3.2. Rôles paradoxale du microenvironnement de la tumeur de la LNH

Le microenvironnement tumoral de NHL consiste en un nombre variable de cellules immunitaires, des cellules stromales, des vaisseaux sanguins et de la matrice extracellulaire. Une tumeur peut changer son microenvironnement, et le micro-environnement peut affecter la façon dont une tumeur se développe et se propage. Les modifications génétiques des cellules malignes et la mesure dans laquelle ces cellules restent dépendants de stimuli externes pour la survie, la prolifération et l'évasion immunitaire déterminent la composition et l'arrangement spatial du microenvironnement de la tumeur. L'interaction entre ces éléments aboutit à la large gamme de micro-environnements tumoraux entre les différents types de cellules de lymphome B.

3.3. Myéloïdes cellules suppressives

Cellules myéloïdes suppressives (MDSC) représentent une population hétérogène de cellules myéloïdes immunitaire immature qui sont produites à des niveaux élevés dans le cancer. Ils sont définis chez la souris, sur la base de l'expression des marqueurs de surface CD11b et Gr-1. La population Gr-1 + MDSC CD11b est constituée d'au moins deux sous-ensembles: granulocytaire (Ly6G +) et des cellules monocytaires (Ly6C +), éventuellement avec des propriétés immunosuppressives.

Bien qu'il soit facile de définir MDSC chez la souris à base de CD11b et Gr-1, les phénotypes de définir cette population chez l'homme sont très divergents dans les études employant différents types de tissus. Ainsi, l'absence de consensus dans la définition MDSCs rend difficile de quantifier cette population dans les échantillons de patients.

Différentes études ont montré que les MDSC sont fortement représentés dans les cancers et suppriment les cellules NK et T, soit par contact avec la cellule ou cytokines sécrétées. MDSC ont également été montré pour soutenir l'angiogenèse et la métastase des cellules tumorales

promouvoir. Ils ont été étudiés de manière intensive dans des tumeurs solides telles que le cancer du pancréas; Cependant, peu d'études ont été réalisées dans les hémopathies malignes.

Il y a accord substantiel sur l'activité immunosuppressive de la sous-ensemble MDSC monocyttaire. Cependant, il est toujours contrasté sur le rôle de la fraction granulocytaire. Alors que certains ont montré que les MDSC granulocytes ont immunosuppression des propriétés similaires à la fraction monocyttaire, d'autres ont signalé qu'ils sont moins immunosuppresseur.

3.4. Macrophages: un acteur clé dans microenvironnement de la tumeur

La majorité des tumeurs malignes contiennent de nombreux macrophages en tant que composant majeur du leucocyte hôte infiltrate. Ces macrophages sont appelés macrophages associés aux tumeurs recrutés dans la masse tumorale par des cytokines chimiotactiques produites principalement par la tumeur.

On a pensé tout d'abord que les macrophages tuent les cellules tumorales. Cependant, les preuves cliniques et expérimentales indiquent que, dans certains cas, les macrophages favorisent la progression de la malignité des tumeurs. Ce rôle paradoxal des macrophages dans le cancer trouve une explication dans leur plasticité fonctionnelle qui peut entraîner l'expression polarisée de fonctions soit pro ou anti-tumorales. Les principaux acteurs dans le cadre de leur phénotype sont les signaux de micro-environnement auxquelles les macrophages sont exposés, qui sélectionne leurs fonctions au sein d'un spectre fonctionnel englobant les extrêmes M1 et M2.

CHAPITRE IV

4. Les neutrophiles: Cendrillon du système immunitaire inné

4.1. Définition

Les neutrophiles sont la population leucocytaire prédominant dans le sang humain, ce qui représente 50 à 70% des leucocytes circulants. Les neutrophiles sont caractérisés classiquement par leur aptitude à agir en tant que cellules phagocytaires, pour libérer enzymes lytiques de leurs granules et pour produire des intermédiaires réactifs de l'oxygène (ROI) ayant un potentiel antimicrobien. Les neutrophiles peuvent également être induits pour exprimer des gènes codant pour des médiateurs inflammatoires clés, y compris les composants du complément, des récepteurs Fc, des chimiokines et des cytokines.

Bien que les neutrophiles sont traditionnellement considérés dans le contexte de leurs fonctions antibactériennes, il devient de plus en plus clair que les TAN et de leurs précurseurs myéloïdes (MDSC de granulocytes) dans la rate, la moelle osseuse et le sang jouent un rôle important dans la biologie du cancer.

4.2. Les neutrophiles et les cancers

4.2.1. Vue d'ensemble

Les neutrophiles constituent une partie importante de l'infiltrat de cellules inflammatoires dans de nombreux modèles de cancer. Comme tous les autres leucocytes, ils se déplacent dans des tissus sous l'influence des cytokines, des chimiokines spécifiques et des molécules d'adhésion cellulaire libérées par le microenvironnement tumoral. Ils ont été vus *in vivo* en association étroite avec les cellules tumorales.

En plus du rôle bien décrit des neutrophiles d'engloutir les bactéries, d'activer le système immunitaire et d'induire des lésions tissulaires dans les infections, il devient clair que les neutrophiles ont un rôle principal dans le cancer. Cependant, le rôle des neutrophiles dans le cancer reste l'objet de controverses. Récemment, les neutrophiles ont été décrits pour posséder une activité de protumor, désignée sous le phénotype N2 par analogie aux macrophages (décrits précédemment). Toutefois, dans certaines conditions, les neutrophiles peuvent exercer une activité anti-tumorale décrite comme le phénotype N1.

4.2.2. Neutrophiles polarisation

Plusieurs lignes de documents de preuve l'existence de deux phénotypes de neutrophiles: la N1 (N2) et antitumoraux (protumor) phénotypes. Cette plasticité des neutrophiles est régulée par des molécules dans le microenvironnement tumoral.

Le facteur de croissance transformant cytokine immunosuppressive (TGF) β induit les neutrophiles à acquérir un phénotype protumor N2 alors que l'IFN- β induit les neutrophiles à acquérir un phénotype tumoral N1. De plus, les neutrophiles expriment des récepteurs fonctionnels pour d'autres cytokines telles que l'IL-1 β , TNF- α (162) et l'IL-10. Ces cytokines sont connus pour agir sur les neutrophiles et il est bien établi que l'IL-1 β et TNF- α sont des cytokines pro-inflammatoires et immunostimulants, alors que l'IL-10 a la capacité d'atténuer l'inflammation et comme immunosuppresseur.

4.2.3. Double rôle de neutrophiles

Les interactions entre une tumeur et son micro-environnement environnant promouvoir des divers processus cellulaires sous-jacents moléculaires et de multiples étapes qui définissent la progression du cancer. Ces mesures comprennent prolifération des cellules tumorales, la néovascularisation de la croissance de la tumeur en outre, la migration locale et la croissance dans le nouvel environnement. Les neutrophiles sont associés et font des contributions fonctionnelles à plusieurs de ces différentes étapes. Cependant, une cytotoxicité, une apoptose des cellules tumorales, rejet de tumeur à médiation immunologique et de la mémoire immunitaire antitumorale peuvent contrecarrer la progression de la tumeur à chaque étape.

CHAPITRE V

5. Interactions entre la thérapie du cancer et le système immunitaire

5.1. Traitement du cancer: la réingénierie de l'immunité tumorale

La plupart des agents anticancéreux ont été initialement développés pour cibler les cellules tumorales. Dans cette perspective, les médicaments chimiothérapeutiques classiques ont été choisis pour leurs effets cytotoxiques directs contre les cellules cancéreuses hautement prolifératives. Ces agents, tels que les anthracyclines ou des agents d'alkylation de l'ADN sont associés à des effets toxiques significatifs et leur efficacité est limitée par l'apparition de clones de cellules tumorales résistantes aux médicaments. Merci à la compréhension croissante de la biologie des cellules cancéreuses au niveau moléculaire où les agents anticancéreux plus spécifiques ont été développés et désignés comme agents thérapeutiques ciblés. Les thérapies ciblées sont conçus pour inhiber voies oncogéniques essentiels pour la prolifération et la survie des cellules tumorales. Ils ont montré une efficacité thérapeutique comme agents simples; Cependant, elles sont limitées par l'acquisition de mutations secondaires qui donnent lieu à des clones de tumeurs résistantes aux médicaments et la rechute du patient.

Plusieurs études ont rapporté un effet d'agents anti-cancéreux sur microenvironnement tumoral qui peut modifier la réponse de la tumeur à la thérapie du cancer, ce qui entraîne dans la promotion ou l'inhibition de la progression tumorale. Par exemple, différentes études ont suggéré que la thérapie du cancer peut altérer les fonctions immunosuppressives des cellules immunosuppressives, les lymphocytes T principalement réglementaires (Treg), MDSC, ou DCS tolérogènes. Il a été bien établi que les tumeurs peuvent favoriser ces cellules immunosuppresseurs qui inhibent l'immunité anti-cancer et significativement compromettre l'efficacité des thérapies basées sur le système immunitaire. Leur élimination et / ou inactivation sont devenus un objectif thérapeutique important pour modifier le microenvironnement tumoral immunosuppresseur et découvrir l'immunité anti-cancer. D'autres études ont rapporté un effet de la thérapie du cancer sur la fonctionnalité des cellules NK, des macrophages et les cellules immunitaires adaptatives.

5.2. Impact du système immunitaire à la thérapie du cancer efficacité

Des populations de cellules immunitaires se sont révélées pour réguler la tumorigenèse, les métastases et la réponse aux agents anticancéreux. Discuté ci-dessous, l'effet de plusieurs cellules immunitaires sur l'efficacité des agents anti-cancéreux.

5.2.1. Système immunitaire inné

Différentes études ont été rapportées en ce qui concerne l'effet des sous-populations de cellules immunitaires innées sur le traitement du cancer efficacité.

Macrophages

Les macrophages représentent une sous-population de cellules de la plus étudiée innée immunitaire en réponse à la chimiothérapie. TAM ont été montrés pour augmenter l'activité thérapeutique de plusieurs types de médicaments chimiothérapeutiques. De nombreuses

études ont montré l'importance de FcR sur les macrophages pour la réponse aux thérapies ciblées à base d'anticorps par ADCC. Utilisation de différents modèles transplantables chez la souris knock-out qui manquent un ou plusieurs FcR, régression de la tumeur médiée par rituximab, anti-cytotoxique protéine de lymphocyte T associée (CTLA) -4, le trastuzumab, ainsi que d'autres anti-erb-B-2 récepteur tyrosine kinase 2 anticorps (erbB-2) est inversée. En revanche, certaines études ont indiqué que l'anticorps thérapeutique ciblant l'EGFR, cetuximab, induit un phénotype immunosuppresseur M2 dans les monocytes humains en culture avec des lignées cellulaires du cancer du côlon dans les résultats in vitro sur la production de la production d'IL-10 et de médiateurs favorisant les tumeurs VEGF.

MDSCs

Les niveaux MDSC sont en corrélation avec une charge tumorale élevée et un plus mauvais pronostic. En outre, l'inhibition ou l'épuisement des MDSC augmente l'activité des vaccins contre le cancer dans des modèles animaux.

5.3. Impact du système immunitaire à la thérapie du cancer efficacité dans NHL

Au-delà de la contribution des éléments du microenvironnement de la tumeur au lymphome prolifération, la survie et l'échappement immunitaire, il y a une preuve directe que les interactions entre les cellules tumorales et le microenvironnement de modifier la sensibilité à des agents cytotoxiques, qui affectent probablement les résultats des patients. Ces mécanismes soit impliquent un contact cellule-cellule ou sont dues à des facteurs solubles libérés dans le microenvironnement de la tumeur.

5.4. Réponse médiée par les neutrophiles à des agents anticancéreux

Les neutrophiles, un autre acteur clé dans le système immunitaire inné, a été étudiée beaucoup moins par rapport aux macrophages en ce qui concerne leur impact sur la réponse de cellules cancéreuses à des agents anti-cancéreux.

Études en corrélation la présence de CD11b + Ly6G + infiltrat de polynucléaires neutrophiles et de résistance au traitement anti-VEGF. Ils ont montré que l'activation oncogénique de la voie RAS favorise la résistance anti-VEGF due à Gr-1 + G-CSF recrutement induit par CD11b + neutrophiles. Ils ont également rapporté que RAS / RAF / MEK voie est actif de manière constitutive dans beaucoup de cellules cancéreuses qui produisent des niveaux élevés de G-CSF et que l'expression du facteur de transcription Ets2 est directement corrélée avec la production élevé du G-CSF. La production du G-CSF est en corrélation avec la mobilisation des neutrophiles qui favorise la résistance anti-VEGF. Un inhibiteur de MEK a réduit la production du G-CSF et de la mobilisation des neutrophiles qui conduit à une survie accrue chez des modèles animaux de cancer, y compris un modèle de souris transgénique de l'adénocarcinome du pancréas.

Le soutien clinique pour un rôle de neutrophiles en réponse à la chimiothérapie viens à partir d'observations dans une variété de patients atteints de cancer, comme le sein et patients atteints de CPNPC, où la neutropénie induite par la chimiothérapie est associée à un meilleur pronostic des patients. Par conséquent, une ciblage des neutrophiles ou leurs médiateurs peut agir en synergie avec des agents chimiothérapeutiques et d'augmenter leurs effets contre les tumeurs.

En revanche, certaines études ont montré que la déplétion des neutrophiles par Gr-1 anticorps altéré l'effet anti-cancer de cyclophosphamide dans un modèle de souris avec des tumeurs fibroblastiques embryonnaires. Mais depuis le GR-1 peut se lier à la fois le granulocytaire et fractions monocytiques, l'épuisement des monocytes peut avoir contribué à cet effet.

L'ensemble de ces études, en dépit de certaines données contradictoires, soulignent la diaphonie entre les cellules tumorales et le microenvironnement tumoral qui conduit à la modulation de la réponse tumorale à des agents anti-cancéreux. Cependant, le rôle des neutrophiles, des acteurs clés dans le système immunitaire inné, dans la régulation de la réponse de lymphome de traitement anti-cancer est inconnu.

Objectifs de l'étude

Les progrès récents dans les chimiothérapies conventionnelles, thérapies ciblées, et des anticorps thérapeutiques ont manifesté le potentiel thérapeutique énorme de traitement du cancer dans le traitement des patients. Cependant, l'efficacité de la thérapie du cancer est souvent limitée par divers mécanismes immuno-régulation pré-établies dans microenvironnement tumoral. Les mécanismes immuno-régulation répandues comprennent les cellules immunitaires différentes, ainsi que des facteurs solubles immuno-régulation et des molécules de check-points.

Neutrophiles, un joueur clé dans le système immunitaire inné, ont été observés en association étroite avec les cellules tumorales et dans la vascularisation des tumeurs. Ils ont été proposés en tant que médiateur clé de la transformation maligne, la progression tumorale et l'angiogenèse et dans la modulation de la réponse de cellules cancéreuses à des agents anti-cancéreux.

Dans cette étude, nous avons étudié l'effet de neutrophiles circulants sur la sensibilité des cellules leucémiques et des cellules LNH primaires fraîches à la thérapie du cancer. Nous émettons l'hypothèse que les neutrophiles, via leur interaction avec les cellules de lymphome B, pourraient modifier la réponse de cellules cancéreuses à différents types de composés cytotoxiques ainsi que des thérapies ciblées. Nous avons constaté que les neutrophiles circulants présentent un effet protecteur sur les cellules B malignes contre une variété de composés anti-lymphome, ce qui constitue encore un nouveau mécanisme par lequel le système immunitaire inné pourrait contribuer négativement à l'issue chez les patients atteints de lymphome.

Ces résultats pourraient être d'une grande importance clinique, étant donné que l'inhibition pharmacologique de la protection médiée par les neutrophiles peut contribuer à améliorer l'activité antitumorale des agents anti-lymphome.

Conclusion and Perspectives

En conclusion, notre étude a démontré que les neutrophiles circulants présentent un effet protecteur sur les cellules B malignes contre une variété de composés anti-lymphome. Ces résultats révèlent un mécanisme de protection roman de cellules B néoplasiques impliquant des cellules immunitaires innées. D'autres investigations seraient nécessaires pour mieux comprendre l'impact des neutrophiles sur la modulation de la réponse d'autres types de cancer et à d'autres thérapies. En outre, d'autres enquêtes sur lesquels des agents thérapeutiques (agents cytotoxiques classiques, l'hormonothérapie, vaccins contre le cancer ou la thérapie ciblée) sont sensibles à l'effet de neutrophiles serait également nécessaire de mieux comprendre les rôles du microenvironnement de la tumeur dans l'induction de la résistance du cancer.

De plus, nous avons pu montrer que la protection induite par les neutrophiles était dépendante de contact cellule-cellule médiée par CD11b / ICAM-1 exprimé par les neutrophiles et les cellules B, respectivement, ainsi que par la molécule d'adhésion CD44. L'effet protecteur a été corrélé avec l'induction de Mcl-1 protéine anti-apoptotique qui a été partiellement abrogée par un-MCL-1 anti- composé. Dans la prochaine étape, nous allons nous concentrer sur la détermination de la voie de signalisation qui active les neutrophiles des voies de signalisation dans les cellules de protection RL. Selon les données publiées précédentes, CD11b / interaction ICAM-1 peut conduire à l'activation de la voie en aval ICAM-Raf-MEK 1-ERK dans les cellules RL. Ces protéines ont un rôle connu dans les cascades de signalisation intracellulaire conduisant à une augmentation de la prolifération cellulaire. En outre, les cellules RL, par contact direct avec les neutrophiles, induisent celui-ci à subir une activation tel que déterminé par l'augmentation de l'expression de différents marqueurs d'activation tels que CD66b. Les neutrophiles activés peuvent libérer ensuite l'IL-6 qui induit une régulation à la hausse Mcl-RL dans les cellules, éventuellement par activation de la voie Jak / Stat. De plus, nous avons pu valider l'effet protecteur des cellules neutrophiles in vivo et ex vivo de travail sur le mécanisme de validation.

Un autre aspect important concerne la pertinence clinique de nos observations. Bien que les données recueillies chez les patients CLL est cohérent avec nos observations faites sur des lignées cellulaires, il sera important de déterminer si les neutrophiles infiltrant les tumeurs présentent un effet protecteur sur les cellules tumorales, de façon similaire à ce que nous avons observé avec les neutrophiles circulants. Autres études corrélatives sont également garantis, en particulier l'étude de l'impact de l'infiltration de neutrophiles dans des échantillons humains de la LNH sur la qualité de la réponse au traitement.

L'identification du mécanisme d'interactions entre les neutrophiles et les cellules B malignes, et la façon dont ces interactions modifient la réponse des cellules de lymphome de traitement du cancer peuvent contribuer au développement de stratégies thérapeutiques pour améliorer l'effet antitumoral de la thérapie conventionnelle ou ciblée. Plus précisément, nous émettons l'hypothèse que les thérapies ciblées d'inhibition du contact cellulaire neutrophiles-tumeur ou réduisant le contenu de Mcl-1 dans des cellules tumorales pourraient contribuer à renforcer l'activité antitumorale de plusieurs agents chimiothérapeutiques.

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LIST OF ABBREVIATIONS

5-FU: 5-Fluorouracil

ABC: Activated B cell

ALL: Acute Lymphoblastic Leukemia

AML: Acute Myeloid Leukemia

ADCC: Antibody-Dependent Cellular Cytotoxicity

B-CLL: B-Chronic Lymphocytic Leukemia

BTK: Bruton tyrosine kinase

BCL-2: B cell chronic lymphocytic leukemia/lymphoma-2

BCR: B cell receptor

BAFF: B cell-activating factor of the TNF family

CLL: Chronic Lymphocytic Leukemia

CAM-DR: Cell Adhesion-Mediated Drug Resistance

CAM-AR: Cell Adhesion-Mediated Antibody Resistance

CSF: Colony-Stimulating Factor

CSF-1R: Colony-Stimulating Factor Receptor

CTLs: cytotoxic CD8⁺ T lymphocytes

CXCL: CXC-chemokine Ligand

CXCR: CXC-chemokine Receptor

CCL: CC-chemokine Ligand

CTLA: Cytotoxic T-Lymphocyte-Associated protein

DLBCL: Diffuse Large B-cell Lymphoma

DC: Dendritic Cells

EGFR: Epidermal Growth Factor Receptor

ECM: ExtraCellular Matrix

ECs: Endothelial Cells

ERBB-2: erb-b-2 receptor tyrosine kinase-2

FDA: Food and Drug Administration

FDCs: Follicular Dendritic Cells

FOXP3: Forkhead box protein P3

fMLP: formylMethionyl-LeucylPhenylalanine

FcRs: Fc receptors

FGF: Fibroblast Growth Factor

Fas-L: Fas-ligand

GCs: Germinal Centers

GrzB: granzyme B

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

G-CSF: Granulocyte-Colony-Stimulating Factor

GBM: Glioblastoma multiforme

hpf: high-power field

HIV: Human Immunodeficiency Virus

Hh: Hedgehog

HRS: Hodgkin Reed-Sternberg

HLA-DR: Human Leukocyte Antigen-DR

HOCl: Hypochlorous acid

HMGB-1: High Mobility Box binding protein-1

HER-2: Human Epidermal Growth Factor Receptor-2

Ig: Immunoglobulin

IPI: International Prognostic Index

IFN: Interferon N

IL: Interleukin

iNOS: inducible Nitric Oxide Synthase

ICAM: InterCellular Adhesion Molecule

IRS-1: Insulin receptor substrate 1

KIRs: Killer-cell Immunoglobulin-like Receptors

LPS: Lipopolysaccharide

LFA-1: Lymphocyte Function-Associated antigen-1

Ly6: lymphocyte antigen 6

LLC: Lewis lung carcinoma

MALT: Mucosa-associated B-cell Lymphoma

MZL: Marginal Zone Lymphoma

MCL: Mantle Cell Lymphoma

mAbs: monoclonal Antibodies

MDSCs: Myeloid-Derived Suppressor Cells

MIF: Macrophage Inhibitory Factor

MIP-1 α : Macrophage-Inflammatory Protein-1 α

MCP-1: Monocyte Chemoattractant Protein-1

MMP: Matrix MetalloProteinase

MAC-1: Macrophage antigen-1

NHLs: Non-Hodgkin Lymphomas

NK: Natural Killer

NN: Naive Neutrophils

NE: Neutrophil Elastase

NLRP-3: NOD-like receptor family, pyrin domain containing-3

NSCLC: Non-Small Cell Lung Cancer

OS: overall survival

PI3K: Phosphoinositide 3-kinase

PSGL-1: P-selectin glycoprotein ligand-1

PIGF: Placental Growth Factor

PRRs: Pattern Recognition Receptors

PFS: Progression-free survival

ROI: Reactive Oxygen Intermediates

ROS: Reactive Oxygen Species

SLL: Small Lymphocytic Leukemia

SleX: Sialyl Lewis X

TFH: Follicular helper T

Th: T helper

TFR: Follicular regulatory T

TNF: Tumor Necrosis Factor

TAM: Tumor-associated macrophages

TAN: Tumor-associated neutrophils

TGF: Transforming Growth Factor

TEM: Transendothelial Migration

TIMP: Tissue Inhibitor of Metalloproteases

Treg: Regulatory T lymphocytes

TLR-4: Toll-like receptor-4

TKIs: Tyrosine Kinase Inhibitors

uPA: Urokinase

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VCAM: Vascular Cell-Adhesion Molecule

VAP-1: Vascular Adhesion Protein-1

WHO: World Health Organization

WM: Waldenstrom's macroglobulinaemia

PUBLICATIONS

From PhD study:

1. **Human neutrophils protect lymphoma cells against cytotoxic and targeted therapies through CD11b/ICAM-1 binding.**

Taghreed Hirz, Eva-Laure Matera, Kamel Chettab, Lars Petter Jordheim, Doriane Mathé, Anne Evesque, Justine Esmenjaud, Charles Dumontet
(Under review in **Blood Journal**)

2. **Neutrophil isolation and analysis to determine their role in lymphoma cell sensitivity to therapeutic agents.**

Taghreed Hirz and Charles Dumontet
(In press, **Journal of Visualized Experiments (JoVE)**).

COMMUNICATIONS

1. Polymorphonuclear neutrophils and cancer: The impact of neutrophils on the sensitivity of B-cell lymphomas to chemotherapy. **(Oral presentation)**
Taghreed Hirz, Cellular and Integrative Molecular Biology doctoral school scientific days 2014, December, Lyon, France.
2. Human neutrophils protect lymphoma cells against cytotoxic and targeted therapies through CD11b/ICAM-1 binding. **(Poster presentation)**
Taghreed Hirz, Eva-Laure Matera, Kamel Chettab, Lars Petter Jordheim, Doriane Mathé, Anne Evesque, Justine Esmenjaud, Charles Dumontet. 2nd International Symposium of Cancer Research Center of Lyon 2015, September, Lyon, France.
3. Neutrophils protect B-cell lymphomas against chemotherapy via cell-cell interaction mediated by CD44 and ICAM1 receptors. **(Poster presentation)**
Taghreed Hirz, Justine Esmenjaud, Anne Evesque, Doriane Mathe-Poloni, Charles Dumontet. AACR annual meeting 2015, April, Philadelphia, Pennsylvania, USA.
4. Effect of polymorphonuclear neutrophils on the sensitivity of lymphoma cells to chemotherapy. **(Poster presentation)**
Taghreed Hirz, Anne Evesque, Charles Dumontet and Florence Depontieu. Scientific Day of the Cancer Research Center of Lyon (CRCL) 2014, March, Lyon, France.

PREFACE

Non-Hodgkin's lymphoma (NHL) is the most common cancer of the lymphatic system, of which there are numerous unique disease subtypes. Over 65,000 cases of NHL are diagnosed annually in the United States of which 85 % are of B cell type. Several studies have described interactions between lymphoma cells and non-malignant cells within the bone marrow, lymph node, and other tumor microenvironments; such as follicular dendritic cells, T cells, human bone marrow stromal cells and derived nurse-like cells. There is a clear impact of the adaptive immune compartment on the outcome of patients with lymphoma, but much less data exist regarding the role of the innate immune cells. Macrophages are important cells of the innate immune system and have been analyzed in the context of NHL where the presence of polarized M2 cells has been shown to be an independent predictor of poor prognosis. Very little data are available regarding the interactions between lymphoma cells and neutrophils which are key players in the innate immune system.

Neutrophils are known to play an established role in host defense and in killing microorganisms. Recently, it is becoming clear that tumor-associated neutrophils and their myeloid precursors play an important role in cancer biology. They have been observed *in vivo* in close association with tumor cells and within tumor vasculature. Current evidences suggest that neutrophils show high functional plasticity and can display either protumor or antitumor activity; a phenomenon designated as "neutrophil polarization". Neutrophils are antitumoral effector cells by inducing cytotoxicity or by mediating tumor rejection and antitumoral immune memory. On the other hand, neutrophils can support tumor progression by promoting angiogenesis, invasion, metastasis and immunosuppression. Few studies indicated the implication of neutrophils in mediating tumor cell response to cancer therapy. Clinical support for a role of neutrophils in chemotherapy response comes from observations in a variety of cancer patients, such as breast and Non-Small Cell Lung Cancer patients, where chemotherapy induced neutropenia is associated with better patient prognosis. However, the role of neutrophils in regulating lymphoma response to cancer therapy has not been previously described.

In the introduction of this thesis, I will present an overview about NHL and the different ways of treatment. Second, I will describe the tumor microenvironment of NHL in addition to the paradoxical roles of its constituents. Third, I will define neutrophils, the Cinderellas of the innate immune system, and their dual roles in cancer. Finally, I will describe the interplay between cancer therapy and the immune system.

In the results section, I will present our work during my PhD study which consists of two parts. The first one demonstrates the impact of neutrophils on the sensitivity of lymphoma B cells to cancer therapy. In the second part, I will describe the neutrophil isolation and analysis to determine their role in lymphoma cell sensitivity to therapeutic agents.

In the final section, discussion, conclusion and perspectives, I will summarize our work and discuss the objectives for future studies.

Overall, our results suggest a new mechanism through which the innate immune system could contribute negatively to the outcome in patients with lymphoma.

CHAPTER I

1. Introduction

The immune system is a complex but well organized system made up of a network of cells, tissues and organs that can identify and eliminate any potentially harmful invaders such as bacteria, viruses, fungi or parasites, and endogenous objects such as immune complexes or transformed cells. This defense has been divided into two general types of reactions: reactions of innate immunity and reactions of adaptative immunity. In general, innate immunity is considered a nonspecific response, whereas the adaptative immune system is thought of as being very specific. In addition, the antigen receptors of the adaptative immune response are commonly viewed as 'master sensors' whose engagement dictates lymphocyte function. The cells of the immune system are usually regulated by hormones and growth promoting and suppressing signals, but all of them are vulnerable to errors, potentially leading to malignant transformation.

Cancer is a class of diseases characterized by uncontrolled division of cells and ability of the cells to invade and damage normal tissues either locally or at distant site of the body. A malignant lymphoma arises when a single B or T lymphocytes is arrested in a specific stage of cell differentiation leading to malignant transformation and clonal expression ¹. Malignancies of hematopoietic cells have been divided into myeloid malignancies and lymphoid malignancies. Lymphoid malignancies comprises more than 67 subtypes of two related cancers that affect the lymphatic system; Hodgkin's disease accounts for approximately 10% of all lymphomas and the remaining 90% are referred to as Non-Hodgkin lymphomas (NHLs) ².

CHAPTER II

2. Non-Hodgkin's Lymphoma

2.1. Definition

Non-Hodgkin's Lymphoma (NHL) is a collective term for a heterogeneous group of lymphoproliferative malignancies with differing patterns of behavior and responses to treatment³. NHL is the tenth most frequently diagnosed neoplasm in the world and ranks seventh in developed countries ⁴. Over 65,000 cases of NHL are diagnosed annually in the United States, and 85 % of these develop from B cells ⁵.

2.2. Incidence and epidemiology

The frequency of specific subtypes of lymphoma varies substantially by geographic region ². The incidence of NHL is currently increasing in many regions. In England, Scotland, and Wales, the age-standardized incidence has increased by 35% over the past 30 years (1988–2007)^{6–8}. A similar tendency has been recorded in the USA, with age-adjusted incidence increased by 77.3 % from 1975 to 2011 ⁹. The incidence in Brazil, India, Japan, Singapore, and western Europe has also increased ^{10–12}.

The reason for this long-term increase is unclear, although the most well established risk factor for the development of NHL is immunosuppression. Infection is also believed to play a part in the development of some lymphomas, either by inhibition of immune function,

or by other mechanisms, such as induction of chronic inflammatory response². Patients with human immunodeficiency virus (HIV), who have had high-dose chemotherapy with stem-cell transplantation, solid organ recipients and those with inherited immunodeficiency syndromes or autoimmune disease all have an increased risk of developing NHL. It is also likely that better diagnostic methods have contributed to this apparent increase in incidence.

Table 1. Chromosomal translocations of Non-Hodgkin Lymphoma (modified from Evans et al., 2003)

NHL histological type	Translocations observed	Cases affected	Proto-oncogene involved	Mechanism behind proto-oncogene activation	Proto-oncogene function
Follicular lymphoma	t(14;18)(q32;q21) t(2;18)(p11;q21) t(18;22)(q21;q11)	80-90%	Bcl2	Transcriptional deregulation	Negative regulator of apoptosis
Mantle-cell lymphoma	t(11;14)(q13;q32)	70%	Bcl1/cyclin D1	Transcriptional deregulation	Cell-cycle regulator
MALT lymphoma	t(11;18)(q21;q21) t(1;14)(p22;q32)	50% Rare	API2/MLT BCL10	Fusion protein Transcriptional deregulation	Antiapoptosis Antiapoptosis?
Diffuse large B-cell lymphoma	Der(3)(q27)	35%	BCL6	Transcriptional deregulation	Transcriptional repressor
Burkitt's lymphoma	t(8;14)(q24;q32) t(2;5)(p11;q24) t(8;22)(q24;q11)	80% 15% 5%	c-MYC	Transcriptional deregulation	Transcription factor regulating proliferation and growth

2.3. Biology of lymphomagenesis

Most lymphoid malignancies are derived from B lymphocytes at various stages of differentiation. Two types of lymphoid tissue exist: central (bone marrow and thymus) and peripheral (blood, spleen, lymph node, and mucosa-associated). To understand the mechanisms by which lymphomas might develop, the events that occur during normal B cell maturation should be considered.

During normal B cell development, cells arise from the central lymphoid tissues where B cell precursors undergo immunoglobulin (Ig) heavy and light chain rearrangement and are equipped with a functional surface antigen receptor¹³. This rearrangement enabled by enzymes that cause breaks in double-stranded DNA¹³. In normal cells, DNA repair processes are activated, but such breaks can contribute to chromosomal translocations in lymphoma that typically result in proto-oncogene activation (Table 1)¹⁴.

The descendants of these precursor cells, called naive B cells, migrate into the peripheral lymphoid tissues. Naive B cells undergo clonal expansion in germinal centers (GC), which are found in the cortex of lymph nodes¹⁵. In the lymph node GC, the Ig genes are further modified by somatic hypermutation, a process by which cells undergo rapid mutations and class-switch recombination¹⁶. Figure 1 shows the stages in B cell development that give rise to chronic lymphocytic leukemia (CLL), follicular lymphoma, diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma, and other B cell lymphomas.

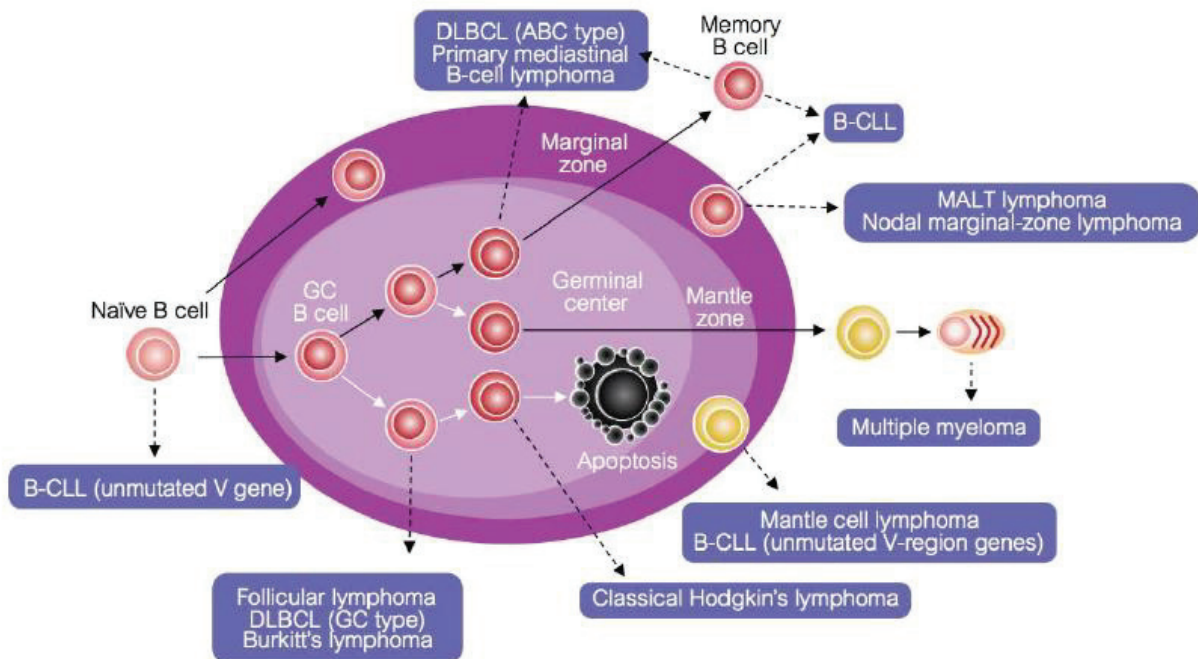


Figure 1. B-cell differentiation and lymphomagenesis.

Distinct types of germinal center (GC)-derived lymphomas originate from cells that are blocked at different stages of maturation.

DLBCL= Diffuse Large B-cell Lymphoma; ABC= Activated B cell; B-CLL= B-Chronic Lymphocytic Leukemia; MALT= Mucosa-associated B-cell Lymphoma

Source: Non-Hodgkin's lymphoma BioOncology

(www.biooncology.com/research-education/bcell/downloads/GA10000083900_NHL_Primer.pdf)

2.4. Classification of NHLs

NHLs encompass a heterogeneous group of cancers, 85-90% of which arise from B lymphocytes, and 10-15% arises from T lymphocytes or Natural Killer (NK) lymphocytes. Different classification systems have grouped these malignancies according to their histological and molecular characteristics². The most recent classifications of subtypes of NHL are described in the World Health Organization (WHO) classification of tumors of haemopoietic and lymphoid tissues, published in 2008 (Table 2)^{2,17}.

This fourth edition of classification takes into account new findings from clinical and laboratory research to provide guidance on how to recognize among categories that share common morphological and immunophenotypical features with other subtypes of lymphoma².

Table 2. Subtypes of Non-Hodgkin Lymphomas according to the 2008 WHO classification (modified form Shankland et al., 2012)

Disease	
B-cell lymphomas	
Precursor B cell	Precursor B-cell lymphoblastic leukemia/lymphoma
Mature B cell	Chronic lymphocytic leukemia/small lymphocytic lymphoma; follicular lymphoma; mantle cell lymphoma; diffuse large B cell lymphoma; Burkitt's lymphoma; lymphoplasmacytic lymphoma; splenic marginal zone lymphoma; nodal marginal zone B-cell lymphoma
B cell proliferations of uncertain malignant potential	Lymphomatoid granulomatosis; post-transplantation lymphoproliferative disorders (polymorphic)
T cell and NK cell lymphomas	
Precursor T cell	Precursor T-cell lymphoblastic leukemia/lymphoma
Extranodal mature T cell and NK cell	Mycosis fungoides; cutaneous anaplastic large-cell lymphoma; extranodal NK-cell or T-cell lymphoma; enteropathy-type lymphoma; hepatosplenic lymphoma; subcutaneous panniculitis-like lymphoma; primary cutaneous CD8-positive lymphoma; primary cutaneous γ/δ T-cell lymphoma; primary cutaneous CD4-positive lymphomarecursor T-cell lymphoblastic leukemia or lymphoma
Nodal mature T cell and NK cell	Peripheral T-cell lymphoma, not otherwise specified; angioimmunoblastic lymphoma; anaplastic large-cell ALK-positive lymphoma; anaplastic large-cell ALK-negative lymphoma; adult T-cell leukemia/lymphoma

2.5. Clinical presentation and staging

Clinical presentation is dependent on the site of involvement, natural history of the lymphoma subtype, and presence or absence of B symptoms (weight loss >10%, night sweats, body temperature >38⁰C) (1). Based on this, NHL is staged with the Ann Arbor classification system according to the principal stages and the modifiers as described in Table 3 ¹⁸.

Table 3. Ann Arbor staging system of NHL (from Shankland et al., 2012)

Definition	
Principal stages	
I	Involvement of one lymph node or one extranodal organ or site (IE)
II	Involvement of two or more lymph node regions on the same side of the diaphragm, or localized involvement of an extranodal site or organ (IIE) and one or more lymph node regions on the same side of the diaphragm
III	Involvement of lymph node regions on both sides of the diaphragm, which might be accompanied by localized involvement of an extranodal organ or site (IIIE) or spleen (IIIS) or both (IIISE); the spleen is regarded as nodal
IV	Diffuse or disseminated involvement of one or more distant extranodal organs with or without associated lymph node involvement
Modifiers	
A	Absence of B symptoms
B	Temperature >38°C, night sweats, and weight loss of greater than 10% of bodyweight in the 6 months preceding admission are defined as systemic symptoms

2.6. Prognostic factors

Parameters used to determine prognosis have been widely investigated. The International Prognostic Index (IPI) is the most widely used prognostic model for patients with NHL¹⁹ and takes into account age (≤ 60 years vs. >60 years), serum lactate dehydrogenase concentration (normal vs abnormal), Ann Arbor stage (I/II vs. III/IV), Eastern Cooperative Oncology Group performance status (<2 vs. ≥ 2) and number of extranodal sites involved (≤ 1 vs. >1). This score has been used to define four risk groups: low risk (zero to one clinical feature), low-intermediate risk (two features), high-intermediate risk (three features), and high risk (four to five features). When applied to a large cohort of patients, these risk groups had 5-year survival rates of 73%, 51%, 43%, and 26%, respectively¹⁹.

Age is a particularly important prognostic marker, and has been repeatedly associated with poorer outcomes^{20,21}. However, elderly patients who are able to receive full-dose chemotherapy have survival rates similar to their younger counterparts^{20,22}.

2.7. NHL treatment

The treatment of NHL is in most cases a combination of multiple modalities, including conventional cytotoxic chemotherapy, radiotherapy, immunotherapy and/or small molecule targeted therapies.

2.7.1. Chemotherapy

Chemotherapy is a systemic treatment using anti-cancer drugs that are given intravenously or orally. It is the main treatment for NHL. Chemotherapy is administered repeatedly (cycles). The chemotherapy regimen used depends on the stage and type of NHL. The most common chemotherapy combination for the initial treatment is called CHOP and contains four drugs: cyclophosphamide, hydroxyl-daunomycin (doxorubicin), Oncovin® (vincristine), and prednisone. Listed in table 4 are examples of chemotherapeutic molecules that are commonly used for NHL treatment according to their mechanisms of action. Patients with poor prognosis aggressive lymphoma may undergo a high-dose chemotherapy treatment followed by autologous stem cell or bone marrow transplantation.

Table 4. Chemotherapeutic molecules used in NHL treatment

Mechanism of action		Example
Modification of DNA structure	Formation of covalent bonds with DNA	Alkylating agents: cyclophosphamide, mafosfamide Platinum: cisplatin, carboplatin
	DNA break	Bleomycin
	Intercalating agents	Anthracyclins: doxorubicin, daunorubicin
Inhibition of DNA synthesis	Interfere with DNA polymerase and ribonucleotide reductase	Fludarabine, pentostatin, cladribine
Inhibition of nucleic acid synthesis	Antimetabolites	Cytarabine, gemcitabine, methotrexate
Mitosis poisons	Inhibition of microtubule depolymerization	Taxane: paclitaxel (Taxol) and docetaxel (Taxotere).
	Destruction of mitotic spindles	Vinca alkaloids: vincristine, vinblastine, vinorelbine

2.7.2. Radiation therapy

Radiation therapy is the use of high energy x-rays or other particles like electrons or protons to destroy cancer cells and shrink cancerous tumors. A radiation therapy regimen usually consists of a specific number of treatments given over a set period of time. It is most often given to patients who have lymphoma that is located in only one area of the body or who have a lymph node that is particularly large, usually more than 10 centimeters across. Depending on the NHL subtype, the radiation therapy is usually given following or in addition to chemotherapy.

2.7.3. Immunotherapy

Immunotherapy is used to improve, target, or restore the function of the immune system. Monoclonal antibodies and interferon are immunotherapies which have been extensively used as treatments for different subtypes of NHL.

2.7.3.1. Monoclonal antibodies

Monoclonal antibodies (mAbs) are directed against a specific antigen preferentially expressed on the surface of cancer cells. Based on the cell surface antigens they recognize, mAbs can be divided into those directed against B cell lineage specific antigens (e.g. CD19, CD20, CD22) and those that are directed against other surface antigens which are not B cell specific (e.g. CD40, CD80, CD30)²³. Several EU/US approved monoclonal antibodies for NHL treatment are listed in table 5.

CD20 is a B cell specific surface antigen and represents an excellent target for the treatment of B cell NHL. This antigen is not expressed at the early stage of B cell development or in terminally differentiated plasma cells; however, it is stably expressed at most stages of B cell differentiation and is present on the cell surface of at least 95% of malignant B cells. CD20 antigen has no known ligand and its function is not completely understood, although it is believed that it acts as anion channel regulating calcium flux and that it may be involved in B cell activation, proliferation and differentiation²⁴. Rituximab was the first approved monoclonal antibody used for the treatment of B cell NHL. Rituximab works by targeting CD20 antigen on the surface of B cell lymphomas leading to cell death.

Rituximab has been shown to enhance the efficacy of the classical CHOP regimen, leading to the now widely used chemo-immunotherapeutic regimen (R-CHOP).

Table 5. Therapeutic monoclonal antibodies approved in the treatment of NHL

International non-proprietary name	Trade name	Type	Indication first approval	First EU (US) approval year
Obtinutuzumab	Gazyva	CD20; Humanized IgG1; Glycoengineered	Chronic lymphocytic leukemia	2014 (2013)
Brentuximab vedotin	Adcetris	CD30; Chimeric IgG1; immunoconjugate	Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2012 (2011)
Ofatumumab	Arzerra	CD20; Human IgG1	Chronic lymphocytic leukemia	2010 (2009)
Ibritumomab tiuxetan	Zevalin	CD20; Murine IgG1	Non-Hodgkin lymphoma	2004 (2002)
Rituximab	MabThera, Rituxan	CD20; Chimeric IgG1	Non-Hodgkin lymphoma	1998 (1997)

2.7.3.2. Interferon

Interferon is a cytokine produced by leukocytes to help the immune system fight infections. Some studies have suggested that giving man-made interferon can make some types of lymphomas to stop growing. Because of its deleterious side effects, interferon is not used very often.

2.7.4. Targeted small molecule inhibitors

Over the last years, there has been a substantial increase in the number of targeted small molecules entering clinical development or approved in lymphomas. Small molecule inhibitors typically interrupt cellular processes by interfering with the intracellular signaling of specific enzymes. These enzymes initiates molecular cascade that can lead to cell growth, proliferation, migration, and angiogenesis in normal and malignant tissues ²⁵.

Figure 2 represents a number of small molecule inhibitors such as Bruton tyrosine kinase inhibitors, phosphoinositide 3-kinase inhibitors and B cell chronic lymphocytic leukemia/lymphoma-2 family inhibitors.

2.7.4.1. Bruton tyrosine kinase inhibitors

B cell receptor signaling in normal and malignant B cells is important for cell proliferation and survival ²⁶. Bruton tyrosine kinase (BTK) is a non-receptor tyrosine kinase that plays a significant role in B cell development and is a unique therapeutic target in B cell malignancies ²⁷. Inhibition of BTK has been associated with reduction in cell migration, proliferation and survival ²⁸⁻³⁰. The encouraging preclinical results of BTK inhibitors has made the pharmacological field rich with several small molecule inhibitors including ibrutinib, LFM-A13, dasatinib, ONO-4059, and CC-292 ³¹⁻³³.

2.7.4.2. Phosphoinositide 3-kinase inhibitors

Phosphoinositide 3-kinase (PI3K) transmits signals that are important in several cellular processes including cell proliferation, survival, growth and motility. Aberrant activation of this pathway has been associated with different types of cancer including lymphomas ^{34,35}. PI3K form a class of enzymes that have recently gained great attention in the development of lymphoma treatment. The isoform δ (PI3K δ) is highly expressed in lymphoid cells and is the most important isoform in CLL ²⁶. Idelalisib is a recently approved (FDA approval 2014) highly selective inhibitor of PI3K δ isoform, showing a high response rate (63% in indolent lymphomas and 48% in MCL). Idelalisib is used with combination with rituximab and chemotherapeutic regimens due to its high activity and acceptable toxicity profile ²³.

2.7.4.3. B cell chronic lymphocytic leukemia/lymphoma-2 family inhibitors

B cell chronic lymphocytic leukemia/lymphoma-2 (BCL-2) family proteins are key apoptotic regulators of the apoptotic process and play a dominant role in normal and malignant lymphoid cells ³⁶. The family comprises pro-survival and pro-apoptotic proteins ³⁷. In malignant lymphoid cells, their balance shift to pro-survival allowing these cells to evade apoptosis ³⁸. ABT-199 is a potent second generation BCL-2 pathway inhibitor that is highly selective for BCL-2 anti-apoptotic protein ³⁹.

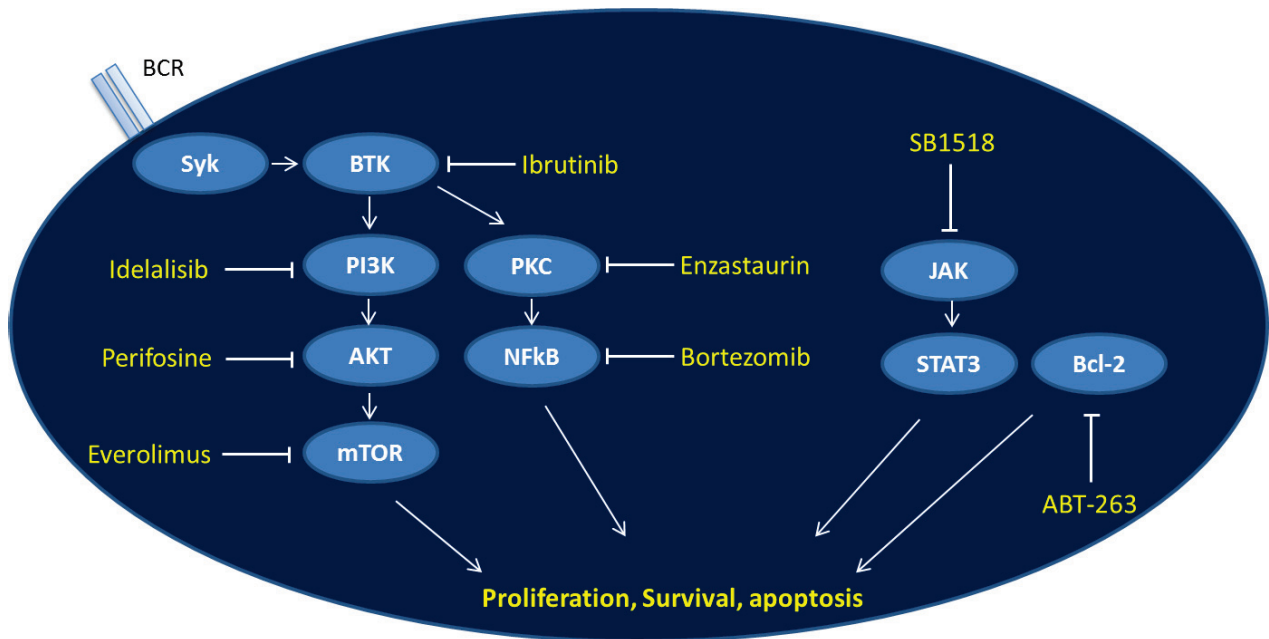


Figure 2. Small molecule inhibitors used for NHL treatment.

Examples for small molecule inhibitors used to interrupt several cellular processes involving cell proliferation, survival and apoptosis.

BCR= B cell receptor

CHAPTER III

3. The tumor microenvironment of NHL

3.1. Overview

Considerable advancement in understanding the pathogenesis or progression of lymphoma has been achieved through the molecular dissection of the malignant cells, which has identified genetic alterations and intracellular pathway dysregulation⁴⁰. However, the importance of the microenvironment where the malignant cells arise and reside has been relatively neglected.

It has been known for over a century that tumors comprise not only malignant cells but also a plethora of non-malignant cells and extracellular matrix (ECM) components which constitute the tumor microenvironment. It has become more evident that the elements of the microenvironment are not only mere bystanders of the host antitumor inflammatory response, but they also have an important impact on tumor pathogenesis and progression⁴¹ or/and tumor suppression⁴².

3.2. Paradoxical roles of the NHL tumor microenvironment

The tumor microenvironment of NHL consists of variable numbers of immune cells, stromal cells, blood vessels and extracellular matrix. A tumor can change its microenvironment, and the microenvironment can affect how a tumor grows and spreads. The genetic alterations of the malignant cells and the degree to which these cells remain dependent on external stimuli for survival, proliferation and immune evasion determined the composition and spatial arrangement of the tumor microenvironment⁴³. The interplay between these elements results in the broad range of tumor microenvironments among different B lymphoma cell types as shown in figure 3.

3.2.1. Stromal cells

Several studies have shown that stromal cells provide essential survival factors that protect the lymphoma cells from apoptosis and support growth in follicular lymphoma^{44,45} and MCL^{44,46}. Among these signals, hedgehog (Hh) ligands that are secreted by the stromal cells and act on inhibiting the spontaneous apoptosis of malignant B cells^{47,48}. In addition, paracrine Hh signaling induces the upregulation of the drug transporter ATP-binding cassette (ABC) G2 in indolent lymphoma thereby inducing chemotolerance⁴⁹.

Previous studies have shown that adhesion to cultured stromal cells can protect malignant B cells from apoptosis induced by chemotherapy drugs, a mechanism known as cell adhesion-mediated drug resistance (CAM-DR)⁵⁰⁻⁵⁴. Later on, Mraz et al. showed a protective effect for the stromal cells against follicular lymphoma apoptosis induced by anti-CD20 antibody rituximab suggesting the existence of a stromal cell adhesion-mediated antibody resistance (CAM-AR)⁵⁵. This effect was VLA-4-dependent which is an integrin highly expressed by lymphoma cells with an important role in the adhesion of B cells to stroma⁵⁶⁻⁵⁹ and provides a pro-survival signaling⁶⁰⁻⁶³.

3.2.2. Innate Immune cells

The innate immune system is composed of many cell types and mediators that interact with tumor cells to either protect or destroy tumors. A novel molecular mechanism for cell adhesion-mediated drug resistance was described for follicular dendritic cells (FDCs), where Lwin and coworkers reported this mechanism on B cell lymphomas, in part through activation of a microRNA-181a-dependent mechanism involving down-regulation of the pro-apoptotic protein BIM expression⁶⁴. In addition, Cui et al. reported that the total loss or partially disrupted FDC networks are correlated with a lower clinical stage⁶⁵.

NK cells are defined as effector lymphocytes of innate immunity endowed with constitutive cytolytic functions⁶⁶. NK cells form an important line of defense against virally infected cells and tumor cells by inducing apoptosis⁶⁷. They are a type of cytotoxic lymphocyte and produce abundant cytokines, such as tumor necrosis factor (TNF)- α and Interferon N (IFN)- γ that can spontaneously lyse susceptible tumor cell targets⁶⁸.

Data obtained from NHL patients found that higher proportions of peripheral blood NK cells were associated with a favorable prognosis^{69,70}. In addition, Clynes et al. described the NK-mediated antibody-dependent cellular cytotoxicity (ADCC) which is an effective antitumor activity seen when rituximab and trastuzumab mAbs are used for treatment of lymphoma and breast cancer, respectively⁷¹. During ADCC, NK cells bind the Fc fragment of mAbs which are recognized by receptors thus allowing recruitment of these NK effector cells to target tumor cells.

3.2.3. Adaptative Immune cells

The adaptative immune system can also differentially regulate NHL through its different components. The function of T cells in the tumor microenvironment is highly variable, and depends on both the T cell subtype and the tumor being investigated. T cells are generally heterogeneous and influence tumor immunity both positively and negatively, depending on the prevalence of various T cell populations within the microenvironment⁶⁷.

Different studies showed an effect for T cells on lymphoma cell survival and proliferation such as in follicular lymphoma and MALT lymphomas^{72,73}. In a study by Umetsu and coworkers, they showed that *in vitro* proliferation of follicular lymphoma cells was induced when these cells were cultured with CD4+ T cell to be enhanced more with Interleukin (IL)-4 addition⁷³, and it is well known that the follicular lymphoma microenvironment is heavily enriched with CD4+ T cells⁷⁴. Moreover, Epron et al. showed a cooperative role for monocytes and T cells to favor follicular lymphoma growth through IL-15 which is presented by macrophages, together with CD40 interactions with T cells⁷⁵.

Intra-tumoral T cells have been shown to induce a substantial impact on antitumor immunity and lymphoma patient outcome⁷⁶. The density of tumor-infiltrating T lymphocytes with cytotoxic and memory phenotypes is highly predictive of favorable clinical outcome in NHL as well as other cancer types⁷⁷⁻⁷⁹. Laurent et al. showed that cytotoxic T cells engaged in target lymphoma cell killing through the secretion of perforin and granzyme B (GrzB)⁸⁰. T helper cells also enhance the killing capacity of macrophages and cytotoxic T cells by producing several cytokines such as IFN- γ , TGF- β , and IL-2⁶⁷.

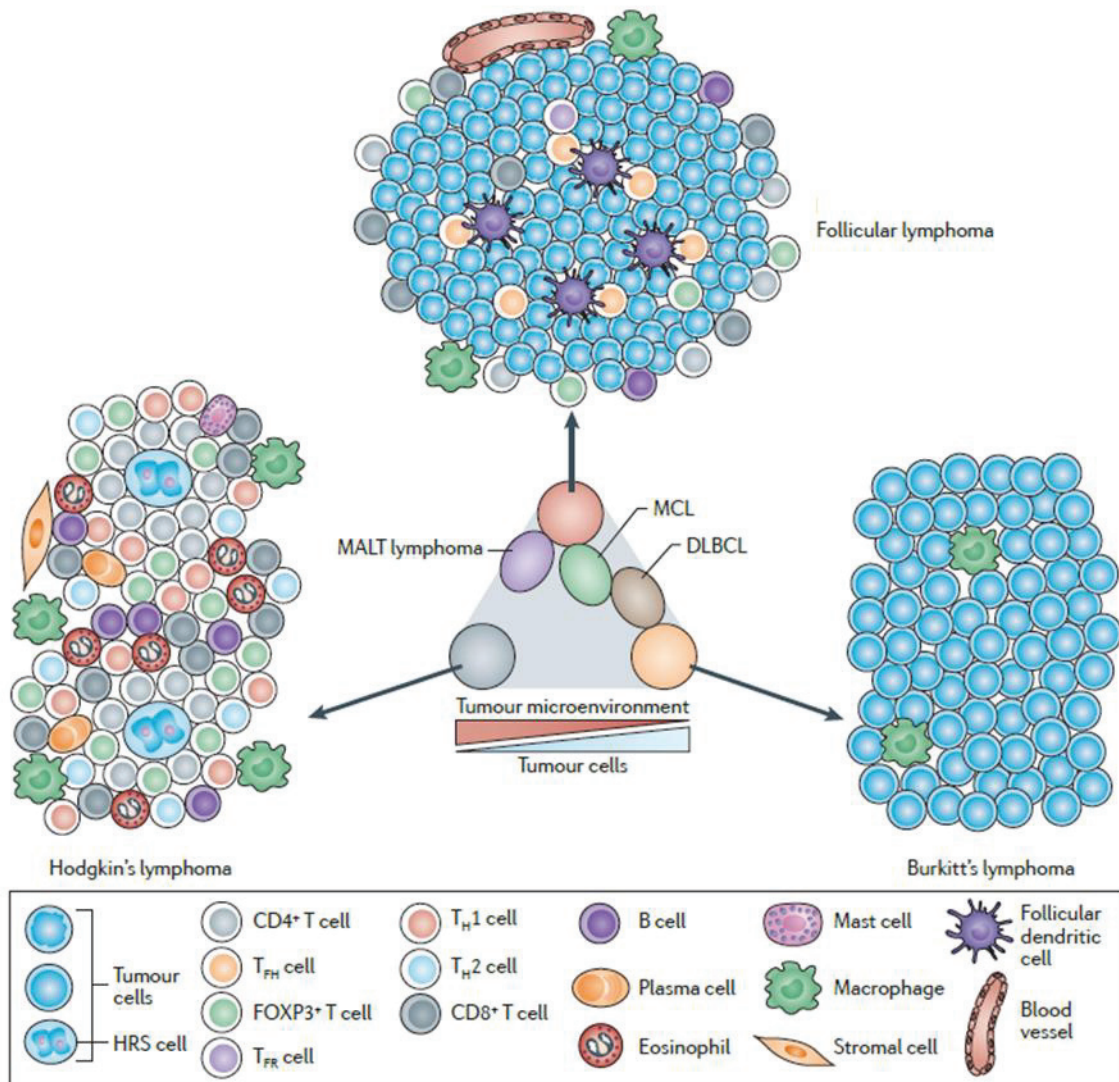


Figure 3. Composition of the B cell lymphomas microenvironment (modified from Scott et al. 2014).

The figure represents the microenvironment of the three B cell lymphoma subtypes that represent the range of tumor cell content, ranging from low-tumor cell content in Hodgkin's lymphoma to high-tumor cell content in Burkitt's lymphoma. The other B cell lymphomas fall within this range, as shown for the most common B cell lymphomas (in the center).

DLBCL= diffuse large B cell lymphoma; FOXP3= forkhead box protein P3; HRS= Hodgkin Reed–Sternberg; MALT= mucosa-associated lymphoid tissue; MCL= mantle cell lymphoma; TFH= follicular T helper; T_H= T helper; T_{FR}= follicular regulatory T.

3.3. Myeloid-Derived Suppressor Cells

Myeloid-Derived Suppressor Cells (MDSCs) represent a heterogeneous population of immature immune myeloid cells that are produced at high levels in cancer⁸¹. They are defined in mice on the basis of expression of the surface markers CD11b and Gr-1⁶⁷. The CD11b+/Gr-1+ MDSC population is comprised of at least two subsets: granulocytic (Ly6G+) and monocytic cells (Ly6C+), possibly with different immunosuppressive properties⁸¹.

While it is easy to define MDSCs in mice based on CD11b and Gr-1, the phenotypes to define this population in humans are quite divergent in studies employing different tissue types. Thus, the lack of consensus in defining MDSCs makes it difficult to quantify this population in patient samples^{82,83}.

Different studies have shown that MDSCs are highly represented in cancers and suppress NK and T cells through either cell contact or secreted cytokines⁸⁴. MDSCs have also been shown to support angiogenesis and promote tumor cell metastasis⁸⁵. They have been intensively investigated in solid tumors such as pancreatic cancer⁸⁵; however, few studies have been performed in hematological malignancies. Lin and coworkers identified CD14+ subpopulation with low or absent human leukocyte antigen DR (HLA-DR) expression in the peripheral blood of patients with B cell NHL. These monocytic subpopulations inhibited T cell function and this suppression was mediated by arginine metabolism⁸⁶. In addition, an elevated frequency of this suppressive monocytic subpopulation correlated with disease progression and overall survival in multiple myeloma and T cell lymphoma^{87,88}.

There is substantial agreement on the immunosuppressive activity of the monocytic MDSC subset. However, there is still contrasting evidence on the role of the granulocytic fraction. Whereas some have shown that granulocytic MDSCs have immunosuppression properties similar to the monocytic fraction^{84,89,90}, others have reported that they are less immunosuppressive.

Kusmartsev et al. showed that adoptively transferred MDSC can enter tumors and differentiate to mature macrophages/tumor-associated macrophages (TAM) or neutrophils/tumor-associated neutrophils (TAN)⁹¹. To investigate whether the majority of TAN are granulocytic MDSCs that were attracted to the tumor or whether they are bone marrow or blood-derived neutrophils that were then converted to TAN by the tumor microenvironment, Fridlender and his coworkers used a transcriptomic approach comparing the phenotype of TAN to naive neutrophils (NN) from the bone marrow and to the granulocytic fraction of MDSCs⁹². It was shown by this study that TAN are not tissue-based granulocytic MDSC, but are a distinct population of neutrophils, differing markedly in their genetic profile from both NN and granulocytic MDSCs, with the NN and granulocytic MDSCs being more closely related to each other than to TAN.

3.4. Macrophages: a key player in tumor microenvironment

The majority of malignant tumors contain numerous macrophages as a major component of the host leukocyte infiltrate⁹³. These macrophages are referred to as tumor-associated macrophages recruited into the tumor mass by chemotactic cytokines produced mostly by the tumor⁹⁴.

It was thought first that macrophages reject and kill tumor cells. However, clinical and experimental evidences indicate that in some cases, macrophages promote the progression and malignancy of tumors⁹⁵⁻⁹⁷. This paradoxical role of macrophages in cancer finds an

explanation in their functional plasticity that may result in the polarized expression of either pro or antitumor functions ⁹⁸. Key players in the setting of their phenotype are the microenvironmental signals to which macrophages are exposed, which selectively tune their functions within a functional spectrum encompassing the M1 and M2 extremes represented in figure 4.

3.4.1. M1-antitumor functions of macrophages

Macrophages are not generally tumoricidal for tumor cells unless the cells are activated. Various stimulants have been shown to activate macrophages such as IFN- γ or antibodies as well as bacterial products such as lipopolysaccharide (LPS) ^{99,100}. Once activated, direct cytotoxicity is exerted on the tumor cells, or indirect cytotoxicity via the secretion of soluble factors ¹⁰¹⁻¹⁰⁴.

Nitric oxide synthesis by macrophages through inducible nitric oxide synthase (iNOS) and their release in the tumor niche has a major role in the antitumor function of macrophages ^{103,105}. The cytokine macrophage inhibitory factor (MIF) has also been implicated in the anti-tumor effect of macrophages. This factor is synthesized by both the macrophages ¹⁰⁶ and the tumor cells ^{107,108} and stimulate a variety of important macrophage functions. Onodera et al. suggested that MIF plays an important role by activating macrophages in autocrine and paracrine fashion to phagocytose foreign particles ¹⁰⁹. Moreover, Pozzi and Weiser demonstrated that recombinant form of human MIF is able to modulate macrophage functions to become cytotoxic for tumor cells ¹¹⁰.

In addition, Shinohara and his colleagues reported that granulocyte-macrophage colony-stimulating factor (GM-CSF) released by the tumor cells stimulates macrophages to secrete macrophage-inflammatory protein-1 α (MIP-1 α) and monocyte chemoattractant protein-1 (MCP-1), which triggers recruitment of mononuclear cells, induces expression of adhesion molecules on macrophages, and enhances contact-dependent cytolysis of tumor cells ¹¹¹.

Also, the interleukins IL-18 and IL-12 secreted by macrophages (M1) ¹¹² have an anti-tumor effect by inducing the cytotoxic activity of NK cells and T cells ^{100,113}. Another indirect route for the anti-tumor effect of macrophages is by their secretion for matrix metalloproteinase (MMP) -12 which is an enzyme that has been shown to be important for the generation of angiostatin ¹¹⁴. Angiostatin is an angiogenesis inhibitor that selectively inhibits the proliferation of endothelial cells and impairs tumor metastasis ¹¹⁵.

3.4.2. M2-protumor functions of macrophages

Macrophages have the potential to secrete various proangiogenic factors, including cytokines and matrix-degrading enzymes ^{100,116} to directly affect tumor growth through promotion of tumor angiogenesis, as well as survival and metastasis of tumor cells ^{96,117-120}.

IL-1 β and TNF- α are TAM-derived cytokines that stimulate the tumor cells to produce angiogenin which is a potent proangiogenic proteins ¹⁰⁰ that has a poor prognosis on patients with colorectal cancer ¹²¹. Moreover, Torisu and colleagues suggested that the production of the potent angiogenic factors IL-8 and vascular endothelial growth factor (VEGF) from melanoma cells is up-regulated through these TAM-derived cytokines, influencing both tumor

growth and angiogenesis in melanomas ¹²². It has been shown also that VEGF expressed by M2 macrophages is a key component of angiogenesis in a variety of human tumors ^{123,124}.

Colony-stimulating factor (CSF)-1 is another stimulating factor produced by the macrophages ¹²⁵. Scholl et al. demonstrated that high level of CSF-1 as well as their receptors has been associated with poor prognosis in breast cancer ¹²⁶. Another study showed that the activation of colony-stimulating factor receptor (CSF-1R) by CSF-1 stimulate anchorage-independent growth and invasiveness in breast carcinoma ¹²⁷.

In addition, urokinase (uPA), a serine protease synthesized by TAM in different types of human tumors ^{128,129}, is thought to be involved in the degradation of ECM which enables tumor angiogenesis, invasion and metastasis. This enzyme has been correlated with poor prognosis in breast cancer patients ¹³⁰. Matrix metalloproteinase-9 (MMP-9) ¹³¹⁻¹³³ and lysosomal proteinases cathepsins ¹³⁴ also have an important role in tumor invasion and metastasis at the level of different tumors.

In gastric MALT lymphoma, Munari et al. showed that TAMs in the proximity of lymphoma cells secrete high levels of APRIL ¹³⁵, which is a proliferation-inducing ligand and this study was the first evidence for an involvement of APRIL in gastric MALT lymphoma development. In addition, Ogden and his colleagues suggested that macrophages, regulated by IL-10 which can be produced by both tumor cells and macrophages, were found to produce markedly higher levels of the B cell-activating factor of the TNF family (BAFF) ¹³⁶. Moreover, the presence of high density of macrophages in the tumor microenvironment has been correlated with poor prognosis in various types of cancer such as lymphoma ¹¹⁶, prostate carcinoma ^{137,138} and non-small cell lung carcinoma ¹³⁹.

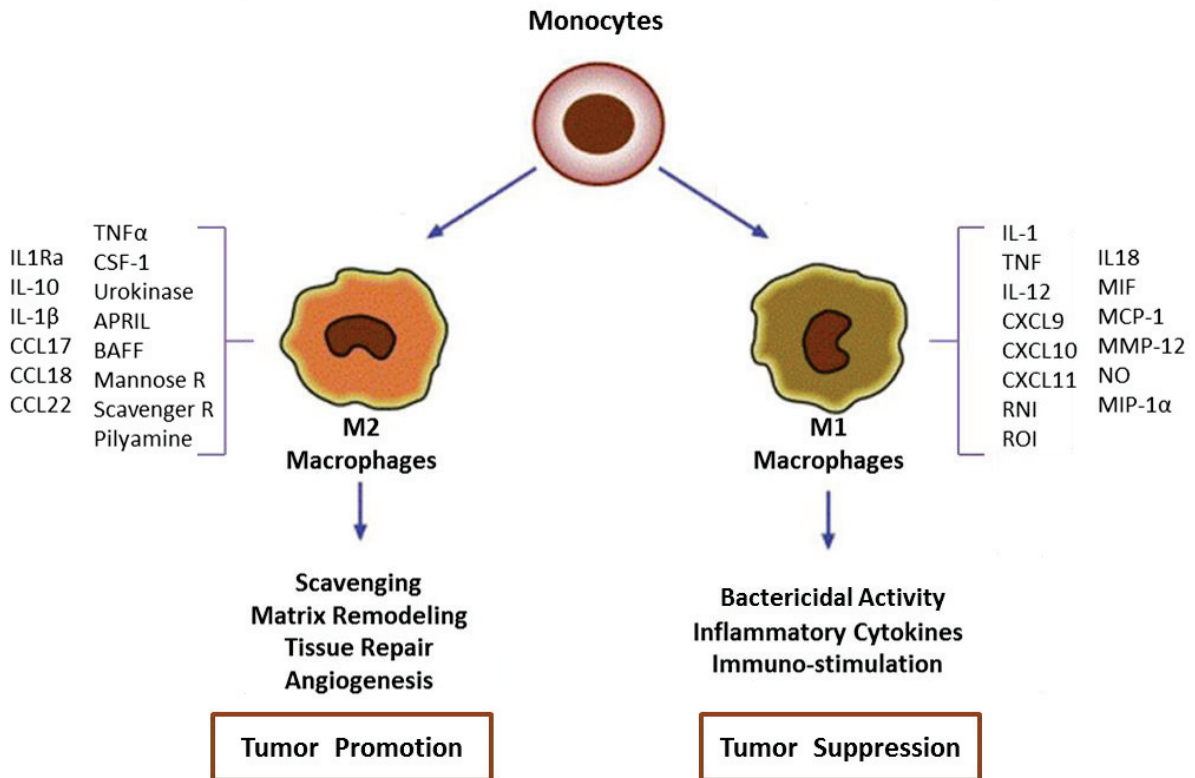


Figure 4. The paradoxical roles of macrophages (modified from Quatromoni et al. 2012).

Macrophages have both protumor and antitumor functions. Tumor microenvironment signals are key players in macrophages polarization. The pleiotropic functions of macrophages influence tumor growth and progression in opposite directions. The outcome depends on the sum of individual functions, dictated by the activation state of macrophages and the intrinsic properties of the tumor cell.

CHAPTER IV

4. Neutrophils: Cinderellas of the innate immune system

4.1. Definition

Neutrophils are the predominant leukocyte population in human blood, accounting for 50-70% of circulating leukocytes. Neutrophils are classically characterized by their ability to act as phagocytic cells, to release lytic enzymes from their granules and to produce reactive oxygen intermediates (ROI) with antimicrobial potential¹⁴⁰. Neutrophils can also be induced to express genes encoding key inflammatory mediators, including complement components, Fc receptors, chemokines and cytokines¹⁴¹.

Although neutrophils are traditionally considered in the context of their antibacterial functions, it is becoming increasingly clear that TANs and their myeloid precursors (granulocytic MDSCs) in the spleen, bone marrow and blood play an important role in cancer biology¹⁴²⁻¹⁴⁷.

4.2. Role in inflammation

Neutrophils participate in the body's primary line of defense against invading pathogens such as bacteria. In non-inflammatory conditions, neutrophils are in their resting state where no toxic intracellular substances will be released. In inflammatory conditions, neutrophils can be activated via a two-stage process¹⁴⁸. First, resting neutrophils will be primed by different bacterial products and cytokines or chemokines, such as TNF- α , GM-CSF, IL-8 and IFN- γ . Primed neutrophils are then mobilized into the site of infection where they encounter activating signals to trigger bacterial killing¹⁴⁹. The priming products will induce neutrophils for the activation of transcription factors that trigger the *de novo* expression of molecules (e.g. receptors and cytokines) and increase their expression for various surface plasma membrane receptors, which enhance neutrophil function and lifespan¹⁴⁸.

The extravasation mechanism of the primed-neutrophils from the circulation is represented in figure 5 where it is controlled by interactions with the vascular endothelium. L-selectin expressed on neutrophil surface interacts with their ligands at the surface of the endothelial cells such as P-selectin, E-selectin or P-selectin glycoprotein ligand-1 (PSGL-1) as it rolls along the endothelium. This induces conformational changes in integrin adhesion expressed at the surface of neutrophils such as lymphocyte function-associated antigen-1 (LFA-1; α L β 2-integrin, CD11a/CD18) and macrophage antigen-1 (MAC-1; α M β 2-integrin, CD11b/CD18) from the inactive form into an active molecules, which are the ligands for the adhesion molecules expressed on the surface of endothelial cells, such as intercellular adhesion molecule (ICAM)-1 and -2 or vascular cell-adhesion molecule (VCAM)-1, leading to high-affinity ligand binding and strong adherence^{150,151}.

Once neutrophils have left the circulation and passed through the endothelium, they migrate towards inflamed tissue along a chemotactic gradient such as N-formylmethionyl-leucylphenylalanine (fMLP) and complement component 5a (C5a). At the site of infection, membrane receptors and immunoglobulins bind to the opsonized bacteria leading to the formation of pseudopodia, phagocytosis of the pathogen and destruction within the intracellular phagosome. Neutrophils contain an arsenal of molecules and proteases stored in

their granules as represented in figure 6, and they can also generate reactive oxygen species (ROS) in order to rapidly kill phagocytosed pathogens ¹⁴⁸.

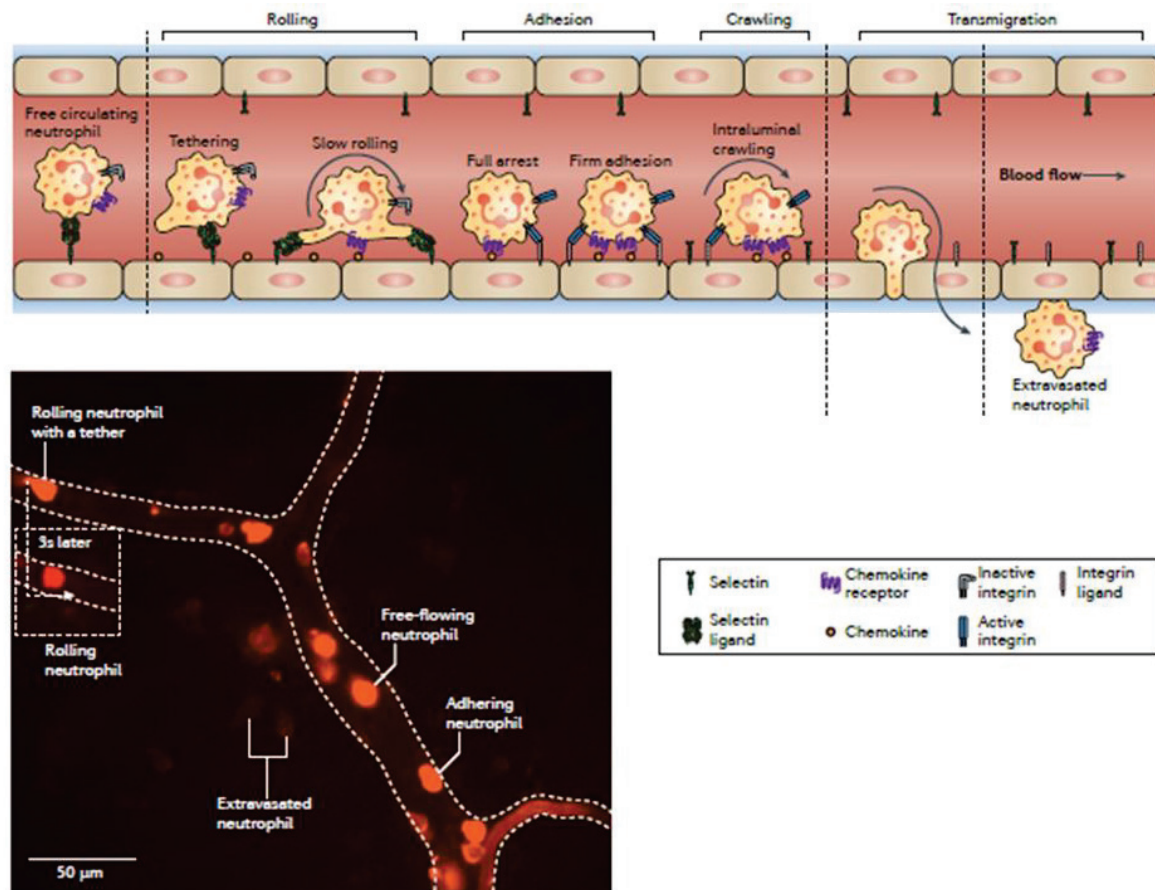


Figure 5. Neutrophil extravasation from the circulation to the site of infection (modified from Kolaczowska et al. 2013).

The neutrophil extravasation process is a cascade of low- and high-affinity adhesive interactions between the neutrophils and the vascular endothelium and involves distinct steps, including tethering, slow rolling, full arrest, firm adhesion of activated neutrophils, intraluminal crawling and transmigration. Rolling is mostly selectin-dependent, whereas adhesion, crawling and transmigration depend on integrin interactions. Chemokines lining the luminal part of endothelium activate rolling neutrophils, thus inducing conformational changes of neutrophil surface integrins and allowing for subsequent events. The intravital microscopy image shows a mouse skin postcapillary venule with neutrophils (LY6G⁺ cells) labelled in red. Mouse skin was infected with *Staphylococcus aureus* and the image was taken 2 hours later. It captured neutrophils at different stages of migration: freely circulating cells, rolling cells extending tethers, adhering neutrophils and the cells that extravasated out of the blood vessel.

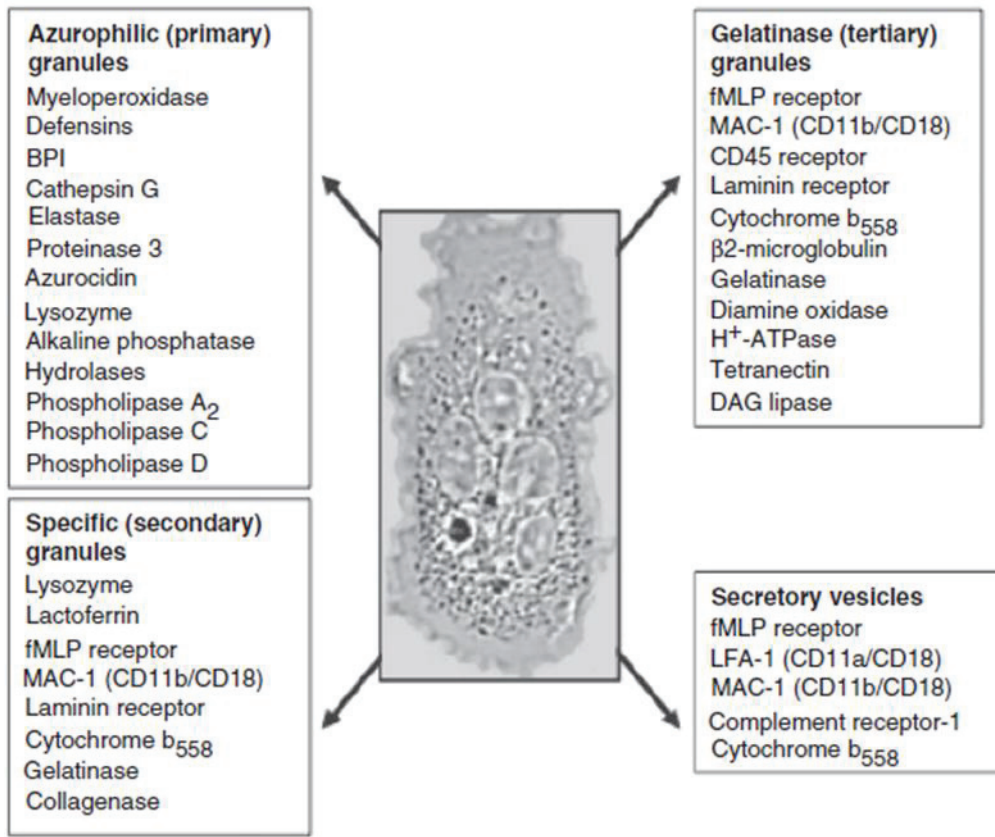


Figure 6. Contents of neutrophil granules (from Wright et al. 2010).

This figure represents a summary of neutrophil granules and their contents. Neutrophil priming induces the mobilization of granules with the secretory vesicles to be mobilized first, followed by gelatinase granules, specific granules and finally azurophilic granules

4.3. Neutrophils and cancer

4.3.1. Overview

Neutrophils make up a significant portion of the inflammatory cell infiltrate in many models of cancer. Like all other leukocytes, they move into tissues under the influence of specific chemokines, cytokines and cell adhesion molecules released by the tumor microenvironment. They have been seen *in vivo* in close association with tumor cells ⁸¹.

In addition to the well-described role of neutrophils to engulf bacteria, activate the immune system and induce tissue damage in infections ¹⁵², it's becoming clear that neutrophils have a main role in cancer ¹⁵³. However, the role of neutrophils in cancer remains the subject of controversy. Recently, neutrophils have been described to have a protumor activity, described as the N2 phenotype by analogy to macrophages (described previously). However, under certain conditions, neutrophils can exert an antitumor activity described as the N1 phenotype ¹⁴⁵.

4.3.2. Recruitment of neutrophils into tumors

Neutrophils, like other leukocytes, move into tissues from the blood under the influence of several factors secreted by the tumor microenvironment as represented in figure 7. Studies have shown an important role for macrophages and endothelial cells in neutrophil recruitment ¹⁵⁴. Also several studies suggested a role for cytotoxic T cells ¹⁵⁵⁻¹⁵⁷ or T helper cells ¹⁵⁸ in addition to tumor cells ¹⁴⁶. Neutrophil recruitment occurs by these cells via specific chemokines (e.g. KC/CXCL1 and MIP2 α /CXCL2), cytokines (e.g. TNF- α and IFN- γ) or cell adhesion molecules located on their own surface (e.g. CD11b) and on the surface of endothelial cells (e.g. selectins, ICAM-1 and Platelet-Endothelial cell adhesion molecule-1) ¹⁵⁹.

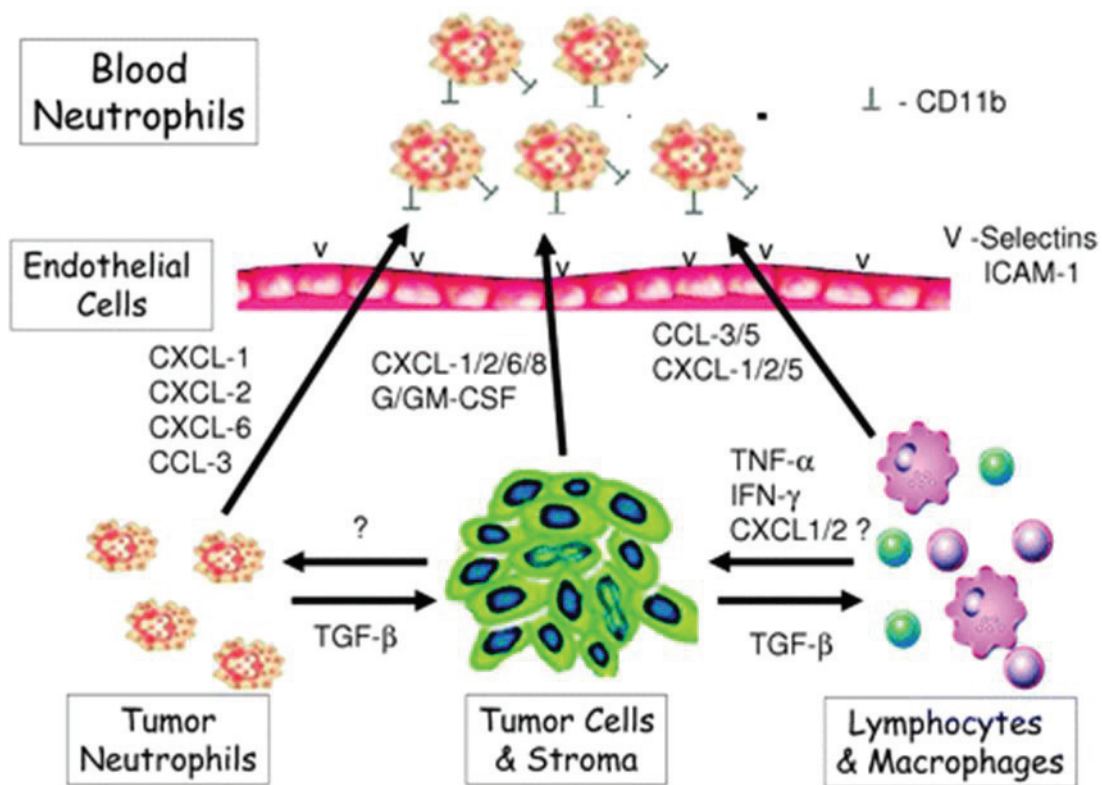


Figure 7. Neutrophil recruitment into tumors (from Fridlender et al. 2012).

A schematic representation of several factors secreted by the cells of tumor microenvironment influencing the recruitment of neutrophils from the blood into the tumor. These factors include several cytokines and chemokines in addition to the role of cell adhesion molecules on the surface of neutrophils and on the surface of endothelial cells.

4.3.3. Neutrophil polarization

Several lines of evidence document the existence of two neutrophil phenotypes: the N1 (antitumor) and N2 (protumor) phenotypes¹⁴⁵. This neutrophil plasticity is regulated by molecules in the tumor microenvironment. Figure 8 represents a simplified scheme of neutrophil polarization.

The immunosuppressive cytokine transforming growth factor (TGF)- β induces neutrophils to acquire an N2 protumor phenotype whereas IFN- β induces neutrophils to acquire an N1 antitumor phenotype¹⁴². In addition, neutrophils express functional receptors for other cytokines such as IL-1 β , TNF- α ¹⁶⁰ and IL-10¹⁶¹. These cytokines are known to act on neutrophils and it is well established that IL-1 β and TNF- α are pro-inflammatory and immunostimulating cytokines, whereas IL-10 has the capacity to dampen inflammation and to be immunosuppressive¹⁶¹.

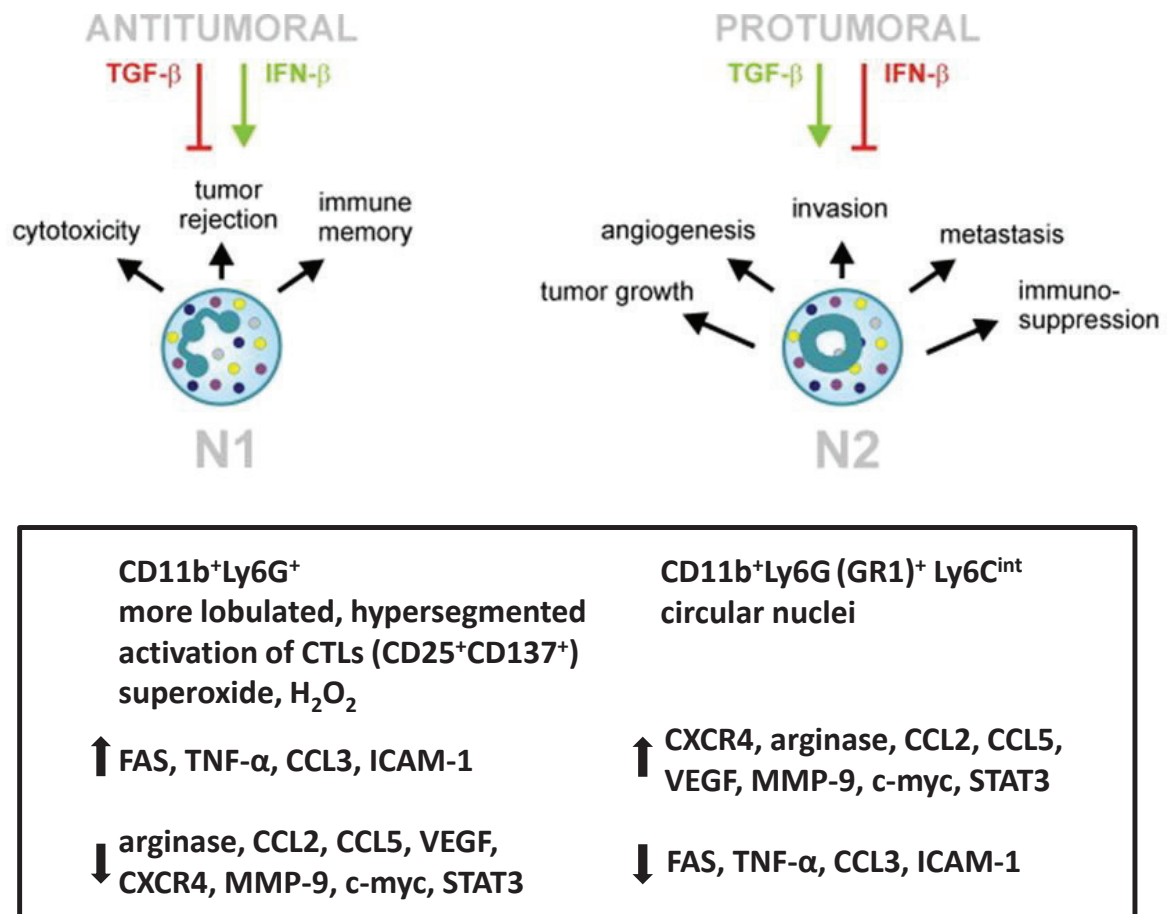


Figure 8. Neutrophil polarization (from Piccard et al. 2012).

Neutrophils exert both antitumoral and protumoral functions in cancer. This phenomenon is denominated “neutrophil polarization” and is influenced by different molecules such as the cytokines TGF-β and IFN-β. N1 neutrophils are antitumoral effector cells by inducing cytotoxicity, tumor cell lysis and apoptosis, and mediating tumor rejection in antitumoral immune memory. N2 neutrophils are protumoral neutrophils inducing tumor progression by promoting angiogenesis, invasion, metastasis and immunosuppression. Differences in characteristics of N1 and N2 phenotypes are indicated in the box.

CTLs= cytotoxic CD8⁺ T lymphocytes; Ly6 (lymphocyte antigen 6) markers: Ly6G (granulocytes) and Ly6C (monocytes) are only defined in mice and antibodies against GR1 (for granulocytes) recognize both Ly6G and Ly6C.

4.3.4. Dual roles of neutrophils

Interactions between a tumor and its surrounding microenvironment promote various molecular and cellular processes underlying multiple steps that define cancer progression. These steps include tumor cell proliferation, neovascularization for further tumor growth, local migration and growth within the new environment ¹⁶².

Neutrophils are associated with and make functional contributions to many of these different steps. Table 6 documents key mechanisms of protumor neutrophils. However, cytotoxicity, tumor cell apoptosis, immunologically mediated tumor rejection and antitumor immune memory may counteract tumor progression at every stage and table 7 documents key mechanisms of antitumor neutrophils.

4.3.4.1. N2-protumor functions of neutrophils

Neutrophils appear to develop a protumorigenic phenotype (N2) and appears to contribute to tumor growth ^{144,145,163,164} and suppression of the antitumor immune response ¹⁶⁵. Therefore, the depletion of these protumoral N2 neutrophils inhibits tumor growth ^{145,163,164} and reduces the level of immunosuppression in the tumor microenvironment. N2 appear to be involved in tumorigenesis and tumor growth through multiple mechanisms.

4.3.4.1.1. Tumorigenesis

Neutrophils have a main role in the initiation process of tumors, mainly by affecting the ECM and the tumor cell microenvironment ¹⁴². Proteases, particularly MMPs secreted by neutrophils, are key players in altering tumor microenvironments through ECM remodeling that help in tumorigenesis.

MMP-9 was reported to be involved in skin tumorigenesis by inducing the regulation of oncogene-induced keratinocyte hyperproliferation and their progression to invasive cancer ¹²⁰. Also, Acuff et al. described the role of MMP-9 in preventing tumor cell apoptosis in lung cancer ¹⁶⁶. Another potential direct effect of neutrophils on tumor growth is described by Houghton and coworkers, through the secretion of neutrophil elastase (NE) that enters tumor cells and binds Insulin receptor substrate-1 (IRS-1), inducing AKT activation ¹⁶⁷. Neutrophils secrete other factors that have an indirect effect on tumor growth by additional recruitment of leukocytes such as KC/CXC-chemokine Ligand(CXCL1)¹⁶⁸, CXCL8 and CC-chemokine Ligand (CCL)-3 ¹⁶⁹.

4.3.4.1.2. Tumor angiogenesis

Tumors require angiogenesis for persistent growth where several angiogenesis factors have been identified ^{41,170}. VEGF ¹⁷¹ and placental growth factor (PIGF) ¹⁷² exemplify the angiogenesis signals in tumors. ECM is a major storage place of angiogenic molecules including chemokines ¹⁷³.

Neutrophils inside tumors form one of the sources of angiogenesis-regulating growth factors, chemokines and proteases ^{174,175} as represented in figure 9, and fibroblast growth factor (FGF)-2 was shown to be a major cytokine involved in neutrophil MMP-9-induced angiogenesis ¹⁷⁶.

Angiogenic switch is a step acquired during tumor development to induce angiogenesis and it is achieved by a change in the balance between inducers and inhibitors of angiogenesis¹⁷⁷. While studying pancreatic islet carcinogenesis, Nozawa et al. revealed that infiltrating neutrophils ‘switch on’ angiogenesis during the early stages of tumorigenesis and exert this capacity through gelatinase B/MMP-9, which likely liberates VEGF sequestered in the ECM¹⁷⁸. MMP-9¹⁷⁸, in addition to MMP-2¹⁷⁹, are involved in the selective remodeling of the ECM and/or basement membranes before the endothelium can sprout and reorganize to form new blood vessels.

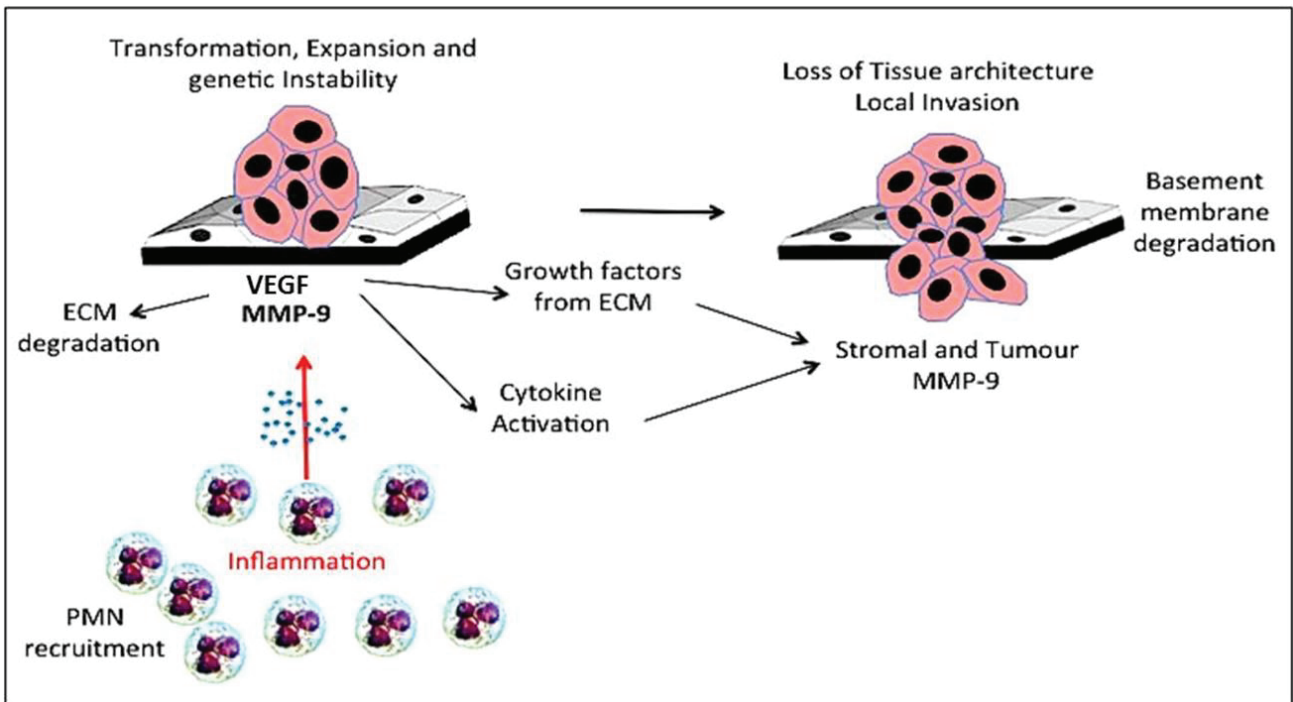


Figure 9. The role of neutrophils in ECM remodeling and tumor cell invasion associated with tumor progression (modified from Farina et al. 2014).

Neutrophils (PMN) secrete several factors (e.g. VEGF and MMP-9) that help in ECM remodeling by inducing the latter degradation. This process helps in tumor invasion and angiogenesis.

MMP= Matrix MetalloProteinase, VEGF= Vascular Endothelial Growth Factor

4.3.4.1.3. Tumor invasion and metastasis

The invasion-metastasis process of tumors consists of a cascade of events represented in figure 10: 1- invade locally through surrounding extracellular matrix and stromal cell layers, 2- intravasate into the lumina of blood vessels, 3- survive the transport through the vasculature, 4- arrest at distant organ sites, 5- extravasate into the parenchyma of distant tissues, 6- initially survive in these foreign microenvironments, and 7- reinitiate their proliferative program at metastatic sites ¹⁸⁰.

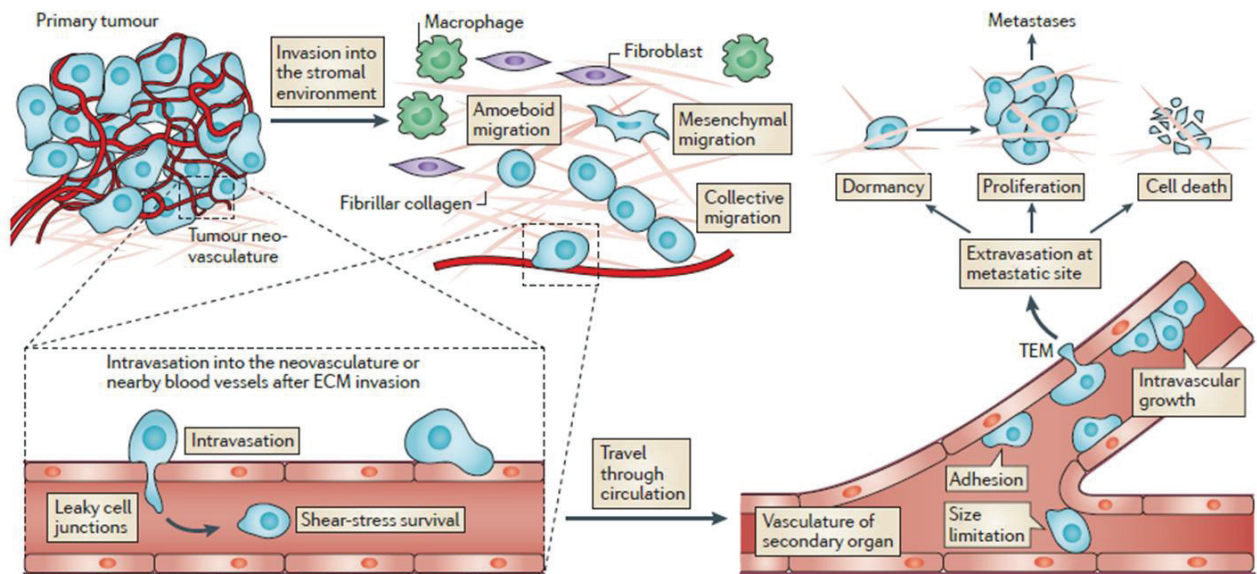


Figure 10. Tumor cell invasion-metastasis process (from Reymond et al. 2013).

Several tumor cells from a primary tumor acquire invasive and migratory properties. These tumor cells leave the primary tumor and invade their surrounding tissues and migrate towards the neighboring blood vessels. Tumor cells enter the bloodstream in a process called intravasation resulting in spreading throughout the body. Then the tumor cells leave the circulation in a process called extravasation at potential secondary tumor sites. Extravasation involves the specific interaction of tumor cells with vascular endothelial cells (ECs) via cell adhesion- and chemokine-related processes. Tumor cells then transmigrate through the endothelial barrier by a process known as transendothelial migration (TEM), resulting in new tumor site. Cells can then enter a state of dormancy or proliferate within this new microenvironment giving rise to micrometastases to be macrometastases. However, some of the tumor cells that extravasate will not colonize these new tissues but will undergo cell death instead. ECM= extracellular matrix

During these processes, neutrophils play important roles, either by increasing tumor invasion and extravasation, remodeling extracellular matrix, or promoting tumor homing.

Neutrophils help tumor invasion and extravasation

Several studies indicate the role of neutrophils in promoting tumor invasion. It was observed that many tumors overproduce specific factors (e.g. chemokines) ¹⁸¹⁻¹⁸³. This results in the classical infiltration of neutrophils into the tumor microenvironment ¹⁴⁶. Neutrophils then induce cell detachment in the tumor microenvironment where several molecules are involved in this process such as TNF- α /TNF- α receptor inhibitor, IL-1 α /IL-1 α receptor and NE ¹⁸⁴. Later, tumor cells that lose the adhesion contacts with the environment will migrate towards blood vessels ^{185,186} and manage to adhere to and pass through the endothelial vessel wall to form a new tumor site, a process known as extravasation represented in figure 11 ¹⁸⁷⁻¹⁹⁰. Huh et al. reported that circulating tumor cells *in vivo* directly anchor to the vascular endothelium, facilitating transendothelial migration of tumor cells, extravasation and formation of new metastases ¹⁹¹. Injection of neutrophils in this model significantly increased cancer cell retention. Other studies showed that neutrophils facilitate the extravasation of melanoma cells ¹⁹² and can augment the capability of tumor cells to extravasate through endothelium ¹⁹³ allowing the adherence of tumor cells into the lungs ¹⁹⁴.

Neutrophils remodel the extracellular matrix

During the acquisition of invasiveness and metastatic ability, protease genes are often up-regulated in the tumor microenvironment, whereas protease inhibitor genes are downregulated and inactive zymogen forms of proteases are converted into active enzymes ¹⁹⁵. In several tumors, such matrix-degrading proteases are not produced by the tumor cells, but rather by stromal and inflammatory cells such as the neutrophils. Welch et al. ¹⁹⁶ demonstrated that neutrophils elicited from tumors secrete high levels of the basement membrane degrading enzymes collagenase-IV and heparanase, assisting tumor cell extravasation during the metastatic process. Paradoxically, not only proteinases but also their inhibitors can correlate with bad prognosis of cancer patients. Tissue inhibitor of metalloproteases (TIMP)-1 may originate from neutrophils, fibroblasts, endothelial cells ¹⁹⁷ and even from the tumor cells themselves ¹⁹⁸. TIMP-1 is inducible by IL-6 and TGF- β ¹⁹⁹ and possesses signaling functions to enhance metastasis ²⁰⁰.

Neutrophils promote tumor homing

Several studies reported the role of neutrophils in tumor homing and metastatic cell survival. Wu et al. described in a study done on lung metastases in 4T1 mammary and Lewis lung carcinoma (LLC) model, a role for type I Interferon N (IFN-I) during metastasis development. Mice with impaired IFN-I signaling develop more lung metastases in both models accompanied by massive neutrophil accumulation in lungs. Elevated granulocyte-colony-stimulating factor (G-CSF) levels in serum and enhanced CXC-chemokine receptor (CXCR)-2 expression on neutrophils are most likely responsible for this phenomenon. In addition, lung-infiltrating neutrophils support tumor extravasation and proliferation due to the enhanced expression of several proteins such as Bv8, MMP9, S100A8 and S100A9 ²⁰¹. Another study done by Acuff et al. reported a role of gelatinase B/MMP-9 in lung metastasis and homing in LLC model. Gelatinase B/MMP-9 released by the neutrophils affects the initial steps of metastatic tumor formation in the lung and a significant increase in tumor cell apoptosis was observed in the lungs of gelatinase B/MMP-9 knockout mice ^{166,202}.

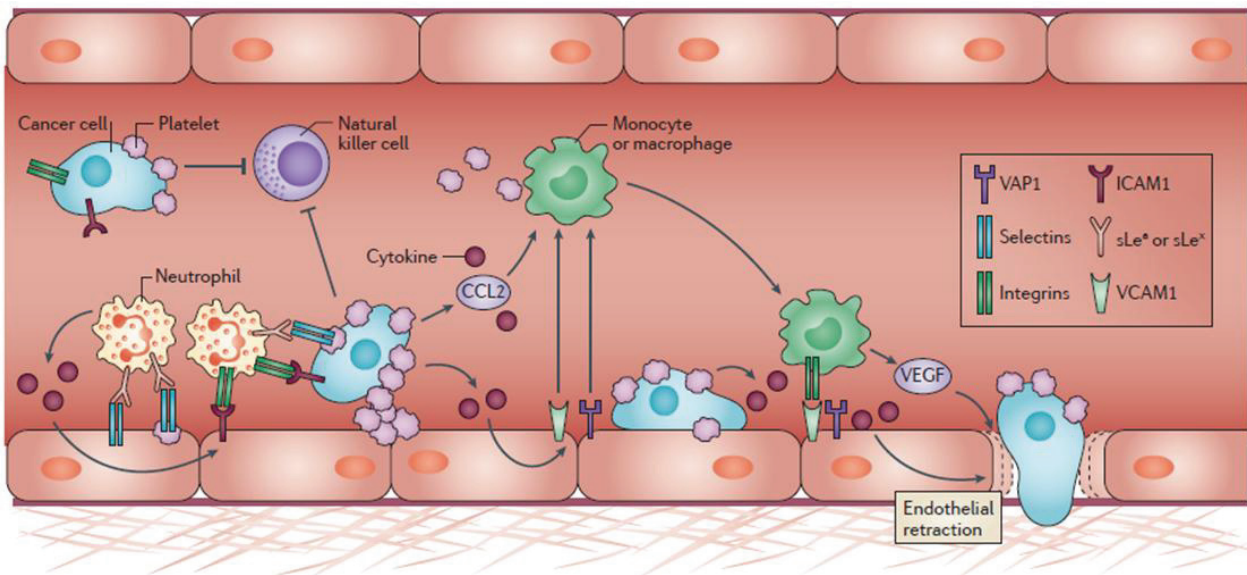


Figure 11. Representative figure for host-tumor cell interactions that contribute to tumor extravasation (from Reymond et al. 2013).

As soon as the tumor cells enter the bloodstream, they interact with platelets that protect tumor cells against lysis by different immune cells such as NK cells. Platelets also induce tumor cell adhesion to endothelial cells (ECs) where they secrete extravasation-enhancing substances and recruit several leukocytes such as neutrophils. Neutrophils then secrete cytokines and enhance the tumor cell adhesion to ECs by acting as bridges between these two cell types through the interaction between $\beta 2$ integrin expressed on neutrophil surface and intercellular adhesion molecule-1 (ICAM-1) expressed on ECs and tumor cell surfaces. Tumor cells then secrete chemokines such as CC-chemokine ligand-2 (CCL-2) or cytokines that activate ECs and induce the expression of several molecules such as vascular cell adhesion molecule-1 (VCAM-1) and vascular adhesion protein 1 (VAP-1), resulting in the induction of monocytes or macrophages recruitment that stimulate also the tumor cell extravasation by secreting VEGF locally that results in the opening in EC junctions.

Table 6. Protumor functions of N2 neutrophils

	Mechanism	Ref
Tumorigenesis	MMP-9 that is predominantly expressed by neutrophils in dysplasias and carcinomas stimulates epithelial carcinogenesis by stimulating hyperproliferation, angiogenesis and tumor progression to more aggressive end-stages	203
Tumor cell apoptosis	Neutrophil infiltration supplies MMP-9 which supports the formation of lung tumors, likely by preventing apoptosis of tumor cells	166
Tumor growth	Association of neutrophil infiltration with increased tumor growth of highly tumorigenic and metastatic melanoma cells upon increasing levels of IL-8/CXCL8	204
	Association between activation of TANs (increased levels of MMP-9) with enhanced tumor growth and increased protumoral angiogenesis of melanoma cells	185
	Association of neutrophils and monocytes through CXCR2 with higher tumoral growth rate of murine squamous cell carcinoma	168
	Infiltrated neutrophils lead to faster tumor growth with better developed blood vessels in an IFN-negative milieu	205
	Neutrophils favor tumor growth in the presence of TGF- β	145
Angiogenesis	Association of neutrophils and monocytes through CXCR2 with enhanced angiogenesis in murine squamous cell carcinoma	168
	Neutrophils within neoplastic lesions switch on angiogenesis during pancreatic islet tumorigenesis, through MMP-9 which likely liberates VEGF	174
	MMP-9-expressing neutrophils are associated with the invasion of new blood vessels into collagen gels, and required together with MMP-2, for vascularization <i>in vivo</i>	179
	Infiltrated neutrophils have higher expression of VEGF, MMP-9 and CXCR4 in an IFN-negative milieu, and cause better developed blood vessels and faster tumor growth	205
Invasion	MMP-9 that is predominantly expressed by neutrophils in carcinomas stimulates tumor progression to more aggressive end-stages	203
	Tumor-elicited neutrophils stimulate invasion of rat mammary adenocarcinoma cells <i>in vitro</i>	196

Metastasis	Association of neutrophil infiltration with more metastatic lung foci of highly tumorigenic and metastatic melanoma cells upon increasing levels of IL-8/CXCL8	204
	Association of neutrophils and monocytes through CXCR2 with more metastasis in murine squamous cell carcinoma	168
	Tumor-elicited neutrophils stimulate metastasis of rat mammary adenocarcinoma cells and associated with neutrophil secretion of MMP-9 and heparanase	196
	Neutrophils induce detachment of bronchioalveolar adenocarcinoma cells. Involved neutrophil molecules are e.g. ICAM-1/LFA-1, TNF-/TNF-receptor inhibitor, IL-1/IL-1 receptor, NE	184
	Neutrophil influx in tumor-bearing lungs is a predominant source of MMP-9 which mediates pulmonary tumor formation, associated with less tumor cell apoptosis	166
	Neutrophils facilitate the extravasation of melanoma cells, by “two-step adhesion” to endothelial and tumor cells via LFA-1, SleX and Mac-1	192
Anticancer therapy	Intra-abdominal neutrophil influx upon tumor surgery enhances local tumor recurrence	206
	CD11b+GR-1+ cells render tumors refractory to blockade of angiogenesis by anti-VEGF Abs, by mediating an angiogenesis pathway that bypasses VEGF	207

SleX, sialyl Lewis X.

4.3.4.2. N1-antitumor functions of neutrophils

Despite the broad protumor effect of neutrophils described above, there are several studies reported an antitumor effect for neutrophils. Neutrophils contain a plethora of cytotoxic mediators, including ROS, membrane-perforating agents, proteases and soluble mediators such as TNF- α , IL-1 β and IFNs¹⁴⁰. Neutrophil cytotoxic mediators have been shown to lead to tumor cell death and regression. Moreover, neutrophils may induce apoptosis and lysis of tumor cells, and may mediate the induction of tumor rejection and antitumoral immune memory. Table 7 documents key mechanisms of antitumoral neutrophil functions.

Neutrophils can assume the cytotoxic N1 phenotype during TGF- β inhibition¹⁴⁵ or after immunologic or cytokine activation (figure 8)²⁰⁸⁻²¹⁰. Thus, the depletion of these N1 neutrophils either enhances tumor growth or reduces the antitumor effects of immunological treatments^{145,155,211,212}. Described below, the different mechanisms for the antitumoral role of neutrophils.

4.3.4.2.1. Tumor cell lysis by tumoricidal molecules

Neutrophils have been shown to have a cytotoxic effect toward several tumor cells such as bladder carcinoma cells, melanoma cells and lymphoid tumor cells *in vitro*^{213,214} and *in vivo*²¹⁵. ROS secreted from activated neutrophils are among the molecules known to have a destructive effect and participate in tumor cell lysis²¹⁶⁻²¹⁸. Zivkovic et al. reported that coculture of tumor cells with neutrophils activates the latter to produce singlet oxygen (1O₂) and to diminish tumor cell proliferation *in vitro*²¹⁸. Another study by Zivkovic et al. showed that isolated neutrophils from peripheral blood of melanoma mice model exert a cytotoxic effect on tumor cells *in vitro* whereas *in vivo* they indicated that neutrophil distraction from tumor using Sephadex G-200 allow the latter fast progression since Sephadex attracted activated neutrophils at the site of its injection at the time when the tumor was transplanted²¹⁹. Moreover, Frindlender and coworkers reported an antitumor role for oxygen radicals (O₂, H₂O₂) released by N1 antitumor phenotype in a TGF- β negative milieu¹⁴⁵. Interestingly, Schaidler et al. suggested that there seems to be a difference between the cytotoxicity of neutrophils towards primary versus metastatic cells where the latter have been shown to be less affected²⁰⁴.

4.3.4.2.2. Tumor cell apoptosis by direct cell contact

Neutrophils enhance tumor cell apoptosis by different mechanisms of direct cell contact. Among the different cell contact mechanisms applied by the neutrophils on tumor-cell lysis and apoptosis, there is the Fas-ligand (Fas-L) associated apoptosis. Fas-L is a member of the TNF superfamily and triggers a death signal after interaction with the Fas molecule on Fas+ cells such as the antitumoral N1 phenotype¹⁴⁵. Chen et al. showed that melanoma cells with suppressed expression of Fas-L grew faster *in vitro* with enhanced lung metastasis *in vivo*²²⁰.

A second mechanism by which neutrophils were shown to be capable of directly inducing tumor cell death is by ADCC represented in figure 12^{71,221,222}. Kushner and colleagues define a monoclonal antibody-mediated neutrophil antihuman tumor cytotoxicity mechanism by which the monoclonal antibody specific for the ganglioside GD2 mediates the tumor cytotoxicity of human melanoma and neuroblastoma GD2+ solid tumor cells by neutrophils *in vitro* and *in vivo*. They stated that the optimal ADCC by human neutrophils requires both integrins CD11b and CD11c. Neutrophil-mediated ADCC is enhanced by GM-CSF treatment, which increases the expression of CD11/CD18 molecules^{223,224}.

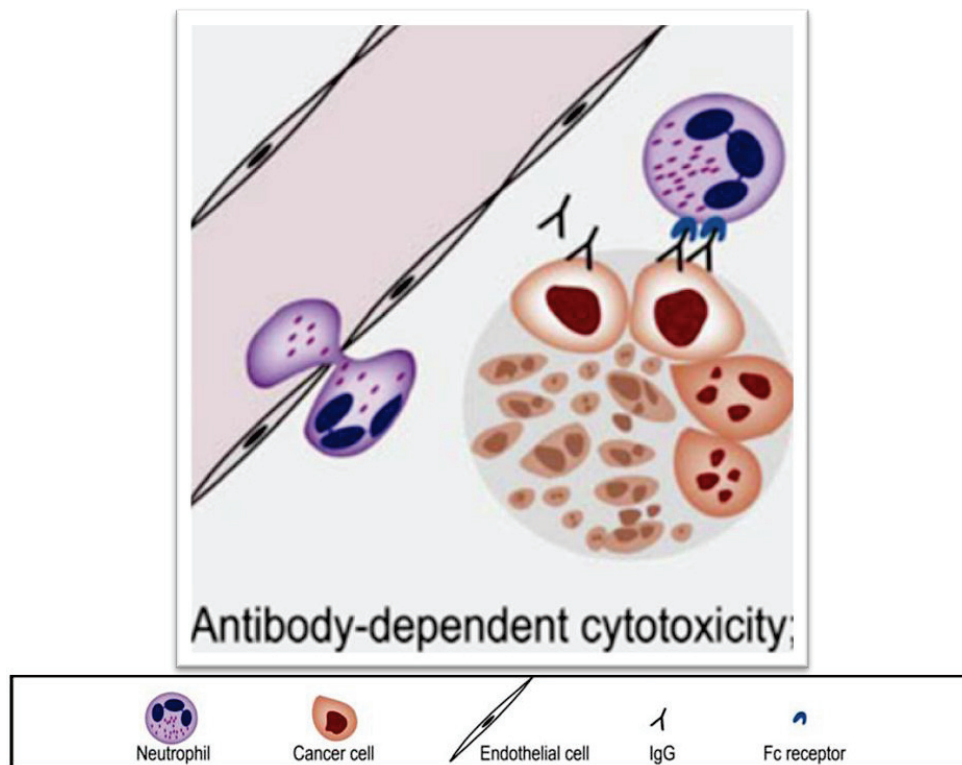


Figure 12. Antibody-dependent cellular cytotoxicity mediated by neutrophils (modified from Ersvaer et al. 2010).

Simplified figure represents the antibody-dependent cellular cytotoxicity (ADCC) mediated by neutrophils. Neutrophils induce tumor cell death by ADCC in the presence of specific monoclonal antibody. The monoclonal antibody directed against a specific antigen overexpressed on the surface of tumor cells binds through its Fc fragment to Fc receptors expressed on neutrophils, thus allowing recruitment of these neutrophil effector cells to target tumor cells.

4.3.4.2.3. Tumor rejection and antitumor immune memory

N1 neutrophils can support tumor rejection. This antitumoral N1 activity against malignant cells is dependent on CD8⁺ T cells since neutrophils have the ability to influence cytotoxic T cells in infections and in cancer. These neutrophils can promote CD8⁺ recruitment and activation by producing T-cell attracting chemokines (e.g. CCL-3, CXCL9 and CXCL10), pro-inflammatory cytokines (e.g. IL-12, TNF- α and GM-CSF)^{145,225} and the activation of dendritic cells²²⁶. Furthermore, neutrophils have been shown to cross-present antigens *in vitro* where these antigen-pulsed neutrophils promote the activation of CD8⁺ T cells²²⁷. Kousis et al. showed that photodynamic therapy-induced CD8⁺ T cell induction required the presence of neutrophils²¹¹. In addition, local release of IL-10 by transfected mouse mammary adenocarcinoma cells enhances antitumor reaction due to the combined action of CD8⁺ T lymphocytes, NK cells, and neutrophils, lead to a complete tumor rejection²²⁸. Tumor rejection by paracrine release of cytokines is typically associated with a massive presence of neutrophils in close contact with tumor cells²⁰⁸.

Neutrophils are involved also in antitumoral immune memory. A study done by Cavallo et al. showed a role for neutrophils together with CD4+ T cells involved in the primary and memory response to murine mammary adenocarcinoma. These tumor cells are rejected immediately, in association with neutrophil infiltration. This neutrophil-dominated rejection leaves a long-lasting, tumor-specific, T lymphocyte-mediated immune memory leading to secondary tumor cell rejection ²²⁹.

Table 7. Antitumoral functions of N1 neutrophils

Mechanism	Ref
Tumor regression	<p>Neutrophil MMP-8 protects against chemical skin carcinogenesis by preventing chronic neutrophilia 230</p> <p>Neutrophils produce singlet oxygen upon coculture with tumor cells and diminish tumor cell proliferation 218</p> <p>Neutrophils are antitumoral in the early phase of carcinoma development, resulting in smaller tumor volumes 218</p>
Tumor cell cytotoxicity	<p>Isolated neutrophils preferentially kill primary instead of metastatic melanoma cells 204</p> <p>Tumor rejection upon paracrine release of cytokines (e.g. IL-2, TNF-α) is associated with degranulated neutrophils with released granules in close contact with damaged tumor cells 208</p> <p>Tumor cell cytotoxicity upon release of G-CSF is associated with cytoplasmatic projections of activated neutrophils in close contact with dead tumor cells and with cell-cell contacts and fusion of cell membranes between neutrophils and G-CSF-producing cells 209</p> <p>Neutrophil-mediated cytotoxicity of tumors upon cytokine release targets tumor-associated blood vessels and leukocyte infiltration is mediated through VCAM-1 and E-selectin on endothelial cells 231</p> <p>Neutrophils are cytotoxic and cytostatic towards various tumor cell types 213–215</p> <p>ROS from activated neutrophils cause tumor cell lysis through oxidative damage 216–218</p> <p>Peripheral blood neutrophils of melanoma-injected mice show an extensive oxidative burst and are cytotoxic for tumor cells 219</p> <p>Neutrophils activated via LFA-1 or Mac-1 release HOCl, resulting in tumor cell lysis, microvessel injury and matrix degradation 217</p> <p>Circulating neutrophils increase after trypan blue treatment of melanoma-</p>

	bearing mice have increased cytotoxicity for tumor cells and produce more toxic oxygen radicals, correlated with accelerated clearance of tumor cells in lungs	232
Tumor cell apoptosis	Association of neutrophils with apoptosis in primary prostate tumors	233
	Binding of antibodies to neutrophil Fc receptors mediates antitumoral cytotoxicity	71
	Neutrophils mediate antibody-dependent cellular cytotoxicity of tumor cells via a series of membrane rupture/resealing events	221
	Granulocytes are main effectors in treatment of renal carcinoma by bispecific antibodies and G-CSF/GM-CSF	224
	Neutrophils mediate ADCC of melanoma and neuroblastoma tumors through CD11b, CD11c (Fc receptor II and III)	223
Tumor rejection and antitumoral immune memory	Association of granulocyte infiltration with rejection of engineered tumor cells which release cytokine such as IL-1 and IL-4 and immunity against parental tumors	234
	Association of neutrophil infiltration upon SPARC expression with melanoma tumor rejection	235
	IL-10-induced rejection of mammary adenocarcinoma by combined action of neutrophils, CD8+ and NK cells and antitumoral memory based on CD8+ cells and neutrophils via tumor-specific antibodies	228
	Association of neutrophil infiltration with rejection of IL-2-secreting mammary adenocarcinoma, followed by T cell-mediated antitumoral memory with activation of CD4+ cells by granulocytes	229
	Granulocytes are involved in rejection of secondary tumors	236
Tumor growth	Association of neutrophil influx with impaired survival and tumor growth of IL-8/CXCL8 transduced primary melanoma cells	204
	Association of neutrophils with decreased <i>in vivo</i> tumor growth of IL-8/CXCL8-expressing human ovarian cancer cells	237
	Association of neutrophils with suppressed subcutaneous tumor growth of huIL-8/CXCL8-, huMIP-1 α /CCL3- or muMIP-1 α -transfected hamster ovarian tumor cells	169
	Neutrophils are primary effectors in tumor growth suppression of IL-4 expressing melanoma and fibrosarcoma cells	238
	Neutrophils impair the growth of various cytokine-expressing tumors	239

	Neutrophils association with slow tumor growth in TGF- β negative milieu	145
Angiogenesis	Slower tumor growth by repressed neutrophil-mediated angiogenesis, associated with inhibited neutrophil expression of VEGF and CXCR4 through downregulation of Stat3 and c-myc, in the presence of constitutive amounts of IFN- β	205
Anticancer therapy	Lysis of cancer cells upon injection with oncolytic viruses is accompanied by indirect killing of uninfected tumor cells through induction of apoptosis by neutrophils	240
	Neutrophils are crucial to establish tumor-specific adaptative immunity upon photodynamic therapy	211

CHAPTER V

5. Interactions between cancer therapy and the immune system

5.1. Cancer therapy: reengineering tumor immunity

Most anticancer agents were originally developed in order to target tumor cells. In this perspective, conventional chemotherapeutic drugs were selected for their direct cytotoxic effects against highly proliferative cancer cells. These agents, such as anthracyclines or DNA-alkylating agents (table 4) are associated with significant toxicities and their efficacy is limited by the emergence of drug resistant tumor cell clones²⁴¹. Thanks to the growing understanding of cancer cell biology at the molecular level where more specific anticancer agents were developed and referred to as targeted therapeutic agents^{241,242}. Targeted therapies (table 5, figure 2) are designed to inhibit oncogenic pathways essential for the proliferation and survival of tumor cells. They showed a therapeutic efficacy as single agents; however, they are limited by the acquisition of secondary mutations that result in drug resistant tumor clones and patient relapse.

Several studies have reported an effect of anti-cancer agents on tumor microenvironment that may alter the tumor response to cancer therapy, resulting in promotion or inhibition of tumor progression. For example, different studies have suggested that cancer therapy can alter the immunosuppressive functions of the immunosuppressive cells, primarily regulatory T lymphocytes (Treg), MDSCs, or tolerogenic DCs. It has been well established that tumors can promote these immunosuppressive cells that inhibit anti-cancer immunity and significantly compromise the efficacy of immune-based therapies^{84,243,244}. Their elimination and/or inactivation have become a major therapeutic objective to alter the immunosuppressive tumor microenvironment and uncover anti-cancer immunity. Other studies reported an effect of cancer therapy on NK cell functionality^{245,246}, macrophages^{247,248} and the adaptative immune cells²⁴⁹⁻²⁵².

Several mechanisms by which cancer therapy can modulate the immune cells (figure 13) and the tumor immunity (figures 14 and 15) are discussed below.

5.1.1. Effect of cancer therapy on tumor immunity

Cancer therapy can promote/inhibit tumor immunity in several ways. They can promote tumor immunity through their therapeutic effect on cancer cells by immunogenic cell death or through disrupting strategies that tumors use to evade the immune response²⁵³.

5.1.1.1. Tumor immunogenicity

Various chemotherapeutic agents can modulate intrinsic tumor cell immunogenicity in multiple ways. Some chemotherapeutic molecules, such as anthracyclines and cyclophosphamide, induce the release of high mobility box binding protein-1 (HMGB-1) or ATP from the tumor cells into their microenvironment. These molecules bind to their respective pattern recognition receptors (PRRs), Toll-like receptor-4 (TLR-4), and the purinergic receptor P2RX7 on malignant cells. This activates the NOD-like receptor family, pyrin domain containing-3 (NLRP-3) inflammasome resulting in IL-1 β secretion and activation of IFN- γ -secreting CD8⁺ T cells^{254,255}. In addition, these chemotherapeutic

molecules increase the exposure of calreticulin on the tumor cell surface, which is critical for the recognition and engulfment of dying tumor cells by DCs ²⁵⁶.

TLR4 and/or P2RX7 loss-of-function polymorphisms are associated with a higher risk of breast cancer relapse after adjuvant anthracycline based chemotherapy ^{257,258}, and fail to affect the response to chemotherapy in patients with non-small cell lung cancer (NSCLC) ²⁵⁹. TLR4 loss-of-function polymorphisms are also associated with shorter progression-free survival (PFS) and overall survival (OS) in patients with advanced colorectal cancer ²⁶⁰ and squamous cell head and neck cancer ²⁶¹.

Chemotherapy can also enhance tumor antigen presentation by up-regulating the expression of tumor antigens themselves, or by up-regulating the MHC-I molecules to which the antigens bind. Some drugs enhance tumor cell death by inducing the expression of co-stimulatory molecules such as B7-1 on the surface of tumor cells and boost their ability to present tumor antigens to achieve effective immune activation. Other drugs decrease the expression of immune checkpoint molecules such as B7-H1/PD-L1 and B7-H4 on the tumor cell surface, thereby preventing infiltrating T cells from being shut down ^{262,263}. Chemotherapy may also increase the sensitivity of the tumor cells to T cell-mediated lysis through Fas-, perforin-, and granzyme B-dependent mechanisms ²⁶⁴. Different mechanisms induced by chemotherapeutic agents are listed in table 8.

Table 8. Modulation of tumor cell immunogenicity by chemotherapy (modified from Chen et al.)

Mechanism	Chemotherapy	Type of tumor	Ref.
Sensitizes tumor cells to CD8+ T cell-mediated apoptosis	Cyclophosphamide	Mesothelioma cells	265
	5-Fluorouracil, Dacarbazine	Melanoma	266
Upregulation/downregulation of antigens	Paclitaxel ,Cisplatin, Doxorubicin	Lymphoma Colon cancer cells	267
	Paclitaxel	Colon cancer cells	268
	Doxorubicin, Ara-C (cytosine arabinoside)	Breast cancer and leukemia cells	262,263
	Ara-C (cytosine arabinoside)	Leukemia cells Mastocytoma and plasma-cytoma cells	262,269,270
	5'-aza-2'deoxyctidine	Renal cell carcinoma, ovarian cancer, melanoma, glioma	271,272
Induction of autophagy	Mitoxantrone, Oxaliplatin	Colon cancer cells	273
Induction of immunogenic cell death	Gemcitabine, Cyclophosphamide, Oxaliplatin, Doxorubicin, Paclitaxel	Colon cancer, melanoma, and fibrosarcoma cells	274

5.1.1.2. Effect of cancer therapy on innate immune cells

Innate immune cells constitute a substantial proportion of the cells within the tumor microenvironment. Several effects of anti-cancer agents have been described on the immunosuppressive cells MDSCs, in addition to DCs, macrophages and NK cells ²⁷⁵.

MDSCs

MDSCs, as described previously, represent a subset of cells characterized by their capacity to suppress immunity ⁸⁴. Several studies with different types of cancer reported that MDSCs elimination, inhibition, or the induction of their differentiation into pro-inflammatory cells using anticancer agents enhance the antitumor immunity and the response to immunotherapy (figure 15). Gemcitabine, an anti-metabolite drug used for the treatment of pancreatic, breast, ovarian, and lung cancers, has been reported by Vincent et al. to deplete MDSCs in tumor-bearing mice, resulting in enhanced antitumor immunity ²⁷⁶. In the same study, they reported an effect of another metabolite, 5-Fluorouracil (5-FU), which enhances MDSCs apoptosis when used at low doses ²⁷⁶. Another chemotherapy agent docetaxel (mitotic inhibitor) was shown to impair the suppressive effect of MDSCs by blocking Stat3 phosphorylation as well as inducing their differentiation into M1 macrophages in a murine model of breast cancer ²⁷⁷. Similarly, it has been reported that low-dose paclitaxel (another mitotic inhibitor) promotes MDSCs differentiation into dendritic cells *in vitro* ²⁷⁸. Doxorubicin is an anthracycline reported to eliminate and inactivates MDSCs in different mouse cancer models. Targeting MDSCs by doxorubicin enhanced CD4+, CD8+ and NK cell activation and proinflammatory cytokine production result in efficient immunotherapy ²⁷⁹. In an interesting study by Diaz-Montero and colleagues, the authors reported an increase in MDSC levels in breast cancer patients treated with standard doxorubicin and cyclophosphamide chemotherapy. These molecules selectively decreased regulatory MDSC while sparing other MDSC and polarizing them toward a functional antitumor phenotype ²⁸⁰.

Sunitinib, a small molecule receptor tyrosine kinase inhibitor, has been shown to reduce circulating MDSC number and suppressive function in renal cell carcinoma patients, which correlates with improved T cell function. This effect was selective since T cells exposed to concentrations of sunitinib that impair MDSC were viable and functional ²⁸¹. Although the molecular mechanisms remain to be fully deciphered, it has been reported that this agent may inhibit VEGF and/or c-KIT-mediating signaling in MDSC. These pathways are known to participate in the expansion of these cells in the tumor ^{281,282}. Other pathways involved in MDSC generation and suppressive activity can be targeted with several other inhibitors such as Celecoxib (cyclooxygenase-2 inhibitor) or sildenafil (phosphodiesterase-5 inhibitor) ²⁸³.

Dendritic Cells

Immunomodulatory effects of anti-cancer agents on DC activities have also been reported. Chen and his coworkers stated that cisplatin can modulate the percentages of myeloid cells by increasing DCs and eliminating MDSCs, thus favoring immune effector responses in melanoma-bearing mice ²⁸⁴. In addition, Soeda et al. reported an increase of CD14+ monocytes, CD11c+ myeloid DCs and CD123+ plasmacytoid DCs in patients with advanced pancreatic cancer receiving gemcitabine for 2 months, in comparison to untreated patients ²⁸⁵. It has also been shown that cyclophosphamide (a cytotoxic alkylating agent) promotes DC maturation ²⁸⁶ and induces the production of IFN-I ²⁸⁷, resulting in the evolution of a durable T-cell memory response. Furthermore, it is reported that low dose paclitaxel promotes the DC maturation in mice ²⁸⁸, inducing proinflammatory cytokine secretion from

CD4+ T helper cells, thereby enhancing the priming and lytic activity of CD8+ cytotoxic T cells²⁸⁹. Cisplatin has also been shown to promote the intratumoral accumulation of CD11c+ DC which are capable to induce tumor specific CTL²⁹⁰.

Regarding targeted therapy, monoclonal antibodies were shown to enhance the activation of DCs through facilitation of antigen uptake and presentation²⁴². Trastuzumab and cetuximab, which is a clinically efficacious mAb directed towards the tumor-associated receptor tyrosine kinase epidermal growth factor receptor (EGFR)²⁹¹, augment tumor antigen presentation by DCs through the formation of immune complexes resulting in enhancing the induction of tumor-specific T cells^{292,293}. In addition, Correal et al. reported an effect for cetuximab in facilitating DC priming, an effect that results in augment tumor immunity²⁹². In an *in vitro* study using colon cancer cell lines, cetuximab promoted DC opsonization of tumor cells and associated maturation with increased expression of MHC-II, CD40, CD80 and CD86²⁹⁴. Moreover, imatinib mesylate, a selective inhibitor of a restricted number of tyrosine kinases, was reported to significantly enhance antitumor immune responses to DC-based immunization against an imatinib-resistant BCR-ABL negative lymphoma²⁹⁵.

Macrophages

Bryniarski et al. demonstrated that low-dose cyclophosphamide can promote the skewing of M2 macrophages into M1 *in vivo*, thus enhancing the production of oxygen radicals, IL-6 and IL-12, and potentiating innate responses²⁴⁷. Similarly, Buhtoiarov and colleagues showed that in mice bearing B16.F10 melanoma, combined treatment with vincristine (a mitotic inhibitor), cyclophosphamide and doxorubicin resulted in substantial enrichment of a TAM subpopulation that can be M1-polarized upon concomitant immunotherapy²⁴⁸. Interestingly, whereas tumor sensitivity to cyclophosphamide or cisplatin *in vitro* is increased when tumor cells are cultivated with macrophages, co-cultures of macrophages with human primary ovarian tumor cells decreased tumor sensitivity to 5-FU²⁹⁶. On the other hand, Dijkgraaf et al. showed that treatment with cisplatin or carboplatin chemotherapies mediate the increase in tumor-promoting M2 macrophages that may form an indirect mechanism for chemoresistance²⁹⁷.

Natural Killer cells

Earlier studies on NK cell function in cancer patients undergoing cytotoxic chemotherapy have shown variable effects, especially in correlation with the clinical outcome^{298,299}. In breast cancer patients with localized and metastatic disease, cytotoxic drug regimens were shown to induce an overall impairment of NK cell responses^{300,301}. In addition, Markasz and his colleagues found that treatment of NK cells with the drugs vinblastine, paclitaxel, docetaxel, cladribine, chlorambucil, bortezomib, and MG-132 effectively inhibited NK cell-mediated killing without affecting the viability of NK cells³⁰². In contrast, other studies showed that cyclophosphamide potently stimulates NK-dependent antitumor immunity in end-stage cancer patients²⁵⁰ and prompt recruitment of DCs, macrophages and NK cells to the tumors in diverse mouse models^{303,304}. Also, Khallouf et al. showed in his study that the combination treatment of 5-Fluorouracil (5-FU) and IFN- α resulted in higher numbers of infiltrating NK cells with enhanced cytotoxicity in a pancreatic tumor model³⁰⁵. A study by Braun et al. analyzed the effects of cytotoxic chemotherapy on NK cell function in a trial of 20 previously untreated solid tumor patients²⁹⁹. Results demonstrated that chemotherapy exerted a differential influence on NK activity which correlated with the pretreatment NK level of function in each individual patient. In patients with depressed NK levels prior to

treatment, chemotherapy augmented NK function; in patients with normal levels prior to treatment, chemotherapy depressed NK function.

Several studies also described the effect of radiotherapy on NK cells. For example, Park et al. showed that low-dose of radiation alone augmented natural cytotoxicity of NK cells against tumor targets cells²⁴⁶. In addition, low-dose radiation was shown by Nakagawa et al. to enhance the cytotoxic effects of NK cells against tumor cells *in vivo*, when NK cells were inoculated as a mixture with tumor cells into mice after radiation³⁰⁶. Another *in vivo* study demonstrated that mice exposed to low-dose radiation exhibited stimulation of innate immunity while suppressing pro-inflammatory responses³⁰⁷.

In addition, tumor-targeted antibody strategies have been proposed to enhance NK cell activity or targeting such as rituximab, trastuzumab, alemtuzumab (mAb directed against CD52 on CLL), and cetuximab³⁰⁸. Furthermore, Romagne et al. generated a human monoclonal antibody called 1-7F9 that recognizes the inhibitory Killer-cell immunoglobulin-like receptors KIRs that inhibit NK cell function³⁰⁹. During preclinical characterization, blocking KIRs with 1-7F9 increased lysis of primary acute myeloid leukemia (AML) blasts and this antibody is currently undergoing clinical evaluation.

5.1.1.3. Effect of cancer therapy on adaptative immune cells

Cancer therapy can promote or suppress functions attributed to immunosuppressive Tregs and other T cell subsets as shown in figure 15. Tregs expand within various tumors, potently suppressing the antitumor immune response³¹⁰.

T cell subsets

Cyclophosphamide was one of the first drugs reported for its ability to interfere with suppressive T cells and to improve immunotherapy²⁴⁹. Ghiringelli et al. showed an effect of cyclophosphamide in depleting Tregs and restoring T and NK cell effector functions in advanced cancer patients³¹¹. Moreover, Ding et al. reported that adoptive transfer of tumor-specific CD4+ T cells after cyclophosphamide gave rise to polyfunctional CD4+ effector cells, which in turn intensified the inflammatory milieu and enhanced the activation of CD8+ T cells in the tumor microenvironment³¹². Paclitaxel has been also reported to selectively reduce Treg number and function while sparing effector T lymphocytes in patients with advanced NSCLC³¹³. In addition, Chang et al. reported that low-dose cisplatin and paclitaxel synergize to generate strong tumor-specific CD8+ T cell responses, through IL-2 and IFN- γ secretion, and high therapeutic efficacy on platinum-resistant ovarian cancers in both mice and patients³¹⁴. 5-FU was also reported to increase IFN- γ production by tumor-specific CD8+ T cell infiltrating the tumor, and to boost T-cell-dependent antitumor responses by *in vivo* elimination of MDSCs²⁷⁶. In experimental carcinogen-induced adenocarcinomas and fibrosarcomas, doxorubicin treatment enhanced tumor-specific proliferation of CD8+ T cells in tumor-draining lymph nodes and promoted tumor infiltration of activated, IFN- γ -producing CD8 T-cells³¹⁵. Lenalidomide and pomalidomide anticancer molecules also alter Treg proliferation and function by reducing FoxP3 expression in Tregs through unclear molecular mechanisms³¹⁶. In addition, Sakamaki et al. reported that lenalidomide has been shown to reduce both Treg and MDSC number in A20 lymphoma model³¹⁷. Furthermore, Banissi and coworkers suggested that low dose metronomic regimens of temozolomide which is an alkylating agent, can also reduce Treg to total CD4+ T lymphocyte ratios and impair Tregs suppressive activity in glioma-bearing rats³¹⁸. Temozolomide has also been reported to

specifically reduce Tregs in advanced melanoma patients ³¹⁹, an effect associated with improved clinical response ³²⁰.

Regarding small molecule targeted therapy, several tyrosine kinase inhibitors (TKIs) have been described for their ability to impair the immunosuppressive Tregs. Sunitinib (a multityrosine kinase inhibitor) has been reported to decrease the number of peripheral and tumor infiltrating Tregs in patients with renal cell carcinoma ³²¹. Terme et al. reported a mechanism by which sunitinib reduces Treg through targeting of the VEGF-VEGFR axis in the mouse CT26 tumor model ²⁵¹. Similar effects for sorafenib (a multikinase inhibitor) on reducing Tregs accumulation were observed in renal cell carcinoma patients ³²². Dasatinib is a second-generation TKI used against imatinib-resistant leukemia and malignancies characterized by c-KIT mutations. Dasatinib was shown to decrease the frequency of circulating Tregs in a mastocytoma mouse model where dasatinib efficiently synergizes with immunotherapy, resulting in improved antitumor effects ³²³. Moreover, Larmonier et al. showed in his study an effect of imatinib mesylate in a mice model with lymphoma, where imatinib impaired Treg immunosuppressive function by reducing the activation of the transcription factors STAT3 and STAT5 in Tregs ²⁹⁵.

B cells

Treatment of cancer patients with intensive chemotherapy results in profound depletion of all lymphocytic populations, especially of B cells ²⁷⁵. A study performed in a mouse model of malignant mesothelioma showed a decrease in B cell frequencies and induced a suppression of antigen-specific IgG antibody responses due to repeated cycles of gemcitabine ²⁵². A study by Shebzukhov and coworkers reported that 5-FU-based adjuvant chemotherapy induced prominent tumor-specific antibody responses in colon cancer patients ³²⁴. In addition, a controversial effect of cyclophosphamide on humoral responses was indicated. In some reports, cyclophosphamide, even at low-dose regimens, exerted suppressive effects on humoral responses while boosting cellular responses, suggesting that B cells are particularly sensitive to cyclophosphamide-induced cytotoxic effects ^{325,326}. In other reports, low-dose cyclophosphamide was shown to increase the relative percentages of B and T-cells in mice bearing SW1C melanoma ³⁰³, and the cellular and antibody responses in patients with advanced cancer ³²⁷.

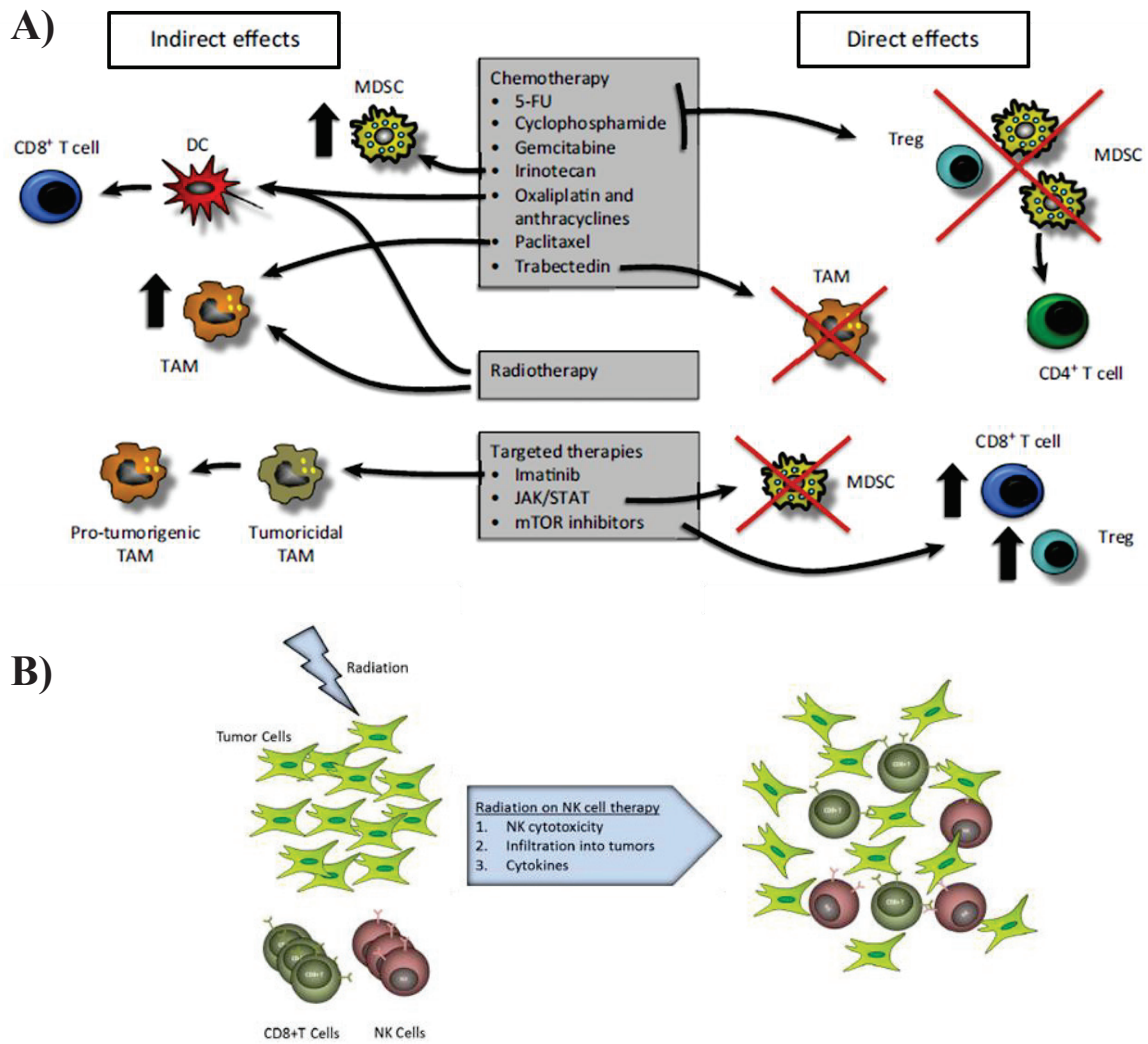


Figure 13. The effects of cancer therapy on immune cells (modified from Coffelt et al. 2015 and Park et al. 2014).

A) Several cancer therapies, such as chemotherapy, radiotherapy and targeted therapy, have been shown to modulate various immune cell populations in direct and indirect manners. B) Radiation increases NK cells cytotoxicity against tumors as well as lymphocytes trafficking into tumors and cytokine production.

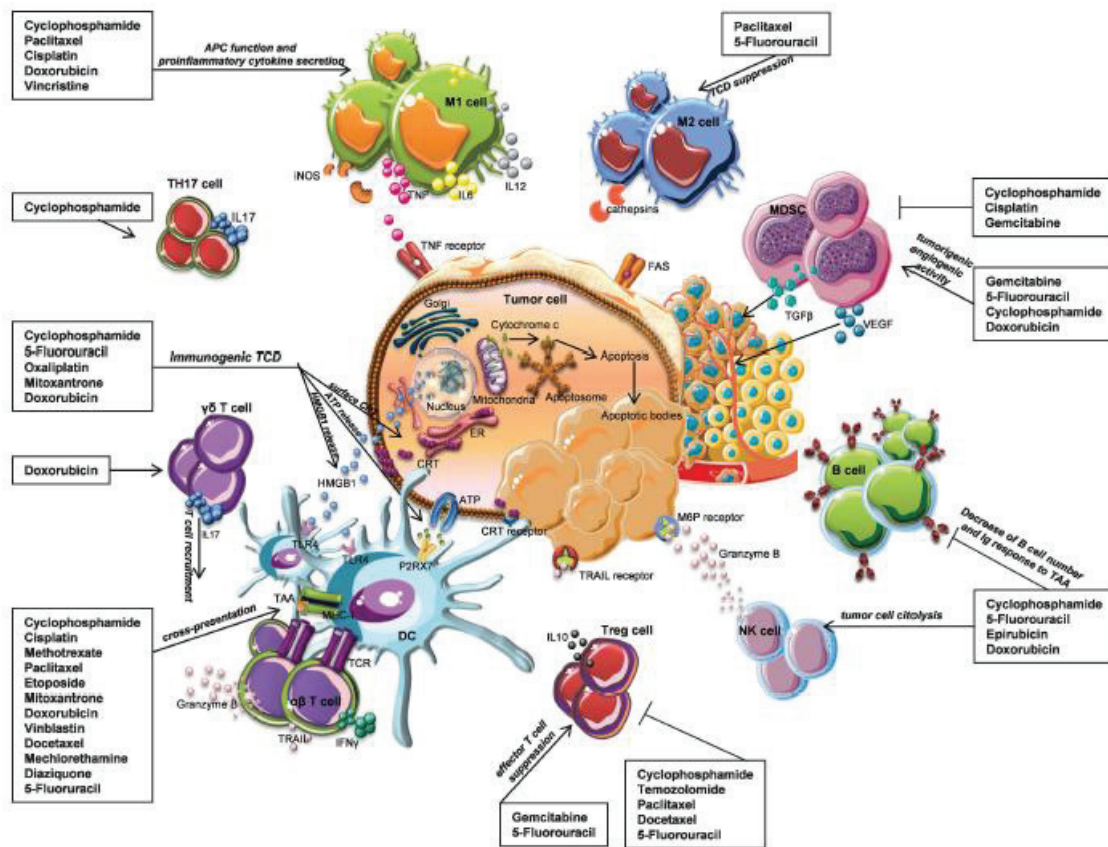


Figure 14. Immunomodulation by conventional cytotoxic drugs (form Bracci et al. 2014).

Conventional cytotoxic drugs can modulate the immune responses through several mechanisms.

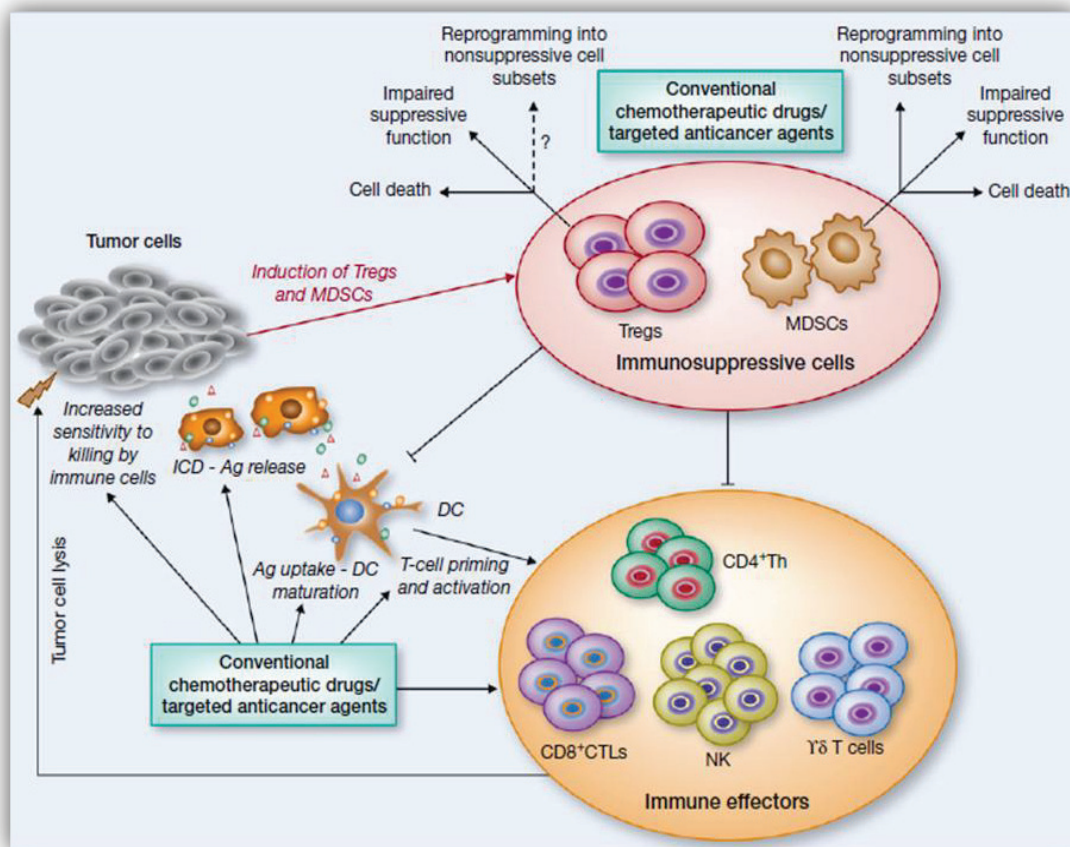


Figure 15. Immunomodulation by conventional chemotherapeutic drugs or targeted therapeutic agents on immunosuppressive cells (from Alizade et al. 2014).

MDSCs and Tregs are known to contribute to the immunosuppressive environment through different mechanisms. These cells blunt antitumor immunity by suppressing effector CD4⁺ Th, CD8⁺ CTL, NK, macrophages, or dendritic cells. Several conventional chemotherapeutic drugs or targeted therapeutic molecules enhance the function of immune effector cells or reverse the immunosuppression pathways induced by MDSCs or Tregs resulting in boosting the efficacy of immunotherapeutic interventions. The conventional chemotherapeutic drugs or targeted therapeutic agents can selectively trigger the death of suppressive cells, inhibit their suppressive function, or foster their differentiation into proinflammatory subsets devoid of inhibitory properties.

5.2. Impact of the immune system on cancer therapy efficacy

Immune cell populations have been shown to participate in and regulate tumorigenesis, metastasis and response to anticancer agents^{328,329}. Discussed below, the effect of several immune cells on the efficacy of anti-cancer agents.

5.2.1. Innate immune cells

Different studies were reported concerning the effect of innate immune cell subpopulations on cancer therapy efficacy.

Macrophages

Macrophages represent one of the most extensively studied innate immune cell subpopulation in response to chemotherapy. TAMs were shown to modulate the therapeutic activity of several types of chemotherapeutic drugs^{330,331}. Mantovani was one of the first who studied the impact of macrophages on tumor response to chemotherapy where he showed that macrophage-inactivating agents released by TAMs in mice transplanted with leukemia or lymphomas, reduce the efficacy of doxorubicin³³². In addition, DeNardo et al. showed that an increase in TAM infiltration with low CD8+ T cell abundance in human breast tumors is associated with poor response to neoadjuvant chemotherapy³³³. Paclitaxel treatment of mammary tumor-bearing mice increases the macrophages infiltration into tumors. These cells counteract the chemotherapy efficacy via several mechanisms, including the inhibition of cytotoxic CD8+ T cell functions via IL-10-mediated suppression of DCs^{333,334}, in addition to the secretion of chemoprotective survival signals such as cathepsins³³⁵. Moreover, Houthuijzen et al. showed that splenic macrophages can be involved in cisplatin resistance in subcutaneous cell line models via secretion of lysophospholipids that alter the DNA damage response³³⁶. A study by Germano et al. described an apoptotic effect of trabectedin chemotherapy, which acts by DNA cleavage, specifically on monocytes and macrophages and this forms a key component of its antitumor activity³³⁷.

Many studies have shown the importance of FcR on macrophages for the response to antibody-based targeted therapies through ADCC. Using various transplantable models in knockout mice that lack one or more FcRs, tumor regression mediated by rituximab, anti-cytotoxic T-lymphocyte-associated protein (CTLA)-4, trastuzumab, and other anti-erb-b-2 receptor tyrosine kinase-2 (ERBB-2) antibodies is reversed^{71,338-342}. In contrast, a study by Pander and his colleagues reported that the therapeutic antibody targeting EGFR, cetuximab, induces an immunosuppressive phenotype M2 in human monocytes cultured with colon cancer cell lines *in vitro* results in the production of production of IL-10 and VEGF tumor-promoting mediators³⁴³.

Natural Killer cells

Cytotoxic lymphocytes, including NK and CD8+ T cells, have been reported to contribute to the efficacy of particular chemotherapeutics. Several studies showed that the depletion of NK cells abolishes the tumor-shrinking ability of cyclophosphamide in tumor-bearing immunodeficient mice³⁴⁴ and in an experimental melanoma metastasis model³⁴⁵. Moreover, Kohlapp and his coworkers reported that NK cells and CD8+ T cells cooperate to improve therapeutic responses in melanoma treated with IL-2 and CTLA-4 blockade³⁴⁶. Furthermore, Ferrari de Andrade et al. reported that NK cells mediate the antitumor effects of a BRAF inhibitor in BRAFV600E-mutant metastatic melanoma³⁴⁷.

Dendritic cells

For many years it was thought that DCs in the tumor bed are immature and lack the ability to prime cytotoxic T cells³⁴⁸. However, this concept has been changed since several transplantable tumors have shown to contain a DC population (CD11b- population) that has the ability to induce cytotoxic T cells^{334,349}. Intravital imaging has shown that DCs are outcompeted by TAMs for T cell interaction, lessening the likelihood of a robust antitumor immune response^{349,350}. This antitumor immunity can be reconsidered by combining paclitaxel with TAM depletion or neutralization of their tolerizing abilities (i.e., blocking IL-10)³³⁴. A study by Ma et al. reported the presence of another DC population (CD11b+ population) that had a greater role in chemotherapy-induced immunogenic cell death in

transplantable models ³⁵¹. These data emphasize the diverse influence of DC subsets on chemotherapeutic efficacy.

VEGF-targeted therapy has been shown to have an immunomodulatory effect on DCs ^{352,353}. Gabrilovich and his coworkers reported an effect of VEGF on DC differentiation. They showed that peptide-pulsed DCs alone resulted in considerable slowing of tumor growth but only during the period of anti-VEGF therapy, and tumor growth resumed after the end of the therapy. Combined treatment with peptide-pulsed DCs and anti-VEGF antibody resulted in a prolonged and much more pronounced antitumor effect ³⁵².

MDSCs

MDSC levels correlate with a higher tumor burden and a worse prognosis ^{82,281,354}. Malmberg and his colleagues demonstrated that MDSCs may impair the efficacy of cancer vaccines via direct effects on T cell activation and antigen presentation by DC ³⁵⁵. Moreover, Mundy-Bosse et al. showed that inhibition of MDSC in murine models may enhance anti-tumor immunity by increasing responsiveness to interferon stimulation ³⁵⁶. Also, inhibition or depletion of MDSC enhances the activity of cancer vaccines in animal models ³⁵⁷⁻³⁶⁶.

5.2.2. Adaptative immune cells

The role of T and B cells in chemotherapy response is highly dependent on the class of chemotherapy used and the tumor type. Their role is paradoxical because these cells can promote or prevent chemotherapeutic potency.

T cell subsets

Studies focused on the role of T cell subsets provide an example of the complexity surrounding adaptative immune cells in chemotherapy response. Bruchard et al. reported a role for CD4+ T cells which limit the ability of 5-FU on EL4 thymoma cells. 5-FU induce the production of IL-1 β by MDSCs which in its role stimulate CD4+ T cells to express IL-17 which blunted the anticancer efficacy of the chemotherapy ³⁶⁷. In contrast, IL-17 is required for therapeutic efficacy of doxorubicin in a subcutaneous sarcoma model, and $\gamma\delta$ T cells (a small subset of T cells possess distinct T-cell receptor), not CD4+ T cells, are the source of IL-17 in this scenario ³⁶⁸. Also, Hannesdottir and his colleagues showed that CD4+ T cell depletion delays tumor growth in a mice model of breast cancer treated with doxorubicin and lapatinib (EGFR inhibitor) ³⁶⁹. In addition, Sevko et al. reported that the antitumor function of CD8+ T cells increased upon treatment with non-cytotoxic doses of paclitaxel in a spontaneous melanoma ³⁷⁰. Similarly, CD8+ T cells contribute to cancer cell killing by immunogenic cell death-inducing chemotherapeutics in a variety of transplantable and carcinogen-induced tumors ^{258,371,372}.

These adaptative immune cells can also modulate the tumor responses to targeted therapies. Knight et al. reported that CD8+ T cells are required for the response of BRAF inhibitors in transplantable melanoma models (364). The same effect was obtained by Cooper et al. who stated that CD8+ T cells were found to play a critical role in the therapeutic effect of BRAF inhibition in melanoma with significantly prolonging survival and slowing tumor growth, as well as significantly increasing the number and activity of tumor-infiltrating lymphocytes when BRAF inhibition combined with immune checkpoint blockade ³⁷⁴.

B cells

B cells facilitate the tumor progression in squamous cell carcinoma mice through antibody-mediated activation of FcRs on TAMs results in stimulating their proangiogenic abilities^{375,376}. In a study by Affara and coworkers, they showed that the combination of platinum-based chemotherapy or paclitaxel together with anti-CD20 antibodies in orthotopic squamous cell carcinomas results in stasis of established tumors, whereas chemotherapy or B cell depletion as single agents are completely ineffective. This synergistic effect of the chemotherapy and B cell depletion is dependent on TAMs or CD8+ T cells because depletion of either population desensitizes tumors to the absence of B cells and chemotherapy³⁷⁷. This indicates that inhibiting B cells in combination with chemotherapy may have a strong effect on some tumor types.

5.3. Impact of the immune system on cancer therapy efficacy in NHL

The studies above established that the antitumor efficacy of anticancer agents can be mediated by immune-dependent mechanisms in several cancer types. Below is a discussion for impact of the immune system on cancer therapy efficacy in NHL.

Beyond the contributions of elements of the tumor microenvironment to lymphoma proliferation, survival and immune escape, there is direct evidence that interactions between tumor cells and the microenvironment alter sensitivity to cytotoxic agents, which probably affect patient outcomes³⁷⁸. These mechanisms either involve cell-cell contact or are due to soluble factors released in the tumor microenvironment.

5.3.1. Cell-cell contact

Cell-cell contact provides survival signals, the withdrawal of which induces cell death. Stromal cells are an essential component of the bone marrow microenvironment that regulate or support tumor survival (discussed in chapter 3), and they have been studied intensively for their role in altering tumor cell response to anti-cancer agents. Lwin et al. showed in his study that interaction with stromal cells reduces apoptosis in response to cytotoxic drugs in *ex vivo* DLBCL and MCL models, and this is mediated by activation of NF- κ B signaling⁵⁴. Another study done by Kurtova et al. showed that MCL cells adhere and spontaneously migrate beneath stromal cells in a CXCR4- and integrin VLA-4-dependent fashion results in MCL drug resistance. In these cell co-cultures, the non-adherent lymphoma cells in the supernatant were sensitive to drug-induced apoptosis³⁷⁹. Interaction with bone marrow-derived stromal cells also protected primary lymphoma cells from apoptosis induced by rituximab. This effect was disrupted by natalizumab which blocks VLA-4⁵⁵.

Experiments involving the co-culture of lymphoma cells and other cell types of the lymph node microenvironment indicate that direct effects on the lymphoma cells produced in response to cell-to-cell adhesion can also change the sensitivity of tumor cells to anti-cancer agents. An example of this process is the induction of miR-181a in lymphoma cells upon adhesion to FDCs, which leads to a reduction in the levels of the pro-apoptotic protein BIM, thus protecting B cell lymphoma cells against drug-induced apoptosis⁶⁴. Another example of this process was presented by Lwin et al. when he showed that adhesion of MCL and other NHL cells to lymphoma stromal cells result in a reduction of miR-548m levels in lymphoma

cells, leading to drug resistance³⁸⁰. In addition, cell adhesion of lymphoma cells to stromal cells has been shown to induce cell cycle arrest³⁸¹, with the implication that these dormant cells may be more resistant to cytotoxic agents. These findings may explain the observation that MCL and FL, the two lymphomas that are consistently associated with stromal cells in the microenvironment, show difficulties in their cure with current standard treatment regimens.

5.3.2. Soluble factors

Several studies reported the contribution of soluble factors secreted by several cells in the microenvironment to the drug resistance of lymphoma cells. Lwin et al. showed a protection of lymphoma cells from the chemotherapy mitoxantrone-induced apoptosis by conditioned medium of a stromal-lymphoma cell co-culture, implying a role for BAFF in lymphoma cell survival⁴⁴. Another study performed by Medina and colleagues reported that interactions between MCL and human stromal cells activate BAFF/NF- κ B signaling resulted in stromal cell production of BAFF resulting in MCL protection against drug-induced apoptosis⁴⁶.

5.4. Neutrophil-mediated response to anti-cancer agents

Neutrophils, another key player in innate immune system, had been studied much less compared to macrophages regarding their impact on cancer cell response to anti-cancer agents.

Phan et al. correlated the presence of CD11b+Ly6G+ neutrophil infiltrate and resistance to anti-VEGF treatment. They showed that the oncogenic RAS pathway activation promotes anti-VEGF resistance due to G-CSF-induced CD11b+Gr-1+ neutrophil recruitment. In this study they reported that RAS/RAF/MEK pathway is constitutively active in many cancer cells that produce high levels of G-CSF and that the expression of Ets2 transcription factor is directly correlated with high G-CSF production. G-CSF production is correlated with neutrophil mobilization that promotes anti-VEGF resistance. A MEK inhibitor reduced G-CSF production and neutrophil mobilization leading to increased survival in animal models of cancer, including a genetically engineered mouse model of pancreatic adenocarcinoma³⁸².

Another study by Liang et al. showed that an increase in neutrophil infiltration into tumors was significantly correlated with glioma grade and in glioblastoma with acquired resistance to anti-VEGF therapy. In this model the effect was mediated by neutrophil-mediated overexpression of S100A4 in tumor cells, since downregulation of S100A4 inhibited tumor progression independently of the infiltration of neutrophils into the tumor and increased the effectiveness of anti-VEGF therapy³⁸³.

Moreover, Shojaei et al. reported that accumulation of CD11b+Gr-1+ cells in tumors, which include neutrophils, macrophages, and MDSCs, renders tumors refractory to angiogenic blockade by VEGF antibodies. The secreted protein Bv8 (prokineticin-2) is one of the players in this pathway and is up-regulated in CD11b+Gr-1+ cells by G-CSF. Hence, G-CSF may promote CD11b+Gr-1+-mediated tumor angiogenesis through a pathway that bypasses VEGF²⁰⁷.

Moreover, Acharyya and coworkers reported that the combination of doxorubicin, cyclophosphamide, and a CXCR2 inhibitor significantly slows tumor growth and metastasis in human breast cancer xenografts compared to chemotherapy or the CXCR2 inhibitor alone where the latter inhibit the CXCR2, the receptor for CXCL1, 2, and 5, that is expressed on neutrophils and other granulocytes³⁸⁴. In addition, Di Mitri et al. reported that infiltrating neutrophils secrete a IL-1 receptor antagonist which results in enhanced tumor proliferation in prostate cancer³⁸⁵.

Clinical support for a role of neutrophils in chemotherapy response comes from observations in a variety of cancer patients, such as breast and NSCLC patients, where chemotherapy induced neutropenia is associated with better patient prognosis^{386,387}. Therefore, targeting neutrophils or their mediators may synergize with chemotherapeutics and increase their effects against tumors.

In contrast, Guerriero and colleagues showed that the depletion of neutrophils by Gr-1 antibody impaired the anti-cancer effect of cyclophosphamide in a mice model with embryonic fibroblast tumors (345). But since the Gr-1 can bind to both the granulocytic and monocytic fractions, the depletion of monocytes may have contributed to this effect.

Altogether, these studies, in spite of some conflicting data, underline the cross talk between tumor cells and the tumor microenvironment which results in modulating tumor response to anti-cancer agents. However, the role of neutrophils; key players in the innate immune system; in regulating lymphoma response to anticancer treatment is not known.

AIMS OF THE STUDY

Recent advances in conventional chemotherapies, targeted therapies, and therapeutic antibodies have manifested the tremendous therapeutic potential of cancer therapy in treating patients. However, the efficacy of cancer therapy is often restricted by various immunoregulatory mechanisms pre-established in tumor microenvironment. The prevalent immunoregulatory mechanisms include different immune cells as well as immunoregulatory soluble factors and check-point molecules.

Neutrophils, a key player in innate immune system, have been observed in close association with tumor cells and within tumor vasculature. They have been proposed as key mediator of malignant transformation, tumor progression, and angiogenesis and in the modulation of cancer cell response to anti-cancer agents.

In this study, we investigated the impact of circulating neutrophils on the sensitivity of preclinical models of NHL and fresh primary leukemic cells to cancer therapy. We hypothesized that neutrophils, via their interaction with lymphoma B cells, could alter the response of cancer cells to various types of cytotoxic compounds as well as targeted therapies. We found that circulating neutrophils display a protective effect on malignant B cells against a variety of anti-lymphoma compounds, thereby constituting yet a new mechanism through which the innate immune system could contribute negatively to the outcome in patients with lymphoma.

These results could be of major clinical importance, since the pharmacological inhibition of neutrophil-mediated protection may contribute to enhanced antitumor activity of anti-lymphoma agents.

RESULTS

Article I

Human neutrophils protect lymphoma cells against cytotoxic and targeted therapies through CD11b/ICAM-1 binding

Hirz *et al.* Under review in Blood journal

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Introduction and objectives

Neutrophils are the predominant leukocyte population in human blood and form part of the body's innate immunity which involves a series of defense mechanisms that protect the host from infections. Neutrophils have long been viewed as terminally differentiated effector cells, playing a major role during the acute phase of inflammation and resistance against microorganisms. Recent evidence have questioned this limited point of view, indicating that neutrophils can interact with distinct cell populations and produce a wide number of cytokines and effector molecules and may play an immunosuppressive role in sepsis ³⁸⁸.

The role of neutrophils in cancer has long been considered negligible although neutrophils are detected in a variety of tumors ³⁸⁹⁻³⁹¹. They were shown to represent a significant portion of the inflammatory cell infiltrate in many models of cancer. Current evidence suggest that neutrophils show high functional plasticity and they can display either protumor or antitumor activity, a phenomenon designated as "neutrophil polarization" ^{392,393}. Recent studies done on various solid tumors had described tumor-associated neutrophils as a prognostic factor in cancer ³⁹⁴⁻⁴⁰⁴. These studies reported that high levels of intratumoral neutrophils are associated with unfavorable recurrence-free, cancer-specific and overall survival.

Very few studies have evaluated the implication of neutrophils in mediating tumor cell response to cancer therapy ^{382,383} although the effect of different immune cells on tumor progression and their role in regulating tumor response to cancer therapy has been intensively investigated ^{330-333,344,345,347,351,356,357}. Clinical support for a role of neutrophils in chemotherapy response comes from observations in a variety of cancer patients, such as breast and NSCLC patients, where chemotherapy induced neutropenia is associated with better patient prognosis ^{386,387}. These data are not informative on the effect of neutrophils during exposure of tumor cells to chemotherapy since drug-induced neutropenia is delayed after administration. The role of neutrophils in regulating lymphoma response to anticancer treatment has not been previously described.

In this study, we aimed to investigate the mechanisms by which neutrophils can regulate the response of NHL and primary leukemic cells to several anti-cancer agents.

Title:

Human neutrophils protect lymphoma cells against cytotoxic and targeted therapies through CD11b/ICAM-1 binding.

Running title:

Neutrophils protect malignant B cells

Authors:

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Keywords: Neutrophils, lymphoma, chemotherapy, targeted therapy, cell-cell interactions

Key Points:

- Circulating neutrophils display a protective effect on malignant B cells against a variety of anti-lymphoma compounds.
- Pharmacological inhibition of this effect could contribute to enhanced antitumor activity of anti-lymphoma agents.

Abstract:

While the role of innate immune cells on tumor progression is the object of increasing scrutiny, the role of neutrophils on sensitivity to therapy has not been previously described. To this end, we performed cocultures of freshly purified human neutrophils and different non-Hodgkin lymphoma (NHL) cell lines in the presence of various cytotoxic and targeted agents. CFSE and DAPI assays were performed to assess the therapeutic effect on cell proliferation and cell death, respectively, using flow cytometry. Neutrophils and differentiated HL60 cells with neutrophil-like properties attenuated the sensitivity of lymphoma cells to anti-cancer agents both in 2D and 3D models *in vitro*. The protective effect of neutrophils was tested *in vivo* using SCID/CB17 mice inoculated with NHL cells together with neutrophils, and treated with vincristine. The co-inoculation of neutrophils reduced the sensitivity of NHL cells to chemotherapy. Similar findings were made on primary cells, purified from patients diagnosed with chronic lymphocytic leukemia, exposed to cytotoxic agents or recently approved targeted agents (ibrutinib and idelalisib) in the presence of autologous neutrophils. Neutrophil-induced protection was dependent on cell-cell contact mediated by the interaction of CD11b and ICAM-1, expressed by neutrophils and B cells respectively, and by the adhesion molecule CD44. This protective effect was Mcl-1-dependent and was partially abrogated by an anti-Mcl-1 compound. These findings reveal a novel protective mechanism of neoplastic B cells involving innate immune cells which could be pharmacologically inhibited to enhance the antitumor effect of conventional or targeted therapy.

Introduction:

Non-Hodgkin's lymphoma (NHL) is the most common cancer of the lymphatic system, with a large number of disease subtypes. Several studies have described interactions between lymphoma cells and non-malignant cells within the bone marrow or lymph nodes, including follicular dendritic cells^{1,2}, human bone marrow stromal cells³ and derived nurse-like cells⁴. There is a clear impact of the adaptive immune compartment on the outcome of patients with lymphoma⁵, but much less data exist regarding the role of the innate immune cells. Macrophages are important cells of this innate immune system and have been analyzed in the context of NHL where the presence of polarized M2 cells has been shown to be an independent predictor of poor prognosis⁵. Very little data are available regarding the interactions between lymphoma cells and neutrophils which are key players in the innate immune system.

Although neutrophils are traditionally considered in the context of their antibacterial functions, it is becoming increasingly clear that tumor-associated neutrophils (TANs) and their myeloid precursors (granulocytic myeloid-derived suppressor cells (G-MDSC)) in the spleen, bone marrow and blood play an important role in cancer biology⁶⁻¹¹. They have been proposed as key mediators of malignant transformation, tumor progression, angiogenesis and in the modulation of the antitumor immunity¹²⁻¹⁶.

Integrins are important signal transducers for virtually all neutrophil functions¹⁷. LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) are the two most abundant CD11/CD18 integrins expressed by neutrophils¹⁸. Mac-1 is an important mediator of neutrophil adherence, cell-cell interactions and their migration from the peripheral blood into tissues. It is constitutively expressed on circulating neutrophils and its surface expression increases significantly upon cell activation¹⁹.

The effect of different immune cells on tumor progression has been extensively investigated, yet their role in regulating treatment response is poorly understood²⁰. The potent capacity of macrophages and inflammatory monocytes to induce tumor progression has been well established and the mechanisms by which they regulate tumor response to cancer therapies are well defined²¹⁻²⁴. However, the potential role of neutrophils in regulating lymphoma response to anticancer treatment has not been previously described.

In this study, we investigated the mechanisms by which circulating neutrophils regulate the response of NHL and fresh primary leukemic cells to various types of cytotoxic compounds as well as targeted agents.

Methods:

Cell lines

Human non-Hodgkin's lymphoma (NHL) B cell lines (follicular lymphoma RL cells, Burkitt's lymphoma Raji cells and mantle cell lymphoma Granta and Es-moult cells) and acute promyelocytic leukemia HL60 cells were obtained from ATCC. All cell lines were grown in complete RPMI medium (RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin) and cultured at 37°C in humidified 5 % CO₂. HL60 cells were differentiated along the granulocyte pathway by exposure to DMSO and retinoic acid as described in the supplementary data (Method S3).

Antibodies and reagents

The detailed lists of reagents and antibodies used in our study are provided in the Supplementary materials (Reagents and Antibodies Tables 1, 2 and 3).

Cell division and cell death assays

Cell division and death were evaluated using flow cytometry. The detailed information of CFSE cells labeling, DAPI staining, isolation of human peripheral blood neutrophils and flow cytometric analysis of surface antigens are provided in the Method S1. Briefly, CFSE-labeled lymphoma B cells (2×10^5 cells/mL) were cultured alone or co-cultured with fresh human neutrophils isolated from healthy donors at different neutrophil to tumor cell (N:T) ratios. Five different chemotherapeutic molecules were added to the culture system at different concentrations: vincristine, doxorubicin, bortezomib, cisplatin or mafosfamide. After 48 hours of incubation, cells were collected and labeled with anti-human CD19. The CD19 positive population, representing the lymphoma B cells, was analyzed for cell proliferation and cell death using CFSE and DAPI assays, respectively, by flow cytometry (LSRII flow cytometer, Diva software).

In some experiments, 1 µg/mL anti-CD44, 10 nM ABT-199 or 10 µM MIM-1 was added to the culture system. In other experiments, CFSE-labeled RL cells and neutrophils were pre-incubated with blocking antibodies for 1 hour before co-culture. RL cells were incubated with 12 µg/mL anti-ICAM-1/CD54, 10 µg/mL anti-CD11a or anti-CD11c whereas neutrophils were incubated with 10 µg/mL anti-CD11a, anti-CD11b or anti-CD11c. Then both cell types were co-cultured together for 48 hours in the presence of 10 nM vincristine.

3-dimensional (3D) culture

For 3D experiments, 50,000 RL cells were incubated in Matrigel according to the manufacturer's protocol, in the presence or absence of differentiated HL60 cells (HL60*diff*) at a ratio of 10 HL60*diff*: 1 RL. 3D cultures were maintained for 7 days and culture medium was changed every two days. On day 5, vincristine was added at a concentration of 10 nM. On day 7, pictures were taken using DMI3000 fluorescent microscope at magnification 40x then spheroids were dissociated by agitation in ice-cold PBS-5mM EDTA for 1 hour. Later, cells were labeled with anti-human CD19 and anti-human CD38 then stained with annexin V-FITC/PI using Annexing V-FLOUS staining followed by flow cytometry, to determine the proportion of viable RL cells (CD19 positive Annexin V negative/PI negative).

Transwell and conditioned medium experiments

In Transwell experiments, CFSE-labeled RL cells and neutrophils were cultured on opposite sides of microporous polyethylene terephthalate inserts (pore size of 0.4 μm). 2×10^6 neutrophils were placed inside the inserts whereas 2×10^5 CFSE-labeled RL cells were placed in the wells, in the presence or absence of 10 nM vincristine. After 48 hours of incubation, the proliferation and cell death fraction of RL cells were determined using CFSE and DAPI assays, respectively.

In conditioned medium (CM) experiments, neutrophils were incubated alone or with RL cells at a N:T ratio of 10:1, in the presence or absence of 10 nM vincristine for 2 hours. Then cells were harvested, washed and incubated with fresh complete RPMI medium. After another 2 hours of incubation, the supernatants (CM) were collected. Naïve CFSE-labeled RL cells were incubated with 100% of CM and compared to RPMI medium as a control, in the presence of 10 nM vincristine for 48 hours and proliferation and cell death of RL cells were determined.

Intracellular content of anti-apoptotic proteins

RL cells were cultured alone or with neutrophils at a N:T ratio of 10:1, in the presence or absence of 10 nM vincristine. After 48 hours of incubation, the intracellular labeling of anti-apoptotic Bcl-2 family members (Bcl-xL, Bcl-2 and Mcl-1) was performed by flow cytometry. First the cells were labeled with anti-human CD19 and anti-human CD15, then were fixed and permeabilized using BD Cytotfix/Cytoperm Fixation/Permeabilization kit before staining with the appropriate labelled antibodies.

Expression microarray studies

RL cells were cultured alone or together with neutrophils at a N:T ratio of 10:1, in the presence or absence of 10 nM vincristine for 2 hours at 37⁰C. RL cells were purified ($\geq 80\%$ pure) after labeling with anti-human CD15-Biotin (Miltenyi Biotec) then anti-Biotin Microbeads (Miltenyi Biotec) followed by magnetic column separation (LD Columns, Miltenyi Biotec). RNA was extracted using Qiamp RNeasy mini kit (Qiagen). After RNA amplification with totalprep RNA amplification kit (Ambion), cRNA was hybridized on human HT-12 v4 beadchip (Illumina). Chips were scanned with an Illumina iScan and data was analyzed using GeneSpring and Ingenuity softwares. Microarray data have been deposited in the GEO database under accession number GSE69255.

Sensitivity of fresh patient samples

Peripheral blood on EDTA was obtained from patients (aged 58- 90 years) diagnosed with CLL according to standard criteria. Procedures were conducted under the approval of Lyon Hospital Ethics committee with all patients signing informed consent.

CLL cells were isolated by ficoll density-gradient centrifugation and purity was verified by CD19 labelling ($\geq 90\%$ CD19-positive). CLL cells (2×10^5 cells/mL) were cultured alone or together with autologous neutrophils, isolated as described in Method S1, at different N:CLL ratios. Chemotherapeutic agents (vincristine and fludarabine) or targeted therapies (ibrutinib

and idelalisib) were added to the cultures. After 24 hours of incubation, the percentage of viable CLL cells (Annexin V negative/PI negative) was determined by double staining of the cells with annexin V-FITC/PI.

Animal Studies

All animal procedures were performed in accordance with the European Union Directive (86/609/EEC). Experiments were performed under individual permit and in animal care facilities accredited by the French Ministry of Agriculture. The study was approved by the local animal ethical committee.

Studies were conducted using female SCID CB17 mice (4 weeks old, 5 mice/group, Charles River). Mice were injected subcutaneously with 3×10^6 RL cells alone or with freshly human neutrophils isolated from peripheral blood of healthy donors at a N:T ratio of 10:1. Vincristine was injected intraperitoneally at a dose of 1mg/kg on the same day. Tumor growth was monitored by caliper measurement of the tumor volume every 3-4 days.

Statistics

Flow cytometry data were analyzed using BD FACSDiva™ software and FlowJo, LLC data analysis software. Results were expressed as the mean \pm SD of at least three independent experiments, each performed in triplicates. One Way Anova test was used for comparison among groups, and p- values were reported (Sigma Plot®, Systat Software). Some experiments were performed once in triplicate. For *in vivo* experiments, results were expressed as median \pm S.E.M and Student's *t* test was used for significant analysis.

Results:

Freshly purified human neutrophils protect lymphoma B cells against the cytotoxicity of chemotherapeutic

To investigate the effect of neutrophils on B lymphoma cells, purified human neutrophils from peripheral blood of healthy donors were co-cultured with different human NHL cell lines (RL, Granta, Raji and Es-moult) in the presence or absence of various chemotherapeutic agents (vincristine (VCR), doxorubicin, bortezomib, cisplatin, and mafosfamide) at different concentrations. The effect of chemotherapy on lymphoma B cell death and proliferation was assessed by flow cytometry using DAPI and CFSE assays, respectively. Results in Figure 1 (A and B) show that vincristine induced cell death (Fig 1A) and inhibited cell proliferation (Fig 1B) in a dose-dependent manner in RL cells. Vincristine-dependent effect on both parameters was significantly reduced in the presence of neutrophils (Fig 1, A and B). Similar effects were observed with the Granta cell line (Fig 1, C and D). Comparable results were obtained using other lymphoma B cell lines such as Raji and Es-moult treated with vincristine at different concentrations (Figure S1).

Similar results supporting the protective effect of neutrophils were obtained with RL cells treated with doxorubicin (10 nM, 1 μ M), bortezomib (10 nM, 15 nM), cisplatin (5 μ M, 25 μ M), or mafosfamide (50 μ M) (Figure S2). Cisplatin and mafosfamide did not induce RL cell death at these concentrations but caused a significant inhibition of cell proliferation. We did not observe any impact of the different chemotherapeutic agents on neutrophil viability with the concentrations used. Overall, these results show that neutrophils exert a protective effect on several NHL models against a variety of conventional cytotoxic compounds *in vitro*.

We further determined whether the protective effect of neutrophils was influenced by the neutrophil:tumor cell ratio. CFSE-labeled RL cells were co-cultured with different ratios of neutrophils in the presence of vincristine at 10 nM. Figure 1 (E and F) show that the protective effect was dependent on and directly correlated with the neutrophil:tumor cell ratio but that a significant effect could be observed at a ratio as low as 1 neutrophil: 1 RL cell.

We next examined whether the protective role of neutrophils was due to the sequestration of chemotherapy. RL cells were incubated alone or together with neutrophils in the presence or absence of doxorubicin (Method S2). The amount of doxorubicin accumulating in RL cells was examined by flow cytometry. Results show that the uptake of doxorubicin by RL cells was not reduced in the presence of neutrophils although neutrophils also showed an uptake of doxorubicin (Figure S3, A and B).

Neutrophil-like HL60diff cells protect RL lymphoma cells against vincristine in 3D culture

We aimed to investigate the effect of neutrophils on lymphoma cells using 3-Dimensional (3D) culture. Given the short half-life of neutrophils *in vitro*, experiments with fresh human neutrophils were not conclusive. We thus chose to use differentiated HL60 cells (HL60diff) which showed a strong increase in CD11b and CD38 expression indicative of granulocytic differentiation, in addition to the morphological changes detected by the appearance of multi-lobed nuclei (Figure S4, A and B). HL60diff stopped proliferating after five days of inducing

differentiation. When RL cells were co-cultured with HL60*diff* cells in a classical 2D system at a ratio of 10 HL60*diff*:1 RL cell, a protective effect against vincristine was observed which was similar to that induced by fresh neutrophils (Figure S5).

RL cells were cultured alone or with HL60*diff* in Matrigel in the presence or absence of vincristine. As shown in Figure 2A, vincristine induced RL spheroid dissociation. The presence of HL60*diff* cells reduced the sensitivity of RL spheroids to vincristine as determined by the size of spheroids after seven days of culture. These results were confirmed by Annexin V/PI staining of the cells obtained after spheroid dissociation, with a significantly greater proportion of CD19 viable cells (CD19 positive, Annexin V negative /PI negative) in the presence of HL60*diff* cells (Fig 2B).

Neutrophil-induced protection requires cell-cell contact

The requirement of cell-cell interaction for the protective effect of neutrophils on tumor cells was explored using transwell experiments and neutrophil-conditioned media. The presence of neutrophils inside the inserts did not modify the effect of vincristine on RL cell death (Fig 3A) or cell proliferation (Fig 3B). Similar results were obtained in experiments in which RL cells were cultured for 48 hours with conditioned medium (CM) harvested from cultures of neutrophils alone or exposed to RL cells and in the presence or absence of vincristine (Figure 3, C and D). Similar results were obtained using CM harvested at different periods of time (data not shown). These results indicate that the neutrophil-induced protection of lymphoma B cells against vincristine requires a direct contact between the two cell types.

CD11b/ICAM-1 interaction and CD44 are implicated in the protective effect of neutrophils

Since cell-cell contact was determined to be important for neutrophil-mediated protection of RL cells, we examined the expression of different surface markers on neutrophils cultured alone or with RL cells in the presence or absence of vincristine. Results in Figure 4A and Table S1 show that neutrophils spontaneously express CD11a, CD11b, CD18, CD32 and CD66b at high levels with lower expression of CD64. Exposure to vincristine did not modify the expression level of these activation markers whereas the co-culture of neutrophils with RL cells (either alone or with vincristine) increased the expression of CD11b, CD18, CD64 and CD66b (6.4-fold, 3.8-fold, 3-fold and 4.5-fold increases, respectively) and decreased CD32 (2-fold decrease), whereas the expression of CD11a remained unchanged.

It has previously been shown that CD11a, CD11b and CD11c integrins at the surface of neutrophils interact with the adhesion molecule ICAM-1²⁵⁻²⁷ which is involved in the regulation of cell proliferation^{28,29}. We determined whether these receptors were implicated in the protective effect of neutrophils. To this end, we used blocking antibodies against these surface antigens and ascertained their effect on RL cell growth in the presence of vincristine. Blocking antibodies recognizing ICAM-1 expressed by RL cells or CD11b expressed by neutrophils inhibited neutrophil-induced protection on RL cell proliferation against vincristine, whereas blocking antibodies recognizing CD11a or CD11c (expressed both by RL cells and neutrophils) had no effect (Fig 4B). These results underline the importance of CD11b/ICAM-1 binding in the neutrophil-mediated protective effect.

In order to obtain insight into the changes induced in RL cells by neutrophils and vincristine, we used a transcriptomic approach on RL cells. Among the different genes that found to be up-regulated in cells co-cultures with neutrophils and vincristine, *CD44* was among the most strongly over-expressed. As CD44 is another adhesion molecule involved in regulating cell proliferation^{30,31} we used blocking antibody recognizing CD44 and observed abrogation of the neutrophil-induced protection of RL cell proliferation in the presence of vincristine (Fig 4C). No effect was obtained for blocking these receptors on RL cell death (data not shown). Taken together, these data support the fact that direct contact involving several adhesion molecules are required to allow the protective effect of neutrophils on lymphoma cells.

Neutrophil-induced protection is dependent on Mcl-1

We examined the effect of neutrophils on the expression by NHL cells of several proteins known to regulate apoptosis of tumor cells, such as anti-apoptotic Bcl-2 family members. For these experiments, RL cells were cultured alone or with neutrophils in the presence or absence of 10 nM vincristine. Vincristine increased significantly the content of Bcl-2 in RL cells, whereas Bcl-xL and Mcl-1 expressions did not change significantly (Fig 5A). The incubation of neutrophils and RL cells induced a slight but non-significant downregulation of Bcl-xL and Mcl-1 contents in RL cells. Conversely, the effect of neutrophils on RL cells incubated with vincristine was a significant increase in Mcl-1 expression (Fig 5A). Thus the effect of neutrophils on the anti-apoptotic protein content of NHL cells was significantly different when these NHL cells were simultaneously exposed to chemotherapeutic agents.

To determine the role of these anti-apoptotic proteins in the protective effect of neutrophils, co-cultures were performed in the presence or absence of small molecule inhibitors of Bcl-2 (ABT-199) and Mcl-1 (MIM-1). ABT-199 (10 nM) significantly enhanced the cytotoxic effect of vincristine as determined by the increase in RL cell death (Fig 5B), and this effect was not influenced by the presence of neutrophils. On the other hand, MIM-1 at 10 μ M significantly reduced the protective effect of neutrophils on vincristine-induced RL cell death (Fig 6C) suggesting that the protective effect of neutrophils is Mcl-1 dependent.

Neutrophils attenuate the sensitivity of RL cells to vincristine *in vivo*

We examined the effect of neutrophils on the sensitivity of RL cells to vincristine *in vivo*. Purified human neutrophils from healthy donors and RL cells were co-injected subcutaneously into SCID CB17 mice, and mice were then treated with vincristine (1 mg/kg) on the same day. Results in Figure 6 show that vincristine significantly reduced RL tumor growth in SCID mice, compared to control mice. Unexpectedly, a similar effect was obtained when neutrophils were co-injected with RL cells in control mice. In mice treated with vincristine, the inoculation of neutrophils together with RL cells reduced the sensitivity of RL cells to vincristine resulting in significantly enhanced tumor growth. This result confirms the protective role of neutrophils previously observed *in vitro* (2D and 3D).

Autologous neutrophils protect primary chronic lymphocytic leukemic cells against anti-leukemic agents

To investigate the effect of neutrophils on primary leukemic cells, freshly isolated CLL cells were cultured alone or with autologous neutrophils for 24 hours, in the presence or absence of different anti-leukemic agents. The percentage of viable CLL cells (Annexin V negative/PI

negative) was measured by double staining with annexin V–FITC and PI, followed by flow cytometric analysis. As shown in Figure 7A, vincristine at 10 nM reduced the percentage of viable CLL cells, an effect which was significantly inhibited in the presence of autologous neutrophils (46 ± 4.9 and 68 ± 3.8 , $p < 0.001$, respectively, mean \pm SEM). Similar results for neutrophil-induced protection were obtained in the presence of fludarabine at 100 μ M (Fig 7B).

Since targeted cancer therapy is becoming a major modality of CLL treatment, we aimed to investigate whether neutrophils induce protection on primary leukemic cells in the presence of two recently approved targeted therapies, ibrutinib (a Bruton's tyrosine kinase inhibitor) and idelalisib (a phosphoinositide 3-kinase delta inhibitor). Results in Figures 7 (C and D) show that the decrease of cell viability induced by ibrutinib and Idelalisib was significantly inhibited by the presence of neutrophils (40.1 ± 3.1 vs 60.1 ± 3.5 and 49.9 ± 3.5 vs 62 ± 3.2 , $p < 0.001$ and $p = 0.01$, respectively). This neutrophil-mediated protection of primary leukemic cells against ibrutinib and idelalisib was ratio-dependent (Fig 7, E and F).

Discussion:

Innate immune cells can constitute a substantial proportion of the cells within the tumor microenvironment and have been associated with tumor progression in patients and animal models of cancer³³. Macrophages are important innate immune cells that have been shown to directly regulate cancer cell response to chemotherapy^{21,23,34,35}. However, the role of neutrophils, key players in the innate immune system, in regulating lymphoma response to anticancer treatment has not been previously described. In the current study, we have shown that circulating neutrophils significantly reduce the sensitivity of malignant B cells to a variety of anticancer therapies, thereby constituting yet a new mechanism through which the innate immune system could contribute negatively to the outcome in patients with lymphoma.

Tumor cells have previously been shown to influence the phenotype and properties of cells present in their microenvironment, in particular through the polarization of tumor infiltrating neutrophils^{9,36}. In our study, exposure to cytotoxic chemotherapy did not modify the expression levels of activation markers of neutrophils such as CD11a, CD11b, CD18, CD32 and CD66b, while their expression levels (except for CD11a and CD32) were significantly increased when neutrophils were co-cultured with RL cells (either alone or in the presence of chemotherapy). These results strongly suggest that NHL cells influence the phenotype and possibly the function of neutrophils with which they come into contact.

Neutrophil-induced protection on NHL cell proliferation in presence of chemotherapy was reduced in the presence of antibodies directed against ICAM-1 (expressed on NHL cells) or CD11b (expressed on neutrophils). The effect of blocking antibodies was only observed when each cell type was previously exposed to cognate specific antibody but not when the antibody was added to the co-culture, suggesting that the activation of ICAM-1 down-stream signaling pathways occurred rapidly, as has previously been described²⁸. ICAM-1 is an adhesion molecule of the immunoglobulin superfamily which has been shown to bind to several ligands including LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and CD11c/CD18³⁷. While it was originally suggested that ICAM-1 played only an adhesive role, recent evidence indicates that it is involved in the regulatory process of cell proliferation^{28,29}. Holland et al.²⁸ observed that cross-linking of ICAM-1 on the B lymphoma cell line A20 induces an increase in tyrosine phosphorylation of several cellular proteins and results in activation of Raf-1 and mitogen-activated protein kinases. Based on these previously published data, we hypothesize that the interaction between ICAM-1 and CD11b in our system activates intracellular pathways in malignant B cells allowing their enhanced proliferation in the presence of both neutrophils and anticancer agents.

Co-culture of RL follicular lymphoma cells in the presence of neutrophils and vincristine up-regulated the expression of CD44 by RL cells. Blocking CD44 was also found to reverse neutrophil-induced protection of RL cell proliferation against vincristine. CD44 is another adhesion molecule involved in cell proliferation^{30,31}. Smith et al.³⁰ reported that CD44 repression via NF- κ B inhibition decreased proliferation and invasiveness of breast cancer cells. These published data support our observations and a role for CD44 in the protective effect of neutrophils on RL cells.

The effect of exposing NHL cells to neutrophils and vincristine in combination on the expression of selected anti-apoptotic proteins in RL cells, differed significantly from those obtained when cells were exposed to neutrophils or vincristine separately. While vincristine increased the content of Bcl-2, neutrophils alone did not induce a significant modification. Conversely neutrophils together with vincristine strongly increased the expression of Mcl-1 in RL cells. Using a specific inhibitor of Mcl-1, we found that the protective effect of neutrophils against vincristine was Mcl-1-dependent as it could be strongly reduced in the

presence of MIM-1. Puthier *et al.*³⁸ have described an upstream pathway leading to Mcl-1 upregulation in human myeloma cells by IL-6, a cytokine which is known to be produced and released by activated neutrophils³⁹.

Taken together, these results suggest a possible mechanism by which neutrophils activate protective signaling pathways in RL cells (Figure S6). RL cells, by direct contact with neutrophils, induce the latter to undergo activation as determined by the increase in the expression of different activation markers (Fig 4A, Table S1). The activated neutrophils then release IL-6³⁹ that induces Mcl-1 upregulation in RL cells, possibly through activation of the Jak/Stat pathway³⁸. CD11b/ICAM-1 interaction may result in the activation of Raf-MEK-ERK pathway^{28,29} in RL cells. These proteins have a known role in intracellular signaling cascades resulting in increased cellular proliferation²⁹.

In an effort to evaluate the relevance of the protective effect of neutrophils in more complex models, we developed a microsphere co-culture system using differentiated promyelocytic cells. We also performed xenograft experiments with co-injection of RL cells and human neutrophils from healthy donors in immune-compromised mice. Both systems allowed us to confirm the protective effect against vincristine-induced cytotoxicity in the RL NHL model. Importantly, most of our results were obtained with circulating neutrophils from healthy donors, and not with tumor-infiltrating neutrophils. In cancer patients, neutrophils migrate into solid tumors under the influence of a variety of cytokines (e.g. KC/CXCL1), a phenomenon which involves cell-adhesion molecules expressed by neutrophils themselves and endothelial cells. When peripheral blood neutrophils penetrate the tumor, immune-sculpting, which results from the cross-talk between immune cells and tumor cells, changes the phenotype of neutrophils into either anti-tumor (N1-TAN) or pro-tumor phenotype (N2-TAN) depending on which cytokines are present in the tumor microenvironment¹⁰. In our *in vivo* model, peripheral blood neutrophils were mixed together with RL cells before injection into mice that could result in the modification of peripheral blood neutrophils phenotype into TAN phenotype. Since we obtained a protective effect for neutrophils *in vivo*, this suggests that TAN may also exert a protection on tumor cells against cancer therapies.

Using samples collected from CLL patients, we obtained a similar protective effect by autologous peripheral blood neutrophils on primary CLL cells, by reducing their sensitivity to vincristine and fludarabine, and the two recently approved targeted therapies ibrutinib and idelalisib. Our study suggests that neutrophils may play a role in protecting CLL cells from anticancer agent-induced apoptosis.

In conclusion, using *in vitro* (2D and 3D models) as well as *in vivo* and *ex vivo* approaches, we showed that cell-cell contact between neutrophils and malignant B cells protect the latter from the cytotoxic effect of anticancer agents used in the treatment of lymphoproliferative diseases. CD11b/ICAM-1 interaction is implicated in neutrophil-mediated protection of malignant B cells, in addition to CD44. This protective effect is dependent on the Mcl-1 antiapoptotic protein. Taken together, our data present an intriguing insight into the relevance of neutrophils in cancer cell sensitivity to anticancer agents and suggest that pharmacological modulation, for example with anti-Mcl1 compounds, could be used to enhance the effect of cytotoxic agents in patients with NHL.

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Authorship

TH participated in the conception and design of the study, performed the experiments, acquired the data, analyzed the results and drafted the manuscript; ELM and JE performed *in vitro* experiments; DM and AE performed the *in vivo* experiments and interpreted their data; KC analyzed the microarray results and contributed to critical revision of the manuscript; LPJ participated in drafting and gave critical comments on the manuscript; CD participated in the conception and design of the study and drafted the manuscript. All authors have read and approved of the final version of the manuscript.

Disclosure of Conflicts of Interest

The authors have declared that no conflict of interest exists.

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Figure Legends

Figure 1. Neutrophils protect lymphoma B cells against vincristine in a ratio-dependent manner. CFSE-labeled lymphoma B cells were cultured alone or together with freshly purified human neutrophils (N) at different N:T ratios, in the presence or absence of 10 nM vincristine (VCR). After 48 h of incubation, cells were labeled with anti human-CD19 then resuspended in DAPI (2 µg/ml) followed by flow cytometric analysis. Cell death (**A, C, E**) and cell proliferation (**B, D, F**) of CD19 positive population were measured using DAPI and CFSE assays, respectively. Data are expressed as mean ± SD of at least three independent experiments performed in triplicates. The data are presented relative to their cognate controls. For the ratio-dependent figures (**E-F**), data are expressed as mean ± SD of one experiment performed in triplicate. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Figure 2. Neutrophil-like HL60diff cells protect RL lymphoma cells against vincristine in 3D culture. RL cells were cultured alone or together with HL60diff cells at HL60diff:RL ratio 10: 1 for 7 days in BD matrigel. On day 5, VCR was added at a concentration of 10 nM. **A)** Pictures were taken on day 7 with a DMI3000 fluorescent microscope at magnification 40x, representing several experiments. Scale bar 40 µm. **B)** Spheroids were dissociated on day 7 using PBS-5mM EDTA and cells were labeled with anti-human CD19 and anti-human CD38. The number of events in CD19+ Annexin V-/PI- population was measured by double staining with annexin V-FITC and PI, followed by flow cytometric analysis. Data are expressed as mean ± SD of three independent experiments performed in triplicates. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. ** $p \leq 0.01$

Figure 3. Protective effect of neutrophils on RL lymphoma cells against vincristine was not due to soluble factors. **A-B)** CFSE-labeled RL cells and freshly purified human neutrophils were co-cultured on opposite sides of porous PET inserts. Neutrophils (N) were placed inside the inserts whereas CFSE-labeled RL cells were placed in the wells, or to inserts with complete RPMI medium as a control. VCR at 10 nM was incorporated at the initiation of culture on CFSE-labeled RL cells. After 48 h of incubation, CFSE-labeled RL cells were resuspended in DAPI (2 µg/ml) followed by flow cytometric analysis. Cell death (**A**) and cell proliferation (**B**) were measured using DAPI and CFSE assays, respectively. Data are expressed as mean ± SD of three independent experiments. **C-D)** CFSE-labeled RL cells were cultured in the presence of 10 nM VCR in a complete RPMI medium, or with conditioned medium harvested from cultures of neutrophils alone or their co-cultures with RL cells and in the absence or presence of VCR. After 48 h of incubation, CFSE-labeled RL cells were resuspended in DAPI (2 µg/ml) followed by flow cytometric analysis. Cell death (**C**) and cell proliferation (**D**) were measured using DAPI and CFSE assays, respectively. Data are expressed as mean ± SD of one experiment performed in triplicate. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. *** $p \leq 0.001$

Figure 4. ICAM1, CD11b and CD44 are required for neutrophil-induced protection on RL cells against vincristine. **A)** Neutrophil phenotype after exposure to VCR and/or neutrophils. Neutrophils were cultured alone or together with RL cells at N:T ratio 10:1 in the presence or absence of 10 nM VCR. After 48 h of incubation, cells were collected and labeled with anti-human CD15. Additionally, they were labeled with anti-human CD11a and CD11b. Graphs are representative of three independent experiments. **B)** CFSE-labeled RL cells were blocked against ICAM1 (12µg/ml), CD11a or CD11c (10µg/ml) and neutrophils were blocked against CD11a, CD11b or CD11c (10µg/ml) for 1 hour. Anti-IgG1 served as a control. CFSE-labeled RL cells were cultured alone or together with neutrophils (N) at N:CFSE-labeled RL ratio 10:1 in the presence of 10 nM VCR for 48 h. **C)** CFSE-labeled RL cells were cultured alone or together with neutrophils in the presence or absence of CD44 blocking antibody (1 µg/ml) treated with 10 nM VCR. Cell proliferation of CD19 positive population was measured using CFSE assay followed by flow cytometric analysis. Data are expressed as mean ± SD of at least four independent experiments performed in triplicates. The data are presented relative to the control. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. **p≤ 0.01, ***p≤ 0.001, ns= not significant

Figure 5. Neutrophil-induced protection is dependent on Mcl-1 antiapoptotic protein. **A)** Relative expression of Bcl-2 family members (Bcl-xL, Bcl-2, Mcl-1) in RL cells. RL cells were cultured alone or together with neutrophils (N) at N:T ratio 10:1, in the presence or absence of 10 nM VCR. After 48 hours of incubation, the cells were collected and labeled with anti-human CD19 and anti-human CD15 then were fixed and permeabilized. Later, the intracellular labeling of anti-apoptotic Bcl-2 family members was performed by flow cytometry. **B-C)** RL cells were cultured alone or together with neutrophils at N:T ratio 10:1, in the presence or absence of 10 nM VCR. ABT-199 (10 nM) (**B**) or MIM1(10 µM) (**C**) inhibitors were added to the culture system. After 48h of incubation, cells were labeled with anti human-CD19 then resuspended in DAPI (2 µg/ml) followed by flow cytometric analysis. Cell death of CD19 positive population was measured using DAPI assay. Data are expressed as mean ± SD of at least three independent experiments performed in triplicates. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. *p≤ 0.05, ***p≤ 0.001, Anova.

Figure 6. Neutrophil-induced protection on RL cells *in vivo*. Mice (5 mice/group) were injected subcutaneously with 3x10⁶ RL cells alone or together with freshly purified human neutrophils (N) at N:T ratio 10:1. VCR was injected intraperitoneally to mice at a dose of 1 mg/kg at the same day. Tumor growth was monitored by caliper measurement of the tumor volume. **p≤ 0.01, ***p≤ 0.001, Student's *t* test.

Figure 7. Autologous neutrophils protect primary leukemia cells against cancer therapy in a ratio-dependent manner. **A-D)** Blood was collected from patients diagnosed with chronic lymphocytic leukemia (CLL). Primary leukemic cells (CLL cells) were isolated and cultured alone or together with autologous neutrophils (N) at N:CLL ratio 10:1 for 24 h, in the presence or absence of (**A**)10 nM VCR (8 patients), (**B**) 100 µM fludarabine (14 patients), (**C**) 25 µM ibrutinib (19 patients) or (**D**) 50 µM idelalisib (16 patients). **E-F)** CLL cells were co-cultured with autologous neutrophils at different N:CLL ratios for 24 h, in the presence or

absence of **(E)** 25 μ M Ibrutinib (10 patients) or **(F)** 50 μ M Idelalisib (10 patients). The percentage of viable CLL cells (Annexin V negative/PI negative) was measured by double staining with annexin V-FITC and PI, followed by flow cytometric analysis. Dashes represent the mean and each dot represent the mean of triplicate. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method.* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Figure 1

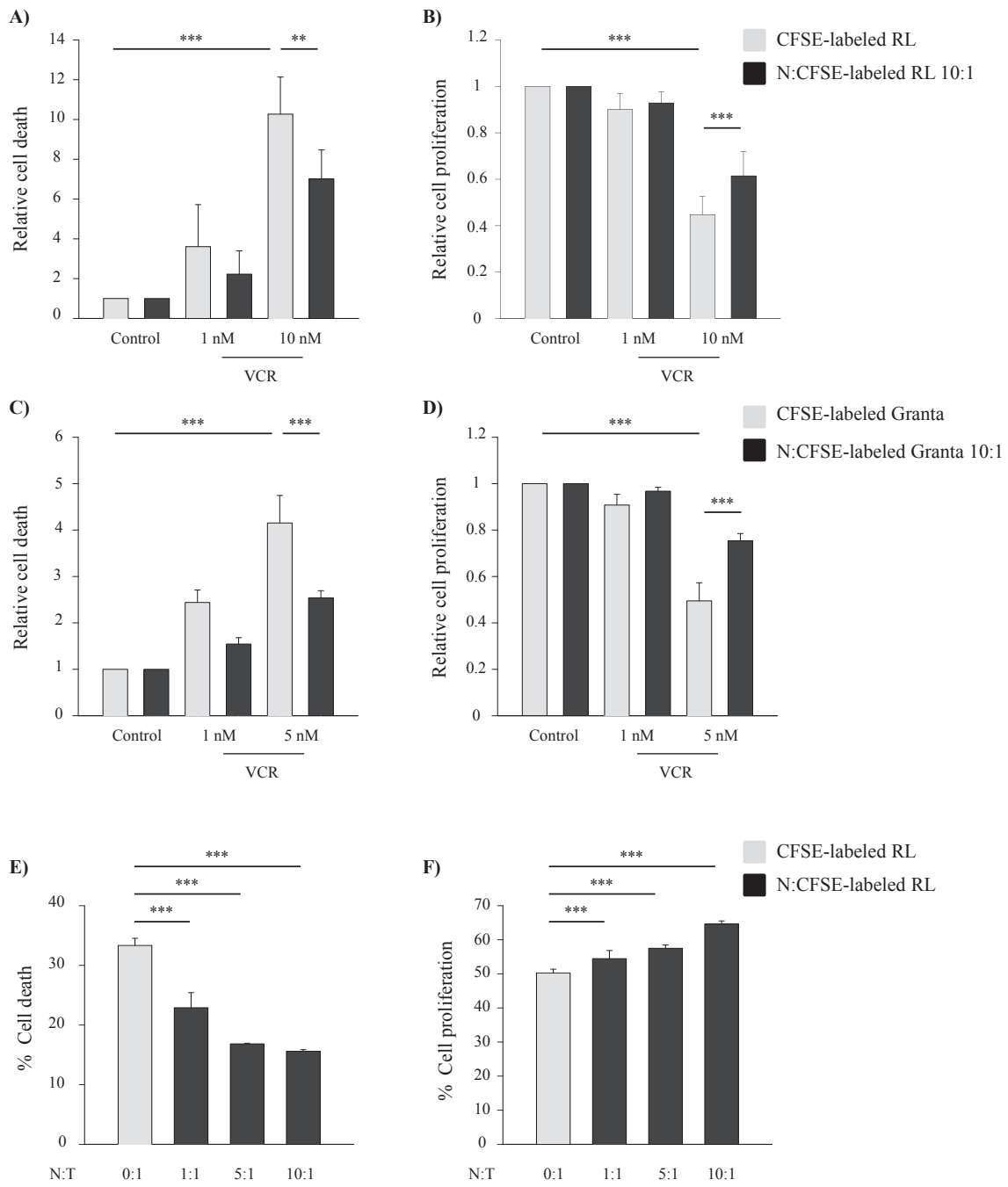


Figure 2

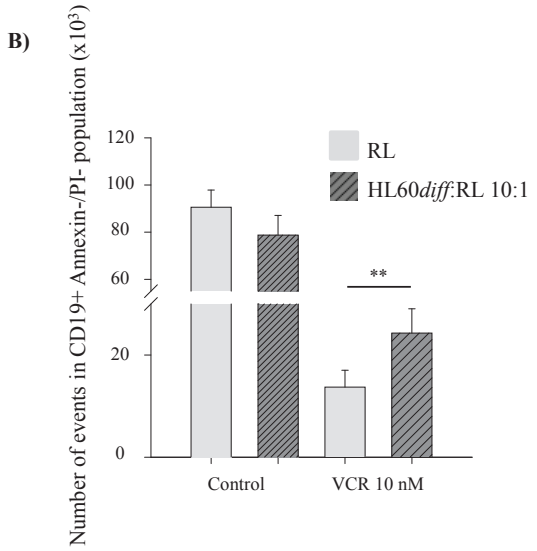
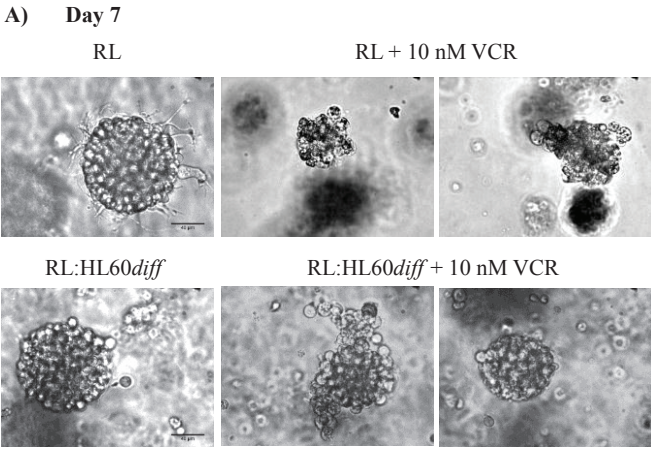


Figure 3

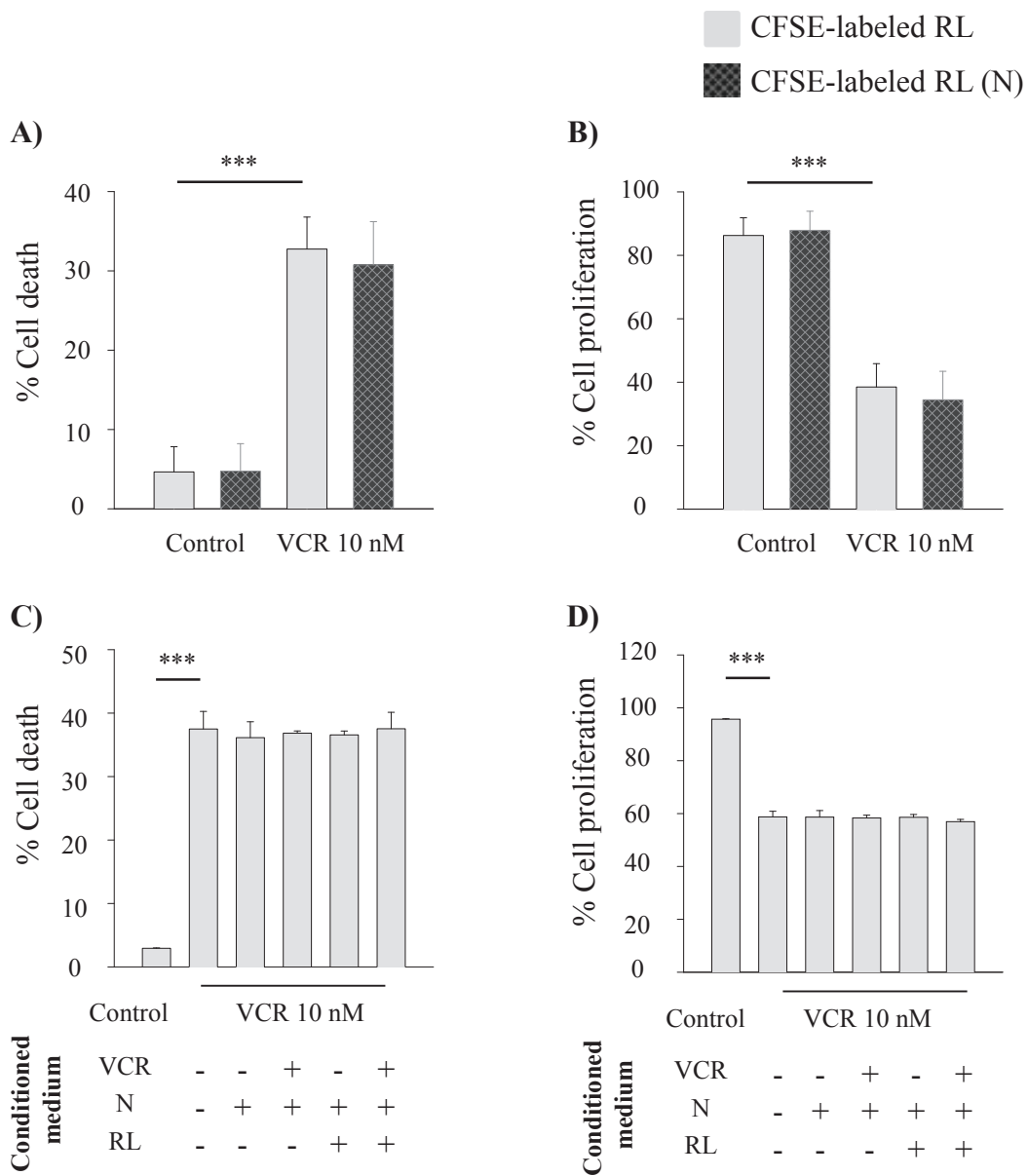


Figure 4

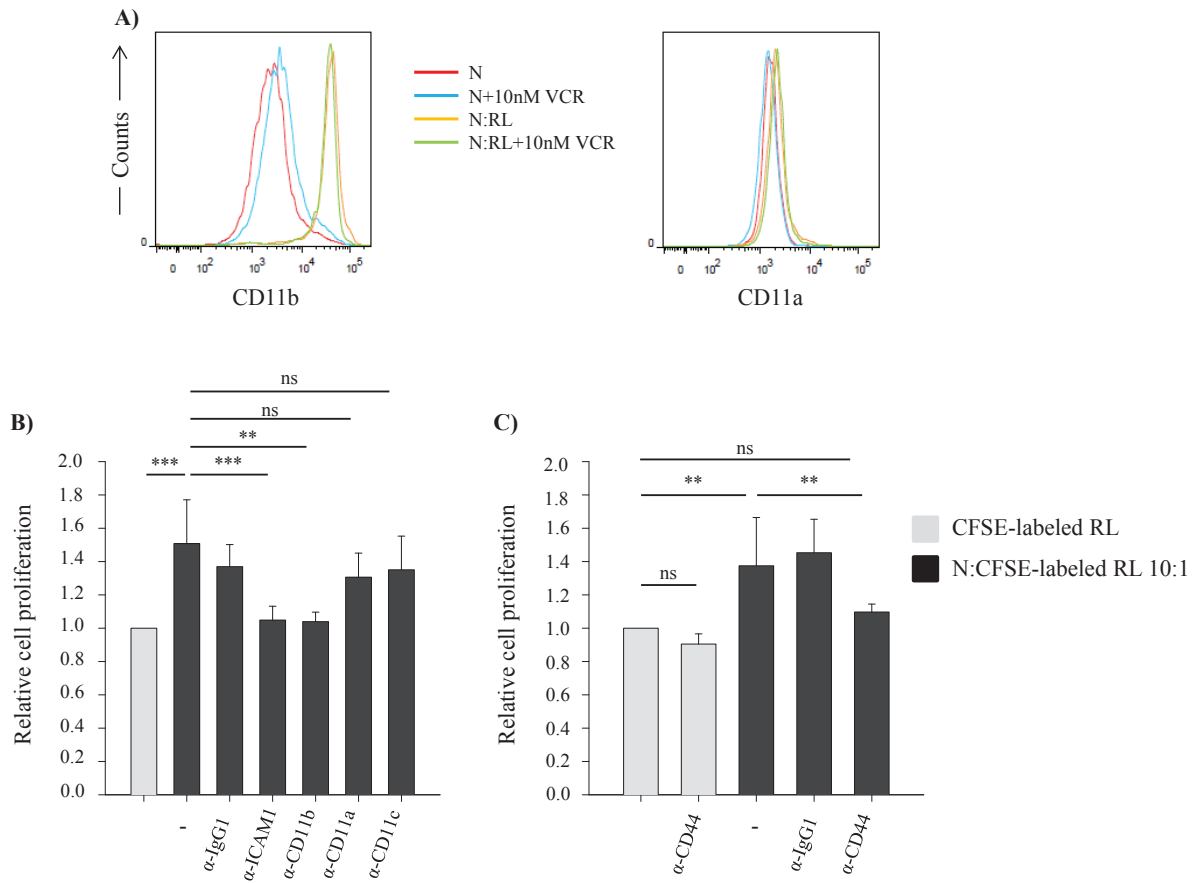


Figure 5

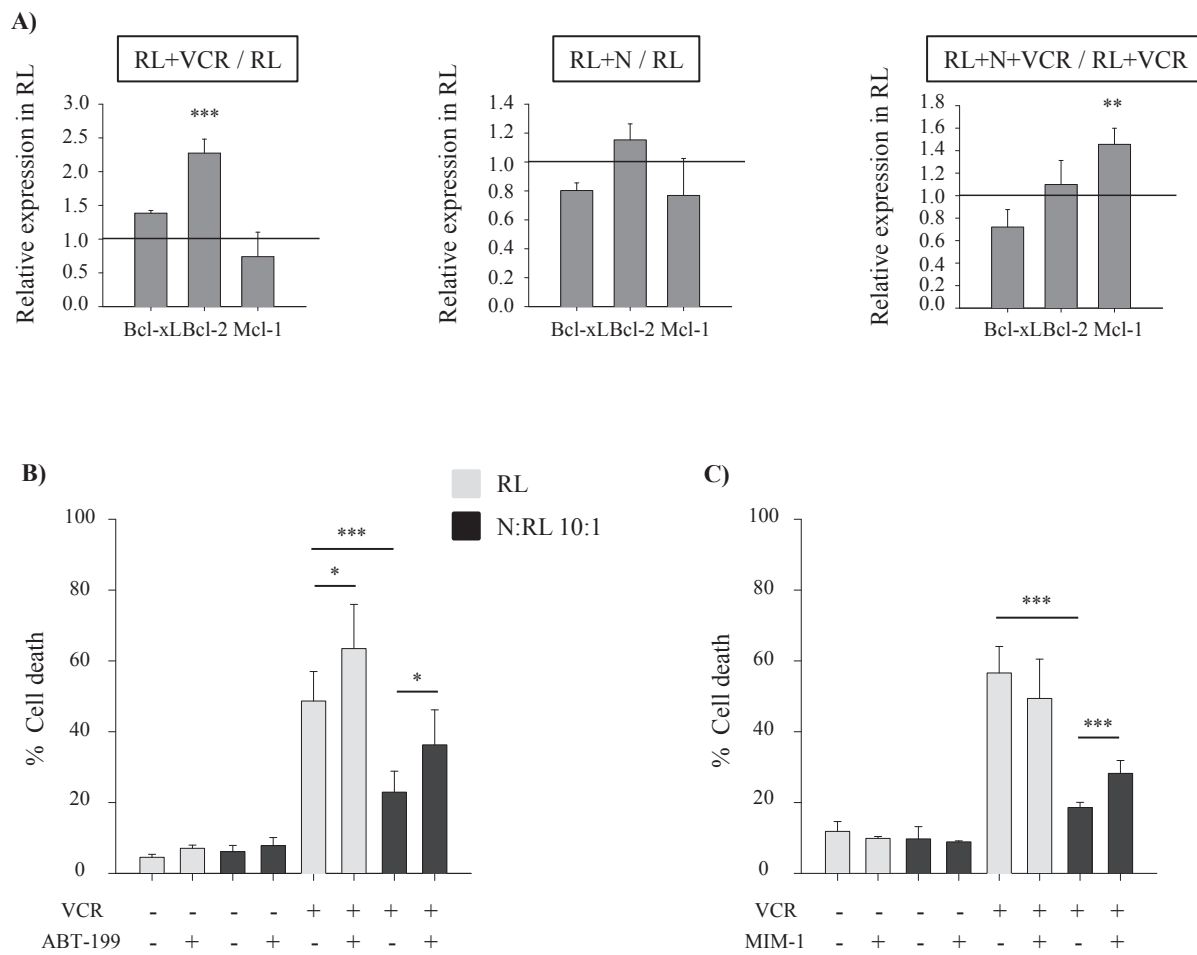


Figure 6

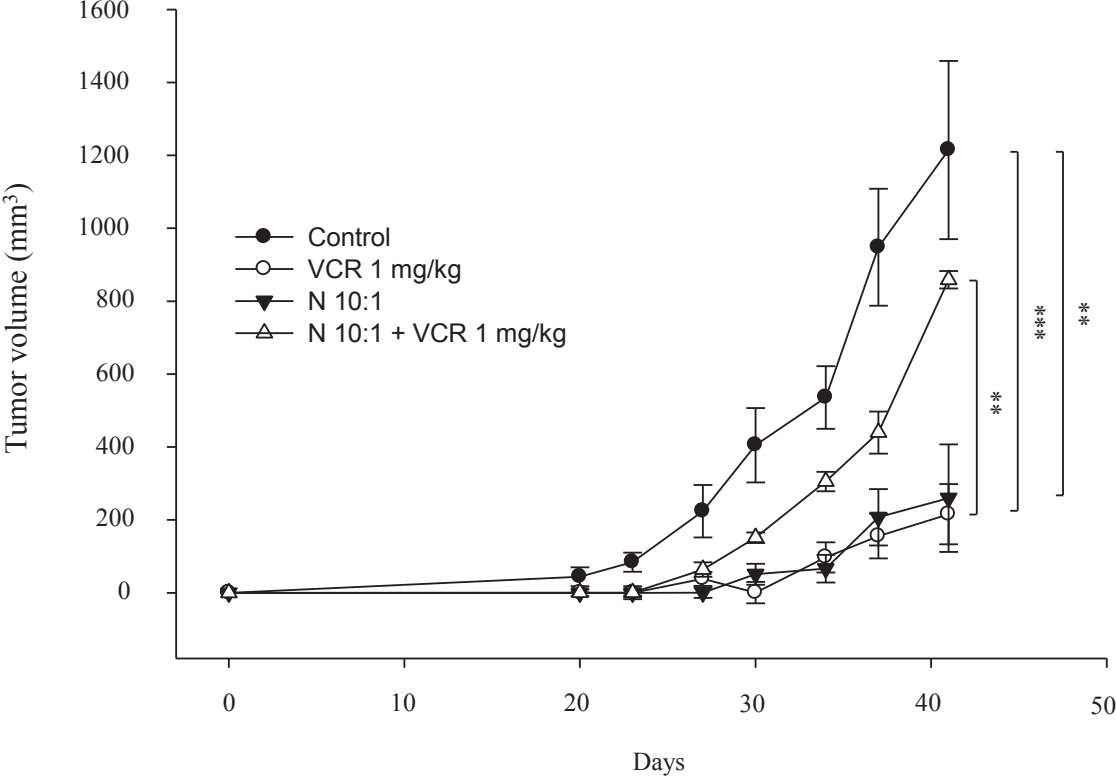
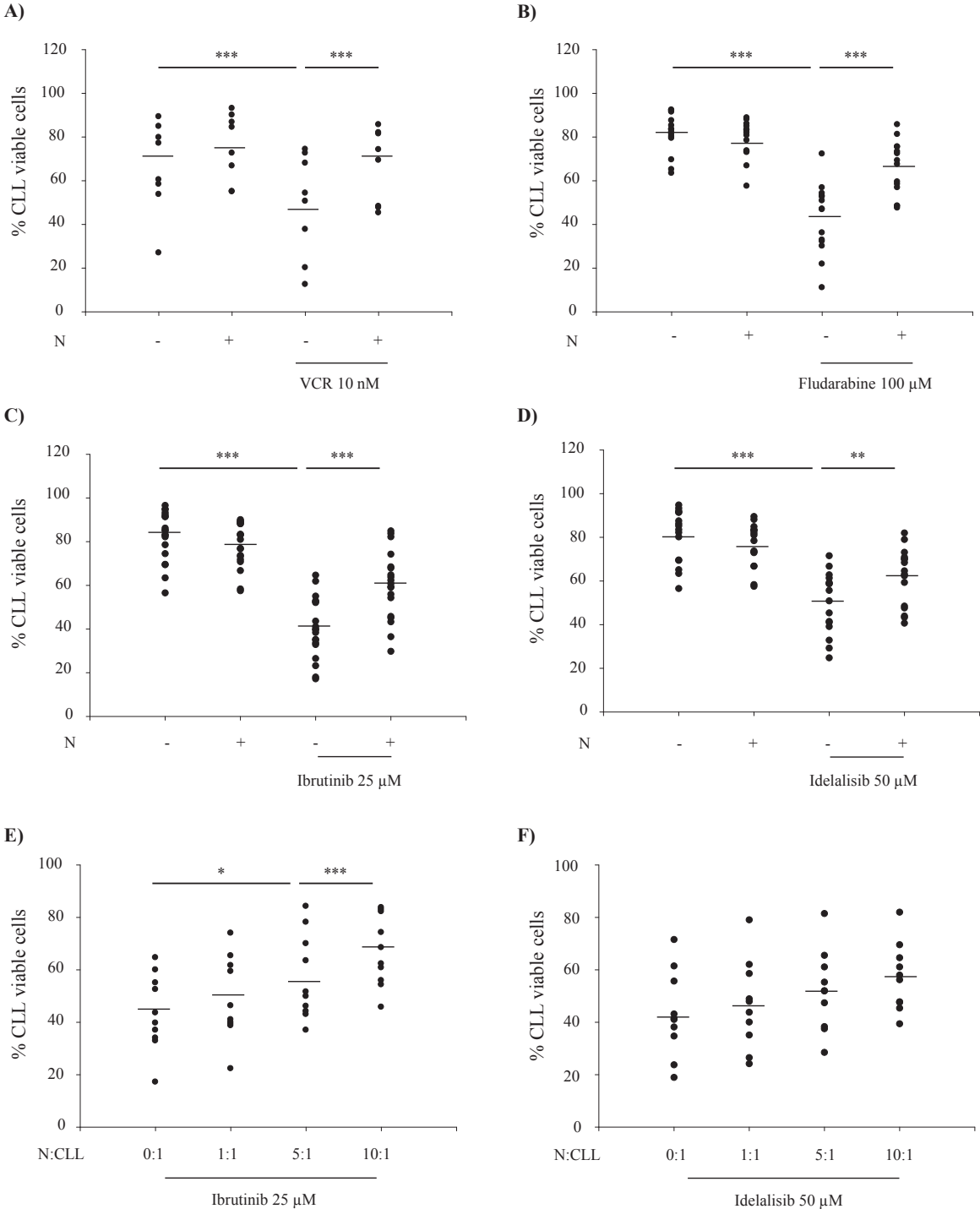


Figure 7



Supplementary Data

Supplementary Materials

Reagents

Chemotherapeutic agents: Vincristine was obtained from EG labo, doxorubicin from accord, bortezomib from Janssen, cisplatin from Mylan, mafosfamide from Santa Cruz and fludarabine from Schering.

Targeted therapies: Bruton's tyrosine kinase (Btk) inhibitor (Ibrutinib) from CliniSciences and phosphoinositide 3-kinase (PI3Kdelta) inhibitor (Idelalisib) from Santa Cruz.

Small molecule inhibitors: ABT-199 was obtained from ChemieTek and MIM-1 from Tocris bioscience.

Others: Red cell lysis buffer (ref 555899), BD Matrigel basement membrane matrix (ref 354234) and BD Cytotfix/Cytoperm Fixation/Permeabilisation kit (ref 554715) were from BD Biosciences. Ficoll (Pancoll, ref P04-60500) obtained from PAN Biotech. Dimethyl sulfoxide (DMSO, ref D8418), dextran (ref D8906), Ethylenediaminetetraacetic acid (EDTA, ref E5134) and bovine serum albumin (BSA, ref A7906) were from Sigma-Aldrich. 4',6-diamidino-2-phenylindole (DAPI, ref 6484) and Annexing V-FLOUS staining kit (ref 11 988 549 001) were from Roche. Sodium chloride (NaCl, ref S3014) was form Euromedex. RPMI 1640 (ref 21875-034), fetal bovine serum (FBS, ref 10270-106) and phosphate-buffered saline (PBS, ref 14040-091) were from Gibco Invitrogen. CarboxyFluorescein diacetate succinimidyl Ester (CFSE, ref C34554), N-acetyl-L-alanyl-L-glutamine (L-Glutamine, ref 25030-024) and penicillin streptomycin (Pen Strep, ref 15140-122) were from Life technologies. Millicell cell culture PET inserts (24-well Millicell, ref PIHT12R48) were from Millipore.

Antibodies

Antibodies Table 1. Function blocking purified mouse anti-human

Antigen	Clone	Antibody supplier
CD11a	HI111	Biologend
CD11b	ICRF44	Biologend
CD11c	3.9	Biologend
CD44	A3D8	Sigma-Aldrich
ICAM-1/CD54	BBIG-11	R and D systems
IgG1	X40	BD Biosciences

Antibodies Table 2. Fluorochrome-conjugated purified monoclonal antibodies

Antigen	Fluorochrome	Clone	Antibody supplier
CD11a	PE	G43-25B	BD Biosciences
CD11b	BV650	M1/70	Biologend
CD15	Alexa Fluor 700	W6D3	Biologend
CD15	FITC	VIMC6	Miltenyi biotech
CD18	BV421	6.7	BD Biosciences
CD19	APC	SJ25C1	BD Biosciences
CD19	PE-Cy7	SJ25C1	BD Biosciences
CD32	PE	3D3	BD Biosciences
CD38	APC	HB7	BD Biosciences
CD64	APC	10.1	BD Biosciences
CD66b	PerCP/Cy5.5	G10F5	Biologend
BCL-2	PE	Bcl-2/100	BD Biosciences
Bcl-xL	Alexa Fluor 488	54H6	Cell signaling
Mcl-1*	APC	Poly6136	Biologend

*Purified anti-Mcl-1 antibody was labeled with APC fluorochrome using LYNX Rapid APC Antibody Conjugation kit (Bio-Rad).

Antibodies Table 3. Fluorochrome-conjugated purified monoclonal antibodies for neutrophils purity. All antibodies were obtained from Miltenyi biotech.

Antigen	Fluorochrome	Clone
CD3	VioBlue	BW264/56
CD14	APC-Vio770	TÜK4
CD15	APC	VIMC6
CD16	FITC	VEP13
CD19	PE-Vio770	LT19
CD45	PerCP-Vio700	5B1
CD56	PE	AF12-7H3

Supplementary Methods

Method S1. Cell preparation and flow cytometry

CFSE cells labeling- Lymphoma B cells at 10^7 cells/mL were incubated in PBS- containing 0.1% BSA with 5 μ M CFSE for 10 min at 37 °C. Labeling was stopped by adding 1 volume ice-cold FBS with 5 minutes of incubation on ice. Then cells were washed three times and incubated at 37 °C with complete RPMI medium. Experiments started 24 hours after CFSE labeling. The entire procedure was carried out in dark.

Isolation of human peripheral blood neutrophils- Human neutrophils were isolated from fresh blood of healthy donors supplied by Etablissement Français du Sang (EFS) (Lyon, France) or from patients diagnosed with chronic lymphocytic leukemia (CLL). Ficoll was carefully added to the diluted blood without mixing the phases. After a short centrifugation step (300 g for 35 minutes) at room temperature (RT), neutrophils together with red blood cells (RBCs) sediment at the bottom of the centrifuge vial. Neutrophils were extracted by 1:1 addition of 3% dextran in 0.9% NaCl while RBCs were removed by red cell lysis buffer. Neutrophils then washed and checked for their purity using a mixture of fluorochrome-conjugated purified monoclonal antibodies (see **Antibodies** section above). Neutrophils were ≥ 90 % pure in all experiments.

Flow cytometric analysis of surface antigens- Cells were collected and washed in PBS with 4% FBS. Cells were incubated with fluorochrome-conjugated purified monoclonal antibodies at 4 °C for 30 minutes. Later, cells were washed with PBS- 4% FBS and the relative levels of surface antigens were assessed by FACS analysis on LSRII (BD Biosciences) using FACS Diva or Flowjo softwares.

Method S2. Cell uptake of doxorubicin

RL cells were incubated alone or with neutrophils at a ratio of N:T 10:1, in the presence or absence of 1 μ M doxorubicin. After 2 hours of incubation, cells were collected on ice and washed with cold PBS then labeled with anti-human CD19 (for 1 hour on ice). Mean doxorubicin-related fluorescence was collected by flow cytometry.

Method S3. Induction of Human promyelocytic (HL60) cells granulocytic differentiation

HL60 cells were maintained at a logarithmic growth rate prior to induction of differentiation. HL60 cells (3×10^5 cells/mL) were induced to differentiate along the granulocyte pathway by 1 μ M retinoic acid (RA, ref R2625, Sigma-Aldrich) and 1.25 % DMSO for 5 days. Medium was renewed on day 2 in the presence of RA and DMSO at the same concentrations.

Differentiation of HL60 cells was analyzed by two different parameters: cell-surface markers expression and morphological changes. On day 5, the cells were collected and HL60 or differentiated HL60 (HL60diff) cells were incubated with anti-human CD11b and anti-human CD38 for cell-surface markers expression analysis. In addition, cells were stained for morphological analysis using Kit RAL 555 Modified Giemsa staining kit (ref CB361550-0000, Cosmos Biomedical).

Supplementary Figures and tables

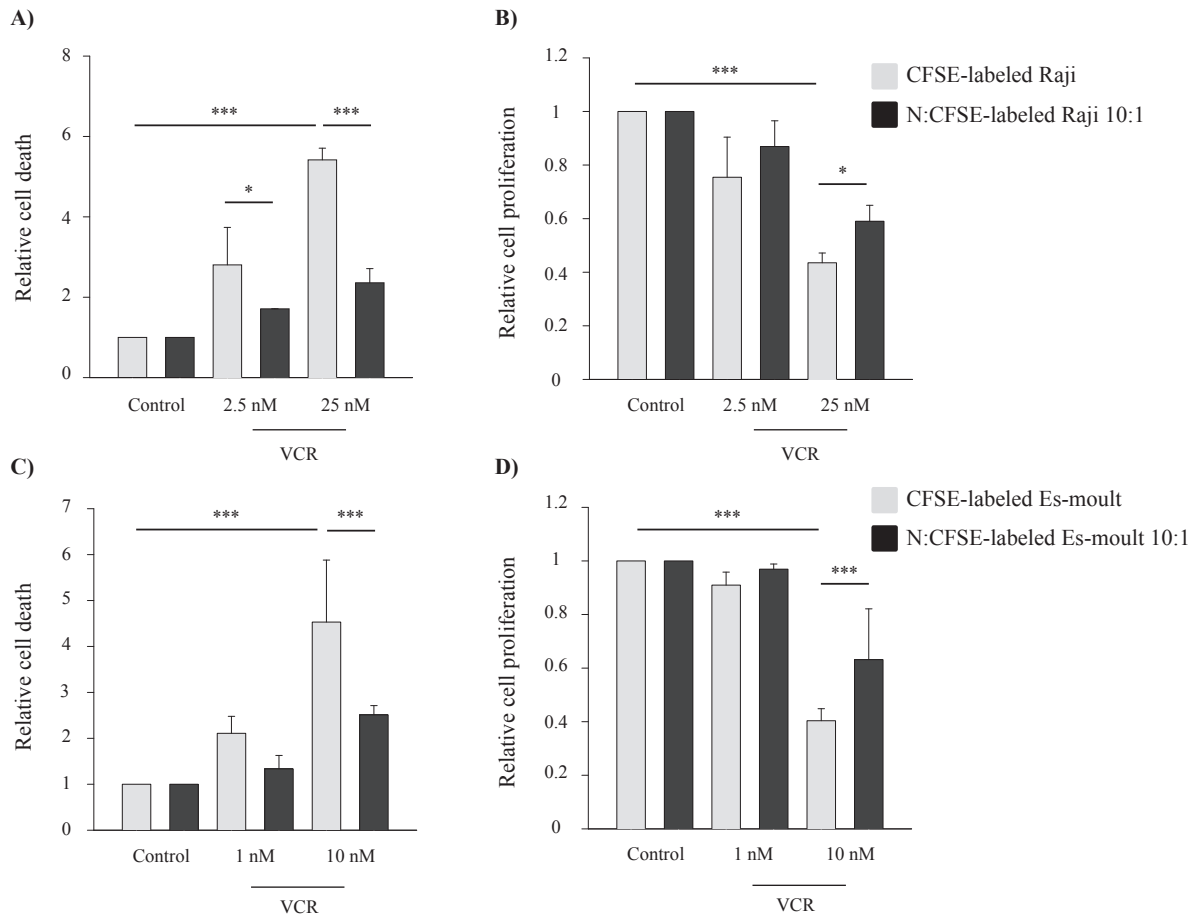


Figure S1. Neutrophils protect lymphoma B cells against vincristine. CFSE-labeled lymphoma B cells were cultured alone or together with neutrophils (N) at N:T ratio 10:1, in the presence or absence of different concentrations of vincristine (VCR). After 48 h of incubation, cells were labeled with anti human-CD19 then resuspended in DAPI (2 μ g/ml) followed by flow cytometric analysis. Cell death (A, C) and cell proliferation (B, D) of CD19 positive population were measured using DAPI and CFSE assays, respectively. Data are expressed as mean \pm SD of at least two independent experiments performed in triplicates. The data are presented relative to their cognate controls. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. * $p \leq 0.05$, *** $p \leq 0.001$

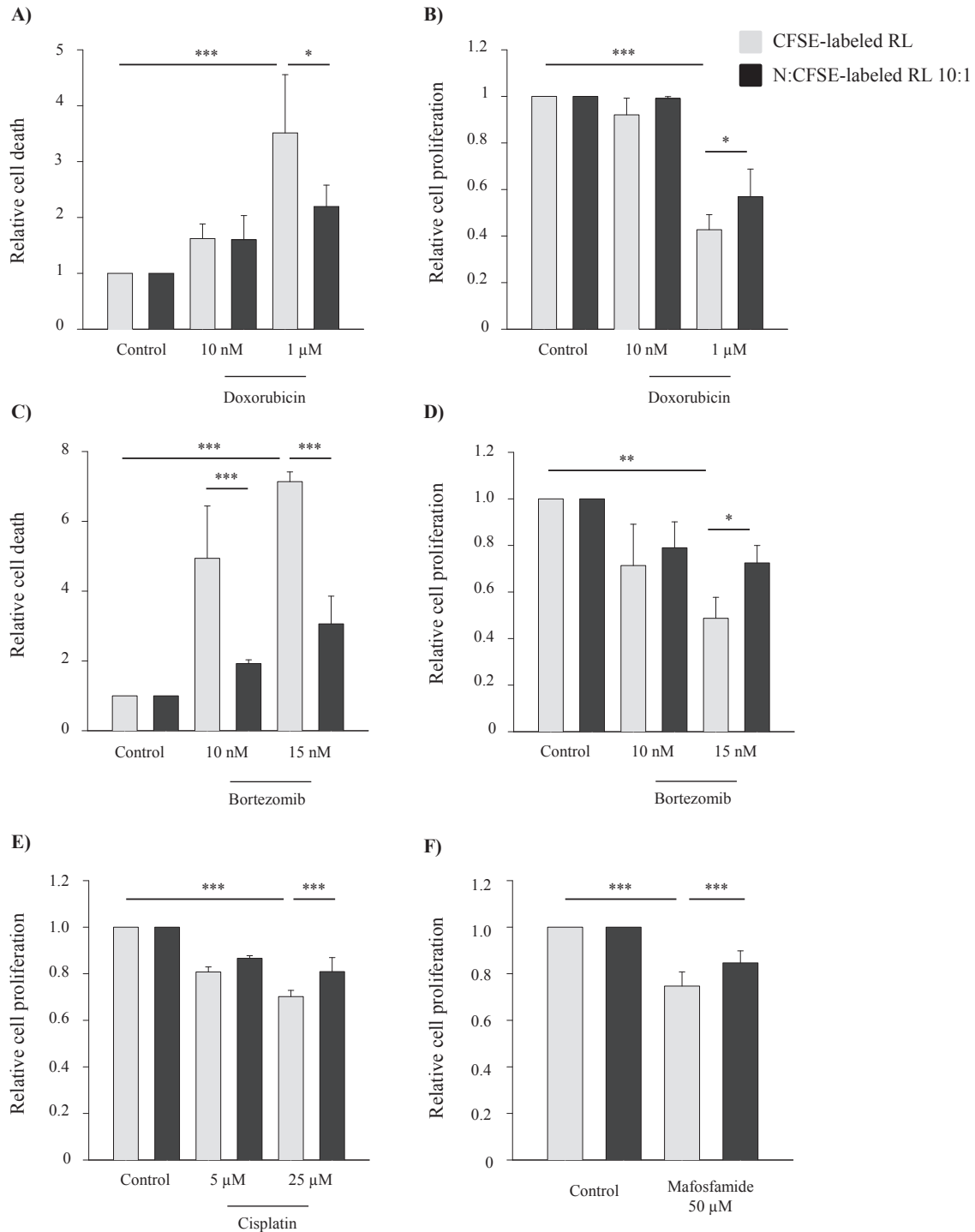


Figure S2. Neutrophils protect RL lymphoma cells against chemotherapeutic agents. CFSE-labeled RL cells were cultured alone or together with freshly purified human neutrophils (N) at N:CFSE-labeled RL ratio 10:1, in the presence or absence of different chemotherapeutic agents. After 48 h of incubation, cells were labeled with anti human-CD19 then resuspended in DAPI (2 μg/ml) followed by flow cytometric analysis. Cell death (A, C) and cell proliferation (B, D, E, F) of CD19 positive population were measured using DAPI and CFSE assays, respectively. Data are expressed as mean ± SD of at least three independent experiments performed in triplicates. The data are presented relative to their cognate controls. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

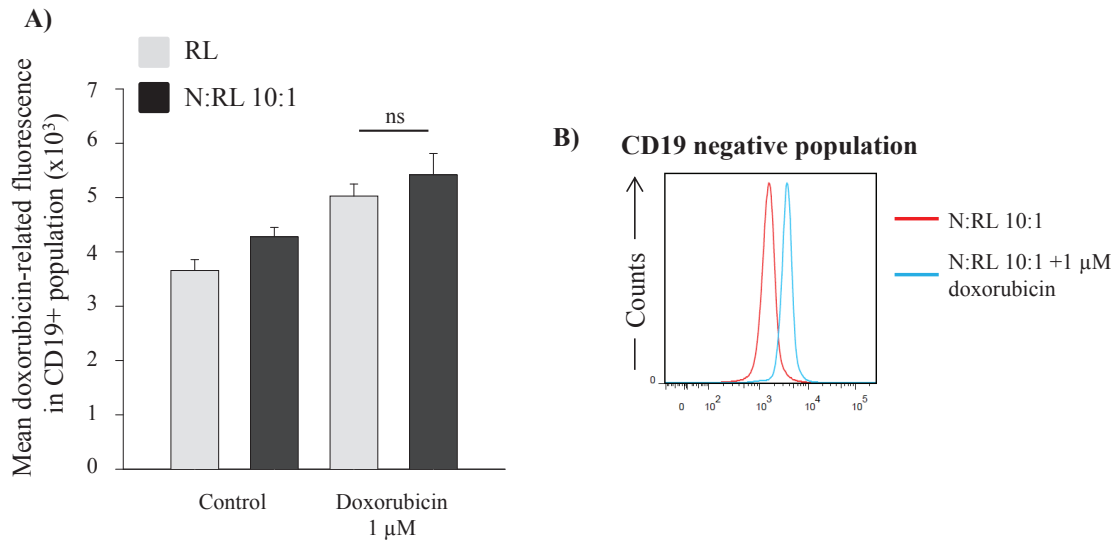


Figure S3. Flow cytometry analysis of doxorubicin accumulation in RL cells and neutrophils. RL cells were cultured alone or together with neutrophils (N) at N:RL ratio 10:1, in the presence or absence of 1 μM doxorubicin. After 2 hours of incubation, cells were labeled with anti human-CD19 followed by flow cytometric analysis. **A)** CD19 positive population was analyzed for doxorubicin accumulation represented by FITC Mean Fluorescence Intensity (MFI). **B)** Histogram reveal doxorubicin accumulation by CD19 negative population in the co-culture system. Data are expressed as mean ± SD of one experiment performed in triplicates. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. ns= not significant

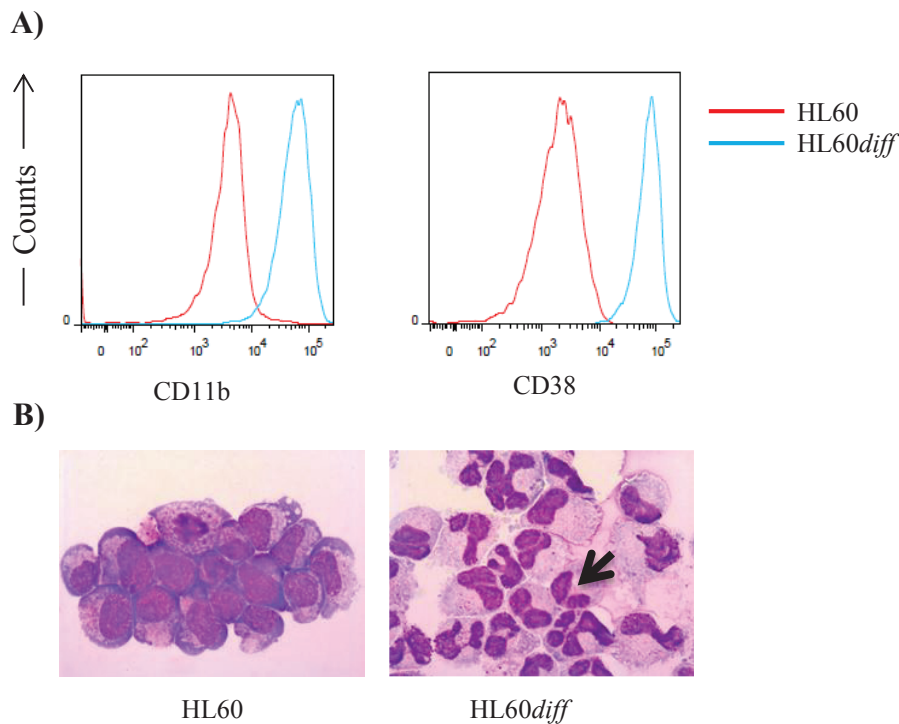


Figure S4. Structural parameters for differentiated HL60 cells. **A)** HL60 and differentiated HL60 cells (HL60diff) cells were labeled with anti-human CD11b and anti-human CD38 antibodies followed by flow cytometry analysis. Mean fluorescence intensity are shown. **B)** HL60 cells show morphological changes consistent with differentiation toward granulocytes when treated with 1 μ M retinoic acid (RA) and 1.25 % DMSO. Photos were taken using Leica DMR-XA microscope with 100x magnification. Bold black arrow point the multi-lobed nucleus. Graphs are representative of five independent experiments.

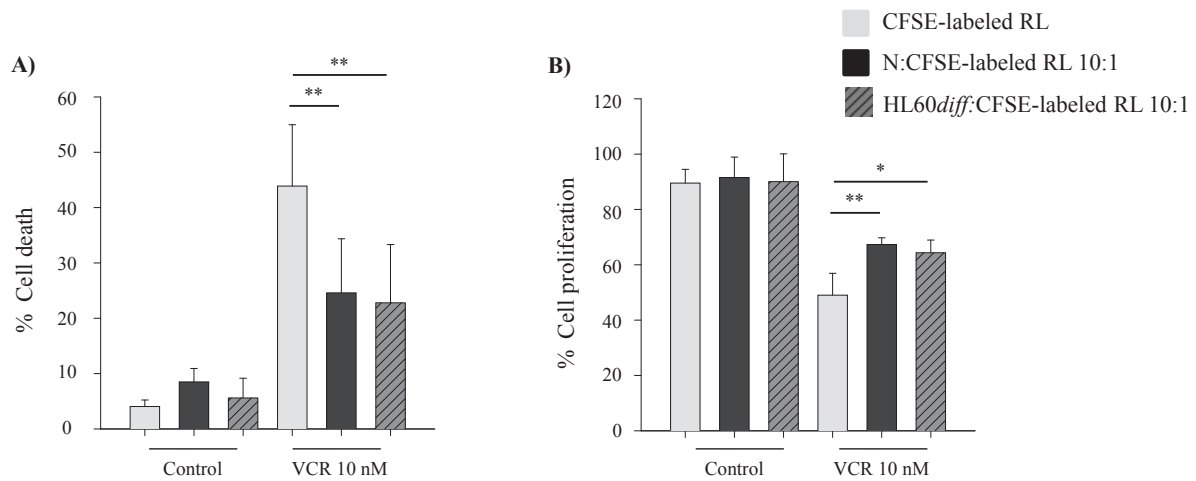


Figure S5. Neutrophil-like *HL60diff* cells protect RL lymphoma cells against vincristine in 2D culture. CFSE-labeled RL cells were cultured alone or together with neutrophils (N) or *HL60diff* cells at N:CFSE-labeled RL or *HL60diff*:CFSE-labeled RL ratio 10: 1, in the presence or absence of 10 nM VCR. After 48 h of incubation, cells were labeled with anti human-CD19 then resuspended in DAPI (2 μ g/ml) followed by flow cytometric analysis. Cell death (**A**) and cell proliferation (**B**) of CD19 positive population were measured using DAPI and CFSE assays, respectively. Data are expressed as mean \pm SD of three independent experiments performed in triplicates. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method. * $p \leq 0.05$, ** $p \leq 0.01$

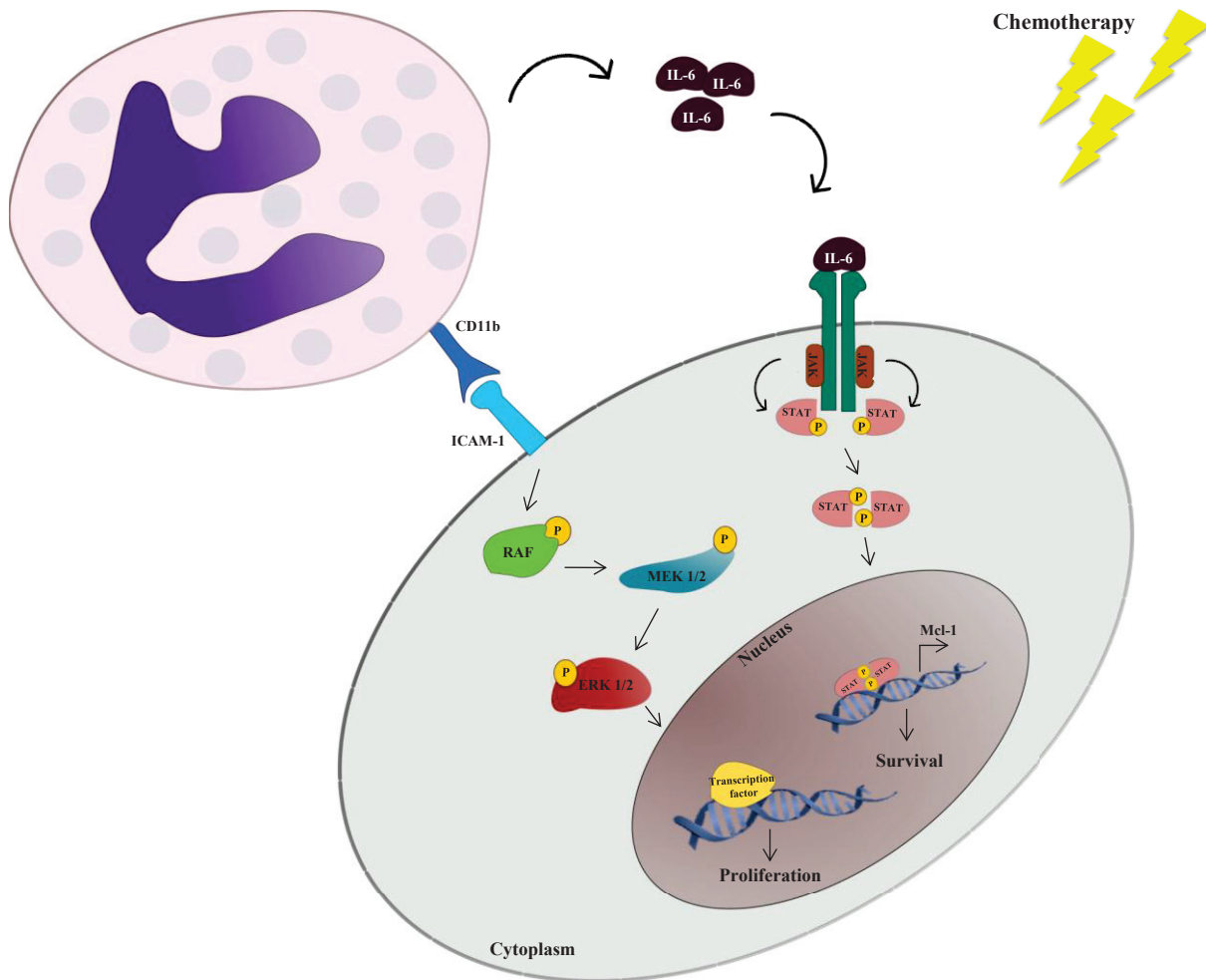


Figure S6. Schematic model representing possible mechanisms for neutrophil-induced protection on lymphoma cells against chemotherapy. Neutrophils interact with lymphoma cells by CD11b/ICAM-1 binding leads to Raf-MEK-ERK pathway activation results in enhancing cell proliferation. As well, direct binding between neutrophils and lymphoma cells leads to IL-6 secretion by the activated neutrophils. IL-6 binding to their receptors leads to Jak/Stat pathway activation that induces Mcl-1 upregulation in lymphoma cells results in enhancing cell survival.

Antigen	N	N + 10 nM VCR	N:RL	N:RL + 10 nM VCR
		mean ± SD		
CD11a	2380 ± 862	2122 ± 596,2	3266 ± 959	2955 ± 737
CD11b	4825 ± 1786	6407 ± 1280	35733 ± 3092***	37050 ± 5020 ###
CD18	10645 ± 3046	12250 ± 1202	45300 ± 707**	43600 ± 1697 #
CD32	2168 ± 202	2404 ± 340,8	1420 ± 355	946 ± 241 #
CD64	705 ± 94	731 ± 67	2380 ± 885	1893 ± 883
CD66b	4423 ± 1350	4764 ± 1075	23200 ± 7114**	18133 ± 6800 ##

Table S1. Mean fluorescence intensity of neutrophils activation markers. Neutrophils (N) were cultured alone or together with RL cells at N:T ratio 10:1 in the presence or absence of 10 nM VCR. After 48 h of incubation, cells were collected and labeled with anti-human CD15. Additionally, they were labeled with anti-human CD11a, CD11b, CD18, CD32, CD66b and CD64. Mean fluorescence intensity is depicted for CD15 positive population. Data are expressed as mean ± SD of three independent experiments. One-way ANOVA statistical test was used for multiple comparisons applying Holm-Sidak method.. **p≤ 0.01, ***p≤ 0.001 (N vs. N:RL), #p≤ 0.05, ##p≤ 0.01, ###p≤ 0.001 (N vs. N:RL + 10 nM VCR).

References

1. Choe J, Li L, Zhang X, Gregory CD, Choi YS. Distinct role of follicular dendritic cells and T cells in the proliferation, differentiation, and apoptosis of a centroblast cell line, L3055. *J. Immunol. Baltim. Md 1950.* 2000;164(1):56–63.
2. Petrasch S, Brittinger G, Wacker HH, Schmitz J, Kosco-Vilbois M. Follicular dendritic cells in non-Hodgkin's lymphomas. *Leuk. Lymphoma.* 1994;15(1-2):33–43.
3. Panayiotidis P, Jones D, Ganeshaguru K, Foroni L, Hoffbrand AV. Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *Br. J. Haematol.* 1996;92(1):97–103.
4. Burger JA, Tsukada N, Burger M, et al. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood.* 2000;96(8):2655–2663.
5. Disis ML. Immune regulation of cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 2010;28(29):4531–4538.
6. Piccard H, Muschel RJ, Opdenakker G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. *Crit. Rev. Oncol. Hematol.* 2012;82(3):296–309.
7. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* 2011;11(8):519–531.
8. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc. Natl. Acad. Sci. U. S. A.* 2008;105(7):2640–2645.
9. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell.* 2009;16(3):183–194.
10. Gregory AD, Houghton AM. Tumor-associated neutrophils: new targets for cancer therapy. *Cancer Res.* 2011;71(7):2411–2416.
11. Peranzoni E, Zilio S, Marigo I, et al. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr. Opin. Immunol.* 2010;22(2):238–244.
12. Mantovani A. Macrophages, Neutrophils, and Cancer: A Double Edged Sword. *New J. Sci.* 2014;2014:e271940.
13. Bekes EM, Schweighofer B, Kupriyanova TA, et al. Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinately the levels of tumor angiogenesis and efficiency of malignant cell intravasation. *Am. J. Pathol.* 2011;179(3):1455–1470.
14. Houghton AM, Rzymkiewicz DM, Ji H, et al. Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. *Nat. Med.* 2010;16(2):219–223.
15. Welch DR, Schissel DJ, Howrey RP, Aeed PA. Tumor-elicited polymorphonuclear cells, in contrast to "normal" circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. *Proc. Natl. Acad. Sci. U. S. A.* 1989;86(15):5859–5863.
16. Teramukai S, Kitano T, Kishida Y, et al. Pretreatment neutrophil count as an independent prognostic factor in advanced non-small-cell lung cancer: an analysis of Japan Multinational Trial Organisation LC00-03. *Eur. J. Cancer Oxf. Engl.* 1990. 2009;45(11):1950–1958.
17. Williams MA, Solomkin JS. Integrin-mediated signaling in human neutrophil functioning. *J. Leukoc. Biol.* 1999;65(6):725–736.

18. Ding ZM, Babensee JE, Simon SI, et al. Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J. Immunol. Baltim. Md 1950.* 1999;163(9):5029–5038.
19. Klink M, Jastrzemska K, Nowak M, et al. Ovarian cancer cells modulate human blood neutrophils response to activation in vitro. *Scand. J. Immunol.* 2008;68(3):328–336.
20. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. *Cell.* 2010;140(6):883–899.
21. Mitchem JB, Brennan DJ, Knolhoff BL, et al. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. *Cancer Res.* 2013;73(3):1128–1141.
22. Stockmann C, Doedens A, Weidemann A, et al. Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. *Nature.* 2008;456(7223):814–818.
23. DeNardo DG, Brennan DJ, Rexhepaj E, et al. Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer Discov.* 2011;1(1):54–67.
24. Challagundla KB, Wise PM, Neviani P, et al. Exosome-Mediated Transfer of microRNAs Within the Tumor Microenvironment and Neuroblastoma Resistance to Chemotherapy. *J. Natl. Cancer Inst.* 2015;107(7.):
25. Marlin SD, Springer TA. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell.* 1987;51(5):813–819.
26. Luscinskas FW, Brock AF, Arnaout MA, Gimbrone MA. Endothelial-leukocyte adhesion molecule-1-dependent and leukocyte (CD11/CD18)-dependent mechanisms contribute to polymorphonuclear leukocyte adhesion to cytokine-activated human vascular endothelium. *J. Immunol. Baltim. Md 1950.* 1989;142(7):2257–2263.
27. Kuzu MA, K oksoy C, Kuzu I, et al. Role of integrins and intracellular adhesion molecule-1 in lung injury after intestinal ischemia-reperfusion. *Am. J. Surg.* 2002;183(1):70–74.
28. Holland J, Owens T. Signaling through intercellular adhesion molecule 1 (ICAM-1) in a B cell lymphoma line. The activation of Lyn tyrosine kinase and the mitogen-activated protein kinase pathway. *J. Biol. Chem.* 1997;272(14):9108–9112.
29. Gardiner EE, D’Souza SE. Sequences within fibrinogen and intercellular adhesion molecule-1 (ICAM-1) modulate signals required for mitogenesis. *J. Biol. Chem.* 1999;274(17):11930–11936.
30. Smith SM, Lyu YL, Cai L. NF- B affects proliferation and invasiveness of breast cancer cells by regulating CD44 expression. *PLoS One.* 2014;9(9):e106966.
31. Park YS, Huh JW, Lee JH, Kim HR. shRNA against CD44 inhibits cell proliferation, invasion and migration, and promotes apoptosis of colon carcinoma cells. *Oncol. Rep.* 2012;27(2):339–346.
32. Zhang L-S, Ma H-W, Greyner HJ, Zuo W, Mummert ME. Inhibition of cell proliferation by CD44: Akt is inactivated and EGR-1 is down-regulated. *Cell Prolif.* 2010;43(4):385–395.
33. Gocheva V, Wang H-W, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev.* 2010;24(3):241–255.
34. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell.* 2013;23(3):277–286.
35. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity.* 2014;41(1):49–61.
36. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis.* 2012;33(5):949–955.

37. Gardner MJ, Jones LM, Catterall JB, Turner GA. Expression of cell adhesion molecules on ovarian tumour cell lines and mesothelial cells, in relation to ovarian cancer metastasis. *Cancer Lett.* 1995;91(2):229–234.
38. Puthier D, Bataille R, Amiot M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK / STAT rather than ras / MAP kinase pathway. *Eur. J. Immunol.* 1999;29(12):3945–3950.
39. Ericson SG, Zhao Y, Gao H, et al. Interleukin-6 production by human neutrophils after Fc-receptor cross-linking or exposure to granulocyte colony-stimulating factor. *Blood.* 1998;91(6):2099–2107.

Perspectives

Our results have shown that circulating neutrophils exert a protective effect on lymphoma B cells against different anti-cancer agents. Further investigations would be necessary to better understand the impact of neutrophils on modulating the response of other cancer types and to other therapies. In addition, to determine which therapeutic agents (conventional cytotoxic agents, hormone therapy, cancer vaccines or targeted therapy) are sensitive to the effect of neutrophils would be interesting to better understand the roles of the tumor microenvironment in induction of cancer resistance.

Regarding the mechanisms of interaction, we have been able to show that neutrophil-induced protection was dependent on cell-cell contact mediated by CD11b/ICAM-1 expressed by neutrophils and B cells, respectively, as well as by the adhesion molecule CD44. Based on previously published data^{144,163,165,391}, we hypothesize that the interaction between ICAM-1 and CD11b in our system activates intracellular pathways in malignant B cells allowing their enhanced proliferation in the presence of both neutrophils and anticancer agents. In the next step, we will try to determine these intracellular pathways by which neutrophils activate the protective signaling pathways in RL cells such as Raf/MAPK pathway that play a critical role in the regulation of cell proliferation and it was shown by Holland et al.⁴⁰⁵ to be the downstream pathway of ICAM-1 receptor.

In addition, we have been able to show that the protective effect was correlated with induction of Mcl-1 anti-apoptotic protein that was partially abrogated by an anti-Mcl-1 compound. So we will focus on examining the upstream pathway leading to Mcl-1 upregulation. Depending on previous published data^{406,407}, ongoing experiments are taking place to examine whether in our co-culture system the activated neutrophils release IL-6 which will be quantified by ELISA. Then we will detect whether IL-6-induced Mcl-1 upregulation requires signaling through Jak/Stat pathway. Briefly, we will evaluate the role of IL-6 in Stat-3 activation, Mcl-1 upregulation and neutrophil-mediated protection. Later, we will evaluate the requirement for Jak2 activity in IL-6-induced Stat-3-phosphorylation and Mcl-1 upregulation using Jak2 kinase inhibitor. In keeping with this hypothesis we have detected a deregulation of Jak/Stat pathway by transcriptomic analysis of RL cells co-cultured with neutrophils in the presence of chemotherapy compared to the absence of neutrophils.

Moreover, we will try to validate the implication of CD11b/ICAM-1 interaction and Mcl-1 anti-apoptotic protein in the protective effect of neutrophils against cancer therapy *in vivo* and *ex vivo* after confirming the neutrophil-mediating protective role in SCID CB17 mice and using blood withdrawn from patients diagnosed with chronic lymphocytic leukemia, respectively. Another interesting point is to determine whether tumor-infiltrating neutrophils display a protective effect on tumor cells, similarly to what we have observed with circulating neutrophils. This is technically challenging since these cells are difficult to obtain in significant amounts and are quite fragile after purification. Microsystems, including single cell systems, may be required to show the protective effect of tumor infiltrating neutrophils on tumor cells.

A clinical study is taking place in Pathology Department in Lyon SudHospital, collaborating with physicians working on Hodgkin's disease, a lymphoma disorder with frequent neutrophils infiltrate. Our aim is to determine whether there is a correlation between the number of neutrophils in patient's samples and their response to therapy. We are also aiming to confirm these preclinical data in the setting of patients with CLL.

Article II

Neutrophil isolation and analysis to determine their role in lymphoma cell sensitivity to therapeutic agents

Hirz *et al.* In-Press, Journal of Visualized Experiments (JoVE)

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Publishing in JoVE allows authors to dynamically present their methods, data analyses and results clearly, accurately, and professionally where every article is accompanied by a video. By showing rather than just telling peers about methods, the scientists can make the work easier to reproduce.

The journal covers many areas of what they designate the life sciences, each area understood as distinct from the others including the following categories: General; Neuroscience; Immunology and Infection; Clinical and Translational Medicine; Bioengineering and Basic Protocols.

Introduction and objectives

Neutrophils are associated with and make functional contributions to multiple steps that define cancer progression such as tumor angiogenesis and in the modulation of the antitumor immunity^{144,163,165,391}. In contrast, cytotoxicity, tumor cell apoptosis, immunologically mediated tumor rejection and antitumor immune memory have been shown to be key mechanisms for neutrophils^{211–215}.

Neutrophils can act as protumor or antitumor effector cells through their release of soluble factors or their interaction with tumor cells. To characterize the specific functions of neutrophils, a fast and credible method is needed for *in vitro* isolation of neutrophils from human blood. Density gradient separation method is an ideal technique in which a high yield of pure cells is obtained. The main advantage of the density gradient separation method over the other existing ones is the ability to isolate pure populations of mononuclear cells and neutrophils in a short period of time and without exposing these cell populations to stimulation by either labeling agents or magnetic fields that could potentially alter their function.

3-dimensional cell culture models represent an intermediate stage between *in vitro* 2D models and *in vivo* models. Creating a 3D culture is clearly more relevant compared to 2D where cells form natural cell-cell attachments making this model more mature and relevant to human and animal physiology⁴⁰⁸. For this reason, 3D co-culture models, especially between cancer cells and other cell types, have been very useful for indicating their contribution to tumor growth, angiogenesis, and metastasis.

Title:

Neutrophil isolation and analysis to determine their role in lymphoma cell sensitivity to therapeutic agents.

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Keywords

Neutrophils, HL60*diff*, Lymphoma, 3D culture, Anti-lymphoma agents, Flow cytometry

Short abstract

Neutrophils are the most abundant type of white blood cells. The isolation of neutrophils from human blood by density gradient separation method and the differentiation of human promyelocytic (HL60) cells along the granulocytic pathway are described here; to test their role on sensitivity of lymphoma cells to anti-lymphoma agents.

Long abstract

Neutrophils are the most abundant (40% to 75%) type of white blood cells and among the first inflammatory cells to migrate towards the site of inflammation. They are key players in the innate immune system and play major roles in cancer biology. Neutrophils have been proposed as key mediators of malignant transformation, tumor progression, angiogenesis and in the modulation of the antitumor immunity; through their release of soluble factors or their interaction with tumor cells. To characterize the specific functions of neutrophils, a fast and reliable method is coveted for *in vitro* isolation of neutrophils from human blood. Here, a density gradient separation method is demonstrated to isolate neutrophils as well as mononuclear cells from the blood. The procedure consists of layering the Ficoll solution carefully above the diluted blood obtained from patients diagnosed with chronic lymphocytic leukemia (CLL), followed by centrifugation, isolation of mononuclear layer, separation of neutrophils from RBCs by dextran then lysis of residual erythrocytes. This method has been shown to isolate neutrophils ≥ 90 % pure. To mimic the tumor microenvironment, 3-dimensional (3D) experiments were performed using Matrigel basement membrane matrix. Given the short half-life of neutrophils *in vitro*, 3D experiments with fresh human neutrophils cannot be performed. For this reason promyelocytic HL60 cells are differentiated along the granulocytic pathway using the differentiation inducers dimethyl sulfoxide (DMSO) and retinoic acid (RA). The aim of our experiments is to study the role of neutrophils on the sensitivity of lymphoma cells to anti-lymphoma agents. However these methods can be generalized to study the interactions of neutrophils or neutrophil-like cells with a large range of cell types in different situations.

Introduction

Innate immune cells constitute an essential proportion of the cells within the tumor microenvironment and have been associated with tumor malignancy in patients and animal models of cancer¹. Recently, it has become more widely appreciated that chronic immune responses play critical roles in promoting tumor progression, metastasis and resistance to chemotherapies². Macrophages are important innate immune cells that have been shown to directly regulate tumor cell response to chemotherapy^{3,4}. However, the role of neutrophils, key players in the innate immune system, in regulating tumor response to anti-cancer treatment is not known. The aims of these protocols are to use a fast and credible method to separate neutrophils from CLL patient's blood samples and to differentiate HL60 cells along the granulocytic pathway in order to study their role in regulating the sensitivity of lymphoma cells to anti-lymphoma agents.

Neutrophils are the most abundant cellular component of the innate immune system in blood⁵ and act as a first line of defense against invading microorganisms⁶. Neutrophils have an essential role in rising effective innate immune responses in addition to variable effector functions in several pathological conditions⁷. Therefore, a fast and credible method to isolate neutrophils from other blood cells, such as density gradient separation method, is required for *in vitro* studies. Using this method for neutrophil isolation will facilitate further research on neutrophil-mediated immunological functions *in vivo* and *ex vivo*.

The ability to obtain pure populations of neutrophils is an important first step for the investigation of patients with immunological diseases⁸. Density gradient separation method is an ideal technique in which a high yield of cells is obtained. The method involves the addition of Ficoll solution in the bottom of a tube containing diluted human blood followed by centrifugation at 300g for 35 minutes without break. The ring of the mononuclear cells appears at the interface and the neutrophils reside below the former. This method have significant advantages with respect to other available methods such as neutrophil isolation kits which are much more expensive⁹. In addition, isolating neutrophils from human blood by commercial kits using antibodies directed to a surface marker specific for human neutrophils, increase the risk of cell activation or differentiation. Density gradient separation method allows the isolation of neutrophils within a short period of time. Within the same step, mononuclear cells are also separated and recovered. It's a fundamental technique in which a high yield of pure cells is obtained in order to achieve functional integrity.

In order to mimic the tumor microenvironment, 3D experiments were performed. Given the short half-life of neutrophils *in vitro*, the 3D experiments with fresh human neutrophils are not conclusive. For this reason, promyelocytic (HL60) cells are induced to differentiate into neutrophil-like cells using the differentiation inducers dimethyl sulfoxide (DMSO) and retinoic acid (RA). Using differentiated HL60 cells (HL60*diff*) will prevent having different responses of neutrophils due to isolation from different donors.

In vitro 3D culture models represent an intermediate stage between *in vitro* 2D models and *in vivo* models. In 2D culture, the cells spread on plastic surface forming unnatural cell attachments to deposited proteins that are denatured on this synthetic surface. Conversely, the cells in 3D culture form natural cell-cell attachments since the cells and the extracellular matrix they synthesize are the natural material to which they are attached. For this reason, 3D coculture models, especially between cancer cells and other cell types, have been very useful for indicating their contribution to tumor growth, angiogenesis, and metastasis. As a result, 3D cultures make the cell culture mimic the physiological conditions that exist *in vivo*¹⁰.

Protocol

1. Neutrophil isolation and coculture with primary leukemic cells

NOTE: Procedures were conducted under the approval of Lyon Hospital Ethics committee with all patients signing informed consent.

1.1. Isolation of primary leukemic cells and neutrophils

1.1.1. Collect tubes of peripheral blood on EDTA (1.8mg EDTA per milliliter of blood) from patients diagnosed with chronic lymphocytic leukemia (CLL).

1.1.2. Add each 15 ml of blood to a sterile 50 ml tube and dilute with 15 ml RPMI (dilution 1:1), then carefully and slowly add 15 ml of Ficoll solution to the bottom of the tube without mixing the phases. Ensure that the Ficoll is at room temperature for the preparation.

1.1.3. Centrifuge at 300 g for 35 minutes at room temperature and without brake. The blood should separate into four distinct phases as shown in **Figure 1A**, top to bottom: platelet and plasma, mononuclear cells (white ring), Ficoll, granulocytes and erythrocytes.

1.1.4. Collect the white ring which represents the primary leukemic cells with a plastic Pasteur pipette and transfer to a new 50 ml tube.

1.1.4.1. Fill the tube with PBS (contains calcium and magnesium) up to 50 ml in total and centrifuge at 300 g for 10 minutes at room temperature.

1.1.4.2. Resuspend the pellet with 5 ml PBS then add PBS up to 50 ml in total and centrifuge at 300 g for 10 minutes at room temperature.

1.1.4.3. Resuspend the pellet with complete RPMI medium (RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin) for counting using cell viability counter.

1.1.5. Aspirate the upper phases leaving the granulocytes and erythrocytes phase and add PBS up to 25 ml then add 3% dextran in 0.9% NaCl up to 50 ml in total.

1.1.6. Mix the tubes 10 times and keep them for 30 minutes at room temperature without mixing.

1.1.7. Collect the upper RBC-poor neutrophil layer and place them into a clean sterile 50 ml tube and centrifuge the tubes at 300 g for 10 minutes at room temperature.

1.1.8. Resuspend each pellet with 5 ml PBS then add red cell lysis buffer up to 50 ml in total.

1.1.9. Keep the tubes in dark for 15 minutes at room temperature then centrifuge at 500 g for 10 minutes at room temperature.

1.1.10. Wash the pellets with PBS (add PBS up to 50 ml in total) then centrifuge at 500 g for 10 minutes at room temperature.

1.1.11. Resuspend the pellets with complete RPMI medium for counting using cell viability counter.

1.1.12. Keep 3×10^5 of each cell type in 50 μ l PBS-FBS (4%) in 5 ml plastic tubes to determine the purity of their isolation using flow cytometry.

1.2. Analyze the morphological appearances of the isolated cell populations

1.2.1.1. To do so, resuspend each 3×10^4 neutrophils and 3×10^4 mononuclear cells in 150 μ l complete RPMI medium.

1.2.1.2. Assemble the glass slides, filter cards, and sample chambers in the cytospin centrifuge, ensuring that the centrifuge is well balanced.

1.2.1.3. Place each cell type into sample chamber and cytocentrifuge at 750 rpm for 10 minutes at room temperature.

1.2.1.4. Remove the slides, filter cards, and sample chambers from the centrifuge. Disassemble carefully, so as not to damage the cells on the slide. Discard filter cards and sample chambers.

- 1.2.1.5. Stain the cells using Giemsa staining kit and keep the slides for 1 hour to dry.
- 1.2.1.6. Examine the cells by microscope with 100x magnification.

1.3. Test the purity of isolated cells by FACS

- 1.3.1. Label the isolated primary leukemic cells with anti-human CD19 antibody conjugated to APC (5 μ l/10⁶ cells).
- 1.3.2. Label the purified neutrophils with mixture of anti-human antibodies listed in Table 1 (5 μ l/10⁶ cells).
- 1.3.3. Incubate the cells in dark for 30 minutes at 4 °C.
- 1.3.4. Wash the cells with 500 μ l PBS-FBS (4%) then centrifuge the tubes at 300 g for 5 minutes at room temperature.
- 1.3.5. Resuspend each pellet in 200 μ l PBS-FBS (4%).
- 1.3.6. Analyze on a flow cytometer using the following optical configuration: 488 nm laser: FSC-A (488 nM), SSC-A (488/10BP), FITC (530/30 BP), PerCP-Cy5.5 (695/40 BP), PE (575/26 BP); 633 nm laser: APC (660/20 BP), APC-Cy7 (780/60 BP). Ensure the color compensation.

1.4. Coculture of primary leukemic cells with autologous neutrophils

- 1.4.1. Seed 2x10⁵ cells/ml of primary leukemic cells alone or with autologous neutrophils at ratio 1:10 in complete RPMI medium. To do so, adjust cell numbers by diluting cell suspensions and add 2 x 10⁵ cells/ml of primary leukemic cells to 2 x 10⁶ cells/ml neutrophils. Mix carefully.
- 1.4.2. Add 25 μ M of Bruton's tyrosine kinase (Btk) inhibitor (ibrutinib) to the cells.
- 1.4.3. Incubate for 24 hours at 37 °C with 5% CO₂.

1.5. Cell Harvest and FACS analysis

- 1.5.1. Collect the cells in 5 ml plastic tubes for FACS analysis and centrifuge the tubes at 300 g for 5 minutes at room temperature.
- 1.5.2. Wash the pellets with 1 ml PBS-FBS (4%) then centrifuge at 300 g for 5 minutes at room temperature.
- 1.5.3. Resuspend pellets in Annexin V and PI using commercial kit and incubate the cells in dark for 10 minutes at room temperature.
- 1.5.4. Analyze on a flow cytometer using the gating strategy of **Figure 2A** and the following optical configuration: 488 nm laser: FSC-A (488 nM), SSC-A (488/10BP), FITC (530/30 BP), PI (610/20 BP). Ensure the color compensation.

2. Differentiation of HL60 cells along the granulocytic pathway and their coculture with RL lymphoma B cells in 3D model

2.1. Differentiation of human promyelocytic (HL60) cells into neutrophil-like cells

- 2.1.1. Adjust HL60 cell number to 3x10⁵ cells/ml then add 1 μ M retinoic acid (RA) and 1.25% DMSO to induce their differentiation.
- 2.1.2. Seed each 3x10⁵ HL60 cells per well in 48-well plate then incubate for 48 hours at 37 °C with 5% CO₂.
- 2.1.3. Later, collect the cells and centrifuge at 300 g for 5 minutes at room temperature.
- 2.1.4. Resuspend the cells with complete RPMI medium for counting using cell viability counter.
- 2.1.5. Then dilute cell suspension in complete RPMI medium to adjust HL60 cell number to

3×10^5 cells/ml and induce again their differentiation by adding 1 μ M retinoic acid (RA) and 1.25 % DMSO.

2.1.6. Seed each 3×10^5 HL60 cells per well in 48-well plate and incubate for another 48 hours at 37 °C with 5% CO₂.

2.2. Analysis of HL60 differentiation (HL60diff)

2.2.1. Collect the cells and centrifuge the tubes at 300 g for 5 minutes at room temperature.

2.2.2. Resuspend the pellet with complete RPMI medium for counting using cell viability counter.

2.2.3. To analyze the changes in cell-surface markers expression by flow cytometry:

2.2.3.1. Place each 3×10^5 undifferentiated HL60 cells (from cell culture) and 3×10^5 HL60diff cells in 5 ml plastic tubes for FACS analysis and centrifuge the tubes at 300 g for 5 minutes at room temperature.

2.2.3.2. Resuspend the pellets in 50 μ l PBS-FBS (4%).

2.2.3.3. Label the cells with anti-human CD11b conjugated to AF700 or anti-human CD38 conjugated to APC (5 μ l/ 10^6 cells).

2.2.3.4. Incubate the cells in dark for 30 minutes at 4 °C.

2.2.3.5. Wash with 500 μ l PBS-FBS (4%) then centrifuge at 300 g for 5 minutes at room temperature.

2.2.3.6. Resuspend the pellets in 200 μ l PBS-FBS (4%).

2.2.3.7. Analyze on flow cytometer using the gating strategy of **Figure 3A** and the following optical configuration: 488 nm laser: FSC-A (488 nM), SSC-A (488/10BP); 633 nm laser: APC (660/20 BP), Alexa Fluor 700 (730/45 BP).

2.2.4. To test the morphological changes in HL60diff, immobilize the cells onto glass slides for microscopic examination:

2.2.4.1. To do so, resuspend each 3×10^4 undifferentiated HL60 cells (from cell culture) and 3×10^4 HL60diff in 150 μ l complete RPMI medium.

2.2.4.2. Assemble the glass slides, filter cards, and sample chambers in the cytospin centrifuge, ensuring that the centrifuge is balanced.

2.2.4.3. Place each cell type into sample chamber and cyto-centrifuge at 1500 rpm for 10 minutes at room temperature

2.2.4.4. Remove the slides, filter cards, and sample chambers from the centrifuge. Disassemble carefully, so as not to damage the cells on the slide. Discard filter cards and sample chambers.

2.2.4.5. Stain the cells using Giemsa staining kit and keep the slides for 1 hour to dry.

2.2.4.6. Examine the cells by microscope with 100x magnification.

2.3. 3-dimensional (3D) culture

NOTE: Ensure that the materials used in this experiment are cold and the experiment is taking place on ice

2.3.1. Resuspend each 5×10^4 RL cells, either alone or mixed with HL60diff at ratio RL:HL60diff 1:10, with 300 μ l matrigel using 1 ml pipette tip cut with a sterile scissor in order to widen the opening to about 2 to 3 mm. Avoid bubbles during this step.

2.3.2. Seed each 300 μ l of cell suspension/well in 24-well plate. Avoid bubbles during this step.

2.3.3. Incubate the plate for 30 minutes at 37 °C with 5% CO₂ then add 1 ml complete RPMI medium for each well and incubate for 7 days at 37 °C with 5% CO₂.

2.3.4. Change the medium every two days.

2.3.5. Add 10 nM vincristine at day 5.

2.4. FACS analysis

2.4.1. After 7 days of culture, aspirate the medium and wash each well with 1ml ice-cold PBS twice.

2.4.2. Add 3 ml/well of ice-cold PBS-EDTA (5mM).

2.4.3. Detach the gel from the bottom of the well by scraping using the bottom of 200 μ l pipette tip.

2.4.4. Shake the plate gently on ice for 30 minutes.

2.4.5. Transfer cell suspension into sterile 15 ml tube and shake the tubes gently on ice for another 30 minutes. Check for the appearance of homogeneous cell suspension. (If it's not the case, then shake the cells for longer time or add more PBS-EDTA).

2.4.6. Centrifuge the tubes at 300 g for 10 minutes at room temperature.

2.4.7. Wash the pellets with PBS then centrifuge at 300 g for 10 minutes at room temperature.

2.4.8. Resuspend with PBS-FBS (4%) and label with anti-human CD19 antibody conjugated to PE-Cy7 and anti-human CD38 antibody conjugated to APC (5 μ l/ 10^6 cells).

2.4.9. Incubate in dark for 30 minutes at 4 °C.

2.4.10. Wash with 500 μ l PBS-FBS (4%) then centrifuge tubes at 300 g for 5 minutes at room temperature.

2.4.11. Resuspend the cells with Annexin V and PI using commercial kit.

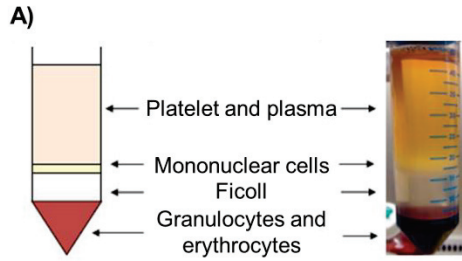
2.4.12. Analyze on flow cytometer using the gating strategy of **Figure 4A** and the following optical configuration: 488 nm laser: FSC-A (488 nM), SSC-A (488/10BP), FITC (530/30 BP), PI (610/20 BP), PE-Cy7 (780/60 BP); 633 nm laser: APC (660/20 BP). Ensure the color compensation.

Results

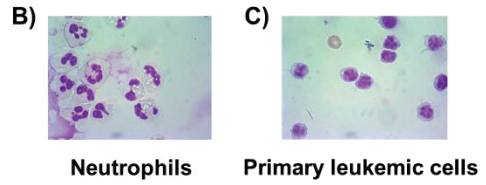
Density gradient separation method described here provides primary leukemic cells and unstimulated neutrophils isolated from the blood of CLL patients. Figure 1A represents the different blood layers obtained after density gradient centrifugation (from top to bottom: platelets and plasma, white ring represents the mononuclear cells, Ficoll, granulocytes and erythrocytes). Figure 1B and 1C show the differences in the morphological appearances between neutrophils (multi-lobed nuclei cells) and mononuclear cells respectively.

Results in Figure 1D represent the forward-scatter (FSC) vs side-scatter (SSC) plot of primary leukemic cells (mononuclear cells). Labeling this population with anti-human CD19 conjugated to APC shows a complete right shift of the histogram (Figure 1E) with only one peak which indicates that this population is positive for CD19 and pure.

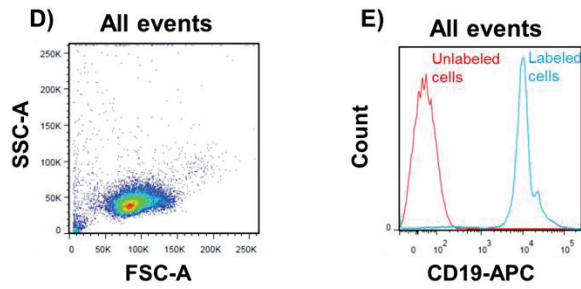
Neutrophils are also $\geq 90\%$ pure as verified by flow cytometry after labeling the isolated neutrophils with a mixture of fluorochrome-conjugated monoclonal antibodies listed in Table 1. Figure 1F represents FSC vs SSC scatter plot of the isolated neutrophils which are positive for CD45 (Figure 1G), positive for CD15 and negative for CD14 (Figure 1H), positive for both CD15 and CD16 (Figure 1I), positive for CD16 and negative for CD56 (Figure 1J).



Isolated cell morphologies



Primary leukemic cells purity



Neutrophils purity

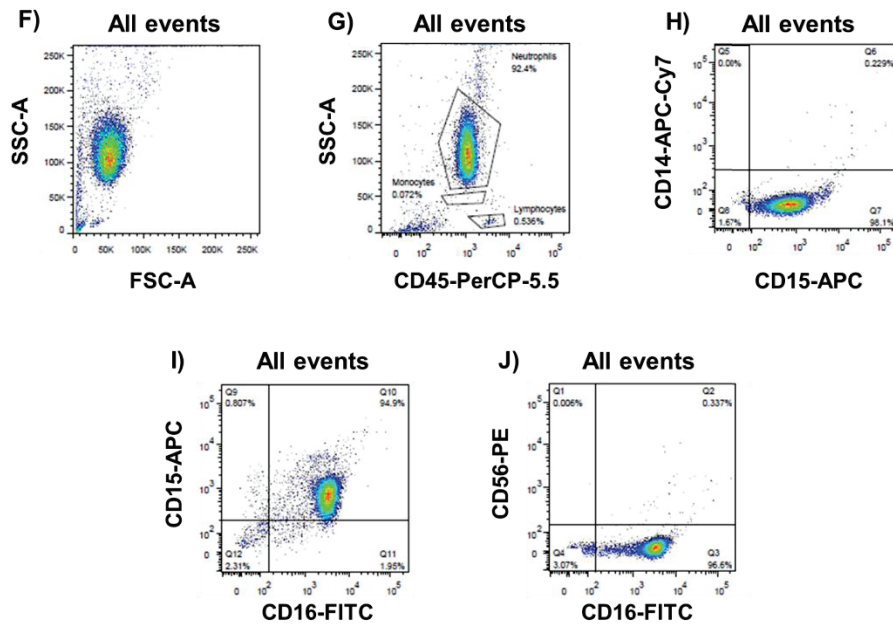


Figure 1: Analysis of morphological appearances and purity of primary leukemic cells and neutrophils isolated from patients' blood. **A)** Schematic view represents different blood layers after density gradient centrifuging. **B-C)** Giemsa staining represent the morphological differences between **B)** neutrophils and **C)** primary leukemic cells (mononuclear cells). Photos were taken by microscope with 100x magnification. **D)** Forward-scatter (FSC) and side-scatter (SSC) plot represents the isolated primary leukemic cell population. **E)** Isolated primary leukemic cells were labeled with anti-CD19-APC and analyzed for the expression of CD19. The red line indicates the unlabeled cells and the blue line indicates cells labeled with anti-CD19-APC. **F)** FSC vs SSC scatter plot represents the isolated neutrophil population. **G-J)** Isolated neutrophils were labeled with a mixture of antibodies listed in Table 1 and analyzed for the expression of **G)** CD45, **H)** CD14 and CD15, **I)** CD15 and CD16, **J)** CD16 and CD56.

Table 1: Fluorochrome-conjugated purified monoclonal antibodies used to test the purity of isolated neutrophils. All antibodies were obtained from Miltenyi biotech.

Antigen	Fluorochrome	Clone
CD14	APC-Vio770 (Cy-7)	TÜK4
CD15	APC	VIMC6
CD16	FITC	VEP13
CD45	PerCP-Vio700 (Cy5.5)	5B1
CD56	PE	AF12-7H3

To investigate the effect of neutrophils on sensitivity of primary leukemic cells (CLL) to ibrutinib, the latter were cultured alone or with autologous neutrophils (N) for 24 hours at ratio N:CLL 10:1, in the presence or absence of ibrutinib at 25 μ M. The percentage of alive primary leukemic cells (Annexin V negative/PI negative) was measured by double staining with annexin V–FITC and PI, followed by flow cytometric analysis. FSC vs SSC scatterplot in Figure 2A represents the gates of neutrophils and primary leukemic cells. Neutrophils are much more granular than the primary leukemic cells so they appear with higher SSC. Gating on the primary leukemic cells cocultured with neutrophils in the presence of ibrutinib, results show higher percentage of alive cells (Figure 2C, 84.8%) compared to the cells cultured alone (Figure 2B, 53.1%). The box graph in Figure 2D shows that the decrease of cell viability induced by ibrutinib was significantly inhibited by the presence of neutrophils (40.1 ± 3.1 vs 60.1 ± 3.5 , $p < 0.001$, 19 patients).

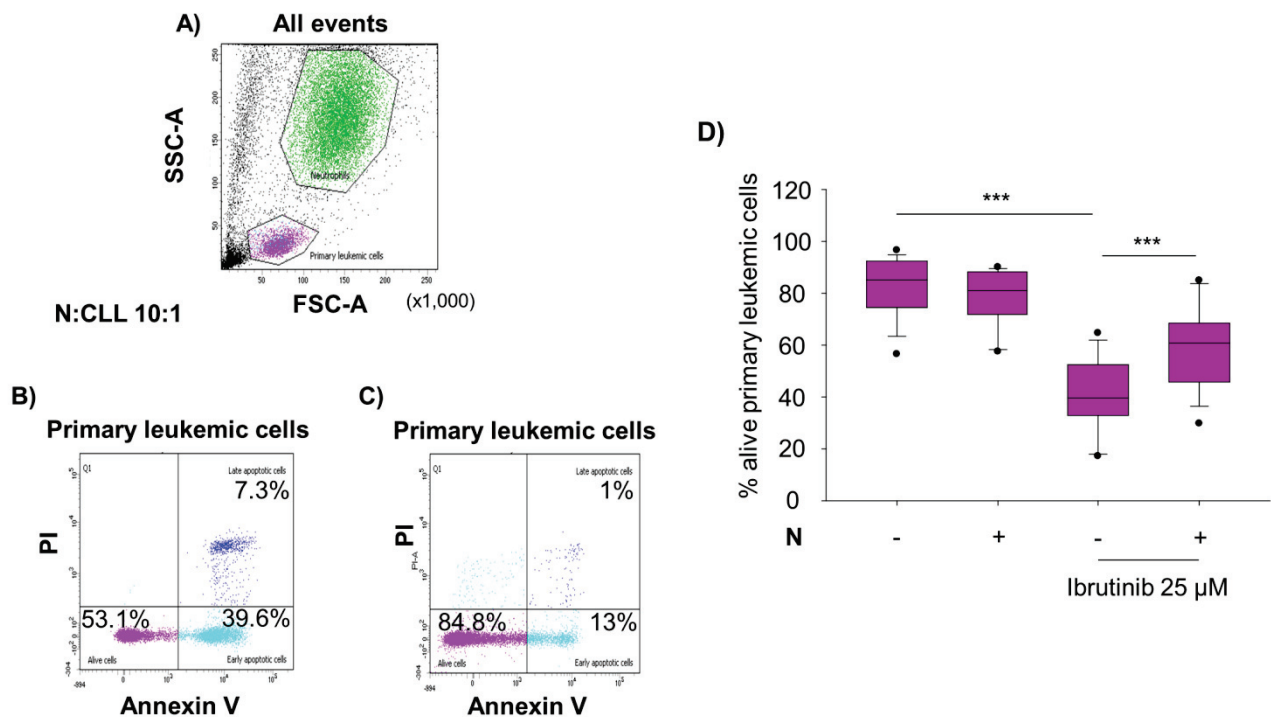


Figure 2: Autologous neutrophils protect primary leukemic cells against ibrutinib. Blood was collected from patients diagnosed with chronic lymphocytic leukemia (CLL). Primary leukemic cells were isolated and cultured alone or together with autologous neutrophils (N) at primary leukemic cells:Nratio 1:10 for 24 h, in the presence or absence of 25 μ M ibrutinib. The percentage of alive primary leukemic cells (Annexin V negative/PI negative) was measured by double staining with annexin V–FITC and PI, followed by flow cytometric analysis. **A)** Forward-scatter (FSC) and side-scatter (SSC) plot represents the gates of neutrophils and primary leukemic cells. **B)** Bidimensional dot-plot shows the percentages of alive and apoptotic primary leukemic cells cultured alone and treated with 25 μ M ibrutinib. **C)** Bidimensional dot-plot shows the percentages of alive and apoptotic primary leukemic cells cocultured with neutrophils and treated with 25 μ M ibrutinib. **D)** Box plot represents the percentage of alive primary leukemic cells of 19 patients. Data are expressed as mean \pm SD. *** $p < 0.001$

The short half-life of neutrophils *in vitro* makes their use in 3D culture ineffectual. Differentiation of HL60 cells along the granulocytic pathway was induced by differentiation inducers. Two different parameters (cell-surface markers expression and morphological changes) were used to measure the differentiation of HL60. FSC vs SSC scatter plot in Figure 3A show that HL60 cells are larger in size compared to HL60*diff* cells with higher FSC. Labeling the cells (HL60 or HL60*diff*) with anti-human CD11b antibody conjugated to AF700 and anti-human CD38 antibody conjugated to APC shows an increase in CD11b and CD38 expression (Figure 3B) upon differentiation which is indicative of granulocytic differentiation. HL60*diff* cells also show morphological changes detected by the appearance of multi-lobed nuclei (Figure 3C).

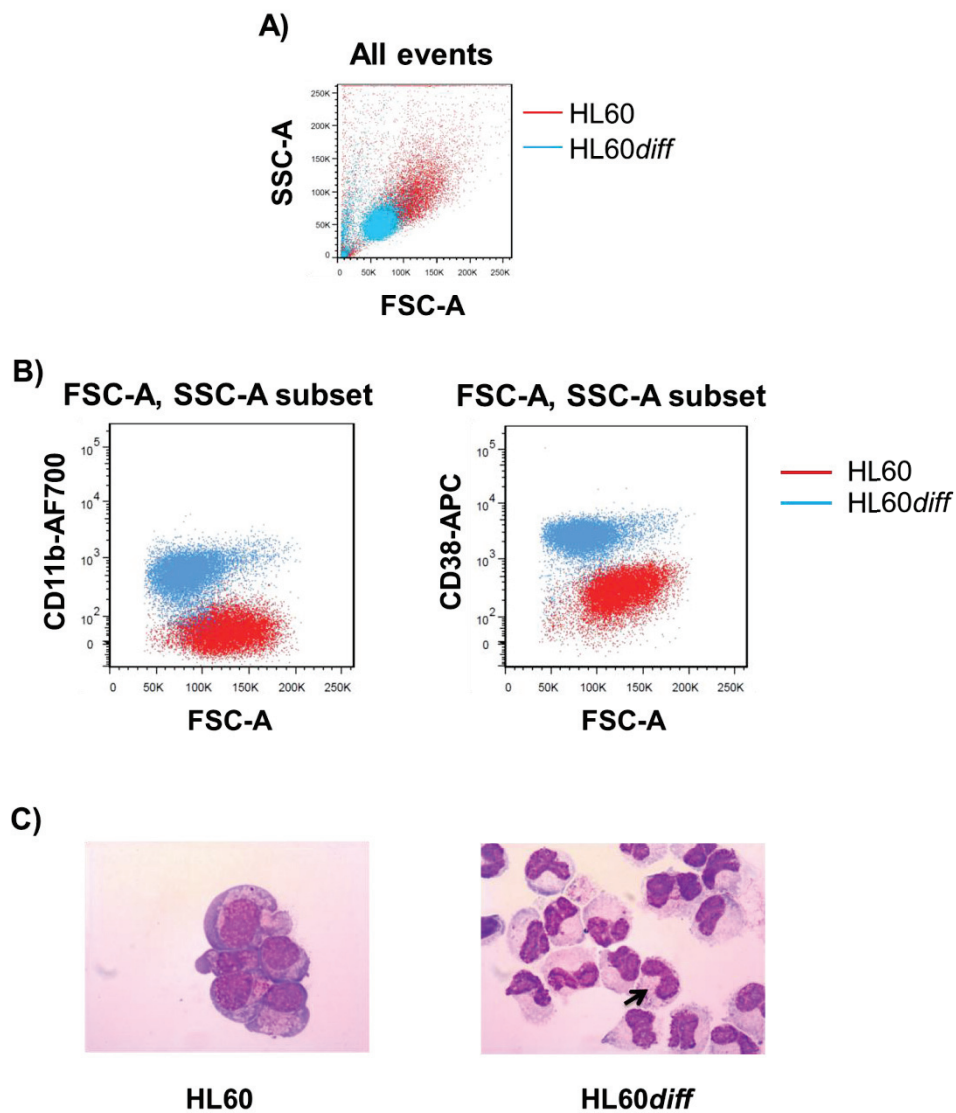


Figure 3: Structural parameters of differentiated HL60 cells. **A)** Forward-scatter (FSC) and side-scatter (SSC) plots represent HL60 and differentiated HL60 cells (HL60diff). **B)** HL60 and HL60diff cells were labeled with anti-CD11b-AF700 or anti-CD38-APC followed by flow cytometry analysis. After gating on each cell population in the FSC-A vs. SSC-A scatter plot (A), the cells were analyzed for the expressions of CD11b or CD38. **C)** HL60 cells show morphological changes consistent with differentiation toward granulocytes. Photos were taken by microscope with 100x magnification. Bold black arrow point the multi-lobed nucleus. Graphs are representative of five independent experiments.

To test the effect of HL60*diff* cells on regulating RL cell response to vincristine in 3D model, RL cells were cultured alone or with HL60*diff* in matrigel in the presence or absence of vincristine at 10 nM. Using the gating strategy shown in Figure 4A, both populations are discriminated based on CD19 and CD38 expression; RL cells positive for CD19 and HL60*diff* cells positive for CD38. Gating on RL cells cocultured with HL60*diff* cells in the presence of vincristine, results show higher percentage of alive cells (Figure 4C, 35.9%) compared to RL cells cultured alone (Figure 4B, 19.7%). The bar graph in Figure 4D shows a significant increase in the percentage of alive RL cells (CD19 positive Annexin V negative/PI negative) in the presence of HL60*diff* cells (19.5 ± 0.2 vs 33.1 ± 1.0 , $p < 0.001$).

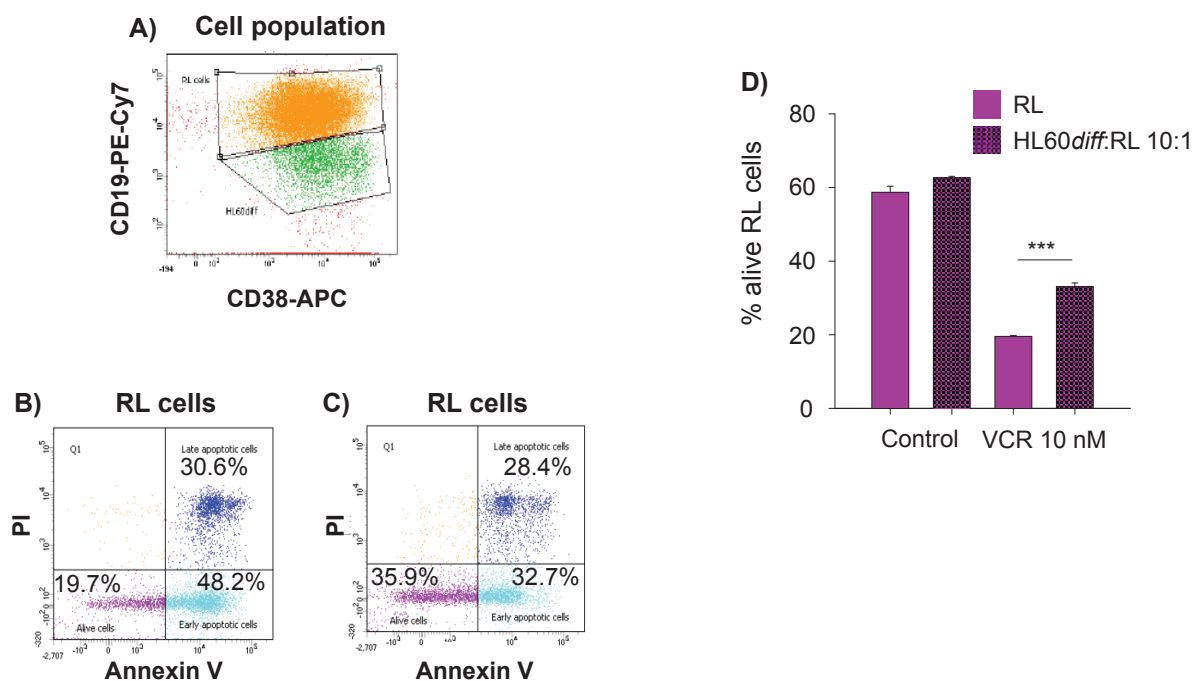


Figure 4: Neutrophil-like HL60*diff* cells protect RL lymphoma cells against vincristine in 3D culture. RL cells were cultured alone or together with HL60*diff* cells at RL:HL60*diff* ratio 1:10 for 7 days in matrigel. On day 5, vincristine (VCR) was added at a concentration of 10 nM. Spheroids were dissociated on day 7 and cells were labeled with anti-CD19-PECy7 and anti-CD38-APC then resuspended with annexin V-FITC and PI followed by flow cytometric analysis. **A)** Forward-scatter (FSC) and side-scatter (SSC) plot represents the gates of RL cells and HL60*diff* cells. **B)** Bidimensional dot-plot shows the percentages of alive and apoptotic RL cells cultured alone and treated with 10 nM vincristine. **C)** Bidimensional dot-plot shows the percentages of alive and apoptotic RL cells cocultured with HL60*diff* and treated with 10 nM vincristine. **D)** The bar graph represents the percentage of alive RL cells. Data are expressed as mean \pm SD. *** $p \leq 0.001$.

Discussion

Described here, an effective, simple, fast and unexpensive protocol for the isolation of neutrophils from human blood with high purity using density gradient centrifugation approach and within the same step mononuclear cells are also separated and recovered. The isolated cell populations are $\geq 90\%$ pure.

Several methods are available for neutrophil isolation from human blood. These include similar methods using discontinuous Percoll gradients^{11,12}, or using commercial kits for neutrophil isolation by positive immuno-magnetic selection during which neutrophils are immuno-magnetic labeled with specific antibody such as anti-human CD16 then neutrophils enriched by binding of the CD16-positive cells on a magnetic column¹³. Commercial kits with negative immuno-magnetic selection are also available during which the whole blood or granulocyte suspension are labeled with a cocktail of antibodies that bind on cells other than neutrophils⁹ (*i.e.* antibody complexes that label RBCs, platelets and unwanted cells such as CD2, CD3, CD9, CD19, CD36, CD56, glycophorin A and dextran-coated magnetic particles), then neutrophils are enriched by elution as the antibody-negative fraction of cells that does not bind on the magnetic column. In addition, neutrophils can be isolated by Fluorescence Activated Cell Sorting¹⁴.

Some techniques have been shown to provide high yields of pure neutrophils, such as positive immuno-magnetic selection. However, this method has disadvantages compared to density gradient centrifugation methods due to the labeling agents that bind on the surface of neutrophils and this could potentially alter their function by inducing their activation or differentiation. Also using the positive immuno-magnetic selection method, the antibody-labeled neutrophils bind on a magnetic column for separation and this may also affect their function. Fluorescence Activated Cell Sorting has another limitation insofar as this method requires longer time to collect the cells which may negatively affect neutrophil survival due to their short half-life.

Density gradient centrifugation method using Ficoll or Percoll medium are very comparable and the neutrophil purity can exceed 90 % using both procedures, but the former is a two-layer gradient which is technically less challenging compared to layering Percoll which consist of 40%, 60% and 80% vol/vol in PBS. It was mentioned by Swamydas et al.¹⁵ that intermixing of the Percoll interfaces often takes place due to the small density differences between the three layers. With respect to the negative immuno-magnetic selection approaches, they have also been reported to be effective for neutrophil isolation with high purity and viability⁹. Their advantage over positive immuno-magnetic selection and Fluorescence Activated Cell Sorting is that neutrophils are not labeled with any labeling agents and do not bind to magnetic column, thus avoiding cell activation but this method is much more expensive and more time consuming compared to density gradient method.

Neutrophils normally undergo rapid spontaneous apoptosis both *in vitro* and *in vivo*^{16,17}. HL60 promyelocytic cells retain many characteristics of human leukocyte progenitors, such as the potential to undergo differentiation into neutrophils¹⁸. Unlike neutrophils, HL60*diff* cells do not rapidly undergo apoptosis and using these cells solves the trouble of having different responses of neutrophils due to their isolation from different donors.

Recently, 3D cultures become more mature and pertinent to human and animal physiology, and an attractive model for different biological research especially in the field of cancer. The pattern shift from 2D to 3D culture is progressing rapidly since the latter is more conspicuous during studying various pathological conditions such as cancer¹⁹. There is an increasing

awareness of the drawbacks of 2D culture where adding a third dimension to a cell's environment is important in order to take a closer look at the importance of cellular interactions²⁰ and to study the differences in cellular behavior and characteristics. 3D culture creates an environment similar to conditions in a living organism (e.g. human being) leading to more relevant research. However, 3D culture involves the complex interplay between different partners such as cells, extracellular matrices and interstitial fluids which do not present in 2D culture. Thus, efforts will be required in order to establish method calibration and to count on good laboratory practices as well as effective supplies especially the commercial brands that are extensively tested and monitored.

The tumor microenvironment consists of multiple cell types, including many immune cell populations that participate in and regulate tumorigenesis, metastasis and response to anticancer agents^{21,22}. Several studies have shown that an increase of neutrophil infiltration in tumors is significantly correlated with acquired resistance to several anti-cancer agents such as anti-VEGF therapy^{23,24}. Furthermore, elevation in the pretreatment neutrophil count in patients with metastatic renal cell carcinoma²⁵, as well as the presence of a high level of intratumoral neutrophils in patients with different solid tumors,²⁶ have been proposed as prognostic factors for poor survival. Our study suggests that neutrophils may play a role in protecting lymphoma B cells from anticancer agent-induced apoptosis.

In summary, the reliable and reproducible method described here can be employed by any laboratory to collect neutrophils and mononuclear cells from human blood. In addition, the differentiation of HL60 cells along the granulocytic pathway is presented in order to avoid some neutrophil problems such as the rapid spontaneous apoptosis. These approaches were used to study the role of neutrophils on sensitivity of lymphoma cells to anti-lymphoma agents where neutrophils show a protective effect on lymphoma cells against these agents using 2D and 3D culture systems. These approaches can be used for a variety of neutrophil functional studies that should deepen our understanding of neutrophil role in cancer biology. In particular, this method can be used to better understand the cross-talk between neutrophils and tumor cells or between neutrophils and other components of the microenvironment.

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Disclosures

The authors have nothing to disclose.

References

1. Gocheva, V., Wang, H.-W., *et al.* IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes & Development* **24** (3), 241–255, doi:10.1101/gad.1874010 (2010).
2. Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, Inflammation, and Cancer. *Cell* **140** (6), 883–899, doi:10.1016/j.cell.2010.01.025 (2010).
3. Mitchem, J. B., Brennan, D. J., *et al.* Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. *Cancer Research* **73** (3), 1128–1141, doi:10.1158/0008-5472.CAN-12-2731 (2013).
4. DeNardo, D. G., Brennan, D. J., *et al.* Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer Discovery* **1** (1), 54–67, doi:10.1158/2159-8274.CD-10-0028 (2011).
5. Di Carlo, E., Forni, G., Lollini, P., Colombo, M. P., Modesti, A. & Musiani, P. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. *Blood* **97** (2), 339–345 (2001).
6. Kumar, V. & Sharma, A. Neutrophils: Cinderella of innate immune system. *International Immunopharmacology* **10** (11), 1325–1334, doi:10.1016/j.intimp.2010.08.012 (2010).
7. Mantovani, A., Cassatella, M. A., Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nature Reviews. Immunology* **11** (8), 519–531, doi:10.1038/nri3024 (2011).
8. Kelbaek, H. Sterile isolation of polymorphonuclear leukocytes from large blood volumes. *Journal of Clinical Chemistry and Clinical Biochemistry. Zeitschrift Für Klinische Chemie Und Klinische Biochemie* **23** (1), 17–20 (1985).
9. Aynaud, M.-M., Suspène, R., *et al.* Human Tribbles 3 protects nuclear DNA from cytidine deamination by APOBEC3A. *The Journal of Biological Chemistry* **287** (46), 39182–39192, doi:10.1074/jbc.M112.372722 (2012).
10. Ziółkowska, K., Stelmachowska, A., Kwapiszewski, R., Chudy, M., Dybko, A. & Brzózka, Z. Long-term three-dimensional cell culture and anticancer drug activity evaluation in a microfluidic chip. *Biosensors & Bioelectronics* **40** (1), 68–74, doi:10.1016/j.bios.2012.06.017 (2013).
11. Eggleton, P., Gargan, R. & Fisher, D. Rapid method for the isolation of neutrophils in high yield without the use of dextran or density gradient polymers. *Journal of Immunological Methods* **121** (1), 105–113 (1989).
12. Behnen, M., Leschczyk, C., *et al.* Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcγRIIIB and Mac-1. *Journal of Immunology (Baltimore, Md.: 1950)* **193** (4), 1954–1965, doi:10.4049/jimmunol.1400478 (2014).
13. Hirsch, G., Lavoie-Lamoureux, A., Beauchamp, G. & Lavoie, J.-P. Neutrophils are not less sensitive than other blood leukocytes to the genomic effects of glucocorticoids. *PLoS One* **7** (9), e44606, doi:10.1371/journal.pone.0044606 (2012).
14. Dorward, D. A., Lucas, C. D., *et al.* Technical advance: autofluorescence-based sorting: rapid and nonperturbing isolation of ultrapure neutrophils to determine cytokine production. *Journal of Leukocyte Biology* **94** (1), 193–202, doi:10.1189/jlb.0113040 (2013).
15. Muthulekha Swamydas, Michail S. Lionakis Isolation, Purification and Labeling of Mouse Bone Marrow Neutrophils for Functional Studies and Adoptive Transfer Experiments. **77**, e50586 (2013).

16. Scaife, H., Woldehiwet, Z., Hart, C. A. & Edwards, S. W. Anaplasma phagocytophilum reduces neutrophil apoptosis in vivo. *Infection and Immunity* **71** (4), 1995–2001 (2003).
17. Maianski, N. A., Maianski, A. N., Kuijpers, T. W. & Roos, D. Apoptosis of neutrophils. *Acta Haematologica* **111** (1-2), 56–66, doi:10.1159/000074486 (2004).
18. Dalton, W. T., Ahearn, M. J., McCredie, K. B., Freireich, E. J., Stass, S. A. & Trujillo, J. M. HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood* **71** (1), 242–247 (1988).
19. Hogan, C., Kajita, M., Lawrenson, K. & Fujita, Y. Interactions between normal and transformed epithelial cells: Their contributions to tumourigenesis. *The International Journal of Biochemistry & Cell Biology* **43** (4), 496–503, doi:10.1016/j.biocel.2010.12.019 (2011).
20. Page, H., Flood, P. & Reynaud, E. G. Three-dimensional tissue cultures: current trends and beyond. *Cell and Tissue Research* **352** (1), 123–131, doi:10.1007/s00441-012-1441-5 (2013).
21. Mantovani, A. Macrophages, Neutrophils, and Cancer: A Double Edged Sword. *New Journal of Science* **2014**, e271940, doi:10.1155/2014/271940 (2014).
22. Hanahan D, Coussens LM Accessories to the crime: functions of cells recruited to the tumor microenvironment. **3**, 309–22 (2012).
23. Shojaei F, Ferrara N Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. **68**, 5501–5504 (2008).
24. Neutrophils promote the malignant glioma phenotype through S100A4. **20**, 187–198 (2014).
25. Teramukai, S., Kitano, T., *et al.* Pretreatment neutrophil count as an independent prognostic factor in advanced non-small-cell lung cancer: an analysis of Japan Multinational Trial Organisation LC00-03. *European Journal of Cancer (Oxford, England: 1990)* **45** (11), 1950–1958, doi:10.1016/j.ejca.2009.01.023 (2009).
26. Zhu, Q., Zhang, X., *et al.* The IL-6-STAT3 axis mediates a reciprocal crosstalk between cancer-derived mesenchymal stem cells and neutrophils to synergistically prompt gastric cancer progression. *Cell Death & Disease* **5**, e1295, doi:10.1038/cddis.2014.263 (2014).

Perspectives

The overall goal of these methods is to isolate neutrophils from human blood with high purity in a short period of time using density gradient centrifugation in order to investigate their role in lymphoma cell sensitivity to cancer therapy using 2-dimensional and 3-dimensional cell culture models.

These techniques paved the way for researchers in the field of cancer to explore the impact of various white blood cells on tumor malignancy in preclinical models and patient samples. They can help answer key questions involving the interaction between the innate immune system and cancer, such as neutrophil-mediated tumor cell response to cancer therapy.

GENERAL DISCUSSION

Role of neutrophils in regulating tumor response to cancer therapy

In 1989, Stephen Paget proposed the “seed and soil” theory, in which cancer cells tend to metastasize in a location with similar characteristics as their region of origin⁴⁰⁹. This theory underlines the crucial roles of the tumor microenvironment during tumorigenesis at not only metastatic sites but also primary sites. Neutrophils make up a significant portion of the inflammatory cell infiltrate in many models of cancer and they have been seen *in vivo* in close association with tumor cells⁸¹. Raising data have pointed out the roles of neutrophils in promoting tumor growth and invasion^{174,175,181–183}. Few studies have indicated the implication of neutrophils in mediating tumor cell response to cancer therapy^{207,344,384,385}. However, the role of neutrophils in regulating lymphoma response to anticancer treatment is not known.

In the scope of this study, we demonstrated that neutrophils reduce the sensitivity of lymphoma cells to cancer therapy. This is in keeping with results obtained by Phan’s group who showed that neutrophils mediate resistance to anti-VEGF targeted therapy in animal models of cancer, including a genetically engineered mouse model of pancreatic adenocarcinoma³⁸². Another study by Liang et al. showed that an increase in neutrophil infiltration into tumors is significantly correlated with glioma grade and in glioblastoma with acquired resistance to anti-VEGF therapy³⁸³. Therefore, it is tempting to speculate that neutrophils induce cancer resistance to various types of therapy.

Role of adhesion molecules in neutrophil-mediated protection

Tumor cells have previously been shown to influence the phenotype and properties of cells present in their microenvironment, in particular through the polarization of tumor infiltrating neutrophils^{81,145}. In our study, we found that the expression levels of the activation markers of neutrophils such as CD11b, CD18, and CD66b were significantly increased when neutrophils were co-cultured with lymphoma cells (either alone or in the presence of chemotherapy). In a study by Perobelli and his coworkers, they reported that the markers CD11b and CD66b can be upregulated in suppressor neutrophils⁴¹⁰.

ICAM-1 is an adhesion molecule of the immunoglobulin superfamily which has been shown to bind to the ligands expressed on the surface of neutrophils such as LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and CD11c/CD18⁴¹¹. In our study, blocking this receptor reduced the neutrophil-induced protection on NHL cell proliferation in the presence of chemotherapy. This result is in keeping with several studies which showed that ICAM-1 is involved in the regulatory process of cell proliferation^{405,412}. A study done by Holland et al. showed that cross-linking of ICAM-1 on the B lymphoma cell line A20 activate Raf-MEK-ERK pathway⁴⁰⁵ that has a known role in inducing cell proliferation⁴¹².

We also found that CD44 gene expression was rapidly induced in NHL cells exposed to neutrophils and chemotherapy. CD44 is another adhesion molecule involved in the process of cell proliferation^{413,414}. Blocking this receptor also reverse the protective effect of neutrophils on NHL cell proliferation which is in keeping with a work for Smith et al. who reported that CD44 repression decreased the proliferation and invasiveness of breast cancer cells⁴¹⁴. In addition, Park et al.⁴¹³ cited that cell proliferation, migration, and invasion were markedly inhibited in CD44 shRNA transfected colon cancer cells.

Role of Bcl-2 family members in neutrophil-mediated protection

Bcl-2 protein family, through its role in regulation of apoptotic pathways, is possibly related to cancer pathophysiology and resistance to conventional chemotherapy. In our study, chemotherapy increased the content of Bcl-2 anti-apoptotic protein in NHL cells whereas neutrophils alone did not induce any significant modification. In contrast, neutrophils together with chemotherapy strongly increased the expression of Mcl-1 in tumor cells. Using a specific inhibitor of Mcl-1, we found that the protective effect of neutrophils against chemotherapy was Mcl-1-dependent as it could be strongly reduced in the presence of MIM-1.

Puthier et al.⁴⁰⁶ have described an upstream pathway leading to Mcl-1 upregulation in human myeloma cells by IL-6, a cytokine which is known to be produced and released by activated neutrophils⁴⁰⁷. We suggest a possible mechanism by which neutrophils activate protective signaling pathways in NHL cells. Since we obtained a significant increase in several activation markers expressed by the neutrophils after cell-cell contact with lymphoma cells, it is possible that these activated neutrophils may secrete the IL-6 cytokine as shown by Ericson and his coworkers⁴⁰⁷. IL-6 will induce NHL cells to upregulate Mcl-1 anti-apoptotic protein probably through Jak/Stat pathway as suggested by Puthier et al.⁴⁰⁶.

TAN vs. NN

Fridlender and his colleagues described distinct population of neutrophils that have differences in their transcriptomic profile. They found that TAN are a distinct population of neutrophils, differing markedly in their transcriptomic profile from both naive neutrophils (NN) and the granulocytic fraction of MDSCs⁹².

Most of our results were obtained with circulating neutrophils from healthy donors, and not with tumor-infiltrating neutrophils. In cancer patients, neutrophils migrate into solid tumors under the influence of a variety of cytokines (KC/CXCL1, MIP2 α /CXCL2, TNF- α and IFN- γ), a phenomenon which involves cell adhesion molecules expressed by neutrophils themselves (such as CD11b) and endothelial cells. Also, Jablonska et al.⁴¹⁵ reported a role for CXCR2 ligands in the homing of neutrophils into developing tumors, while Brackett *et al.*⁴¹⁶ reported a role for IL-17 on the migration of neutrophils to tumor draining lymph nodes. When peripheral blood neutrophils penetrate the tumor, immune-sculpting, which results from the cross-talk between immune cells and tumor cells, changes the phenotype of neutrophils into either antitumor (N1-TAN) or protumor phenotype (N2-TAN) depending on which cytokines are present in the tumor microenvironment¹⁴⁶. In our *in vivo* model, peripheral blood neutrophils were mixed together with NHL cells before injection into mice that could result in the modification of peripheral blood neutrophils phenotype into TAN phenotype. Since we obtained a protective effect for neutrophils *in vivo*, this suggests that TAN may also exert a protection on tumor cells against cancer therapies.

Validation of the protective role of neutrophils *ex vivo*

Using samples collected from CLL patients, we obtained a similar protective effect by autologous peripheral blood neutrophils on primary CLL cells, by reducing their sensitivity to chemotherapy and targeted therapy. Our results suggest that neutrophils may play a role in protecting primary cells from anticancer agent-induced apoptosis. Further studies would be necessary to detect the phenotypical differences and changes between neutrophils withdrawn from healthy donors and CLL patients before and after co-culture with lymphoma cells.

Different studies defined the molecular mechanisms that regulate apoptosis in CLL cells as complex mechanisms ⁴¹⁷, but the importance of the Bcl-2 family of apoptosis-regulating proteins in CLL has been established for more than a decade ⁴¹⁸. In our study, analysis of the expression of anti-apoptotic Bcl-2 family members in primary cells showed a variation in the results among patients. Further studies are required to better understand the correlation between the expression of Bcl-2 family members and the protective effect of neutrophils against anticancer agents.

.... and in 3D model

To evaluate the relevance of the protective effect of neutrophils in more complex models, we developed a microsphere co-culture system using differentiated promyelocytic cells. We also performed xenograft experiments with co-injection of NHL cells and human neutrophils from healthy donors in immune-compromised mice. Both systems allowed us to confirm the protective effect against chemotherapy-induced cytotoxicity in NHL model. These results suggest that neutrophils are able to protect tumor cells in a 3D-environment.

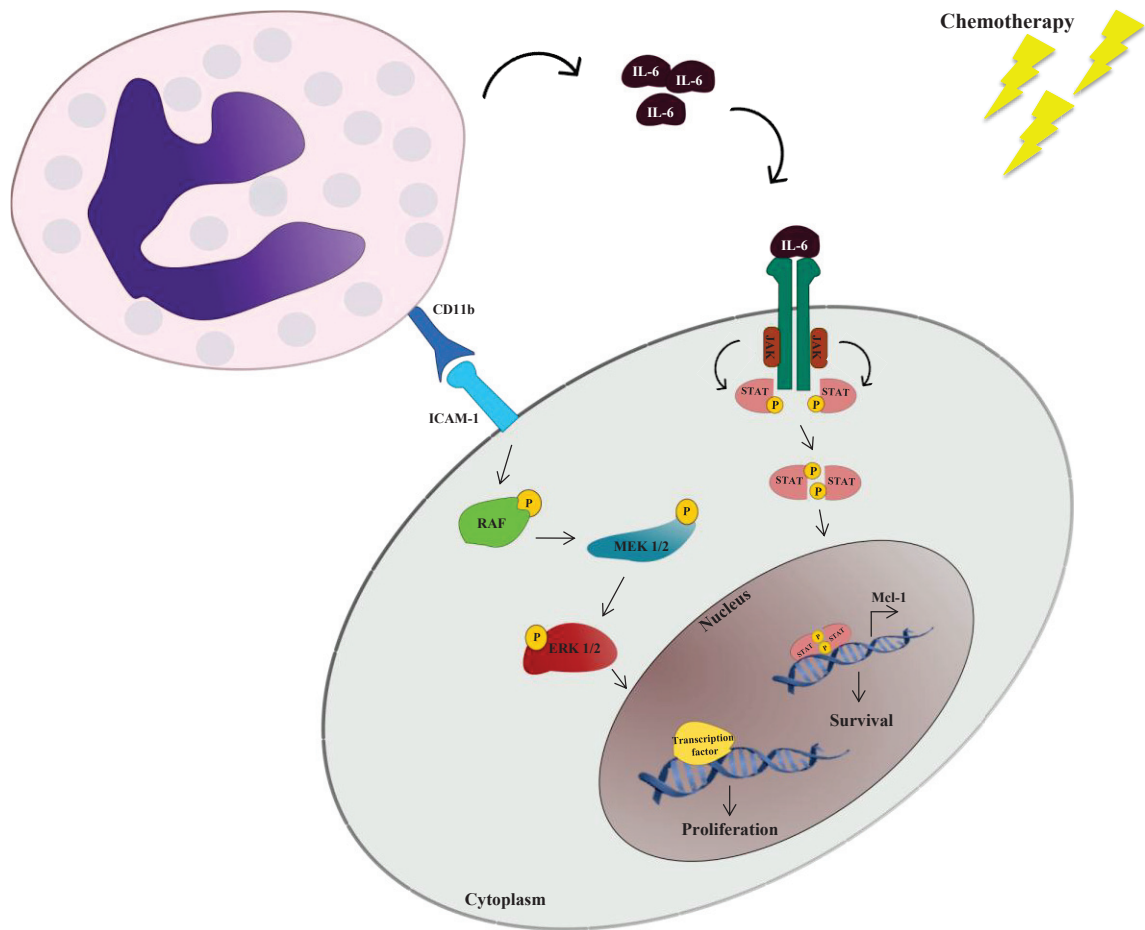


Figure 16. Schematic model representing possible mechanisms for neutrophil-induced protection on lymphoma cells against chemotherapy (Hirz et al. 2015, to be submitted). Neutrophils interact with lymphoma cells by CD11b/ICAM-1 binding leads to Raf-MEK-ERK pathway activation results in enhancing cell proliferation. As well, direct binding between neutrophils and lymphoma cells leads to IL-6 secretion by the activated neutrophils. IL-6 binding to their receptors leads to Jak/Stat pathway activation that induces Mcl-1 upregulation in lymphoma cells results in enhancing cell survival.

CONCLUSION AND PERSPECTIVES

In conclusion, our study has demonstrated that circulating neutrophils display a protective effect on malignant B cells against a variety of anti-lymphoma compounds. These findings reveal a novel protective mechanism of neoplastic B cells involving innate immune cells. Further investigations would be necessary to better understand the impact of neutrophils on modulating the response of other cancer types and to other therapies. In addition, further investigations on which therapeutic agents (conventional cytotoxic agents, hormone therapy, cancer vaccines or targeted therapy) are sensitive to the effect of neutrophils would be also necessary to better understand the roles of the tumor microenvironment in induction of cancer resistance.

Interestingly, we have been able to show that neutrophil-induced protection was dependent on cell-cell contact mediated by CD11b/ICAM-1 expressed by neutrophils and B cells, respectively, as well as by the adhesion molecule CD44. The protective effect was correlated with induction of Mcl-1 anti-apoptotic protein that was partially abrogated by an anti-Mcl-1 compound. In the next step, we will focus on determining the signalization pathway by which neutrophils activate protective signaling pathways in RL cells. According to previous published data, CD11b/ICAM-1 interaction may result in the activation of the ICAM-1 downstream Raf-MEK-ERK pathway in RL cells. These proteins have a known role in intracellular signaling cascades resulting in increased cellular proliferation. In addition, RL cells, by direct contact with neutrophils, induce the latter to undergo activation as determined by the increase in the expression of different activation markers such as CD66b. The activated neutrophils then may release IL-6 that induces Mcl-1 upregulation in RL cells, possibly through activation of the Jak/Stat pathway. Moreover, we were able to validate the protective effect of neutrophils *in vivo* and *ex vivo* working on the mechanism validation.

Another important aspect concerns the clinical relevance of our observations. While data gathered in CLL patients is coherent with our observations made on cell lines, it will be important to determine whether tumor-infiltrating neutrophils display a protective effect on tumor cells, similarly to what we have observed with circulating neutrophils. Other correlative studies are also warranted, in particular the study of the impact of neutrophil infiltration in human NHL samples on the quality of response to therapy.

Identifying the mechanism of interactions between neutrophils and malignant B cells, and how these interactions alter the response of lymphoma cells to cancer therapy may contribute to the development of therapeutic strategies to enhance the antitumor effect of conventional or targeted therapy. Specifically we hypothesize that targeted therapies inhibiting neutrophil-tumor cell contact or reducing Mcl-1 content in tumor cells might contribute to enhancing the antitumor activity of several chemotherapeutic agents.

REFERENCES

1. Shaffer, A. L., Rosenwald, A. & Staudt, L. M. Lymphoid malignancies: the dark side of B-cell differentiation. *Nat. Rev. Immunol.* **2**, 920–932 (2002).
2. Shankland, K. R., Armitage, J. O. & Hancock, B. W. Non-Hodgkin lymphoma. *Lancet Lond. Engl.* **380**, 848–857 (2012).
3. Armitage, J. O. Treatment of non-Hodgkin's lymphoma. *N. Engl. J. Med.* **328**, 1023–1030 (1993).
4. Hainsworth, J. D. *et al.* Rituximab as first-line and maintenance therapy for patients with indolent non-hodgkin's lymphoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **20**, 4261–4267 (2002).
5. Smith, S. D. *et al.* Diffuse large B-cell lymphoma in adults aged 75 years and older: a single institution analysis of cause-specific survival and prognostic factors. *Ther. Adv. Hematol.* **4**, 349–353 (2013).
6. Office for National Statistics. Cancer statistics registrations: registrations of cancer diagnosed in 2007, England. Series MB1 no. 38. Surrey, UK: Office of Public Sector Information, 2010.
7. Cancer Research UK. Non-Hodgkin lymphoma (NHL) statistics— key facts. <http://info.cancerresearchuk.org/cancerstats/keyfacts/non-hodgkin-lymphoma/> (accessed June 26, 2012).
8. Jemal, A., Siegel, R., Xu, J. & Ward, E. Cancer statistics, 2010. *CA. Cancer J. Clin.* **60**, 277–300 (2010).
9. National Cancer Institute. SEER stat fact sheets: non-Hodgkin lymphoma. <http://seer.cancer.gov/statfacts/html/nhl.html> (accessed June 26, 2012).
10. Cartwright, R. *et al.* The rise in incidence of lymphomas in Europe 1985-1992. *Eur. J. Cancer Oxf. Engl.* **1990** **35**, 627–633 (1999).
11. Devesa, S. S. & Fears, T. Non-Hodgkin's lymphoma time trends: United States and international data. *Cancer Res.* **52**, 5432s–5440s (1992).
12. Naresh, K. N., Srinivas, V. & Soman, C. S. Distribution of various subtypes of non-Hodgkin's lymphoma in India: a study of 2773 lymphomas using R.E.A.L. and WHO Classifications. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **11 Suppl 1**, 63–67 (2000).
13. Lenz, G. & Staudt, L. M. Aggressive lymphomas. *N. Engl. J. Med.* **362**, 1417–1429 (2010).
14. Jung, D., Giallourakis, C., Mostoslavsky, R. & Alt, F. W. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu. Rev. Immunol.* **24**, 541–570 (2006).
- 15.
16. Küppers, R. Mechanisms of B-cell lymphoma pathogenesis. *Nat. Rev. Cancer* **5**, 251–262 (2005).
17. Swerdlow SH, Campo E, Harris NL, *et al.* WHO classification of tumours of haematopoietic and lymphoid tissues, 4th edn. Lyon, France: IARC Press, 2008.
18. Carbone, P. P., Kaplan, H. S., Musshoff, K., Smithers, D. W. & Tubiana, M. Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res.* **31**, 1860–1861 (1971).
19. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. *N. Engl. J. Med.* **329**, 987–994 (1993).

20. Dixon, D. O. *et al.* Effect of age on therapeutic outcome in advanced diffuse histiocytic lymphoma: the Southwest Oncology Group experience. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **4**, 295–305 (1986).
21. Vose, J. M. *et al.* The importance of age in survival of patients treated with chemotherapy for aggressive non-Hodgkin's lymphoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **6**, 1838–1844 (1988).
22. Coiffier, B. What treatment for elderly patients with aggressive lymphoma? *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **5**, 873–875 (1994).
23. Stathis, A. & Ghielmini, M. New agents for the treatment of lymphoma. *Ann. Oncol.* **23**, x92–x98 (2012).
24. Cheson, B. D. & Leonard, J. P. Monoclonal antibody therapy for B-cell non-Hodgkin's lymphoma. *N. Engl. J. Med.* **359**, 613–626 (2008).
25. Gerber, D. E. Targeted therapies: a new generation of cancer treatments. *Am. Fam. Physician* **77**, 311–319 (2008).
26. Siddiqi, T. & Rosen, S. T. Novel biologic agents for non-Hodgkin lymphoma and chronic lymphocytic leukemia-part 2: adoptive cellular immunotherapy, small-molecule inhibitors, and immunomodulation. *Oncol. Williston Park N* **29**, 299–308 (2015).
27. Aalipour, A. & Advani, R. H. Bruton's tyrosine kinase inhibitors and their clinical potential in the treatment of B-cell malignancies: focus on ibrutinib. *Ther. Adv. Hematol.* **5**, 121–133 (2014).
28. de Rooij, M. F. M. *et al.* The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* **119**, 2590–2594 (2012).
29. Cheng, S. *et al.* BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. *Leukemia* **28**, 649–657 (2014).
30. Herman, S. E. M. *et al.* Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* **117**, 6287–6296 (2011).
31. Akinleye, A., Chen, Y., Mukhi, N., Song, Y. & Liu, D. Ibrutinib and novel BTK inhibitors in clinical development. *J. Hematol. Oncol. J Hematol Oncol* **6**, 59 (2013).
32. Burger, J. A. Bruton's tyrosine kinase (BTK) inhibitors in clinical trials. *Curr. Hematol. Malig. Rep.* **9**, 44–49 (2014).
33. Aalipour, A. & Advani, R. H. Bruton tyrosine kinase inhibitors: a promising novel targeted treatment for B cell lymphomas. *Br. J. Haematol.* **163**, 436–443 (2013).
34. Courtney, K. D., Corcoran, R. B. & Engelman, J. A. The PI3K pathway as drug target in human cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **28**, 1075–1083 (2010).
35. Altman, J. K. & Plataniias, L. C. Exploiting the mammalian target of rapamycin pathway in hematologic malignancies. *Curr. Opin. Hematol.* **15**, 88–94 (2008).
36. Llambi, F. *et al.* A unified model of mammalian BCL-2 protein family interactions at the mitochondria. *Mol. Cell* **44**, 517–531 (2011).
37. Wei, M. C. *et al.* Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727–730 (2001).
38. Bodur, C. & Basaga, H. Bcl-2 inhibitors: emerging drugs in cancer therapy. *Curr. Med. Chem.* **19**, 1804–1820 (2012).
39. Souers, A. J. *et al.* ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat. Med.* **19**, 202–208 (2013).
40. Shaffer, A. L., Young, R. M. & Staudt, L. M. Pathogenesis of human B cell lymphomas. *Annu. Rev. Immunol.* **30**, 565–610 (2012).
41. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–674 (2011).

42. Mantovani, A. Macrophages, Neutrophils, and Cancer: A Double Edged Sword. *New J. Sci.* **2014**, e271940 (2014).
43. Scott, D. W. & Gascoyne, R. D. The tumour microenvironment in B cell lymphomas. *Nat. Rev. Cancer* **14**, 517–534 (2014).
44. Lwin, T. *et al.* Lymphoma cell adhesion-induced expression of B cell-activating factor of the TNF family in bone marrow stromal cells protects non-Hodgkin's B lymphoma cells from apoptosis. *Leukemia* **23**, 170–177 (2009).
45. Amé-Thomas, P. *et al.* Human mesenchymal stem cells isolated from bone marrow and lymphoid organs support tumor B-cell growth: role of stromal cells in follicular lymphoma pathogenesis. *Blood* **109**, 693–702 (2007).
46. Medina, D. J. *et al.* Mesenchymal stromal cells protect mantle cell lymphoma cells from spontaneous and drug-induced apoptosis through secretion of B-cell activating factor and activation of the canonical and non-canonical nuclear factor κ B pathways. *Haematologica* **97**, 1255–1263 (2012).
47. Sacedón, R. *et al.* Sonic hedgehog is produced by follicular dendritic cells and protects germinal center B cells from apoptosis. *J. Immunol. Baltim. Md 1950* **174**, 1456–1461 (2005).
48. Dierks, C. *et al.* Essential role of stromally induced hedgehog signaling in B-cell malignancies. *Nat. Med.* **13**, 944–951 (2007).
49. Singh, R. R. *et al.* ABCG2 is a direct transcriptional target of hedgehog signaling and involved in stroma-induced drug tolerance in diffuse large B-cell lymphoma. *Oncogene* **30**, 4874–4886 (2011).
50. Damiano, J. S., Cress, A. E., Hazlehurst, L. A., Shtil, A. A. & Dalton, W. S. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* **93**, 1658–1667 (1999).
51. Taylor, S. T., Hickman, J. A. & Dive, C. Survival signals within the tumour microenvironment suppress drug-induced apoptosis: lessons learned from B lymphomas. *Endocr. Relat. Cancer* **6**, 21–23 (1999).
52. Dalton, W. S. Drug resistance and drug development in multiple myeloma. *Semin. Oncol.* **29**, 21–25 (2002).
53. Kay, N. E. *et al.* Bone biopsy derived marrow stromal elements rescue chronic lymphocytic leukemia B-cells from spontaneous and drug induced cell death and facilitates an 'angiogenic switch'. *Leuk. Res.* **31**, 899–906 (2007).
54. Lwin, T. *et al.* Bone marrow stromal cells prevent apoptosis of lymphoma cells by upregulation of anti-apoptotic proteins associated with activation of NF-kappaB (RelB/p52) in non-Hodgkin's lymphoma cells. *Leukemia* **21**, 1521–1531 (2007).
55. Mraz, M. *et al.* Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin α 4- β 1 (VLA-4) with natalizumab can overcome this resistance. *Br. J. Haematol.* **155**, 53–64 (2011).
56. Baldini, L. *et al.* Differential expression of very late activation antigen-3 (VLA-3)/VLA-4 in B-cell non-Hodgkin lymphoma and B-cell chronic lymphocytic leukemia. *Blood* **79**, 2688–2693 (1992).
57. Lúcio, P. J. *et al.* Expression of adhesion molecules in chronic B-cell lymphoproliferative disorders. *Haematologica* **83**, 104–111 (1998).
58. Jacob, M. C. *et al.* Quantification of cellular adhesion molecules on malignant B cells from non-Hodgkin's lymphoma. *Leukemia* **13**, 1428–1433 (1999).
59. Rossi, D. *et al.* CD49d expression is an independent risk factor of progressive disease in early stage chronic lymphocytic leukemia. *Haematologica* **93**, 1575–1579 (2008).

60. Koopman, G. *et al.* Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. *J. Immunol. Baltim. Md 1950* **152**, 3760–3767 (1994).
61. Weekes, C. D., Kuszynski, C. A. & Sharp, J. G. VLA-4 mediated adhesion to bone marrow stromal cells confers chemoresistance to adherent lymphoma cells. *Leuk. Lymphoma* **40**, 631–645 (2001).
62. Matsunaga, T. *et al.* Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nat. Med.* **9**, 1158–1165 (2003).
63. Zucchetto, A. *et al.* CD38/CD31, the CCL3 and CCL4 chemokines, and CD49d/vascular cell adhesion molecule-1 are interchained by sequential events sustaining chronic lymphocytic leukemia cell survival. *Cancer Res.* **69**, 4001–4009 (2009).
64. Lwin, T. *et al.* Follicular dendritic cell-dependent drug resistance of non-Hodgkin lymphoma involves cell adhesion-mediated Bim down-regulation through induction of microRNA-181a. *Blood* **116**, 5228–5236 (2010).
65. Cui, W. *et al.* Nodal follicular lymphoma without complete follicular dendritic cell networks is related to localized clinical stage. *Pathol. Int.* **61**, 737–741 (2011).
66. Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44–49 (2011).
67. Yang, Z.-Z. & Ansell, S. M. The tumor microenvironment in follicular lymphoma. *Clin. Adv. Hematol. Oncol. HO* **10**, 810–818 (2012).
68. Walzer, T., Jaeger, S., Chaix, J. & Vivier, E. Natural killer cells: from CD3(-)NKp46(+) to post-genomics meta-analyses. *Curr. Opin. Immunol.* **19**, 365–372 (2007).
69. Palmer, S. *et al.* Prognostic importance of T and NK-cells in a consecutive series of newly diagnosed patients with chronic lymphocytic leukaemia. *Br. J. Haematol.* **141**, 607–614 (2008).
70. Plonquet, A. *et al.* Peripheral blood natural killer cell count is associated with clinical outcome in patients with aaPI 2-3 diffuse large B-cell lymphoma. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **18**, 1209–1215 (2007).
71. Clynes, R. A., Towers, T. L., Presta, L. G. & Ravetch, J. V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med.* **6**, 443–446 (2000).
72. Hussell, T., Isaacson, P. G., Crabtree, J. E. & Spencer, J. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to *Helicobacter pylori*. *Lancet Lond. Engl.* **342**, 571–574 (1993).
73. Umetsu, D. T., Esserman, L., Donlon, T. A., DeKruyff, R. H. & Levy, R. Induction of proliferation of human follicular (B type) lymphoma cells by cognate interaction with CD4+ T cell clones. *J. Immunol. Baltim. Md 1950* **144**, 2550–2557 (1990).
74. Johnson, P. W. *et al.* Isolated follicular lymphoma cells are resistant to apoptosis and can be grown in vitro in the CD40/stromal cell system. *Blood* **82**, 1848–1857 (1993).
75. Epron, G. *et al.* Monocytes and T cells cooperate to favor normal and follicular lymphoma B-cell growth: role of IL-15 and CD40L signaling. *Leukemia* **26**, 139–148 (2012).
76. Dave, S. S. *et al.* Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N. Engl. J. Med.* **351**, 2159–2169 (2004).
77. Ansell, S. M., Stenson, M., Habermann, T. M., Jelinek, D. F. & Witzig, T. E. Cd4+ T-cell immune response to large B-cell non-Hodgkin's lymphoma predicts patient outcome. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **19**, 720–726 (2001).
78. Lee, A. M. *et al.* Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **24**, 5052–5059 (2006).

79. Wahlin, B. E. *et al.* T cells in tumors and blood predict outcome in follicular lymphoma treated with rituximab. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **17**, 4136–4144 (2011).
80. Laurent, C. *et al.* Distribution, function, and prognostic value of cytotoxic T lymphocytes in follicular lymphoma: a 3-D tissue-imaging study. *Blood* **118**, 5371–5379 (2011).
81. Fridlender, Z. G. & Albelda, S. M. Tumor-associated neutrophils: friend or foe? *Carcinogenesis* **33**, 949–955 (2012).
82. Montero, A. J., Diaz-Montero, C. M., Kyriakopoulos, C. E., Bronte, V. & Mandruzzato, S. Myeloid-derived suppressor cells in cancer patients: a clinical perspective. *J. Immunother. Hagerstown Md 1997* **35**, 107–115 (2012).
83. Pastuła, A. & Marcinkiewicz, J. Myeloid-derived suppressor cells: a double-edged sword? *Int. J. Exp. Pathol.* **92**, 73–78 (2011).
84. Gabrilovich, D. I. & Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* **9**, 162–174 (2009).
85. Goedegebuure, P. *et al.* Myeloid-derived suppressor cells: general characteristics and relevance to clinical management of pancreatic cancer. *Curr. Cancer Drug Targets* **11**, 734–751 (2011).
86. Lin, Y. *et al.* Immunosuppressive CD14+HLA-DR(low)/- monocytes in B-cell non-Hodgkin lymphoma. *Blood* **117**, 872–881 (2011).
87. Brimnes, M. K. *et al.* Increased level of both CD4+FOXP3+ regulatory T cells and CD14+HLA-DR^{low} myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. *Scand. J. Immunol.* **72**, 540–547 (2010).
88. Wilcox, R. A. *et al.* Monocytes promote tumor cell survival in T-cell lymphoproliferative disorders and are impaired in their ability to differentiate into mature dendritic cells. *Blood* **114**, 2936–2944 (2009).
89. Youn, J.-I., Nagaraj, S., Collazo, M. & Gabrilovich, D. I. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J. Immunol. Baltim. Md 1950* **181**, 5791–5802 (2008).
90. Movahedi, K. *et al.* Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood* **111**, 4233–4244 (2008).
91. Kusmartsev, S., Nagaraj, S. & Gabrilovich, D. I. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. *J. Immunol. Baltim. Md 1950* **175**, 4583–4592 (2005).
92. Fridlender, Z. G. *et al.* Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. *PLoS One* **7**, e31524 (2012).
93. van Ravenswaay Claasen, H. H., Kluin, P. M. & Fleuren, G. J. Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. *Lab. Investig. J. Tech. Methods Pathol.* **67**, 166–174 (1992).
94. Stephens, T. C., Currie, G. A. & Peacock, J. H. Repopulation of gamma-irradiated Lewis lung carcinoma by malignant cells and host macrophage progenitors. *Br. J. Cancer* **38**, 573–582 (1978).
95. Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S. & Ruco, L. The origin and function of tumor-associated macrophages. *Immunol. Today* **13**, 265–270 (1992).
96. Condeelis, J. & Pollard, J. W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263–266 (2006).

97. Mantovani, A., Schioppa, T., Porta, C., Allavena, P. & Sica, A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev.* **25**, 315–322 (2006).
98. Quatromoni, J. G. & Eruslanov, E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *Am. J. Transl. Res.* **4**, 376–389 (2012).
99. Young, H. A. & Hardy, K. J. Role of interferon-gamma in immune cell regulation. *J. Leukoc. Biol.* **58**, 373–381 (1995).
100. Bingle, L., Brown, N. J. & Lewis, C. E. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J. Pathol.* **196**, 254–265 (2002).
101. Urban, J. L., Shepard, H. M., Rothstein, J. L., Sugarman, B. J. & Schreiber, H. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5233–5237 (1986).
102. Hibbs, J. B., Taintor, R. R., Vavrin, Z. & Rachlin, E. M. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157**, 87–94 (1988).
103. Keller, R., Geiges, M. & Keist, R. L-arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res.* **50**, 1421–1425 (1990).
104. Clauss, M. *et al.* Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J. Exp. Med.* **172**, 1535–1545 (1990).
105. Klimp, A. H. *et al.* Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages. *Cancer Res.* **61**, 7305–7309 (2001).
106. Calandra, T., Bernhagen, J., Mitchell, R. A. & Bucala, R. The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J. Exp. Med.* **179**, 1895–1902 (1994).
107. Meyer-Siegler, K. & Hudson, P. B. Enhanced expression of macrophage migration inhibitory factor in prostatic adenocarcinoma metastases. *Urology* **48**, 448–452 (1996).
108. Shimizu, T. *et al.* High expression of macrophage migration inhibitory factor in human melanoma cells and its role in tumor cell growth and angiogenesis. *Biochem. Biophys. Res. Commun.* **264**, 751–758 (1999).
109. Onodera, S. *et al.* Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion. *Immunology* **92**, 131–137 (1997).
110. Pozzi, L. A. & Weiser, W. Y. Human recombinant migration inhibitory factor activates human macrophages to kill tumor cells. *Cell. Immunol.* **145**, 372–379 (1992).
111. Shinohara, H., Yano, S., Bucana, C. D. & Fidler, I. J. Induction of chemokine secretion and enhancement of contact-dependent macrophage cytotoxicity by engineered expression of granulocyte-macrophage colony-stimulating factor in human colon cancer cells. *J. Immunol. Baltim. Md 1950* **164**, 2728–2737 (2000).
112. Trinchieri, G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv. Immunol.* **70**, 83–243 (1998).
113. Sica, A. *et al.* Macrophage polarization in tumour progression. *Semin. Cancer Biol.* **18**, 349–355 (2008).
114. O'Reilly, M. S. *et al.* Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79**, 315–328 (1994).

115. Gorrin-Rivas, M. J. *et al.* Implications of human macrophage metalloelastase and vascular endothelial growth factor gene expression in angiogenesis of hepatocellular carcinoma. *Ann. Surg.* **231**, 67–73 (2000).
116. Disis, M. L. Immune regulation of cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **28**, 4531–4538 (2010).
117. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* **23**, 549–555 (2002).
118. Lewis, C. E. & Pollard, J. W. Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* **66**, 605–612 (2006).
119. Pollard, J. W. Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* **4**, 71–78 (2004).
120. Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860–867 (2002).
121. Etoh, T., Shibuta, K., Barnard, G. F., Kitano, S. & Mori, M. Angiogenin expression in human colorectal cancer: the role of focal macrophage infiltration. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **6**, 3545–3551 (2000).
122. Torisu, H. *et al.* Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. *Int. J. Cancer J. Int. Cancer* **85**, 182–188 (2000).
- 123.
124. Leek, R. D., Harris, A. L. & Lewis, C. E. Cytokine networks in solid human tumors: regulation of angiogenesis. *J. Leukoc. Biol.* **56**, 423–435 (1994).
125. Rambaldi, A., Young, D. C. & Griffin, J. D. Expression of the M-CSF (CSF-1) gene by human monocytes. *Blood* **69**, 1409–1413 (1987).
126. Scholl, S. M. *et al.* Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. *J. Natl. Cancer Inst.* **86**, 120–126 (1994).
127. Kacinski, B. M. CSF-1 and its receptor in ovarian, endometrial and breast cancer. *Ann. Med.* **27**, 79–85 (1995).
128. Xu, Y. *et al.* Endothelial and macrophage upregulation of urokinase receptor expression in human renal cell carcinoma. *Hum. Pathol.* **28**, 206–213 (1997).
129. Hildenbrand, R., Wolf, G., Böhme, B., Bleyl, U. & Steinborn, A. Urokinase plasminogen activator receptor (CD87) expression of tumor-associated macrophages in ductal carcinoma in situ, breast cancer, and resident macrophages of normal breast tissue. *J. Leukoc. Biol.* **66**, 40–49 (1999).
130. Hildenbrand, R., Dilger, I., Hörlin, A. & Stutte, H. J. Urokinase and macrophages in tumour angiogenesis. *Br. J. Cancer* **72**, 818–823 (1995).
131. Davies, B. *et al.* Activity of type IV collagenases in benign and malignant breast disease. *Br. J. Cancer* **67**, 1126–1131 (1993).
132. Davies, B. *et al.* Levels of matrix metalloproteases in bladder cancer correlate with tumor grade and invasion. *Cancer Res.* **53**, 5365–5369 (1993).
133. Naylor, M. S., Stamp, G. W., Davies, B. D. & Balkwill, F. R. Expression and activity of MMPs and their regulators in ovarian cancer. *Int. J. Cancer J. Int. Cancer* **58**, 50–56 (1994).
134. Lipponen, P. K. Expression of cathepsin D in transitional cell bladder tumours. *J. Pathol.* **178**, 59–64 (1996).
135. Munari, F. *et al.* Tumor-associated macrophages as major source of APRIL in gastric MALT lymphoma. *Blood* **117**, 6612–6616 (2011).

136. Ogden, C. A. *et al.* Enhanced apoptotic cell clearance capacity and B cell survival factor production by IL-10-activated macrophages: implications for Burkitt's lymphoma. *J. Immunol. Baltim. Md 1950* **174**, 3015–3023 (2005).
137. Lissbrant, I. F. *et al.* Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. *Int. J. Oncol.* **17**, 445–451 (2000).
138. Shimura, S. *et al.* Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. *Cancer Res.* **60**, 5857–5861 (2000).
139. Koukourakis, M. I. *et al.* Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small-cell lung cancer: impact on tumour neoangiogenesis and survival. *Br. J. Cancer* **77**, 1696–1703 (1998).
140. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity* **33**, 657–670 (2010).
141. Cassatella, M. A. Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* **73**, 369–509 (1999).
142. Piccard, H., Muschel, R. J. & Opdenakker, G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. *Crit. Rev. Oncol. Hematol.* **82**, 296–309 (2012).
143. Mantovani, A., Cassatella, M. A., Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* **11**, 519–531 (2011).
144. Shojaei, F., Singh, M., Thompson, J. D. & Ferrara, N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 2640–2645 (2008).
145. Fridlender, Z. G. *et al.* Polarization of tumor-associated neutrophil phenotype by TGF-beta: 'N1' versus 'N2' TAN. *Cancer Cell* **16**, 183–194 (2009).
146. Gregory, A. D. & Houghton, A. M. Tumor-associated neutrophils: new targets for cancer therapy. *Cancer Res.* **71**, 2411–2416 (2011).
147. Peranzoni, E. *et al.* Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr. Opin. Immunol.* **22**, 238–244 (2010).
148. Wright, H. L., Moots, R. J., Bucknall, R. C. & Edwards, S. W. Neutrophil function in inflammation and inflammatory diseases. *Rheumatol. Oxf. Engl.* **49**, 1618–1631 (2010).
149. Hallett, M. B. & Lloyds, D. Neutrophil priming: the cellular signals that say 'amber' but not 'green'. *Immunol. Today* **16**, 264–268 (1995).
150. Ley, K., Laudanna, C., Cybulsky, M. I. & Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* **7**, 678–689 (2007).
151. Hajishengallis, G., Chavakis, T., Hajishengallis, E. & Lambris, J. D. Neutrophil homeostasis and inflammation: novel paradigms from studying periodontitis. *J. Leukoc. Biol.* (2014). doi:10.1189/jlb.3VMR1014-468R
152. Ashtekar, A. R. & Saha, B. Poly's plea: membership to the club of APCs. *Trends Immunol.* **24**, 485–490 (2003).
153. Nagaraj, S., Schrum, A. G., Cho, H.-I., Celis, E. & Gabrilovich, D. I. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J. Immunol. Baltim. Md 1950* **184**, 3106–3116 (2010).
154. Maus, U. A. *et al.* Monocytes are potent facilitators of alveolar neutrophil emigration during lung inflammation: role of the CCL2-CCR2 axis. *J. Immunol. Baltim. Md 1950* **170**, 3273–3278 (2003).
155. Stoppacciaro, A. *et al.* Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T cell-produced interferon gamma. *J. Exp. Med.* **178**, 151–161 (1993).

156. Aruga, A., Aruga, E., Cameron, M. J. & Chang, A. E. Different cytokine profiles released by CD4⁺ and CD8⁺ tumor-draining lymph node cells involved in mediating tumor regression. *J. Leukoc. Biol.* **61**, 507–516 (1997).
157. Sherwood, E. R., Enoh, V. T., Murphey, E. D. & Lin, C. Y. Mice depleted of CD8⁺ T and NK cells are resistant to injury caused by cecal ligation and puncture. *Lab. Investig. J. Tech. Methods Pathol.* **84**, 1655–1665 (2004).
158. Himmel, M. E. *et al.* Human CD4⁺ FOXP3⁺ regulatory T cells produce CXCL8 and recruit neutrophils. *Eur. J. Immunol.* **41**, 306–312 (2011).
159. Kobayashi, Y. The role of chemokines in neutrophil biology. *Front. Biosci. J. Virtual Libr.* **13**, 2400–2407 (2008).
160. Ferrante, A. Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors. *Immunol. Ser.* **57**, 417–436 (1992).
161. Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19**, 683–765 (2001).
162. Nelson, A. R., Fingleton, B., Rothenberg, M. L. & Matrisian, L. M. Matrix metalloproteinases: biologic activity and clinical implications. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **18**, 1135–1149 (2000).
163. Pekarek, L. A., Starr, B. A., Toledano, A. Y. & Schreiber, H. Inhibition of tumor growth by elimination of granulocytes. *J. Exp. Med.* **181**, 435–440 (1995).
164. Tazawa, H. *et al.* Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression. *Am. J. Pathol.* **163**, 2221–2232 (2003).
165. Schmielau, J. & Finn, O. J. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res.* **61**, 4756–4760 (2001).
166. Acuff, H. B., Carter, K. J., Fingleton, B., Gorden, D. L. & Matrisian, L. M. Matrix metalloproteinase-9 from bone marrow-derived cells contributes to survival but not growth of tumor cells in the lung microenvironment. *Cancer Res.* **66**, 259–266 (2006).
167. Houghton, A. M. *et al.* Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. *Nat. Med.* **16**, 219–223 (2010).
168. Loukinova, E. *et al.* Growth regulated oncogene-alpha expression by murine squamous cell carcinoma promotes tumor growth, metastasis, leukocyte infiltration and angiogenesis by a host CXC receptor-2 dependent mechanism. *Oncogene* **19**, 3477–3486 (2000).
169. Hirose, K. *et al.* Chemokine gene transfection into tumour cells reduced tumorigenicity in nude mice in association with neutrophilic infiltration. *Br. J. Cancer* **72**, 708–714 (1995).
170. Loges, S., Roncal, C. & Carmeliet, P. Development of targeted angiogenic medicine. *J. Thromb. Haemost. JTH* **7**, 21–33 (2009).
171. Carmeliet, P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3**, 4–10 (2005).
172. Ribatti, D. The discovery of the placental growth factor and its role in angiogenesis: a historical review. *Angiogenesis* **11**, 215–221 (2008).
173. Nyberg, P., Salo, T. & Kalluri, R. Tumor microenvironment and angiogenesis. *Front. Biosci. J. Virtual Libr.* **13**, 6537–6553 (2008).
174. Nozawa, H., Chiu, C. & Hanahan, D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 12493–12498 (2006).
175. Farina, A. R. & Mackay, A. R. Gelatinase B/MMP-9 in Tumour Pathogenesis and Progression. *Cancers* **6**, 240–296 (2014).

176. Ardi, V. C. *et al.* Neutrophil MMP-9 proenzyme, unencumbered by TIMP-1, undergoes efficient activation in vivo and catalytically induces angiogenesis via a basic fibroblast growth factor (FGF-2)/FGFR-2 pathway. *J. Biol. Chem.* **284**, 25854–25866 (2009).
177. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353–364 (1996).
178. Ardi, V. C., Kupriyanova, T. A., Deryugina, E. I. & Quigley, J. P. Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 20262–20267 (2007).
179. Masson, V. *et al.* Contribution of host MMP-2 and MMP-9 to promote tumor vascularization and invasion of malignant keratinocytes. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **19**, 234–236 (2005).
180. Valastyan, S. & Weinberg, R. A. Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147**, 275–292 (2011).
181. Opdenakker, G. & Van Damme, J. Cytokines and proteases in invasive processes: molecular similarities between inflammation and cancer. *Cytokine* **4**, 251–258 (1992).
182. Opdenakker, G. & Van Damme, J. Chemotactic factors, passive invasion and metastasis of cancer cells. *Immunol. Today* **13**, 463–464 (1992).
183. Vogelstein, B. & Kinzler, K. W. Cancer genes and the pathways they control. *Nat. Med.* **10**, 789–799 (2004).
184. Wislez, M. *et al.* Neutrophils promote aerogenous spread of lung adenocarcinoma with bronchioloalveolar carcinoma features. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **13**, 3518–3527 (2007).
185. Van Coillie, E. *et al.* Tumor angiogenesis induced by granulocyte chemotactic protein-2 as a countercurrent principle. *Am. J. Pathol.* **159**, 1405–1414 (2001).
186. Opdenakker, G. & Van Damme, J. The countercurrent principle in invasion and metastasis of cancer cells. Recent insights on the roles of chemokines. *Int. J. Dev. Biol.* **48**, 519–527 (2004).
187. Liang, S., Slattery, M. J. & Dong, C. Shear stress and shear rate differentially affect the multi-step process of leukocyte-facilitated melanoma adhesion. *Exp. Cell Res.* **310**, 282–292 (2005).
188. Dong, C., Slattery, M. J., Liang, S. & Peng, H.-H. Melanoma cell extravasation under flow conditions is modulated by leukocytes and endogenously produced interleukin 8. *Mol. Cell. Biomech. MCB* **2**, 145–159 (2005).
189. Peng, H.-H., Liang, S., Henderson, A. J. & Dong, C. Regulation of interleukin-8 expression in melanoma-stimulated neutrophil inflammatory response. *Exp. Cell Res.* **313**, 551–559 (2007).
190. Reymond, N., d'Água, B. B. & Ridley, A. J. Crossing the endothelial barrier during metastasis. *Nat. Rev. Cancer* **13**, 858–870 (2013).
191. Huh, S. J., Liang, S., Sharma, A., Dong, C. & Robertson, G. P. Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. *Cancer Res.* **70**, 6071–6082 (2010).
192. Slattery, M. J. & Dong, C. Neutrophils influence melanoma adhesion and migration under flow conditions. *Int. J. Cancer J. Int. Cancer* **106**, 713–722 (2003).
193. Wu, Q. D., Wang, J. H., Condrón, C., Bouchier-Hayes, D. & Redmond, H. P. Human neutrophils facilitate tumor cell transendothelial migration. *Am. J. Physiol. Cell Physiol.* **280**, C814–822 (2001).
194. Orr, F. W. & Wang, H. H. Tumor cell interactions with the microvasculature: a rate-limiting step in metastasis. *Surg. Oncol. Clin. N. Am.* **10**, 357–381, ix–x (2001).
195. Werb, Z. ECM and cell surface proteolysis: regulating cellular ecology. *Cell* **91**, 439–442 (1997).

196. Welch, D. R., Schissel, D. J., Howrey, R. P. & Aeed, P. A. Tumor-elicited polymorphonuclear cells, in contrast to 'normal' circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5859–5863 (1989).
197. Gilbert, L. A. & Hemann, M. T. DNA damage-mediated induction of a chemoresistant niche. *Cell* **143**, 355–366 (2010).
198. Aoudjit, F., Masure, S., Opdenakker, G., Potworowski, E. F. & St-Pierre, Y. Gelatinase B (MMP-9), but not its inhibitor (TIMP-1), dictates the growth rate of experimental thymic lymphoma. *Int. J. Cancer J. Int. Cancer* **82**, 743–747 (1999).
199. Edwards, D. R. *et al.* Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* **6**, 1899–1904 (1987).
200. Schrötzlmair, F. *et al.* Tissue inhibitor of metalloproteinases-1-induced scattered liver metastasis is mediated by host-derived urokinase-type plasminogen activator. *J. Cell. Mol. Med.* **14**, 2760–2770 (2010).
201. Wu, C.-F. *et al.* The lack of type I interferon induces neutrophil-mediated pre-metastatic niche formation in the mouse lung. *Int. J. Cancer J. Int. Cancer* **137**, 837–847 (2015).
202. Redondo-Muñoz, J. *et al.* Matrix metalloproteinase-9 promotes chronic lymphocytic leukemia b cell survival through its hemopexin domain. *Cancer Cell* **17**, 160–172 (2010).
203. Coussens, L. M., Tinkle, C. L., Hanahan, D. & Werb, Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* **103**, 481–490 (2000).
204. Schaider, H. *et al.* Differential response of primary and metastatic melanomas to neutrophils attracted by IL-8. *Int. J. Cancer J. Int. Cancer* **103**, 335–343 (2003).
205. Jablonska, J., Leschner, S., Westphal, K., Lienenklaus, S. & Weiss, S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J. Clin. Invest.* **120**, 1151–1164 (2010).
206. van den Tol, M. P. *et al.* The post-surgical inflammatory response provokes enhanced tumour recurrence: a crucial role for neutrophils. *Dig. Surg.* **24**, 388–394 (2007).
207. Shojaei, F. & Ferrara, N. Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. *Cancer Res.* **68**, 5501–5504 (2008).
208. Di Carlo, E. *et al.* The intriguing role of polymorphonuclear neutrophils in antitumor reactions. *Blood* **97**, 339–345 (2001).
209. Colombo, M. P. *et al.* Granulocyte colony-stimulating factor (G-CSF) gene transduction in murine adenocarcinoma drives neutrophil-mediated tumor inhibition in vivo. Neutrophils discriminate between G-CSF-producing and G-CSF-nonproducing tumor cells. *J. Immunol. Baltim. Md 1950* **149**, 113–119 (1992).
210. Hicks, A. M. *et al.* Transferable anticancer innate immunity in spontaneous regression/complete resistance mice. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 7753–7758 (2006).
211. Kousis, P. C., Henderson, B. W., Maier, P. G. & Gollnick, S. O. Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. *Cancer Res.* **67**, 10501–10510 (2007).
212. Suttman, H. *et al.* Neutrophil granulocytes are required for effective Bacillus Calmette-Guérin immunotherapy of bladder cancer and orchestrate local immune responses. *Cancer Res.* **66**, 8250–8257 (2006).
213. Gerrard, T. L., Cohen, D. J. & Kaplan, A. M. Human neutrophil-mediated cytotoxicity to tumor cells. *J. Natl. Cancer Inst.* **66**, 483–488 (1981).
214. Dallegri, F., Patrone, F., Frumento, G. & Sacchetti, C. Antibody-dependent killing of tumor cells by polymorphonuclear leukocytes. Involvement of oxidative and nonoxidative mechanisms. *J. Natl. Cancer Inst.* **73**, 331–339 (1984).

215. Katano, M. & Torisu, M. Neutrophil-mediated tumor cell destruction in cancer ascites. *Cancer* **50**, 62–68 (1982).
216. Lichtenstein, A., Seelig, M., Berek, J. & Zigelboim, J. Human neutrophil-mediated lysis of ovarian cancer cells. *Blood* **74**, 805–809 (1989).
217. Dallegri, F. *et al.* Tumor cell lysis by activated human neutrophils: analysis of neutrophil-delivered oxidative attack and role of leukocyte function-associated antigen 1. *Inflammation* **15**, 15–30 (1991).
218. Zivkovic, M. *et al.* Oxidative burst and anticancer activities of rat neutrophils. *BioFactors Oxf. Engl.* **24**, 305–312 (2005).
219. Zivkovic, M. *et al.* Oxidative burst of neutrophils against melanoma B16-F10. *Cancer Lett.* **246**, 100–108 (2007).
220. Chen, Y.-L., Wang, J.-Y., Chen, S.-H. & Yang, B.-C. Granulocytes mediates the Fas-L-associated apoptosis during lung metastasis of melanoma that determines the metastatic behaviour. *Br. J. Cancer* **87**, 359–365 (2002).
221. Kindzelskii, A. L. & Petty, H. R. Early membrane rupture events during neutrophil-mediated antibody-dependent tumor cell cytotoxicity. *J. Immunol. Baltim. Md 1950* **162**, 3188–3192 (1999).
222. Ersvaer, E. *et al.* The protein kinase C agonist PEP005 (ingenol 3-angelate) in the treatment of human cancer: a balance between efficacy and toxicity. *Toxins* **2**, 174–194 (2010).
223. Kushner, B. H. & Cheung, N. K. Absolute requirement of CD11/CD18 adhesion molecules, FcRII and the phosphatidylinositol-linked FcRIII for monoclonal antibody-mediated neutrophil antihuman tumor cytotoxicity. *Blood* **79**, 1484–1490 (1992).
224. Elsässer, D. *et al.* Preclinical studies combining bispecific antibodies with cytokine-stimulated effector cells for immunotherapy of renal cell carcinoma. *Anticancer Res.* **19**, 1525–1528 (1999).
225. Sawanobori, Y. *et al.* Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. *Blood* **111**, 5457–5466 (2008).
226. van Gisbergen, K. P. J. M., Geijtenbeek, T. B. H. & van Kooyk, Y. Close encounters of neutrophils and DCs. *Trends Immunol.* **26**, 626–631 (2005).
227. Beauvillain, C. *et al.* Neutrophils efficiently cross-prime naive T cells in vivo. *Blood* **110**, 2965–2973 (2007).
228. Giovarelli, M. *et al.* Local release of IL-10 by transfected mouse mammary adenocarcinoma cells does not suppress but enhances antitumor reaction and elicits a strong cytotoxic lymphocyte and antibody-dependent immune memory. *J. Immunol. Baltim. Md 1950* **155**, 3112–3123 (1995).
229. Cavallo, F. *et al.* Role of neutrophils and CD4+ T lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene. *J. Immunol. Baltim. Md 1950* **149**, 3627–3635 (1992).
230. Freije, J. M. P. *et al.* Matrix metalloproteinases and tumor progression. *Adv. Exp. Med. Biol.* **532**, 91–107 (2003).
231. Colombo, M. P. *et al.* Hypoxic tumor cell death and modulation of endothelial adhesion molecules in the regression of granulocyte colony-stimulating factor-transduced tumors. *Am. J. Pathol.* **148**, 473–483 (1996).
232. Graves, D. Role of polymorphonuclear leukocytes in the pulmonary clearance of arrested cancer cells. *Invasion Metastasis* **3**, 160–173 (1983).
233. Sanford, M. A. *et al.* Independent contributions of GR-1+ leukocytes and Fas/FasL interactions to induce apoptosis following interleukin-12 gene therapy in a metastatic model of prostate cancer. *Hum. Gene Ther.* **12**, 1485–1498 (2001).

234. Di Carlo, E., Forni, G. & Musiani, P. Neutrophils in the antitumoral immune response. *Chem. Immunol. Allergy* **83**, 182–203 (2003).
235. Alvarez, M. J. *et al.* Secreted protein acidic and rich in cysteine produced by human melanoma cells modulates polymorphonuclear leukocyte recruitment and antitumor cytotoxic capacity. *Cancer Res.* **65**, 5123–5132 (2005).
236. Musiani, P. *et al.* Cytokines, tumour-cell death and immunogenicity: a question of choice. *Immunol. Today* **18**, 32–36 (1997).
237. Lee, L. F. *et al.* IL-8 reduced tumorigenicity of human ovarian cancer in vivo due to neutrophil infiltration. *J. Immunol. Baltim. Md 1950* **164**, 2769–2775 (2000).
238. Noffz, G., Qin, Z., Kopf, M. & Blankenstein, T. Neutrophils but not eosinophils are involved in growth suppression of IL-4-secreting tumors. *J. Immunol. Baltim. Md 1950* **160**, 345–350 (1998).
239. Musiani, P. *et al.* Role of neutrophils and lymphocytes in inhibition of a mouse mammary adenocarcinoma engineered to release IL-2, IL-4, IL-7, IL-10, IFN-alpha, IFN-gamma, and TNF-alpha. *Lab. Investig. J. Tech. Methods Pathol.* **74**, 146–157 (1996).
240. Breitbach, C. J. *et al.* Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Mol. Ther. J. Am. Soc. Gene Ther.* **15**, 1686–1693 (2007).
241. Galluzzi, L., Senovilla, L., Zitvogel, L. & Kroemer, G. The secret ally: immunostimulation by anticancer drugs. *Nat. Rev. Drug Discov.* **11**, 215–233 (2012).
242. Vanneman, M. & Dranoff, G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat. Rev. Cancer* **12**, 237–251 (2012).
243. Zitvogel, L., Tesniere, A. & Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat. Rev. Immunol.* **6**, 715–727 (2006).
244. Colombo, M. P. & Piconese, S. Regulatory-T-cell inhibition versus depletion: the right choice in cancer immunotherapy. *Nat. Rev. Cancer* **7**, 880–887 (2007).
245. Rosental, B. *et al.* The effect of chemotherapy/radiotherapy on cancerous pattern recognition by NK cells. *Curr. Med. Chem.* **19**, 1780–1791 (2012).
246. Park, B., Yee, C. & Lee, K.-M. The effect of radiation on the immune response to cancers. *Int. J. Mol. Sci.* **15**, 927–943 (2014).
247. Bryniarski, K., Szczepanik, M., Ptak, M., Zemelka, M. & Ptak, W. Influence of cyclophosphamide and its metabolic products on the activity of peritoneal macrophages in mice. *Pharmacol. Rep. PR* **61**, 550–557 (2009).
248. Buhtoiarov, I. N. *et al.* Anti-tumour synergy of cytotoxic chemotherapy and anti-CD40 plus CpG-ODN immunotherapy through repolarization of tumour-associated macrophages. *Immunology* **132**, 226–239 (2011).
249. North, R. J. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J. Exp. Med.* **155**, 1063–1074 (1982).
250. Ghiringhelli, F. *et al.* Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol. Immunother. CII* **56**, 641–648 (2007).
251. Terme, M. *et al.* VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. *Cancer Res.* **73**, 539–549 (2013).
252. Nowak, A. K., Robinson, B. W. S. & Lake, R. A. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. *Cancer Res.* **62**, 2353–2358 (2002).
253. Chen, G. & Emens, L. A. Chemoimmunotherapy: reengineering tumor immunity. *Cancer Immunol. Immunother. CII* **62**, 203–216 (2013).

254. Mittal, D., Gubin, M. M., Schreiber, R. D. & Smyth, M. J. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr. Opin. Immunol.* **27**, 16–25 (2014).
255. Sistigu, A. *et al.* Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nat. Med.* **20**, 1301–1309 (2014).
256. Sukkurwala, A. Q. *et al.* Immunogenic calreticulin exposure occurs through a phylogenetically conserved stress pathway involving the chemokine CXCL8. *Cell Death Differ.* **21**, 59–68 (2014).
257. Apetoh, L. *et al.* Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat. Med.* **13**, 1050–1059 (2007).
258. Ghiringhelli, F. *et al.* Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat. Med.* **15**, 1170–1178 (2009).
259. Vacchelli, E. *et al.* Loss-of-function alleles of P2RX7 and TLR4 fail to affect the response to chemotherapy in non-small cell lung cancer. *Oncoimmunology* **1**, 271–278 (2012).
260. Tesniere, A. *et al.* Immunogenic death of colon cancer cells treated with oxaliplatin. *Oncogene* **29**, 482–491 (2010).
261. Bergmann, C. *et al.* Toll-like receptor 4 single-nucleotide polymorphisms Asp299Gly and Thr399Ile in head and neck squamous cell carcinomas. *J. Transl. Med.* **9**, 139 (2011).
262. Ghebeh, H. *et al.* Doxorubicin downregulates cell surface B7-H1 expression and upregulates its nuclear expression in breast cancer cells: role of B7-H1 as an anti-apoptotic molecule. *Breast Cancer Res. BCR* **12**, R48 (2010).
263. Vereecque, R., Saudemont, A. & Quesnel, B. Cytosine arabinoside induces costimulatory molecule expression in acute myeloid leukemia cells. *Leukemia* **18**, 1223–1230 (2004).
264. Emens, L. A. & Middleton, G. The Interplay of Immunotherapy and Chemotherapy: Harnessing Potential Synergies. *Cancer Immunol. Res.* **3**, 436–443 (2015).
265. van der Most, R. G. *et al.* Cyclophosphamide chemotherapy sensitizes tumor cells to TRAIL-dependent CD8 T cell-mediated immune attack resulting in suppression of tumor growth. *PLoS One* **4**, e6982 (2009).
266. Yang, S. & Haluska, F. G. Treatment of melanoma with 5-fluorouracil or dacarbazine in vitro sensitizes cells to antigen-specific CTL lysis through perforin/granzyme- and Fas-mediated pathways. *J. Immunol. Baltim. Md 1950* **172**, 4599–4608 (2004).
267. Ramakrishnan, R. *et al.* Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. *J. Clin. Invest.* **120**, 1111–1124 (2010).
268. Kaneno, R. *et al.* Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. *Cell. Oncol. Dordr.* **34**, 97–106 (2011).
269. Donepudi, M., Raychaudhuri, P., Bluestone, J. A. & Mokyr, M. B. Mechanism of melphalan-induced B7-1 gene expression in P815 tumor cells. *J. Immunol. Baltim. Md 1950* **166**, 6491–6499 (2001).
270. Sojka, D. K., Donepudi, M., Bluestone, J. A. & Mokyr, M. B. Melphalan and other anticancer modalities up-regulate B7-1 gene expression in tumor cells. *J. Immunol. Baltim. Md 1950* **164**, 6230–6236 (2000).
271. Adair, S. J. & Hogan, K. T. Treatment of ovarian cancer cell lines with 5-aza-2'-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules. *Cancer Immunol. Immunother. CII* **58**, 589–601 (2009).

272. Fonsatti, E. *et al.* Functional up-regulation of human leukocyte antigen class I antigens expression by 5-aza-2'-deoxycytidine in cutaneous melanoma: immunotherapeutic implications. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **13**, 3333–3338 (2007).
273. Michaud, M. *et al.* Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* **334**, 1573–1577 (2011).
274. Zitvogel, L. *et al.* Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **16**, 3100–3104 (2010).
275. Bracci, L., Schiavoni, G., Sistigu, A. & Belardelli, F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ.* **21**, 15–25 (2014).
276. Vincent, J. *et al.* 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res.* **70**, 3052–3061 (2010).
277. Kodumudi, K. N. *et al.* A novel chemoimmunomodulating property of docetaxel: suppression of myeloid-derived suppressor cells in tumor bearers. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **16**, 4583–4594 (2010).
278. Michels, T. *et al.* Paclitaxel promotes differentiation of myeloid-derived suppressor cells into dendritic cells in vitro in a TLR4-independent manner. *J. Immunotoxicol.* **9**, 292–300 (2012).
279. Alizadeh, D. *et al.* Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. *Cancer Res.* **74**, 104–118 (2014).
280. Diaz-Montero, C. M. *et al.* Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol. Immunother. CII* **58**, 49–59 (2009).
281. Ko, J. S. *et al.* Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **15**, 2148–2157 (2009).
282. Ozao-Choy, J. *et al.* The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. *Cancer Res.* **69**, 2514–2522 (2009).
283. Alizadeh, D. & Larmonier, N. Chemotherapeutic targeting of cancer-induced immunosuppressive cells. *Cancer Res.* **74**, 2663–2668 (2014).
284. Chen, J. *et al.* Preconditioning chemotherapy with cisplatin enhances the antitumor activity of cytokine-induced killer cells in a murine melanoma model. *Cancer Biother. Radiopharm.* **27**, 210–220 (2012).
285. Soeda, A. *et al.* Regular dose of gemcitabine induces an increase in CD14⁺ monocytes and CD11c⁺ dendritic cells in patients with advanced pancreatic cancer. *Jpn. J. Clin. Oncol.* **39**, 797–806 (2009).
286. Wada, S. *et al.* Cyclophosphamide augments antitumor immunity: studies in an autochthonous prostate cancer model. *Cancer Res.* **69**, 4309–4318 (2009).
287. Schiavoni, G. *et al.* Cyclophosphamide induces type I interferon and augments the number of CD44(hi) T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. *Blood* **95**, 2024–2030 (2000).
288. Pfannenstiel, L. W., Lam, S. S. K., Emens, L. A., Jaffee, E. M. & Armstrong, T. D. Paclitaxel enhances early dendritic cell maturation and function through TLR4 signaling in mice. *Cell. Immunol.* **263**, 79–87 (2010).
289. Machiels, J. P. *et al.* Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-

- secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res.* **61**, 3689–3697 (2001).
290. Kang, T. H. *et al.* Chemotherapy acts as an adjuvant to convert the tumor microenvironment into a highly permissive state for vaccination-induced antitumor immunity. *Cancer Res.* **73**, 2493–2504 (2013).
 291. Dougan, M. & Dranoff, G. Immune therapy for cancer. *Annu. Rev. Immunol.* **27**, 83–117 (2009).
 292. Correale, P. *et al.* Cetuximab ± chemotherapy enhances dendritic cell-mediated phagocytosis of colon cancer cells and ignites a highly efficient colon cancer antigen-specific cytotoxic T-cell response in vitro. *Int. J. Cancer J. Int. Cancer* **130**, 1577–1589 (2012).
 293. Wolpoe, M. E. *et al.* HER-2/neu-specific monoclonal antibodies collaborate with HER-2/neu-targeted granulocyte macrophage colony-stimulating factor secreting whole cell vaccination to augment CD8⁺ T cell effector function and tumor-free survival in Her-2/neu-transgenic mice. *J. Immunol. Baltim. Md 1950* **171**, 2161–2169 (2003).
 294. Maréchal, R. *et al.* Putative contribution of CD56 positive cells in cetuximab treatment efficacy in first-line metastatic colorectal cancer patients. *BMC Cancer* **10**, 340 (2010).
 295. Larmonier, N. *et al.* Imatinib mesylate inhibits CD4⁺ CD25⁺ regulatory T cell activity and enhances active immunotherapy against BCR-ABL- tumors. *J. Immunol. Baltim. Md 1950* **181**, 6955–6963 (2008).
 296. Potapov, S. L., Korman, D. B., Shamaev, V. I., Ershova, R. B. & Makarov, O. V. Sensitivity of clonogenic cells of human ovarian ascitic cancer to antitumor drugs. *Arch. Für Geschwulstforsch.* **58**, 99–104 (1988).
 297. Dijkgraaf, E. M. *et al.* Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment. *Cancer Res.* **73**, 2480–2492 (2013).
 298. Mackay, I. R., Goodyear, M. D., Riglar, C. & Penschow, J. Effect on natural killer and antibody-dependent cellular cytotoxicity of adjuvant cytotoxic chemotherapy including melphalan in breast cancer. *Cancer Immunol. Immunother. CII* **16**, 98–100 (1983).
 299. Braun, D. P. & Harris, J. E. Effect of chemotherapy on NK function in the peripheral blood of cancer patients. *Cancer Immunol. Immunother. CII* **21**, 240–245 (1986).
 300. Sewell, H. F. *et al.* Chemotherapy-induced differential changes in lymphocyte subsets and natural-killer-cell function in patients with advanced breast cancer. *Int. J. Cancer J. Int. Cancer* **55**, 735–738 (1993).
 301. Brenner, B. G. & Margolese, R. G. The relationship of chemotherapeutic and endocrine intervention on natural killer cell activity in human breast cancer. *Cancer* **68**, 482–488 (1991).
 302. Markasz, L. *et al.* Effect of frequently used chemotherapeutic drugs on the cytotoxic activity of human natural killer cells. *Mol. Cancer Ther.* **6**, 644–654 (2007).
 303. Liu, P., Jaffar, J., Hellstrom, I. & Hellstrom, K. E. Administration of cyclophosphamide changes the immune profile of tumor-bearing mice. *J. Immunother. Hagerstown Md 1997* **33**, 53–59 (2010).
 304. Doloff, J. C. & Waxman, D. J. VEGF receptor inhibitors block the ability of metronomically dosed cyclophosphamide to activate innate immunity-induced tumor regression. *Cancer Res.* **72**, 1103–1115 (2012).
 305. Khallouf, H. *et al.* 5-Fluorouracil and interferon- α immunochemotherapy enhances immunogenicity of murine pancreatic cancer through upregulation of NKG2D ligands and MHC class I. *J. Immunother. Hagerstown Md 1997* **35**, 245–253 (2012).

306. Nakagawa, K., Yoshida, F., Omori, N., Tsunoda, T. & Nose, T. The effect of radiation therapy combined with natural killer cells against spontaneous murine fibrosarcoma. *Biotherapy Dordr. Neth.* **2**, 69–75 (1990).
307. Sonn, C. H. *et al.* Augmentation of natural cytotoxicity by chronic low-dose ionizing radiation in murine natural killer cells primed by IL-2. *J. Radiat. Res. (Tokyo)* **53**, 823–829 (2012).
308. Golay, J. & Introna, M. Mechanism of action of therapeutic monoclonal antibodies: promises and pitfalls of in vitro and in vivo assays. *Arch. Biochem. Biophys.* **526**, 146–153 (2012).
309. Romagné, F. *et al.* Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. *Blood* **114**, 2667–2677 (2009).
310. Nizar, S. *et al.* T-regulatory cell modulation: the future of cancer immunotherapy? *Br. J. Cancer* **100**, 1697–1703 (2009).
311. Ghiringhelli, F. *et al.* Metronomic cyclophosphamide regimen selectively depletes CD4⁺CD25⁺ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol. Immunother. CII* **56**, 641–648 (2007).
312. Ding, Z.-C. *et al.* Polyfunctional CD4⁺ T cells are essential for eradicating advanced B-cell lymphoma after chemotherapy. *Blood* **120**, 2229–2239 (2012).
313. Zhang, L. *et al.* Differential impairment of regulatory T cells rather than effector T cells by paclitaxel-based chemotherapy. *Clin. Immunol. Orlando Fla* **129**, 219–229 (2008).
314. Chang, C.-L. *et al.* Dose-dense chemotherapy improves mechanisms of antitumor immune response. *Cancer Res.* **73**, 119–127 (2013).
315. Mattarollo, S. R. *et al.* Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors. *Cancer Res.* **71**, 4809–4820 (2011).
316. Galustian, C. *et al.* The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. *Cancer Immunol. Immunother. CII* **58**, 1033–1045 (2009).
317. Sakamaki, I. *et al.* Lenalidomide enhances the protective effect of a therapeutic vaccine and reverses immune suppression in mice bearing established lymphomas. *Leukemia* **28**, 329–337 (2014).
318. Banissi, C., Ghiringhelli, F., Chen, L. & Carpentier, A. F. Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model. *Cancer Immunol. Immunother. CII* **58**, 1627–1634 (2009).
319. Ridolfi, L. *et al.* Low-dose temozolomide before dendritic-cell vaccination reduces (specifically) CD4⁺CD25⁺Foxp3⁺ regulatory T-cells in advanced melanoma patients. *J. Transl. Med.* **11**, 135 (2013).
320. Correale, P. *et al.* Immunity feedback and clinical outcome in colon cancer patients undergoing chemoimmunotherapy with gemcitabine + FOLFOX followed by subcutaneous granulocyte macrophage colony-stimulating factor and aldesleukin (GOLFIG-1 Trial). *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **14**, 4192–4199 (2008).
321. Adotevi, O. *et al.* A decrease of regulatory T cells correlates with overall survival after sunitinib-based antiangiogenic therapy in metastatic renal cancer patients. *J. Immunother. Hagerstown Md 1997* **33**, 991–998 (2010).
322. Desar, I. M. E. *et al.* Sorafenib reduces the percentage of tumour infiltrating regulatory T cells in renal cell carcinoma patients. *Int. J. Cancer J. Int. Cancer* **129**, 507–512 (2011).
323. Yang, Y. *et al.* Antitumor T-cell responses contribute to the effects of dasatinib on c-KIT mutant murine mastocytoma and are potentiated by anti-OX40. *Blood* **120**, 4533–4543 (2012).

324. Shebzukhov, Y. V. *et al.* Humoral immune response to thymidylate synthase in colon cancer patients after 5-FU chemotherapy. *Immunol. Lett.* **100**, 88–93 (2005).
325. Otterness, I. G. & Chang, Y. H. Comparative study of cyclophosphamide, 6-mercaptopurine, azathiopurine and methotrexate. Relative effects on the humoral and the cellular immune response in the mouse. *Clin. Exp. Immunol.* **26**, 346–354 (1976).
326. Smith, S. R., Terminelli, C., Kipilman, C. T. & Smith, Y. Comparative effects of azathioprine, cyclophosphamide and frentizole on cellular immunity in mice. *J. Immunopharmacol.* **3**, 133–170 (1981).
327. Berd, D., Maguire, H. C. & Mastrangelo, M. J. Potentiation of human cell-mediated and humoral immunity by low-dose cyclophosphamide. *Cancer Res.* **44**, 5439–5443 (1984).
328. Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* **19**, 1423–1437 (2013).
329. Hanahan, D. & Coussens, L. M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* **21**, 309–322 (2012).
330. De Palma, M. & Lewis, C. E. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* **23**, 277–286 (2013).
331. Noy, R. & Pollard, J. W. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* **41**, 49–61 (2014).
332. Mantovani, A., Polentarutti, N., Luini, W., Peri, G. & Spreafico, F. Role of host defense mechanisms in the antitumor activity of adriamycin and daunomycin in mice. *J. Natl. Cancer Inst.* **63**, 61–66 (1979).
333. DeNardo, D. G. *et al.* Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer Discov.* **1**, 54–67 (2011).
334. Ruffell, B. *et al.* Macrophage IL-10 blocks CD8⁺ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell* **26**, 623–637 (2014).
335. Shree, T. *et al.* Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev.* **25**, 2465–2479 (2011).
336. Houthuijzen, J. M. *et al.* Lysophospholipids secreted by splenic macrophages induce chemotherapy resistance via interference with the DNA damage response. *Nat. Commun.* **5**, 5275 (2014).
337. Germano, G. *et al.* Role of macrophage targeting in the antitumor activity of trabectedin. *Cancer Cell* **23**, 249–262 (2013).
338. Park, S. *et al.* The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell* **18**, 160–170 (2010).
339. Grugan, K. D. *et al.* Tumor-associated macrophages promote invasion while retaining Fc-dependent anti-tumor function. *J. Immunol. Baltim. Md 1950* **189**, 5457–5466 (2012).
340. Chao, M. P. *et al.* Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* **142**, 699–713 (2010).
341. Minard-Colin, V. *et al.* Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage FcγRI, FcγRIII, and FcγRIV. *Blood* **112**, 1205–1213 (2008).
342. Simpson, T. R. *et al.* Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J. Exp. Med.* **210**, 1695–1710 (2013).
343. Pander, J. *et al.* Activation of tumor-promoting type 2 macrophages by EGFR-targeting antibody cetuximab. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **17**, 5668–5673 (2011).

344. Guerriero, J. L. *et al.* DNA alkylating therapy induces tumor regression through an HMGB1-mediated activation of innate immunity. *J. Immunol. Baltim. Md 1950* **186**, 3517–3526 (2011).
345. Hanna, N. & Burton, R. C. Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastases in vivo. *J. Immunol. Baltim. Md 1950* **127**, 1754–1758 (1981).
346. Kohlhapp, F. J. *et al.* NK cells and CD8+ T cells cooperate to improve therapeutic responses in melanoma treated with interleukin-2 (IL-2) and CTLA-4 blockade. *J. Immunother. Cancer* **3**, 18 (2015).
347. Ferrari de Andrade, L. *et al.* Natural killer cells are essential for the ability of BRAF inhibitors to control BRAFV600E-mutant metastatic melanoma. *Cancer Res.* **74**, 7298–7308 (2014).
348. Gabrilovich, D. I., Ostrand-Rosenberg, S. & Bronte, V. Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* **12**, 253–268 (2012).
349. Broz, M. L. *et al.* Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell* **26**, 638–652 (2014).
350. Engelhardt, J. J. *et al.* Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. *Cancer Cell* **21**, 402–417 (2012).
351. Ma, Y. *et al.* Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity* **38**, 729–741 (2013).
352. Gabrilovich, D. I., Ishida, T., Nadaf, S., Ohm, J. E. & Carbone, D. P. Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **5**, 2963–2970 (1999).
353. Osada, T. *et al.* The effect of anti-VEGF therapy on immature myeloid cell and dendritic cells in cancer patients. *Cancer Immunol. Immunother. CII* **57**, 1115–1124 (2008).
354. Gabitass, R. F., Annels, N. E., Stocken, D. D., Pandha, H. A. & Middleton, G. W. Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. *Cancer Immunol. Immunother. CII* **60**, 1419–1430 (2011).
355. Malmberg, K.-J. Effective immunotherapy against cancer: a question of overcoming immune suppression and immune escape? *Cancer Immunol. Immunother. CII* **53**, 879–892 (2004).
356. Mundy-Bosse, B. L. *et al.* Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. *Cancer Res.* **71**, 5101–5110 (2011).
357. Kusmartsev, S. *et al.* All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res.* **63**, 4441–4449 (2003).
358. Ishizaki, H. *et al.* Modified vaccinia Ankara expressing survivin combined with gemcitabine generates specific antitumor effects in a murine pancreatic carcinoma model. *Cancer Immunol. Immunother. CII* **60**, 99–109 (2011).
359. Weiss, T., Vitacolonna, M. & Zöller, M. The efficacy of an IL-1alpha vaccine depends on IL-1RI availability and concomitant myeloid-derived suppressor cell reduction. *J. Immunother. Hagerstown Md 1997* **32**, 552–564 (2009).
360. De Santo, C. *et al.* Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 4185–4190 (2005).

361. Nagaraj, S. *et al.* Anti-inflammatory triterpenoid blocks immune suppressive function of MDSCs and improves immune response in cancer. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **16**, 1812–1823 (2010).
362. Nakashima, H., Terabe, M., Berzofsky, J. A., Husain, S. R. & Puri, R. K. A novel combination immunotherapy for cancer by IL-13R α 2-targeted DNA vaccine and immunotoxin in murine tumor models. *J. Immunol. Baltim. Md 1950* **187**, 4935–4946 (2011).
363. Bose, A. *et al.* Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. *Int. J. Cancer J. Int. Cancer* **129**, 2158–2170 (2011).
364. Ko, H.-J. *et al.* A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. *Cancer Res.* **67**, 7477–7486 (2007).
365. Tseng, C.-W. *et al.* Pretreatment with cisplatin enhances E7-specific CD8⁺ T-Cell-mediated antitumor immunity induced by DNA vaccination. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **14**, 3185–3192 (2008).
366. Melani, C., Sangaletti, S., Barazzetta, F. M., Werb, Z. & Colombo, M. P. Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. *Cancer Res.* **67**, 11438–11446 (2007).
367. Bruchard, M. *et al.* Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat. Med.* **19**, 57–64 (2013).
368. Ma, Y. *et al.* Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy. *J. Exp. Med.* **208**, 491–503 (2011).
369. Hannesdóttir, L. *et al.* Lapatinib and doxorubicin enhance the Stat1-dependent antitumor immune response. *Eur. J. Immunol.* **43**, 2718–2729 (2013).
370. Sevko, A. *et al.* Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. *J. Immunol. Baltim. Md 1950* **190**, 2464–2471 (2013).
371. Casares, N. *et al.* Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J. Exp. Med.* **202**, 1691–1701 (2005).
372. Mattarollo, S. R. *et al.* Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors. *Cancer Res.* **71**, 4809–4820 (2011).
373. Knight, D. A. *et al.* Host immunity contributes to the anti-melanoma activity of BRAF inhibitors. *J. Clin. Invest.* **123**, 1371–1381 (2013).
374. Cooper, Z. A. *et al.* Response to BRAF inhibition in melanoma is enhanced when combined with immune checkpoint blockade. *Cancer Immunol. Res.* **2**, 643–654 (2014).
375. de Visser, K. E., Korets, L. V. & Coussens, L. M. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* **7**, 411–423 (2005).
376. Andreu, P. *et al.* FcR γ activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* **17**, 121–134 (2010).
377. Affara, N. I. *et al.* B cells regulate macrophage phenotype and response to chemotherapy in squamous carcinomas. *Cancer Cell* **25**, 809–821 (2014).
378. McMillin, D. W., Negri, J. M. & Mitsiades, C. S. The role of tumour-stromal interactions in modifying drug response: challenges and opportunities. *Nat. Rev. Drug Discov.* **12**, 217–228 (2013).
379. Kurtova, A. V., Tamayo, A. T., Ford, R. J. & Burger, J. A. Mantle cell lymphoma cells express high levels of CXCR4, CXCR5, and VLA-4 (CD49d): importance for

- interactions with the stromal microenvironment and specific targeting. *Blood* **113**, 4604–4613 (2009).
380. Lwin, T. *et al.* A microenvironment-mediated c-Myc/miR-548m/HDAC6 amplification loop in non-Hodgkin B cell lymphomas. *J. Clin. Invest.* **123**, 4612–4626 (2013).
381. Lwin, T. *et al.* Cell adhesion induces p27Kip1-associated cell-cycle arrest through down-regulation of the SCFSkp2 ubiquitin ligase pathway in mantle-cell and other non-Hodgkin B-cell lymphomas. *Blood* **110**, 1631–1638 (2007).
382. Phan, V. T. *et al.* Oncogenic RAS pathway activation promotes resistance to anti-VEGF therapy through G-CSF-induced neutrophil recruitment. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 6079–6084 (2013).
383. Liang, J. *et al.* Neutrophils promote the malignant glioma phenotype through S100A4. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **20**, 187–198 (2014).
384. Acharyya, S. *et al.* A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell* **150**, 165–178 (2012).
385. Di Mitri, D. *et al.* Tumour-infiltrating Gr-1+ myeloid cells antagonize senescence in cancer. *Nature* **515**, 134–137 (2014).
386. Han, Y. *et al.* Prognostic value of chemotherapy-induced neutropenia in early-stage breast cancer. *Breast Cancer Res. Treat.* **131**, 483–490 (2012).
387. Di Maio, M. *et al.* Chemotherapy-induced neutropenia and treatment efficacy in advanced non-small-cell lung cancer: a pooled analysis of three randomised trials. *Lancet Oncol.* **6**, 669–677 (2005).
388. Pillay, J. *et al.* A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J. Clin. Invest.* **122**, 327–336 (2012).
389. Eruslanov, E. B. *et al.* Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *J. Clin. Invest.* **124**, 5466–5480 (2014).
390. Fridlender, Z. G. & Albelda, S. M. Tumor-associated neutrophils: friend or foe? *Carcinogenesis* **33**, 949–955 (2012).
391. Tazawa, H. *et al.* Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression. *Am. J. Pathol.* **163**, 2221–2232 (2003).
392. Piccard, H., Muschel, R. J. & Opdenakker, G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. *Crit. Rev. Oncol. Hematol.* **82**, 296–309 (2012).
393. Fridlender, Z. G. *et al.* Polarization of tumor-associated neutrophil phenotype by TGF-beta: ‘N1’ versus ‘N2’ TAN. *Cancer Cell* **16**, 183–194 (2009).
394. Donskov, F. & Maase, H. von der. Impact of immune parameters on long-term survival in metastatic renal cell carcinoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **24**, 1997–2005 (2006).
395. Jensen, H. K. *et al.* Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **27**, 4709–4717 (2009).
396. Jensen, T. O. *et al.* Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer* **118**, 2476–2485 (2012).
397. Rao, H.-L. *et al.* Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients’ adverse prognosis. *PLoS One* **7**, e30806 (2012).
398. Li, Y.-W. *et al.* Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection. *J. Hepatol.* **54**, 497–505 (2011).

399. Kuang, D.-M. *et al.* Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *J. Hepatol.* **54**, 948–955 (2011).
400. Gao, Q. *et al.* CXCR6 upregulation contributes to a proinflammatory tumor microenvironment that drives metastasis and poor patient outcomes in hepatocellular carcinoma. *Cancer Res.* **72**, 3546–3556 (2012).
401. Zhou, S.-L. *et al.* Overexpression of CXCL5 mediates neutrophil infiltration and indicates poor prognosis for hepatocellular carcinoma. *Hepatol. Baltim. Md* **56**, 2242–2254 (2012).
402. Gu, F.-M. *et al.* Intratumoral IL-17⁺ cells and neutrophils show strong prognostic significance in intrahepatic cholangiocarcinoma. *Ann. Surg. Oncol.* **19**, 2506–2514 (2012).
403. Zhao, J. *et al.* The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. *PLoS One* **7**, e33655 (2012).
404. Ino, Y. *et al.* Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer. *Br. J. Cancer* **108**, 914–923 (2013).
405. Holland, J. & Owens, T. Signaling through intercellular adhesion molecule 1 (ICAM-1) in a B cell lymphoma line. The activation of Lyn tyrosine kinase and the mitogen-activated protein kinase pathway. *J. Biol. Chem.* **272**, 9108–9112 (1997).
406. Puthier, D., Bataille, R. & Amiot, M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK / STAT rather than ras / MAP kinase pathway. *Eur. J. Immunol.* **29**, 3945–3950 (1999).
407. Ericson, S. G. *et al.* Interleukin-6 production by human neutrophils after Fc-receptor cross-linking or exposure to granulocyte colony-stimulating factor. *Blood* **91**, 2099–2107 (1998).
408. Ziółkowska, K. *et al.* Long-term three-dimensional cell culture and anticancer drug activity evaluation in a microfluidic chip. *Biosens. Bioelectron.* **40**, 68–74 (2013).
409. Paget, S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev.* **8**, 98–101 (1989).
410. Perobelli, S. M. *et al.* Plasticity of neutrophils reveals modulatory capacity. *Braz. J. Med. Biol. Res. Rev. Bras. Pesqui. Médicas E Biológicas Soc. Bras. Biofísica Al* **48**, 665–675 (2015).
411. Gardner, M. J., Jones, L. M., Catterall, J. B. & Turner, G. A. Expression of cell adhesion molecules on ovarian tumour cell lines and mesothelial cells, in relation to ovarian cancer metastasis. *Cancer Lett.* **91**, 229–234 (1995).
412. Gardiner, E. E. & D'Souza, S. E. Sequences within fibrinogen and intercellular adhesion molecule-1 (ICAM-1) modulate signals required for mitogenesis. *J. Biol. Chem.* **274**, 11930–11936 (1999).
413. Park, Y. S., Huh, J. W., Lee, J. H. & Kim, H. R. shRNA against CD44 inhibits cell proliferation, invasion and migration, and promotes apoptosis of colon carcinoma cells. *Oncol. Rep.* **27**, 339–346 (2012).
414. Smith, S. M., Lyu, Y. L. & Cai, L. NF- κ B affects proliferation and invasiveness of breast cancer cells by regulating CD44 expression. *PLoS One* **9**, e106966 (2014).
415. Jablonska, J., Wu, C.-F., Andzinski, L., Leschner, S. & Weiss, S. CXCR2-mediated tumor-associated neutrophil recruitment is regulated by IFN- β . *Int. J. Cancer J. Int. Cancer* **134**, 1346–1358 (2014).
416. Brackett, C. M., Muhitch, J. B., Evans, S. S. & Gollnick, S. O. IL-17 promotes neutrophil entry into tumor-draining lymph nodes following induction of sterile inflammation. *J. Immunol. Baltim. Md 1950* **191**, 4348–4357 (2013).

417. Pepper, C. *et al.* Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers. *Blood* **112**, 3807–3817 (2008).
418. Schena, M. *et al.* The role of Bcl-2 in the pathogenesis of B chronic lymphocytic leukemia. *Leuk. Lymphoma* **11**, 173–179 (1993).