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Jérôme Moreaux

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**ROLE DES MEMBRES DE LA
FAMILLE BAFF/APRIL DANS LE
MYELOME MULTIPLE :
IMPLICATIONS
PHYSIOPATHOLOGIQUES ET
INTERET THERAPEUTIQUE**

Thèse présentée pour obtenir le grade de
DOCTEUR DE L'UNIVERSITE MONTPELLIER I

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Formation doctorale: Biologie-Santé
Spécialité: Immunologie

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Résumé :

Le myélome multiple (MM) est une néoplasie B caractérisé par l'accumulation d'un clone plasmocytaire dans la moelle osseuse. Cette pathologie demeure incurable d'où la nécessité d'identifier de nouvelles cibles thérapeutiques. C'est notamment dans cette optique que nous avons initié, au sein du laboratoire, un travail de comparaison des profils d'expression génique des plasmocytes tumoraux purifiés de malades avec ceux de plasmocytes normaux et de lymphocytes B, ce qui permettra l'identification de nouvelles voies importantes pour la biologie du MM et donc de nouvelles cibles thérapeutiques potentielles.

Par cette approche, nous avons mis en évidence un rôle essentiel des membres de la famille BAFF/APRIL et de leurs récepteurs (BCMA, BAFF-R et TACI) dans la biologie du MM. Les cellules de MM expriment les récepteurs alors que les ligands sont principalement produits par les cellules de l'environnement médullaire. L'utilisation d'un inhibiteur spécifique de BAFF/APRIL a permis de montrer que ces facteurs de croissance sont importants pour la survie et la prolifération des cellules tumorales. TACI apparaît être le récepteur principal pour médier l'effet de BAFF et APRIL dans le MM. Une forte expression de TACI par les cellules de MM est associée à une signature génique de plasmocytes matures alors que les plasmocytes tumoraux présentant une faibles expression de TACI ont une signature génique de plasmablastes proliférants. Nous avons montré que syndecan-1, un protéoglycane à chaînes héparane sulfate joue un rôle essentiel dans la biologie du MM en permettant l'accumulation de fortes concentrations de facteurs de croissance à la surface des cellules. Nous avons identifié que syndecan-1 joue un rôle de corécepteur pour APRIL et TACI supportant ainsi la croissance des cellules de MM.

Ces travaux offrent de nouvelles perspectives thérapeutiques pour le MM et ont débouché sur un essai clinique de phase I/II, au CHU de Montpellier, utilisant un inhibiteur de la voie BAFF/APRIL dans le MM.

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Liste des principales abréviations

Ac	Anticorps
APRIL	A proliferating-inducing ligand
AREG	Amphiregulin
BAFF	B-cell activating factor
BCMA	B-cell maturation antigen
BMPC	Plasmocyte mature (Bone marrow plasma cell)
CMH	Complexe Majeur d'Histocompatibilité
CCR	Chemokine, CC motif, receptor
CVID	Immunodéficience commune variable
CXCR	CXC chemokine Receptor
DKK1	Dickkopf1
EGF	Epidermal growth factor
Erk	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FISH	Fluorescent in situ hybridization
Frzb	Frizzled-related protein
FTase	Farnesyltransferase
FTI	Inhibiteur de farnesyltransferase
HBEGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte Growth Factor
HS	Héparane sulfate
ICAM	Intercellular cell adhesion molecule
IFN	interferon
Ig	Immunoglobuline
IGF	Insulin like growth factor
IL, IL-R	Interleukine, récepteur d'interleukine
JAK	Janus kinase
KO	Knock out
LB	Lymphocyte B

LT	Lymphocyte T
MAPK	Mitogen activated protein kinase
MGUS	Monoclonal gammopathy of unknown signification
MIP	Macrophage Inflammatory Protein
MM	Myélome Multiple
NF κ B	Nuclear factor κ B
PR	Polyarthrite rhumatoïde
PCL	Plasma cell leukaemia
PI-3K	Phasphatidylinositol-3 kinase
PPC	Polyclonal Plasma Cell
RANKL	Receptor activator of NF- κ B ligand
RC	Réponse Complète
SjS	Syndrome de Sjögren
SLE	Lupus érythémateux systémique
SDF-1	Stromal cell Derived Factor-1
STAT	Signal transducer and activator of transcription
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TGF	Trandforming growth factor
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

INTRODUCTION

I- Généralités

Le myélome multiple (MM) est une néoplasie B caractérisée par l'accumulation d'un clone plasmocytaire tumoral dans la moelle osseuse. Le MM représente 1% des nouveaux cas de cancers et 10 à 15% des hémopathies malignes. On dénombre 15000 nouveaux cas par an en Europe et environ le même nombre aux Etats-unis. L'âge médian au diagnostic est de 67 ans. Malgré une thérapeutique utilisant de hautes doses de chimiothérapie, supportées par une autogreffe de cellules souches hématopoïétiques, le MM reste une néoplasie constamment fatale dont la médiane de survie n'excède pas 6 ans. Le tableau clinique du MM se caractérise par des lésions osseuses lytiques, une anémie, des infections récurrentes et une atteinte rénale. Ces différents symptômes sont dus à l'envahissement médullaire par le plasmocyte tumoral myélomateux sécrétant une immunoglobuline (Ig) monoclonale, et à une forte production de cytokines pro inflammatoires, notamment l'interleukine 6 (IL-6). Nous envisagerons ici les principaux mécanismes qui concourent au développement de cette pathologie, incluant les anomalies génétiques propres au compartiment tumoral ou les modifications du microenvironnement médullaire favorisant la croissance des cellules myélomateuses.

I-1. Origine de la cellule tumorale myélomateuse

La différenciation lymphocytaire B normale commence dans la moelle osseuse avec le stade lymphocyte pro-B où se déroule le réarrangement des gènes des chaînes lourdes d'immunoglobulines (d'abord D_H-J_H puis $V_H-D_HJ_H$), 2) suivi du stade

lymphocyte pré-B défini par l'apparition à la membrane d'un pré-BCR (B-cell receptor). C'est également à ce stade qu'a lieu le réarrangement des chaînes légères (V_L - J_L). Le stade suivant est le lymphocyte B immature qui exprime un BCR sous forme d'IgM membranaires associées aux chaînes Iga/Igb. Puis, le stade lymphocyte B mature naïf caractérisé par la coexpression d'IgD et d'IgM membranaires. Les lymphocytes B naïfs migrent ensuite de la moelle dans le sang périphérique jusqu'aux organes lymphoïdes secondaires où, au contact de l'antigène (Ag), ils vont pouvoir se différencier en lymphocytes B mémoires ou en cellules plasmocytaires immatures de type plasmablastique. Au sein des organes lymphoïdes secondaires, les lymphocytes B naïfs rencontrent l'Ag puis interagissent avec des lymphocytes T et vont initier la formation de centres germinatifs nécessaires à l'obtention de plasmocytes. Au sein des centres germinatifs, les lymphocytes B activés subissent tout d'abord une très forte prolifération et des mutations somatiques des régions hypervariables des gènes des chaînes lourdes et légères d'Ig (stade centroblaste). Puis les lymphocytes B cessent de proliférer (centrocytes). Seuls les centrocytes exprimant une Ig de forte affinité pour l'Ag sont sélectionnés de façon positive. Les autres sont éliminés par apoptose. Après sélection, la commutation isotypique permet de passer d'une IgM à une IgG, une IgA ou une IgE membranaire. Enfin, les lymphocytes B se différencient en lymphocytes B mémoires ou en plasmablastes qui rejoindront la moelle osseuse. Une fois dans la moelle osseuse, les plasmablastes vont se différencier en plasmocytes matures synthétisant de grandes quantités d'Ig. Cependant, la différenciation intramédullaire des plasmablastes en plasmocytes matures reste à l'heure actuelle peu connue.

L'origine du clone tumoral est une cellule tardive de la différenciation lymphocytaire B. En effet, le plasmocyte tumoral produit des Ig d'isotype IgG ou IgA, rarement IgD

ou IgE, donc la cellule tumorale myélomateuse a subi le phénomène de commutation isotypique. De plus, l'analyse des séquences des gènes variables des chaînes lourdes et légères d'Ig des plasmocytes tumoraux a montré un taux élevé de mutations tout au long de la maladie (1, 2). Ces données mettent en évidence l'origine lymphoïde post-folliculaire des cellules de MM. Bien que le MM se caractérise par l'accumulation d'un clone plasmocytaire dans la moelle osseuse, la majorité des études évaluant l'index de prolifération plasmocytaire révèlent qu'il excède rarement 1%. Il existerait donc une fraction proliférante représentative de la « cellule souche myélomateuse ».

I-2. Anomalies cytogénétiques du MM

Hallek *et al.* proposent un modèle multi-étape de l'oncogenèse du MM (3). Les cinq stades en sont le **plasmocyte normal**, les **gammopathies monoclonales de signification indéterminées (MGUS)** qui évoluent dans 16% des cas vers un **myélome à localisation médullaire** puis vers des **formes extramédullaires** terminales à partir desquelles ont été obtenues toutes les **lignées** tumorales. L'instabilité caryotypique est une caractéristique du MM (4). Par une analyse cytogénétique des chromosomes en métaphase, des anomalies chromosomiques peuvent être détectées chez 30 % des patients au diagnostic (5). L'utilisation du FISH sur les plasmocytes tumoraux permet de détecter des anomalies chromosomiques chez les patients au moment du diagnostic (5). Comme représenté dans la figure 1, une division en deux groupes de myélomes se recoupant

partiellement a été proposé en se basant sur l'analyse cytogénétique des chromosomes en métaphase :

- Les myélomes non hyperdiploïdes (comprenant les myélomes hypodiploïdes, pseudodiploïdes, et tetraploïdes) caractérisés des translocations récurrentes mettant en jeu les régions des chaînes lourdes d'Ig (IgH) sur le chromosome 14q32.3 et par une incidence importante de la délétion du chromosome 13q (6, 7).
- Les myélomes hyperdiploïdes associés à des trisomies multiples mettant en jeu les chromosomes 3, 5, 7, 9, 11, 15, 19 et 21 mais avec des incidences plus faibles de translocations IgH et de délétions du chromosome 13q (6, 7).

Les translocations mettant en jeu les gènes d'IgH se retrouvent chez 50% des patients atteints de MGUS, 55-70% des patients avec un myélome médullaire, 80% des patients avec une leucémie à plasmocytes (PCL) primaire et 90% des lignées de MM (8, 9). Les translocations affectant les gènes d'Ig légères (IgL) sont moins fréquentes (< 20%) et impliquent le locus IgL- λ la plupart du temps. Les partenaires chromosomiques ont été identifiés dans 40% des cas et inclus :

- Le chromosome 11q13, engendrant une dérégulation de la cycline D1 (CCND1) et de MYEOV chez 16% des patients.
- Le chromosome 4p16, marqué par une dérégulation de l'expression de FGFR3 et MMSET chez 15% des patients.
- Le chromosome 16q22-23, avec une dérégulation de l'expression de c-maf chez 5% des patients.
- Le chromosome 6p21, caractérisé par une dérégulation de la cycline D3 (CCND3) chez 4% des patients.

Les translocations impliquant les gènes IgH définissent des entités distinctes de MM avec des aspects cliniques, morphologiques, immunophénotypiques et des implications thérapeutiques différentes (5, 10, 11). Tout particulièrement la translocation t(4 ;14)(p16.3 ;q32.3) confère un mauvais pronostic même après une chimiothérapie intensive supportée par une autogreffe de cellules souches hématopoïétiques (12). Les translocations impliquant les gènes IgH sont également trouvées chez les patients atteints de MGUS suggérant que ce sont des événements précoces (5). Récemment un modèle de l'évolution clonale du MM basée sur les résultats du FISH en interphase a été proposé (8). Suivant ce modèle, les translocations t(11 ;14), les caryotypes hyperdiploïdes et non hyperdiploïdes représentent des sous-groupes distincts de la maladie. De plus les patients avec un gain du chromosome 1q21, caractérisé par un taux de B2M significativement plus élevé et un niveau d'hémoglobine plus faible, peuvent représenter un groupe avec un mauvais pronostic. Depuis, le mauvais pronostic associé au gain du chromosome 1q21 dans le MM a été confirmé par d'autres équipes (13-16). Les délétions des chromosomes 22q11, 8p12, 6q21 et 17p13 (locus TP53) sont des événements tardifs apparaissant lors de la progression de la maladie, mais ne définissant pas de sous-groupes de MM (8).

La délétion du chromosome 13q14 se retrouve chez 50% des patients atteints de MM au diagnostic. Cette abération chromosomique a été à plusieurs reprises décrite associée à un mauvais pronostic (5, 12).

La délétion du chromosome 17p13 se retrouve chez environ 10 à 30% des malades atteints de MM au diagnostic (8, 17). La délétion du chromosome 17p13 confère un mauvais pronostic pour les patients traités par une chimiothérapie conventionnelle. De plus, la délétion du 17p13 a également été rapporté comme un mauvais facteur

pronostique chez les patients traités par chimiothérapie intensive et autogreffe de cellules souches hématopoïétiques (12, 18).

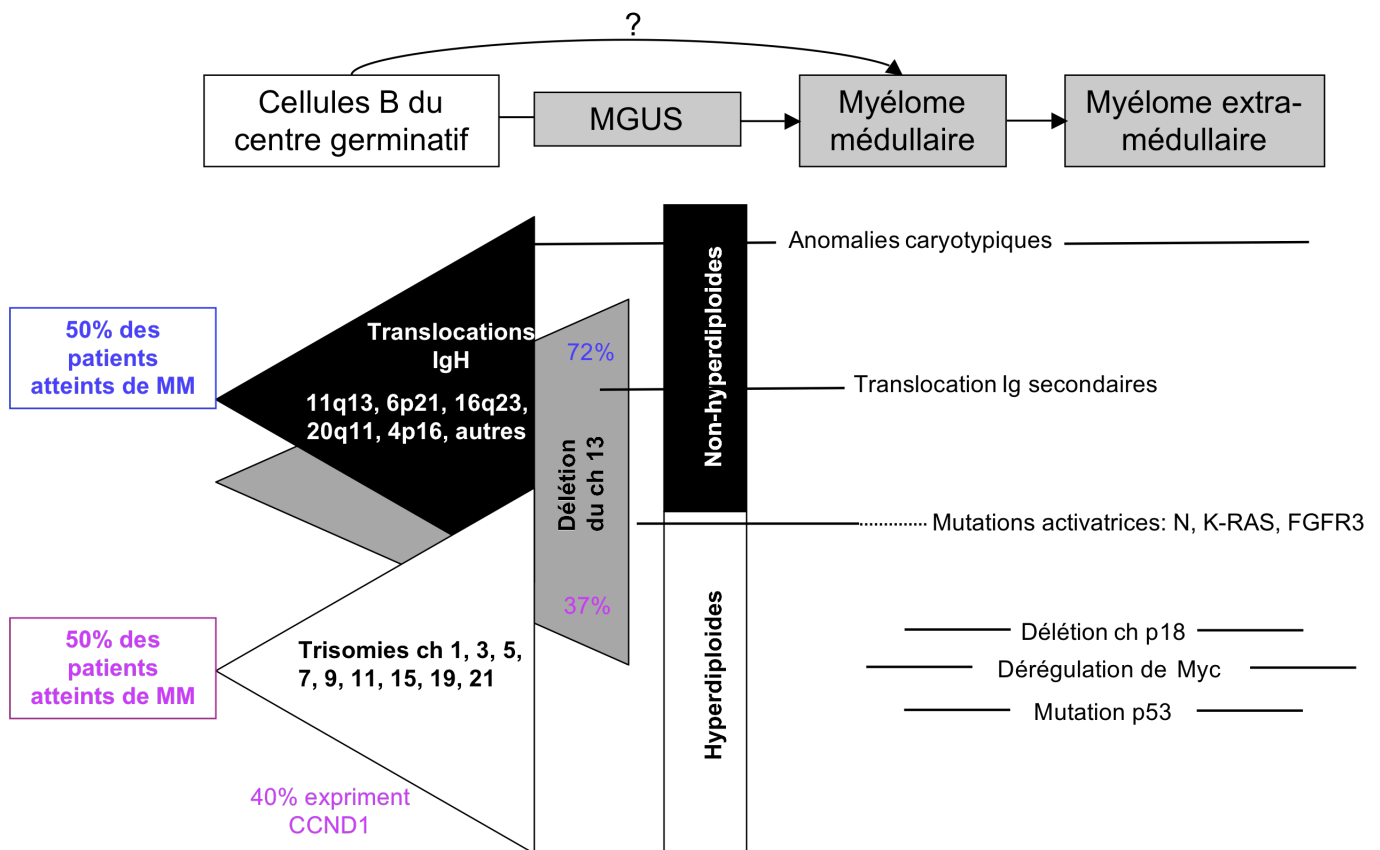


Figure 1 : Stades de la maladie et évènements oncogéniques (Adapté d'après Bergsagel et al (19)).

Les évènements oncogéniques les plus précoces sont présents dans les MGUS et comporte deux catégories : les translocations IgH et les trisomies. Chacun de ces deux groupes peut présenter une délétion du chromosome 13. Les autres anomalies caryotypiques incluant les translocations secondaires et les changements épigénétiques peuvent se produire à tous les stades. Les mutations activatrices de K- ou N-RAS apparaissent marquer, à défaut d'en être une cause, la transition entre le stade MGUS et le MM, mais parfois surviennent durant la progression du MM. Les évènements oncogéniques plus tardifs, qui apparaissent lorsque les tumeurs sont plus agressives, incluent la dérégulation de MYC, la délétion bi-allélique de p18, l'inactivation de Rb et la perte ou la mutation de p53.

On retrouve des mutations de RAS chez 35 à 50% des patients atteints de MM au diagnostic (5). Les mutations de RAS sont rares chez les patients atteints de MGUS et augmentent au cours de la progression de la maladie suggérant qu'elles contribuent à la progression du MM.

I-3. Les traitements du myélome multiple

Le traitement de référence du MM demeure la chimiothérapie standard associant melphalan et corticoïdes. La médiane de survie des patients avec une chimiothérapie standard est d'environ 30 à 36 mois, avec 5% de rémission complète (RC) (les critères de réponse aux traitements sont définis dans la table 1) (20).

Table 1. Critères de réponse aux traitements.

Rémission complète	- disparition complète de l'Ig monoclonale sérique et urinaire - plasmocytose médullaire < 5% sans plasmocytes dystrophiques
Rémission partielle	- diminution du taux d'Ig monoclonale sérique de plus de 50% - diminution du taux de protéinurie monoclonale de plus de 75% - absence de nouvelles lésions ostéolytiques et calcémie normale - correction au moins partielle des douleur osseuse et de l'anémie
Échec	- variation du taux d'Ig monoclonale ne dépassant pas 25% - variation du taux de protéinurie monoclonale ne dépassant pas 50% - stabilité des autres paramètres
Progression	Présence d'un ou plusieurs des signes suivants - augmentation du taux d'Ig monoclonale sérique de plus de 25% - augmentation du taux de protéinurie monoclonale de plus de 75% - apparition de plusieurs lésions ostéolytiques - progression de l'anémie et de l'hypercalcémie - apparition de localisations plasmocytaires extra osseuses

L'intensification de la chimiothérapie associée à une **autogreffe** de moelle osseuse ou de cellules souches périphériques (CSP), a permis d'améliorer significativement le taux de RC (25%) et la médiane de survie (> 50 mois) (21, 22). L'introduction des

biphosphonates de seconde génération dans le traitement du MM permet de réduire les atteintes osseuses des patients mais sans allonger la survie globale (23). Une possibilité attrayante pour éliminer toutes les cellules tumorales est l'**allogreffe** de cellules souches hématopoïétiques et de lymphocytes T. Ce traitement a été proposé chez les patients âgés de moins de 50 ans et aboutit à un taux de réponse de 35% (24, 25). Ce traitement reste néanmoins entaché d'un mortalité et d'une morbidité sévères dues à l'effet de réaction du greffon contre l'hôte (GVH) et à la pancytopénie induite par le traitement myéloablatif. L'effet du greffon contre les cellules de MM (GVM) a été démontré, suggérant une action des lymphocytes T cytotoxiques du donneur contre les cellules myélomateuses (26). Plus récemment, la diminution de l'intensité du conditionnement par des « mini allogreffes » permet de diminuer la morbidité de la greffe allogénique et permet d'étendre l'utilisation des allogreffes à des patients plus âgés. Des études plus récentes ont mis en évidence qu'une autogreffe suivie d'une allogreffe conditionnée par un traitement non myéloablatif permet d'obtenir des taux de réponse de l'ordre de 80% et un taux de morbidité relativement faible (11% au jour 100) (27, 28).

III- Biologie du myélome multiple

)II-1. Interaction des cellules tumorales avec leur environnement

Les plasmocytes tumoraux, comme les plasmocytes normaux, se localisent dans la moelle osseuse où ils entretiennent une communication étroite avec les cellules de l'environnement médullaire qui est essentielle pour la progression de la maladie.

Dans la moelle osseuse, ils coexistent et interagissent avec des **cellules stromales**, les protéines de la matrice extracellulaire, les ostéoblastes, les ostéoclastes, les cellules endothéliales, les monocytes, les polynucléaires et les lymphocytes. Ces interactions vont permettre le recrutement des cellules myélomateuses au sein de la moelle et créer un environnement optimal pour la survie et la prolifération des plasmocytes tumoraux.

A. Chimiockines et localisation médullaire des plasmocytes tumoraux

La différenciation lymphocytaire B est un processus dynamique durant lequel le lymphocyte B exprimant une Ig de forte affinité va quitter le centre germinatif des organes lymphoïdes secondaires pour migrer vers la moelle osseuse qui est le site principal des plasmocytes matures. Le récepteur CXCR4 et son ligand SDF-1 jouent un rôle majeur dans la localisation médullaire des plasmocytes. CXCR4 est exprimé par les plasmocytes normaux et tumoraux (29, 30).

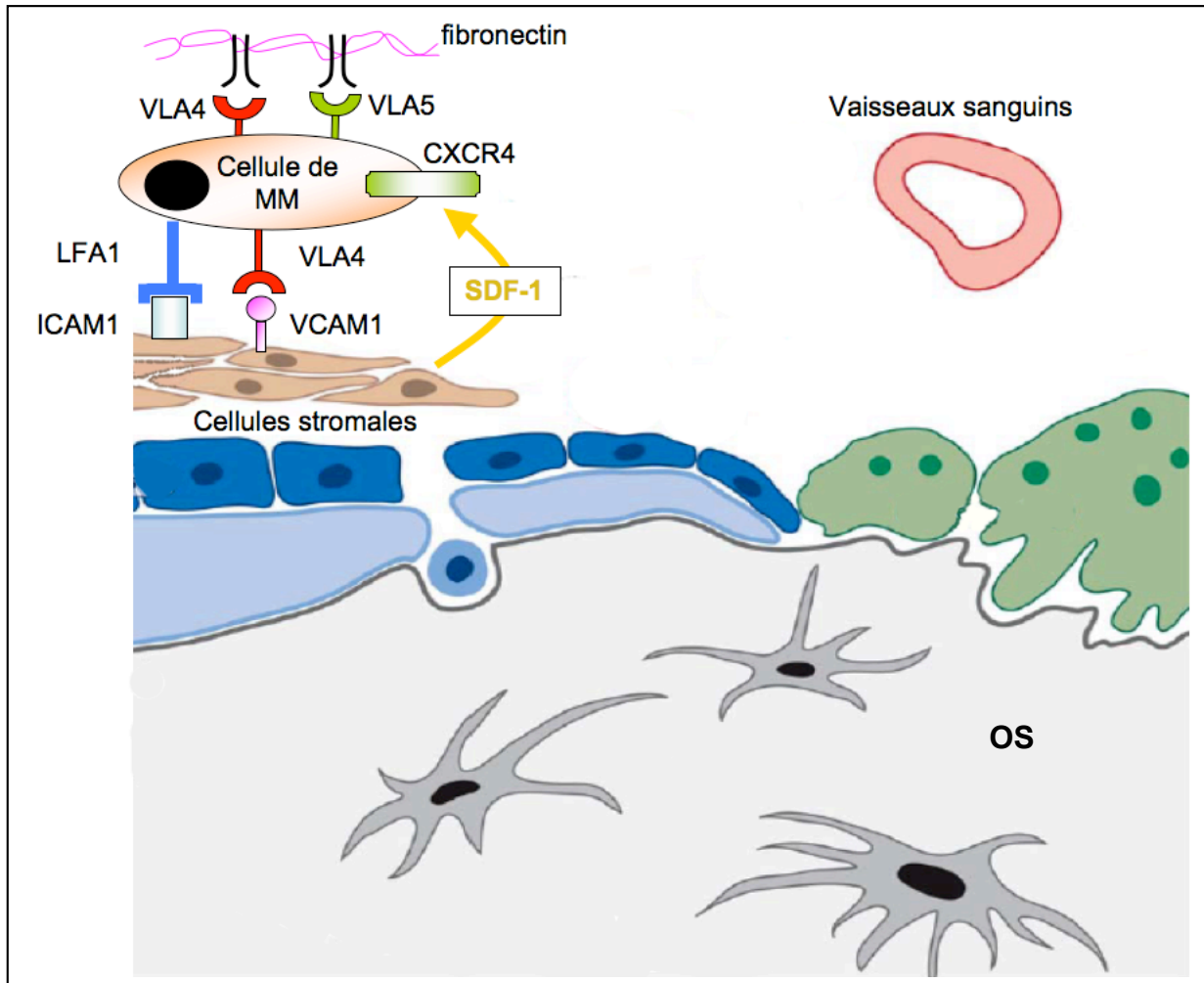


Figure 2 : Molécules d'adhésion et chimiokines dans le MM.

B. Environnement médullaire et myélome multiple

L'environnement médullaire est composé de différentes populations cellulaires comprenant les cellules souches hématopoïétiques, les lymphocytes B et T, les macrophages, les cellules NK, les érythrocytes, les cellules stromales médullaires (BMSC), les cellules endothéliales, les monocytes, les polynucléaires neutrophiles, les ostéoclastes et les ostéoblastes. Ces contacts cellulaires (CAM-DR) et les facteurs de croissance présents dans le milieu médullaire entraînent une résistance des cellules myélomateuses aux thérapies conventionnelles du MM. La liste

grandissante des facteurs de croissance myélomateux comprend l'IL-6, l'insulin-like growth factor (IGF)-1, l'hepatocyte growth factor (HGF), les membres de la famille EGF (epidermal growth factor), l'IL-1 β , vascular endothelial growth factor (VEGF), stromal cell-derived growth factor (SDF)-1, tumor necrosis factor (TNF-) α , macrophage inflammatory protein (MIP-1a), Wnt et les membres de la famille Notch.

C. Les cellules stromales médullaires (BMSC)

Les interactions entre les cellules myélomateuses et les cellules stromales induisent une cascade de voies de signalisation impliquées dans la survie et la prolifération : phosphatidylinositol-3 kinase (PI3K)/Akt ; I kappa B kinase (IKK)/ nuclear factor kappa-B (NF κ B) ; Ras/Raf/mitogen-activated protein kinase (ERK) ; and Janus kinase (JAK) 2/signal transducers and activators of transcription (STAT) 3. Ceci conduit à une induction de l'expression des cyclines D, des protéines antiapoptotiques de la famille Bcl-2 et de l'activité de la télomérase (31-33). Ces événements cellulaires sont médiés par les interactions directes des cellules de MM avec les BMSC (34) ou indirectement par les facteurs de croissances sécrétés par les BMSC et/ou les cellules de MM. Beaucoup de ces facteurs de croissances sont responsables d'effets pléiotrophiques en stimulant l'ostéoclastogénèse (IL-6, IL-1, VEGF, SDF-1 α et MIP-1 α) ou en modulant l'expression des molécules d'adhésion sur les cellules de MM et les BMSC (TNF α) (35). Les interactions entre les cellules de MM et les BMSC supportent la croissance, la survie et la migration des cellules de MM et contribuent également à la progression de la maladie et à la résistance des cellules de MM aux traitements conventionnels.

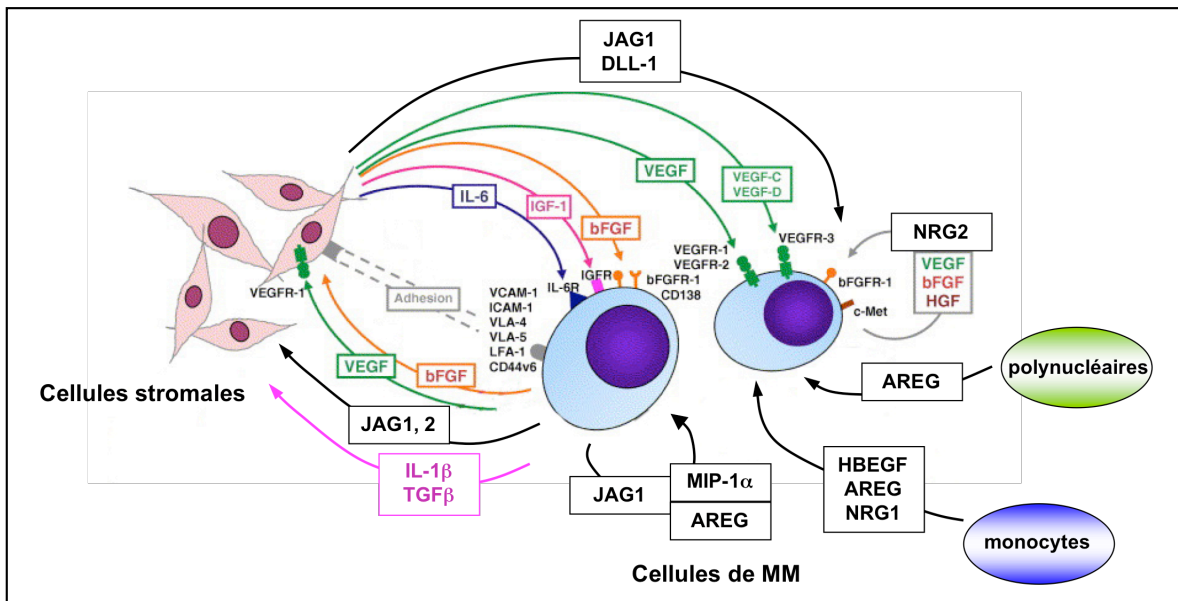


Figure 3 : Facteurs de croissance myélomateux.

D. Cellules endothéliales médullaires

L'angiogénèse supporte la croissance des cellules tumorales en délivrant l'oxygène et les nutriments. Le rôle de l'angiogénèse dans la croissance, la progression et la dissémination métastatique des tumeurs solides est bien établie. De plus, la progression de certains cancers hématologiques, incluant les lymphomes non hodgkiniens, les leucémies lymphoblastiques, les leucémies lymphocytaires chroniques, les leucémies myéloïdes et le MM est aussi corrélé avec le degré d'angiogénèse. Il a été démontré que l'angiogénèse médullaire est un marqueur de la progression du MM et est corrélée avec l'activité de la maladie. L'augmentation de la densité des microvaisseaux médullaires chez les patients atteints de MM est un facteur de mauvais pronostic (36) et la densité en microvaisseaux médullaires au diagnostic est un facteur pronostique important pour la survie des patients traités par

chimiothérapie intensive et autogreffe. L'angiogénèse médullaire est supportée en partie par le VEGF, le basic fibroblast growth factor (bFGF) et les métalloprotéinases (MMP) sécrétées par les cellules de MM (36). De plus, les cellules endothéliales médullaires sécrètent des facteurs de croissances incluant le VEGF et bFGF. Ce rôle majeur de l'angiogénèse dans le MM a conduit à l'utilisation thérapeutique de la thalidomide, connue pour ses propriétés anti-angiogéniques (37). La thalidomide inhibe la sécrétion du VEGF, bFGF et d'HGF ainsi que la prolifération des cellules endothéliales et la formation de capillaires sanguins (38, 39). Des études récentes démontrent que la diminution de la densité des microvaisseaux médullaires se produit uniquement chez les patients répondeurs à la thalidomide (40), suggérant que l'angiogénèse médullaire est une cible thérapeutique du MM.

E. Les ostéoclastes

La lyse osseuse est un des signes cliniques majeur du MM, avec des lésions osseuses présentes chez 70 à 80% des patients. L'os est un tissu en perpétuel renouvellement. Les plasmocytes tumoraux sont, dans les travées osseuses, en contact étroit avec l'os. Les cellules de MM vont entraîner des lésions osseuses en bouleversant l'équilibre entre les ostéoclastes et les ostéoblastes. En effet, les cellules de MM stimulent la différenciation, l'activité et/ou le recrutement des ostéoclastes et inhibent la différenciation ou l'activité des ostéoblastes.

L'interaction du récepteur activateur de $\text{NF}\kappa\text{B}$ (RANK) à la surface des ostéoclastes avec le RANK ligand (RANKL) exprimé par les ostéoblastes et les BMSC, ainsi que $\text{MIP-1}\alpha$ sécrété par les cellules myélomateuses, jouent des rôles importants dans la destruction osseuse du MM (41). A l'opposé, les BMSC produisent l'ostéoprotégérine (OPG), qui prévient l'activation excessive des ostéoclastes en servant de récepteur

compétiteur pour RANKL. Le blocage de la fixation de RANKL à RANK soit par le forme soluble de RANK, soit par l'OPG inhibe la maturation des ostéoclastes et la destruction de l'os chez un modèle murin du MM (42, 43). Par contre, l'interaction de VCAM-1 (vascular cell adhesion molecule 1) exprimé à la surface des BMSC avec l'intégrine $\alpha4\beta1$ exprimé par les cellules de MM diminue la sécrétion d'OPG et induit l'expression de RANKL, provoquant ainsi l'ostéolyse (44, 45). Les cellules de MM sont aussi capables de séquestrer l'OPG en l'internalisant et le dégradant dans les compartiments lysosomaux. Ce mécanisme passe par une interaction avec les chaînes héparane sulfate du protéoglycane syndecan-1 (46). Indépendamment de RANKL, MIP-1 α est un puissant inducteur de la différenciation ostéoclastique (47). Les taux de MIP-1 α chez les patients atteints de MM sont élevés et corrélés avec les lésions osseuses. De plus, un antisens de MIP-1 α induit une diminution des lésions osseuses dans un modèle murin du MM (48). Plus récemment, d'autres facteurs capables de stimuler l'activité des ostéoclastes dans le MM ont été décrits, comme l'IL-3 (49) ou SDF-1 (50). Par ailleurs, notre équipe a montré que le gène codant pour Gas6, le ligand du récepteur tyro-3, est surexprimé par les cellules de MM (51). Tyro3 est exprimé par les ostéoclastes matures et est connu pour être impliqué dans leur activation (52). Gas6 pourrait jouer un rôle critique dans l'augmentation des lésions osseuses dans le MM.

De façon importante, les ostéoclastes sont également capables de supporter efficacement la croissance des cellules de MM *in vitro* (53, 54). Les ostéoclastes sécrètent des facteurs de croissances myélomateux incluant l'IL-6. Une publication récente suggère un rôle important de la chondroïtine synthase-1 (CHSY1) (55). CHSY1 se retrouve en quantité importante dans les surnageants de co-culture cellules de MM/ostéoclastes. L'inhibition de l'expression de CHSY1 par ARN

interférence entraîne une diminution des taux de CHSY1 dans le milieu de co-culture et une induction de l'apoptose des cellules de MM. CHYS1 possède un domaine Fringe, connu pour inhiber l'activité du récepteur Notch. Ce dernier pourrait donc jouer un rôle important dans la communication des cellules de MM avec les ostéoclastes.

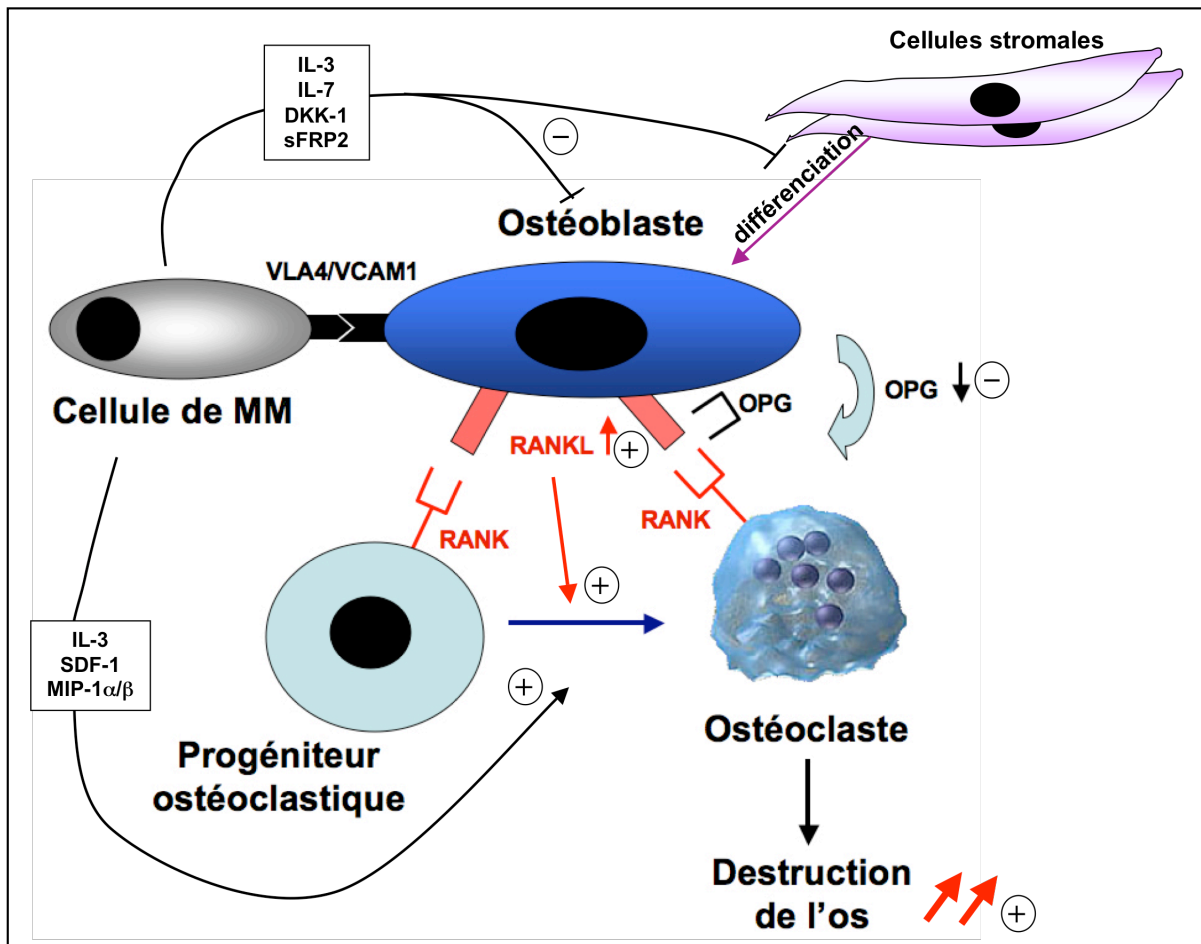


Figure 4 : Résorption osseuse dans le MM.

F. Les ostéoblastes

Des études histologiques ont montré que les ostéoblastes sont moins nombreux et moins actifs chez les patients avec des lésions osseuses importantes. L'équipe de Shaughnessy a démontré que les cellules de MM produisent DKK1 et qu'une forte expression de DKK1 est corrélée à la présence de lésions osseuses (56). DKK1

inhibe la voie Wnt et inhibe la différenciation des BMSC en ostéoblastes. Cependant, une autre étude démontre qu'un autre inhibiteur de la voie Wnt : sFRP2, est produit par les cellules de MM et bloque la différenciation ostéoblastique. Dans cette étude, les auteurs n'observent pas d'inhibition de la différenciation ostéoblastique avec la protéine recombinante DKK1 (57). L'IL-3 jouant un rôle activateur pour les ostéoclastes, serait également capable de bloquer la différenciation ostéoblastique (58). De plus, un blocage de la différenciation ostéoblastique a été observé dans des cocultures de cellules de MM avec des BMSC. Cet effet serait médié par l'IL-7 qui entraîne l'inhibition du facteur de transcription RUNX2/CBFA1 critique pour la différenciation ostéoblastique (59). Un autre inhibiteur de la voie Wnt : Frzb/sFRP3 est fortement exprimé par les cellules de MM (60) et pourrait ainsi jouer un rôle dans la lyse osseuse.

G. Les facteurs de croissance

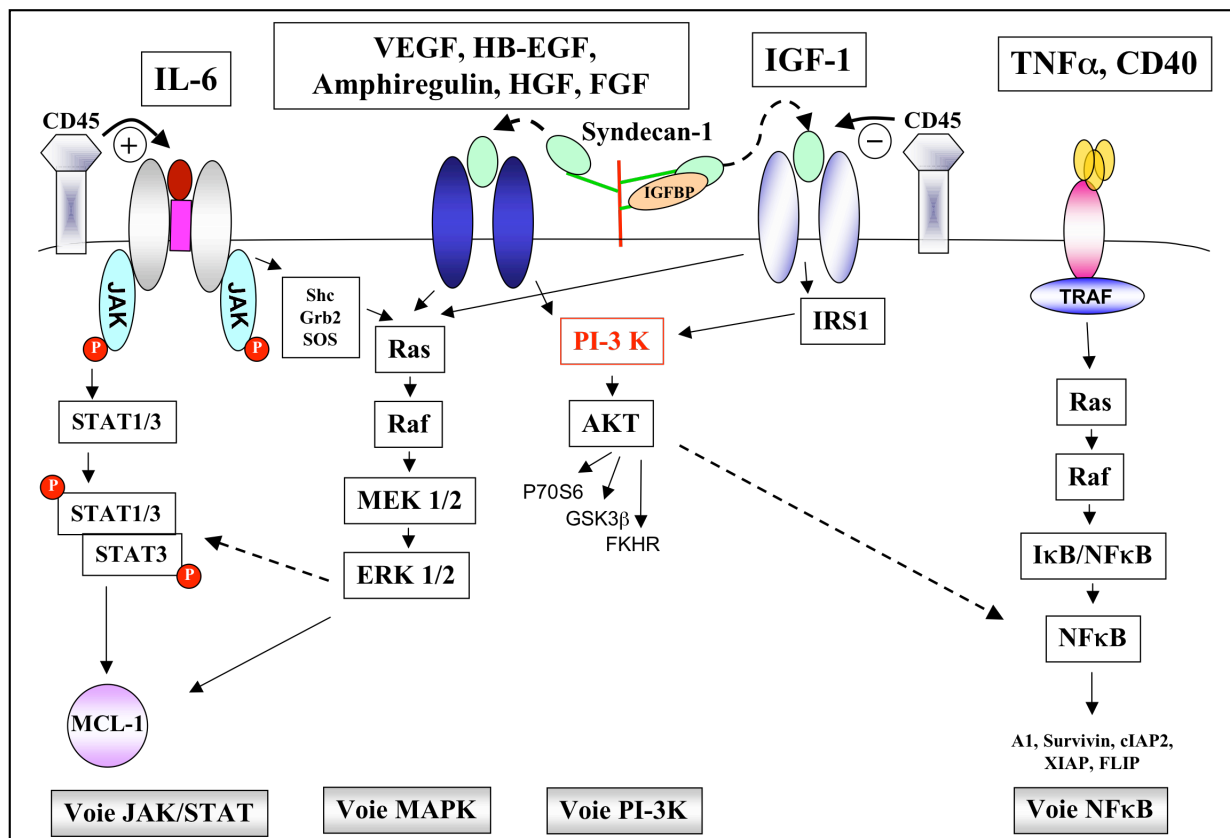


Figure 5 : Principales voies de signalisation impliquée dans la croissance des cellules myélomateuses. L'IL-6 est le seul facteur qui active la voie JAK/STAT. Les autres facteurs de croissance activent les voies PI3K, MAPK et NFκB. Les flèches en pointillées indiquent une communication possible entre les voies de signalisation.

- L'interleukine-6 (IL-6)

L'IL-6 est le facteur de croissance jouant un rôle central dans la physiopathologie du MM (61, 62). L'IL-6 permet la croissance *in vitro* de plasmocytes tumoraux en phase extramédullaire de la maladie et, dans certains cas, l'obtention de lignées de MM (63). La prolifération *in vitro* des plasmocytes tumoraux de malades en phase chronique est bloquée par des Ac anti-IL-6. L'administration d'Ac anti-IL-6 a des effets anti-tumoraux et un taux élevé d'IL-6 circulante, évalué par le dosage de la C-réactive protéin (CRP), est un facteur de mauvais pronostic (64, 65). L'IL-6 inhibe *in vitro* l'apoptose des cellules de MM induite par les agents de chimiothérapie conventionnels comme la dexaméthasone. L'IL-6 interagit avec son récepteur membranaire, la gp80, exprimé constitutivement à la surface des cellules myélomateuses ou avec la forme soluble, sIL-6R, de poids moléculaire 55 kD, qui a un effet agoniste. L'association IL-6/IL-6-récepteur est capable d'entraîner l'homodimérisation de la chaîne transductrice du signal gp130 et d'activer ainsi une cascade de signaux comportant notamment les voies Jak/STAT et ras/MAP kinases. D'autres cytokines utilisant la chaîne gp130, le ciliary neurotrophic factor (CNTF), l'oncostatine M (OSM) ou l'IL-11, peuvent stimuler la croissance de lignées de MM *in vitro* mais les taux circulants de ces cytokines sont extrêmement faibles par rapport au taux d'IL-6 (66). Notre équipe a démontré que l'IL-6 est un facteur de croissance essentiellement paracrine, produit principalement par l'environnement médullaire (62). Chez certains patients, on retrouve une faible production d'IL-6 autocrine par

les cellules tumorales. Cette production d'IL-6 autocrine est associée à un phénotype agressif caractérisé par un index de prolifération élevé et une résistance accrue aux drogues. Dans la moelle osseuse, les BMSC sont la principale source d'IL-6 (3, 67). La transcription et /ou la sécrétion d'IL-6 par les BMSC sont induites par l'adhésion des cellules de MM aux BMSC par l'intermédiaire des molécules d'adhésion VLA4 et LFA1 et de leurs ligands VCAM et ICAM (68). Dans ce cas, l'induction de la production d'IL-6 passe par la voie NF- κ B. Des facteurs de croissances produits principalement par les plasmocytes tumoraux sont également capables d'induire la production d'IL-6 par les BMSC. Parmi ces facteurs, on compte l'IL-1 β , via les PGE2 (69), le TGF- β (70), le TNF- α (71), le VEGF et le FGF (72, 73).

Les taux sériques d'IL-6 et d'IL-6R sont des facteurs pronostiques du MM et sont un reflet de la fraction proliférante de cellules tumorales chez les patients. Des stratégies visant à bloquer l'IL-6 ont été développées avec des anticorps anti-IL-6, anti-IL-6R ou un antagoniste de l'IL-6 (Sant7) qui se fixe à l'IL-6R sans activer les voies de signalisation en aval (74). Bien qu'une activité anti-tumorale ait été observée, cette approche thérapeutique reste encore aujourd'hui limitée par le fait qu'il est difficile de bloquer à long terme l'IL-6 produite en trop grande quantité.

- Insulin-like growth factor-1 (IGF-1)

L'IGF-1 régule la prolifération, la différenciation et l'apoptose des cellules (33, 75). Dans la circulation sanguine, l'IGF-1 se fixe essentiellement à l'IGFBP-3 (IGF binding protein 3). Des études montrent qu'une forte concentration d'IGF-1 circulant est associée avec un risque accru de cancer de la prostate, du sein, du poumon et du colon alors qu'un taux élevé d'IGF1BP-3 est associé à un risque réduit (75). Cependant aucune relation directe entre le taux sérique d'IGF-1 chez les patients et

le pronostic du MM n'a pu être établi. Une étude met en évidence que le taux moyen d'IGF-1 chez les patients atteints de MM est le même que celui des sujets sains. Cependant, les patients un faible taux d'IGF-1 circulant (< 13 nM) ont une survie accrue par rapport à ceux présentant des taux plus élevés, ce qui consolide l'hypothèse d'un rôle de l'IGF-1 dans la progression de la maladie (76). Il a aujourd'hui été établi que les BMSC sont la principale source d'IGF-1 (77).

Des études récentes ont bien décrit le rôle de l'IGF-1 dans la biologie du MM. L'IGF-1 potentialise l'effet de l'IL-6 sur la prolifération et la survie des cellules de MM (78). L'IGF-1 active la voie de signalisation Ras/Raf/MAPK et la voie PI3K/Akt mais pas la voie JAK2/STAT3 contrairement à l'IL-6 (79). L'IGF-1 stimule l'activation des voies de signalisation PI3K/Akt et NF κ B (via PI3K/Akt), induit la phosphorylation du facteur de transcription FKHR (forkhead), induit une surexpression d'une série de protéines anti-apoptotiques incluant FLIP, la survivine, cIAP-2, A1/Bfl-1 et XIAP et diminue la sensibilité des cellules de MM aux drogues (80). De plus, l'IGF-1 stimule la production de cytokines angiogéniques (80, 81). Il a également été démontré que l'IGF-1 médie la migration des cellules de MM via l'activation de la voie de signalisation PI3K/Akt (82). L'IGF-1 inhibe l'apoptose induite par le dexaméthasone sans modifier les niveaux d'expression des protéines Bcl-2 ou Bcl-XL (33, 80). L'IGF-1 protège également les cellules de MM de l'apoptose induite par les agents de chimiothérapie et les inhibiteurs du protéasome (33, 80). L'IGF-1 induit l'activité de la télomérase dans les cellules de MM. Ceci est médié par l'activation de la voie PI3K/Akt et l'inhibition de la voie PI3K/Akt ou de la voie NF κ B bloque cet effet (31). Récemment, il a été démontré que la cavéoline 1, qui est généralement absente des cellules du sang, est exprimée par les cellules de MM et joue un rôle crucial dans les voies de signalisation médiées par l'IL-6 et l'IGF-1 (83). Un traitement par beta-

cyclodextrine qui détruit la structures des cavéoles et inhibe la fonction de la cavéoline, inhibe la survie des cellules de MM induite par l'IL-6 et l'IGF-1 (83). La tyrosine phosphatase CD45 régule de manière négative la phosphorylation d'AKT induite par l'IGF-1 en s'associant directement avec l'IGF-1R pour le déphosphoryler (84). L'expression de CD45 serait donc une signature de plasmocytes non sensibles à l'IGF-1. À l'opposé, le phénotype CD45+ est associé à une dépendance accrue à l'IL-6 (ou à une production d'IL-6 autocrine). De plus, seules les cellules de MM CD45- sont sensibles à l'inhibition de l'IGF-1 par un anticorps monoclonal (85). Les résultats des études précliniques d'inhibiteur de la voie IGF-1/IGF-1R dans le MM sont encourageants. Un inhibiteur spécifique de l'activité kinase de l'IGF-1R (NVP-ADW742), utilisé seul ou en combinaison avec les agents de chimiothérapie, a une activité anti-tumorale significative chez des souris ayant subi une xénogreffe de cellules de MM (33). Ces résultats ont été confirmés avec un autre type d'inhibiteur (Picropodophyllin ou PPP) dans le modèle murin du myélome 5T33MM (86, 87).

- Vascular endothelial growth factor (VEGF)

Le VEGF est connu comme un agent angiogénique chez les tumeurs solides et hématologiques (88). Dans le MM, le VEGF est produit à la fois par les cellules de MM et les BMSC (89, 90) et est responsable, du moins en partie, de l'augmentation de l'angiogénèse dans la moelle osseuse de patients atteints de MM. Cependant, un lien direct entre le taux de VEGF et le pronostic des patients atteints de MM n'est pas formellement établi.

Des études montrent que certaines lignées de MM et cellules tumorales de patients expriment le récepteur du VEGF (Flt-1). Dans ce cas, le VEGF induit la phosphorylation de Flt-1, l'activation de ERK et la prolifération des cellules de MM

(89, 90) qui peut être neutralisé par un anticorps anti-VEGF ou des inhibiteurs du récepteur au VEGF PTK787 (91) et GW654652 (92). Le VEGF induit la migration des cellules de MM et de façon plus efficace chez les patients avec une PCL que chez les patients avec un MM médullaire. Cette migration induite par le VEGF est associée à l'activation de la PI3K-dépendent protein kinase C α (PKC- α) (90) et est neutralisée par un anticorps anti-VEGF. Le VEGF induit aussi la phosphorylation Src-dépendante de la cavéoline-1 qui est requise pour la phosphorylation de p130^{Cas} et la migration des cellules de MM. L'inhibiteur du protéasome (Bortézomide) inhibe la phosphorylation de la cavéoline-1 induite par le VEGF, diminue de façon marquée l'expression de la cavéoline-1 et inhibe la migration des cellules de MM (93). Plus récemment, il a été démontré que le VEGF induit l'expression de Mcl-1 dans les lignées de MM et cellules primaires de patients. Cette induction de Mcl-1 est inhibée par un inhibiteur pan-VEGF (GW654652). Ceci est associé à une diminution de la prolifération et une induction de l'apoptose (94). L'effet direct du VEGF sur les cellules de MM couplé à l'induction de cytokines telle que l'IL-6 et de l'angiogénèse dans la moelle osseuse met en évidence un intérêt de l'inhibition du VEGF dans le traitement du MM. L'approche ayant montré le plus de succès pour cibler le VEGF est l'utilisation d'un anticorps monoclonal humanisé anti-VEGF (bevacizumab) qui a été récemment approuvé par l'agence du médicament américaine pour le traitement du cancer colorectal métastatique (95). Des études sont en cours dans le MM pour analyser l'effet du bevacizumab, avec ou sans thalidomide, chez des patients atteints de MM en rechute ou réfractaires au traitement (88). L'inhibiteur tyrosine kinase du récepteur au VEGF, GW654652, s'est montré également actif dans des études précliniques et va être évalué en clinique dans le MM (91, 92).

- Fibroblast growth factor (FGF)

Les cellules de MM expriment et produisent du bFGF, une cytokine angiogénique qui contribue à augmenter le potentiel angiogénique des cellules de MM dans les myélomes progressifs (36). Les BMSC de patients atteints de MM et de sujets normaux expriment les récepteurs du FGF : FGFR1-R4. La stimulation des BMSC par du bFGF induit une augmentation de la sécrétion d'IL-6. La stimulation de lignées de MM ou de cellules myélomateuses primaires par l'IL-6 induit l'expression et la sécrétion de bFGF (72). Ces données suggèrent que les interactions paracrines de l'IL-6 et du bFGF, entre les cellules de MM et les BMSC, pourraient induire non seulement une néovascularisation mais aussi jouer un rôle sur la croissance des cellules tumorales dans la moelle osseuse (72). Dans le MM, la surexpression du FGFR3 par la translocation t(4 ;14) est un événement primaire chez 10-20% des patients et confère un mauvais pronostic (35, 96). En tant que récepteur membranaire, FGFR3 peut être la cible d'anticorps monoclonaux (97). En tant que tyrosine kinase, le FGFR3 peut être inhibé par des inhibiteurs ciblés de tyrosine kinase. L'inhibition du FGFR3 sauvage ou de la forme constitutivement activée, dans les cellules de MM, par des petites molécules inhibitrices (SU5402, SU10991, PD173074, CHIR-258 ou PKC412) est associée à une diminution de la viabilité et à un blocage de la croissance des cellules tumorales *in vitro* et *in vivo* dans un modèle murin du MM (98-102). Ces études précliniques valident le fait que le FGFR3 est une cible thérapeutique chez les patients ayant une translocation t(4 ;14) et les inhibiteurs du FGFR3 sont actuellement évalués afin d'améliorer le pronostic de ce sous-groupe de patients.

- Tumor necrosis factor- α (TNF- α)

Le TNF- α est un facteur de croissance myélomateux (103). Le TNF- α est plus efficace que le VEGF ou le TGF- β pour stimuler la sécrétion d'IL-6 par les BMSC via la voie NF κ B. NF κ B est un facteur de transcription qui contrôle la transcription de nombreuses cytokines, chimiokines, molécules d'adhésion et des protéines anti-apoptotiques ou impliquées dans la croissance cellulaires (104). NF κ B est généralement un hétérodimère composé des sous-unités p50 et p65 (RelA) et son activité est régulée par son association avec des protéines de la famille I κ B (généralement I κ B α). Après une stimulation par le TNF- α , la protéine I κ B α est phosphorylée puis ubiquitinilée et dégradée par le protéasome, induisant la libération de NF κ B (p50/p65) et sa translocation dans le noyau où il va moduler la transcription de ces gènes cibles. NF κ B contrôle l'expression de molécules d'adhésion comme ICAM-1 et VCAM-1. Le TNF- α induit l'expression de VCAM-1 et ICAM-1 à la fois à la surface des cellules de MM et des BMSC, induisant l'adhésion des cellules de MM aux BMSC, la sécrétion d'IL-6 par les BMSC résultant de cette adhésion et la résistance aux drogues médiée par l'adhésion (CAM-DR) (34). De nouveaux agents de chimiothérapie incluant la thalidomide et l'IMiDs (105) pourraient agir, du moins en partie, en inhibant les fonctions du TNF- α .

- Interferon- α (IFN- α)

L'effet de l'IFN- α dans le MM est controversé. Ceci est dû à des expériences in vitro qui ont montré que l'IFN- α pouvait avoir un effet inhibiteur ou stimulateur de la croissance des cellules tumorales selon les échantillons. Notre équipe ainsi que d'autres équipes ont montré que l'IFN- α est un facteur de croissance pour les cellules de MM (106-108), essentiellement en induisant une surexpression de la protéine anti-apoptotique Mcl-1 (109). Les effets opposés de l'IFN- α observés dans

le myélome pourraient être dus à sa capacité à induire l'expression de l'inhibiteur P19 dans certaines lignées de MM, induisant ainsi l'apoptose (110). L'IFN- α a été utilisé en clinique pour le traitement du MM. Si chez certains patients, un effet anti-tumoral a été observé, d'autres ont développé des PCL au cours du traitement (111, 112).

- Hepatocyte growth factor (HGF)

Le taux sérique d'HGF est très fortement augmenté chez certains patients atteints de MM par rapport aux sujets normaux. De plus, le taux sérique d'HGF est corrélé avec l'évolution de la maladie : les taux d'HGF étant plus élevés dans les myélomes de stades III que dans les myélomes de stades I et II (113). L'HGF est produit par les plasmocytes tumoraux qui expriment aussi son récepteur c-met, suggérant l'existence de boucles autocrines fonctionnelles HGF/c-met (114). L'équipe de *Derksen et al* a montré sur une lignée de MM que l'HGF est un facteur de croissance qui agit via son récepteur c-met et le protéoglycane syndecan-1 (115). HGF est activé dans le milieu extracellulaire par HGFA (hepatocyte growth factor activator), une protéase produite par les cellules de myélome (116). Plus récemment, il a été démontré qu'un inhibiteur spécifique de c-met inhibe la prolifération des cellules de MM purifiées (117). Ces données soulignent le rôle important de la boucle autocrine HGF/c-met dans le MM, mais devront être confirmées. Enfin, deux équipes ont démontrées que l'HGF stimule l'adhésion des cellules de MM à la fibronectine via VLA4 (118, 119).

- Les membres de la famille EGF

La famille EGF comprend 10 membres qui reconnaissent de manière spécifique les 4 récepteurs de la famille ErbB : ErbB1, ErbB2, ErbB3 et ErbB4. Les ligands peuvent être divisés en 3 groupes selon leur spécificité pour les récepteurs. EGF, amphiregulin (AREG) et transforming growth factor- α (TGF- α) reconnaissent ErbB1. La betacellulin (BTC), epiregulin (EPR) et heparin-binding EGF-like growth factor (HB-EGF) reconnaissent ErbB3 et/ou ErbB4. ErbB2 n'a pas de ligand propre, cependant il est le partenaire préférentiel d'hétérodimérisation pour les 3 autres récepteurs. Des anomalies de la voie EGF/EGFR sont retrouvées dans de nombreux types de cancers, et son hyperactivation par rapport aux conditions physiologiques est très fréquente dans les cellules tumorales. Plusieurs mécanismes peuvent conduire à cette hyperactivation : une surproduction des ligands qui va conduire à une activation constitutive anormale des récepteurs, une amplification des gènes ErbBs qui entraîne une surexpression des récepteurs ou une mutation des gènes ErbBs qui entraîne une activation constitutive des récepteurs même en absence de ligand. Cependant une implication de la famille EGF dans le MM n'était pas décrite. Nous avons observé à l'aide de macroarrays, une surexpression d'HB-EGF dans certaines lignées de myélome par rapport à des lignées lymphoblastoïdes autologues (60). Notre équipe a ainsi pu démontrer que HB-EGF est un facteur de croissance pour les cellules de myélome, qui agit en synergie avec l'IL-6 (120). HB-EGF est produit par les cellules de l'environnement médullaire (monocytes et cellules stromales). Un inhibiteur spécifique des récepteurs de la famille ErbB entraîne une réduction massive de la viabilité des cellules de MM cultivées *in vitro* avec leur environnement. Cet inhibiteur ne touche pas les autres populations cellulaires de la moelle osseuse et ne présente pas de toxicité pour les progéniteurs hématopoïétiques. Les récepteurs ErbB apparaissent donc comme une cible

thérapeutique intéressante pour le traitement du MM. **Par la suite, notre équipe a voulu déterminer si la fonction d'HB-EGF dans la croissance des cellules de MM était partagée par d'autres membres de la famille EGF. Nous nous sommes intéressé à l'amphiregulin (AREG) qui a des propriétés biochimiques proches de celles d'HB-EGF, notamment la capacité de se fixer aux héparanes sulfates (Article 1).**

« Expression of EGF-family receptors and amphiregulin in multiple myeloma. Amphiregulin is a growth factor for myeloma cells. » Oncogene 2005;24(21):3512-24 .

Notre équipe a démontré que AREG est surexprimé par les cellules de MM par rapport à des plasmocytes normaux, des plasmablastes et des lymphocytes B. Au même titre que HB-EGF, AREG est aussi produite par les cellules de l'environnement médullaire. Les récepteurs ErbB1 et ErbB2 sont exprimés par les plasmocytes normaux alors qu'ErbB3 et ErbB4 sont exprimés uniquement par les plasmocytes tumoraux et seraient donc liés à l'oncogenèse. AREG agit sur les cellules de l'environnement en stimulant la croissance des cellules stromales et la production d'IL-6 par ces cellules. AREG est également un facteur de croissance autocrine pour les cellules de MM qui expriment son récepteur ErbB1 et ses corécepteurs, CD9 et le protéoglycane syndecan-1. Un inhibiteur pan-ErbB entraîne une apoptose des plasmocytes tumoraux cultivés in vitro chez 71% des patients testés. De plus une combinaison inhibiteur pan-ErbB + dexaméthasone entraîne une élimination presque totale des cellules de MM présentes dans la culture. Ce travail renforce l'intérêt d'utiliser des anti-ErbB comme traitement ciblé du MM.

- Stromal cell-derived factor-1 α (SDF1- α)

Expression of EGF-family receptors and amphiregulin in multiple myeloma. Amphiregulin is a growth factor for myeloma cells

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A hallmark of plasma cells is the expression of syndecan-1, which has major functions in epithelial cells, in particular as the coreceptor of heparin-binding growth factors. We previously found that heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a growth factor for malignant plasma cells. As amphiregulin (AREG) is another heparin-binding factor of the EGF family, we investigated its role in multiple myeloma (MM). Using Affymetrix DNA microarrays, we show here that the *AREG* gene was expressed by purified primary myeloma cells from 65 patients and that the expression was higher than in normal bone marrow (BM) plasma cells or plasmablastic cells. AREG stimulated IL-6 production and growth of BM stromal cells. Using real-time reverse transcriptase-polymerase chain reaction, we found that MM cells expressed *ErbB* receptors and that AREG promoted their growth. Furthermore, PD169540 (a pan-*ErbB* inhibitor) and IRESSA (an *ErbB1*-specific inhibitor) induced apoptosis of primary myeloma cells from 10/14 and 4/14 patients, respectively, and there was a synergistic effect with dexamethasone. Altogether, our data provide strong evidence that AREG plays an important role in the biology of MM and emphasize the advantages of using *ErbB* inhibitors, which might target myeloma cells as well as the tumor environment.

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Introduction

Multiple myeloma (MM) is a B cell neoplasia characterized by the accumulation of clonal malignant plasma cells in the bone marrow (BM). In the majority

of patients, malignant plasma cells require mediators delivered by the tumor environment to survive and proliferate. Interleukin-6 (IL-6) is one of these factors produced by the BM environment (Kawano *et al.*, 1988; Klein *et al.*, 1989), but cannot alone promote the survival of myeloma cells from patients with intramedullary myeloma (Gu *et al.*, 2000). Thus, additional factors produced by the tumor environment are necessary to promote the survival of primary myeloma cells, together with IL-6 (Klein *et al.*, 2003). A hallmark of plasma cells is the expression of the heparan-sulfate proteoglycan (HSPG) syndecan-1 (CD138), which is induced during the differentiation of B cells into plasma cells (Costes *et al.*, 1999). In epithelial cells, syndecan-1 plays a major biological role as a coreceptor for heparin-binding growth factors and chemokines (Rapraeger, 2000; Couchman, 2003). This underscores that heparin-binding growth factors are involved in the biology of normal or malignant plasma cells. We recently found that one of the members of the epidermal growth factor (EGF) family, HB-EGF, is produced by some myeloma cell lines and is a potent growth factor for myeloma cells that express HB-EGF receptors (Wang *et al.*, 2002; Mahtouk *et al.*, 2004). HB-EGF is one of the 10 members of the EGF-related growth factors that include EGF, transforming growth factor- α (TGF- α), amphiregulin (AREG), HB-EGF, betacellulin, epiregulin and the four neuregulins 1–4 (Harris *et al.*, 2003). Both HB-EGF and AREG have a heparin-binding domain (Inui *et al.*, 1997; Nylander *et al.*, 1998). This family binds to and induces the homo- or heterodimerization of three receptors: the EGF receptor *ErbB1*, *ErbB3* and *ErbB4*. *ErbB2* is a fourth member unable to bind EGF ligands. It is the preferred heterodimerization partner for all other *ErbB* members and contributes to the potentiation of *ErbB* receptor signaling (Holbro *et al.*, 2003). Interestingly, *AREG* was recently highlighted in two studies on myeloma cells. Claudio *et al.* (2002) constructed a cDNA library of primary malignant plasma cells and identified several growth factors and cytokines by 5'-sequencing, including *AREG*. In an independent study using HuGeneFL Affymetrix

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microarrays, Zhan *et al.* (2002) reported *AREG* to be one of the 25 genes with 'spiked' expression in a subset of MM cells from newly diagnosed patients. Thus, given the important role of HB-EGF as a myeloma cell growth factor, we investigated the production and biological function of *AREG* in MM.

In this study, we show that purified primary myeloma cells overexpressed *AREG* compared to normal plasmablastic cells (PPCs). In addition, *AREG* was overexpressed in the myeloma cells of a subset of patients compared to normal plasma cells. *AREG* stimulated the growth and IL-6 production of bone marrow stromal cells (BMSCs) and was able to stimulate MM cell growth. PD169540, a pan-ErbB kinase inhibitor, and IRESSA, an ErbB1-specific kinase inhibitor, each induced primary myeloma cell apoptosis in short-term culture for 5 days, alone or in combination with dexamethasone (DEX). These findings provide the framework for future clinical trials with specific ErbB receptor inhibitors, targeting both tumor cells and their microenvironment.

Results

Gene expression of AREG and HB-EGF by DNA microarrays and real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Gene expression profiling (GEP) was performed on seven normal PPC samples, seven normal bone marrow plasma cell (BMPC) samples, purified malignant plasma cells from 72 patients – seven with MGUS and 65 with MM – and 20 human myeloma cell lines (HMCLs) with Affymetrix U133A + B microarrays. Data for *AREG* expression are shown in Figure 1a. *AREG* was a 'present' gene (using Affymetrix call) in myeloma cells of all 65 patients or in all seven BMPC samples, and an 'absent' gene in all seven PPC samples. The median *AREG* expression in primary myeloma cells (median value = 251) was 2.5- and 15-fold higher than that in BMPCs (median value = 97, $P = 0.039$) or PPCs (median value = 16, $P < 10^{-4}$), respectively. It was very high in some primary myeloma cell samples (Figure 1a). No significant difference in *AREG* expression was found between patients with Durie-Salmon stage I, II or III MM (Figure 1a). Median *AREG* expression was also significantly increased in plasma cells from patients with MGUS (median value = 154) compared to PPCs ($P = 0.001$) or BMPCs ($P = 0.039$). HMCLs did not express or weakly expressed *AREG* (median expression value = 9). *AREG* expression was also investigated using real-time RT-PCR in selected samples. Primary myeloma cells and BMPCs expressed *AREG* (respectively, five- and eight-fold less than the A431 carcinoma cell (LDH) line known to produce EGF-family ligands, which was subgroups assigned the arbitrary value of 100), whereas *AREG* expression was undetectable in HMCLs or PPCs, validating the Affymetrix data (Figure 2a).

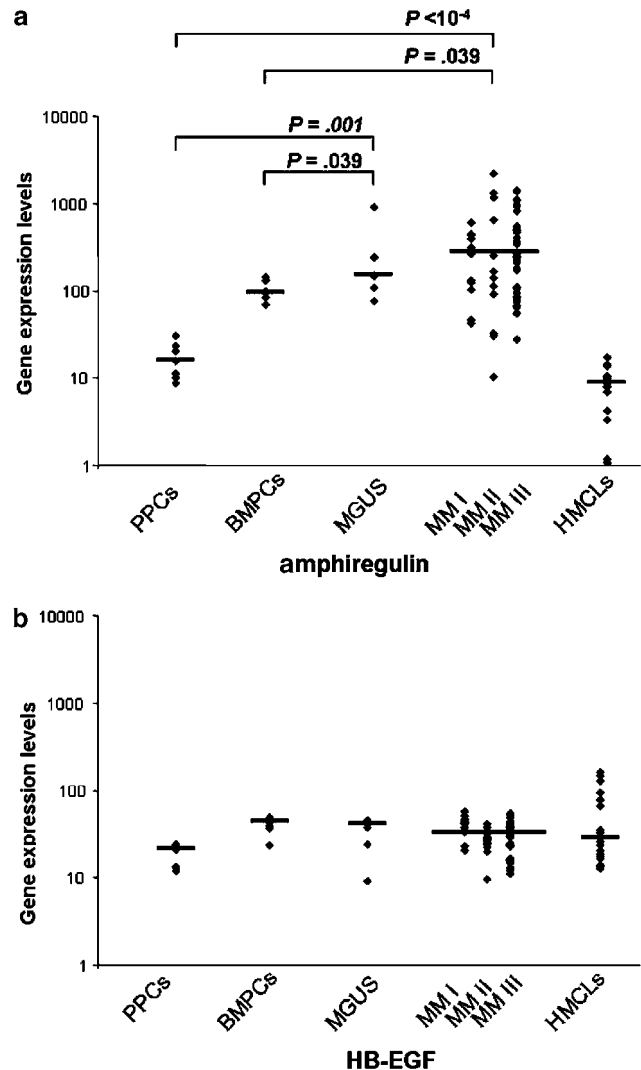


Figure 1 Gene expression profile of *amphiregulin* and *HB-EGF*. Gene expression profiles of *amphiregulin* (a) and *HB-EGF* (b) were determined with Affymetrix U133 A + B DNA microarrays in seven normal plasmablastic cell (PPC) samples, seven normal bone marrow plasma cell (BMPC) samples, purified malignant plasma cells of seven patients with MGUS and 65 patients with MM, and 20 myeloma cell lines (HMCLs). MMI, MMII and MMIII indicate data from myeloma cells of patients with stage I, II or III MM. Statistical comparisons were made with a Mann-Whitney test

To look for a link between *AREG* expression and clinical parameters, the 65 patients with MM were classified into two groups: patients with a low or high *AREG* expression in myeloma cells (*AREG*^{low} or *AREG*^{high}), assayed with Affymetrix microarrays. To find the best way to delimit *AREG*^{low} and *AREG*^{high} groups, we considered subgroups defined by 50, 40, 25 or 15% of the patients with the highest or lowest *AREG* expression in myeloma cells (Table 1). The *AREG*^{low} groups included a higher frequency of patients with lactate dehydrogenase (LDH) ≥ 190 IU/l and with beta-2 microglobulin (β_2m) ≥ 4 mg/l, except for the 15%-group in which the β_2m levels between

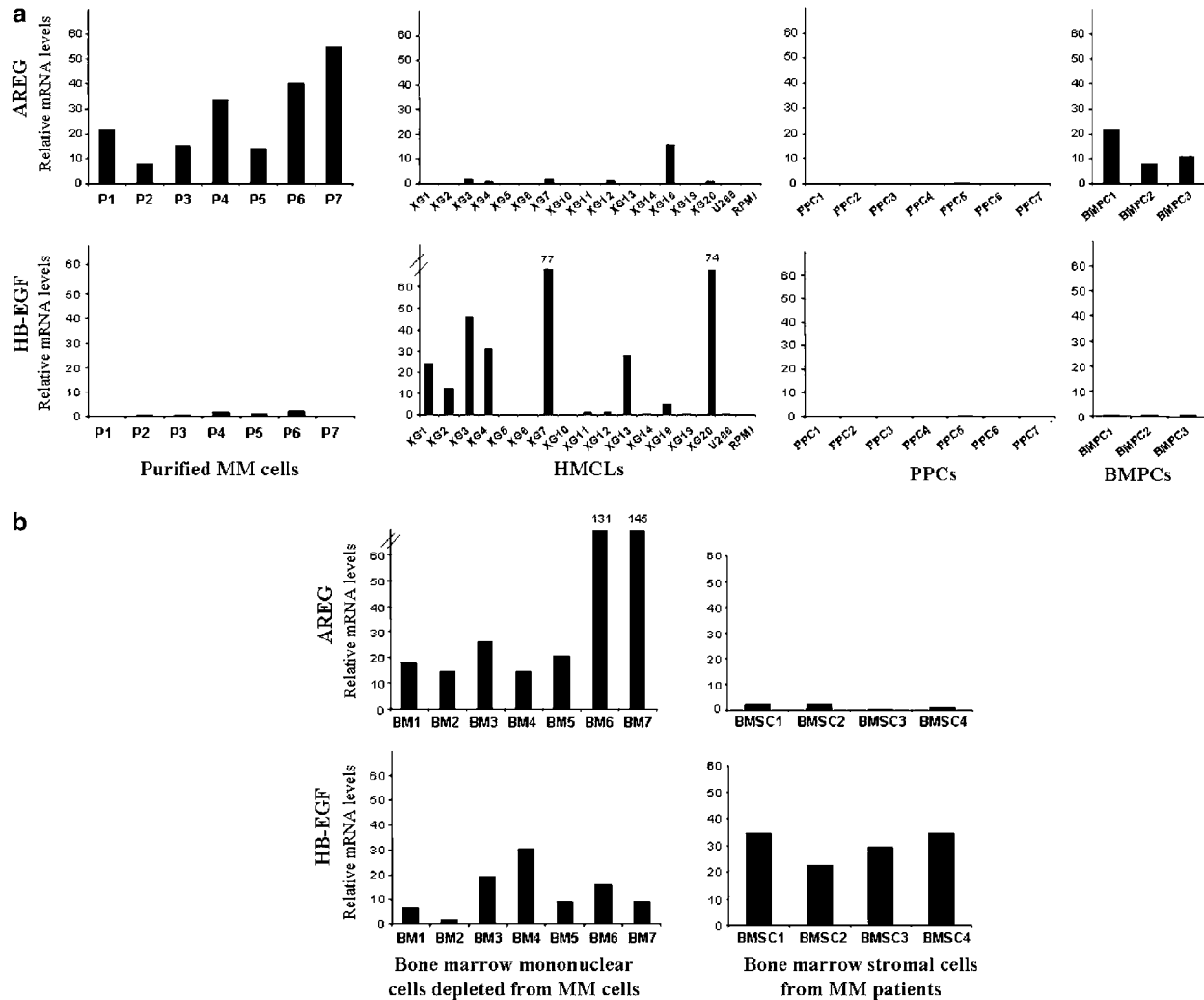


Figure 2 *AREG* or *HB-EGF* mRNA quantification in myeloma cells, normal plasma cells or the bone marrow environment of MM patients using real-time RT-PCR. *AREG* and *HB-EGF* expression was determined on (a) purified primary myeloma cells from seven patients, 20 HMCLs, seven PPC samples, three BMPC samples, (b) bone marrow (BM) mononuclear cells depleted of myeloma cells from seven patients with MM and bone marrow stromal cells (BMSCs) isolated from four patients with MM. Real-time RT-PCR analysis was made as described in Materials and methods

Table 1 Clinical data of the *AREG*^{high} and *AREG*^{low} patients

	Definition of <i>AREG</i> ^{high} and <i>AREG</i> ^{low} subgroups							
	50%-group		40%-group		25%-group		15%-group	
	<i>AREG</i> ^{high} (n = 32)	<i>AREG</i> ^{low} (n = 32)	<i>AREG</i> ^{high} (n = 25)	<i>AREG</i> ^{low} (n = 25)	<i>AREG</i> ^{high} (n = 15)	<i>AREG</i> ^{low} (n = 15)	<i>AREG</i> ^{high} (n = 10)	<i>AREG</i> ^{low} (n = 10)
$\beta_2m \geq 4$ mg/l	31%	56%	32%	60%	47%	80%	50% (NS)	80% (NS)
LDH ≥ 190 IU/l	13%	34%	16%	40%	13%	47%	10%	50%

In total, 65 newly diagnosed patients with MM were separated into two subgroups according to *AREG* gene expression in myeloma cells assayed with Affymetrix microarrays. The two subgroups represent 50% (*n* = 32), 40% (*n* = 25), 25% (*n* = 15) or 15% (*n* = 10) of the patients with the highest (*AREG*^{high}) or lowest (*AREG*^{low}) *AREG* expression in myeloma cells. Data are the percentage of patients within each subgroup with the indicated clinical parameter. The percentages are statistically significantly different between the two groups, with the χ^2 test (*P* ≤ 0.05), except when (NS), i.e., ‘nonsignificant’, is indicated

AREG^{high} and *AREG*^{low} subgroups were not statistically different. No significant difference was found for the other clinical parameters (CRP,

albumin, serum calcium, hemoglobin, age), cytogenetic abnormalities or Durie-Salmon staging (results not shown).

The *HB-EGF* gene showed a different expression pattern from *AREG*. It was weakly expressed by primary myeloma cells, BMPCs and PPCs, with a median value of 31, 50 and 22, respectively, but was more highly expressed by six out of 20 HMCLs (XG-1, XG-3, XG-4, XG-7, XG-13 and XG-20; see Figure 1b), with a signal varying from 81 to 166. *HB-EGF* expression in certain HMCLs was confirmed by real-time RT-PCR (Figure 2a) and was in agreement with our previous RT-PCR data (Mahtouk *et al.*, 2004). In addition, we found that the BM environment of patients with MM highly expressed *AREG*, with a relative expression level from 20 to 145, as well as *HB-EGF* with a relative expression level from 2 to 31 (Figure 2b). It is noteworthy that BMSCs derived from MM patients poorly expressed *AREG* but highly expressed *HB-EGF* (Figure 2b). One putative explanation for the lack of *AREG* expression in HMCLs, as opposed to primary myeloma cells, is that the BM tumor environment might trigger *AREG* expression in primary myeloma cells. In order to test this hypothesis, three GFP-transduced HMCLs were cocultured with BM cells from patients with MM, and GFP-HMCLs were sorted. *AREG* expression could not be induced in the three HMCLs by BM cells of patients with MM (results not shown). Prostaglandin E2 (PGE2), known to induce *AREG* expression in epithelial cancers (Shao *et al.*, 2003), was also unable to induce *AREG* expression in four HMCLs (results not shown).

Gene expression of ErbB receptors in myeloma cells and BM environment cells

AREG preferentially binds to ErbB1, in the form of ErbB1/ErbB1 homodimers or ErbB1/ErbB2 heterodimers (Holbro *et al.*, 2003). As Affymetrix probesets were not sensitive enough for ErbB receptors detection ('absent Affymetrix call' in the majority of myeloma cell samples), we used real-time RT-PCR to study their expression in purified primary myeloma cells from 21 patients with MM, 17 HMCLs, seven PPC samples, three BMPC samples, BM mononuclear cells devoid of myeloma cells from seven patients with MM and BMSCs derived from four patients. *ErbB* expression in each sample was normalized to that of β_2m and compared to the expression in the XG-7 HMCL, which expresses the four ErbB receptors (Mahtouk *et al.*, 2004). The XG-7 HMCL was assigned the arbitrary value of 100. Purified primary myeloma cells from 16 of 21 patients and seven of 17 HMCLs expressed *ErbB1* mRNA (relative expression level ranging from 0.5 to 236). BMPCs also expressed *ErbB1*, unlike PPCs (Figure 3a). *ErbB2* was variably expressed in 19 of 21 primary myeloma cells, 13 of 17 HMCLs and three out of three BMPCs, but was undetectable in six out of seven normal PPCs (Figure 3b). Of 17 HMCLs, 14 expressed *ErbB3* mRNA, whereas it was not expressed or weakly expressed in primary myeloma cells, PPCs and BMPCs (Figure 3c). Finally, nine of 17 HMCLs expressed *ErbB4*. Purified primary myeloma cells from 14 out of 21 patients expressed *ErbB4* at a lower level

than HMCLs, except for two patients whose myeloma cells highly expressed *ErbB4* (relative expression level of 684 and 4734). PPCs and BMPCs did not express *ErbB4* (Figure 3d).

Bone marrow mononuclear cells from six of the seven MM patients, which were depleted of myeloma cells, expressed *ErbB1*, *ErbB2*, but not *ErbB3* and *ErbB4*, at similar levels as those in primary myeloma cells or BMPCs (Figure 4a). Interestingly, all four BMSCs expressed very high levels of *ErbB1*, *ErbB2* (on the average 250- and 200-fold higher, respectively, than XG-7 cells) and *ErbB3* (three-fold higher than XG-7 cells), but not *ErbB4* (Figure 4b).

AREG promotes stromal cell growth and IL-6 production by BMSCs

As BMSCs strongly expressed *ErbB1* and *ErbB2*, we investigated whether *AREG* could stimulate their growth. These cells were cultured at low serum concentration (5% FCS) in order to limit the concentration of EGF members present in FCS. As shown in Figure 5a, *AREG* dramatically promoted the growth of all three BMSCs derived from patients with MM (five-fold for BMSC1, six-fold for BMSC2 and two-fold for BMSC3 after 3 weeks of culture, $P < 0.01$). IRESSA (1 μ M), an ErbB1-specific inhibitor (Manegold, 2003), abrogated the *AREG* effect. In addition, *AREG* induced a slight but significant increase in IL-6 secretion by two BMSCs in five separate experiments: BMSC1 (2.5-fold, $P = 0.0001$) and BMSC2 (1.5-fold, $P = 0.02$) (Figure 5b). IRESSA did not affect the spontaneous IL-6 production by BMSCs but abolished the stimulatory effect of *AREG* ($P = 0.0047$ and 0.01, respectively). Real-time RT-PCR showed an increase in IL-6 transcripts after *AREG* exposure, which was abrogated by IRESSA. These results indicate that the *AREG*-induced stimulation of IL-6 occurred at the mRNA level (Figure 5c).

AREG is a myeloma cell growth factor

Similar to *HB-EGF*, *AREG* is able to bind cell-associated HSPGs and the CD9 tetraspanin (Inui *et al.*, 1997; Sakuma *et al.*, 1997; Harris *et al.*, 2003). The association with these molecules facilitates binding to ErbB receptors (Rapraeger, 2000; Boucheix and Rubinstein, 2001). Using Affymetrix U133 A + B microarrays, we confirmed that the HSPG *syndecan-1* was expressed by all myeloma cells and normal BMPCs, but not by PPCs, as was expected (Tarte *et al.*, 2000) (Figure 6a). *CD9* was expressed ('present' call) by 71% of primary myeloma cell samples (median expression value = 264), with a very high expression in some samples, and by 65% of HMCLs (median expression value = 397). It was also expressed by 100% of BMPCs (median expression value = 469) and 71% of PPCs (median expression value = 100) (Figure 6b). It should be noted that there was a good correlation ($r = 0.88$; $P < 0.05$) between Affymetrix *CD9* expression and CD9 protein levels assayed by FACS on HMCLs (Figure 6c).

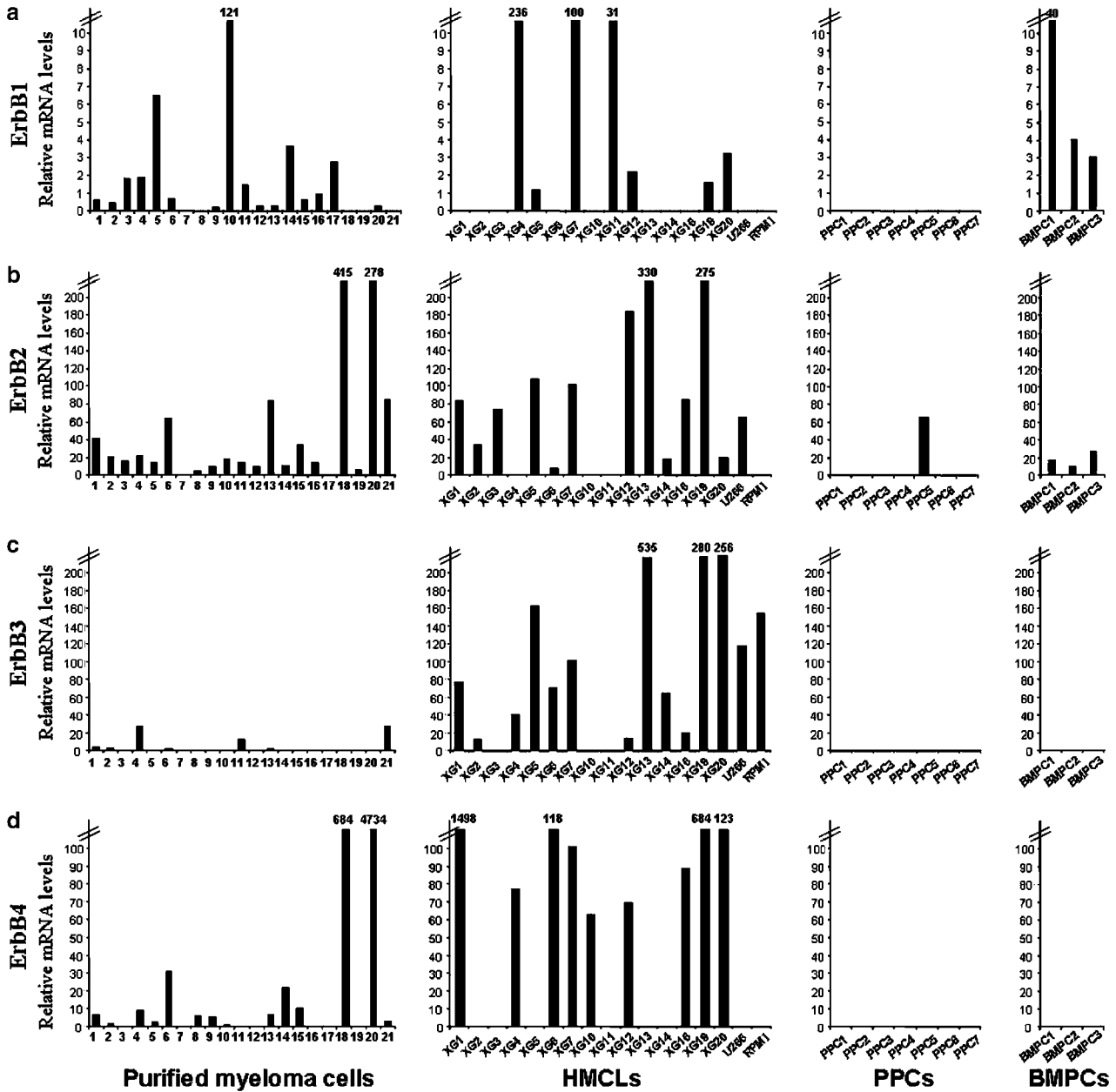


Figure 3 *ErbB* mRNA quantification in myeloma cells, normal plasma cells, and normal plasmablastic cells using real-time RT-PCR. Real-time RT-PCR was performed on RNA samples isolated from purified primary myeloma cells, HMCLs, PPCs, and BMPCs. Real-time RT-PCR analysis was made as described in Materials and methods

Owing to *ErbB1*, *ErbB2*, and coreceptors expression in some myeloma cell lines, we next investigated whether AREG could stimulate their growth. Data shown in Figure 7a illustrate that with a low concentration of IL-6, AREG was a potent myeloma cell growth factor for XG-1 and XG-7 HMCLs. No effect was found without adding exogenous IL-6. A significant effect was found with 100 ng/ml of AREG and a maximum effect with 1000 ng/ml (results not shown). XG-7 expressed *ErbB1* (Figure 3a) and accordingly, the stimulatory effect of AREG was abrogated by IRESSA or AG1478 *ErbB1* inhibitors (1 μ M) and by the PD169540 pan-ErbB

inhibitor (1 μ M) (Figure 7b). No synergistic inhibition was found when the two inhibitors were used at suboptimal concentrations (results not shown). Surprisingly, AREG was also able to promote the proliferation of XG-1 cells that did not express *ErbB1*, but expressed *ErbB4* and *ErbB3* (Figure 3c, d). In agreement with this, the stimulatory effect of AREG on XG-1 cells was not affected by IRESSA or AG1478 *ErbB1* inhibitors, but was inhibited by the pan-ErbB inhibitor PD169540 (Figure 7b). We checked that AREG did not induce *ErbB1* expression in XG-1 cells with real-time RT-PCR (Figure 7c). These data imply that AREG may promote

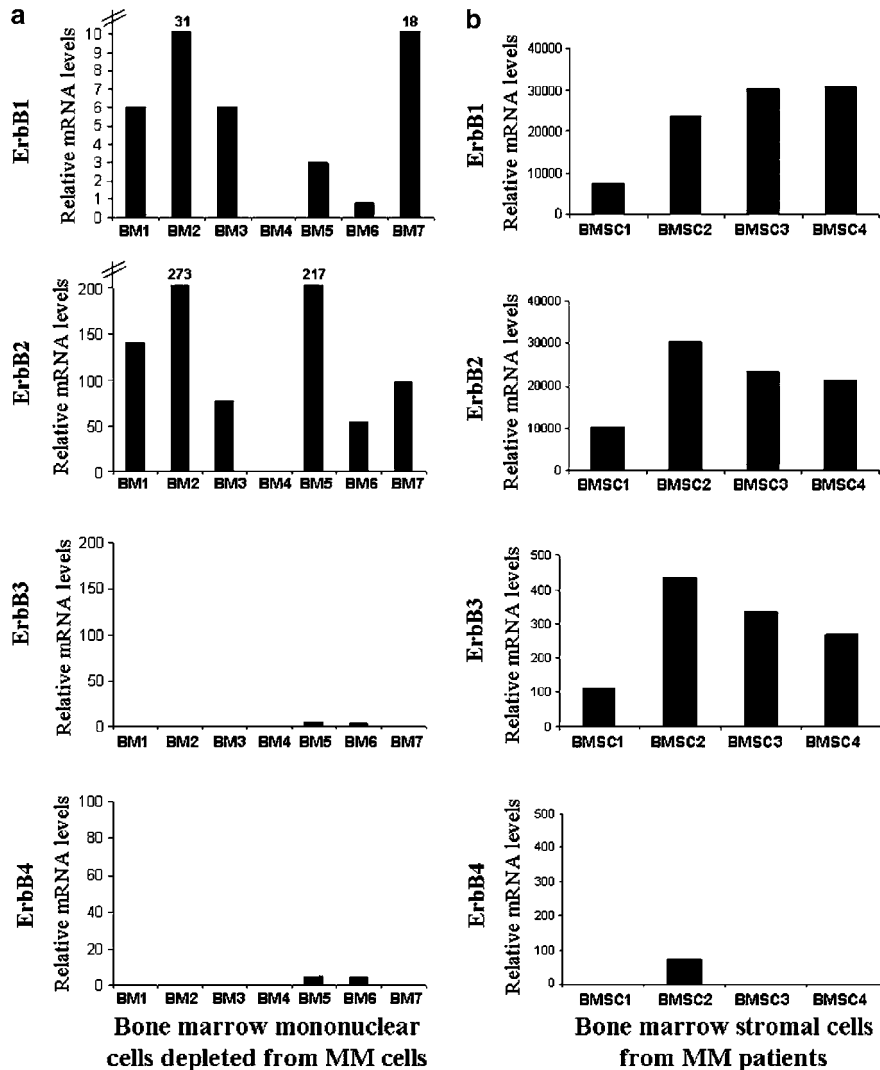


Figure 4 *ErbB* mRNA quantification in the tumor environment and bone marrow stromal cells. Real-time RT-PCR was performed on RNA samples isolated from bone marrow mononuclear cells depleted of myeloma cells from seven patients (a) and bone marrow stromal cells derived from four patients with MM (b). Real-time RT-PCR analysis was made as described in Materials and methods

myeloma cell growth through ErbB1-independent pathways, probably via ErbB3 and/or ErbB4.

The ErbB inhibitors PD169540 and IRESSA inhibit the survival of primary myeloma cells

We investigated the effect of ErbB inhibitors on the survival of primary myeloma cells from 14 patients with intramedullary myeloma. Myeloma cells were not purified because they die within 1 day after removal from the BM environment, as reported (Gu *et al.*, 2000). Data from the 14 patients are summarized in Table 2. The pan-ErbB inhibitor PD169540 significantly reduced the number of viable myeloma cells in 10 of 14 patients (median reduction = 43%, $P < 0.001$, Table 2a). Interestingly, in combination with DEX, myeloma cells were almost completely eliminated (92% reduction in the number of

viable myeloma cells with DEX + PD169540 versus 31% reduction with DEX alone, $P < 0.001$, Table 2a). As illustrated in Figure 8a for patient 1, the inhibitor specifically killed myeloma cells and did not affect cells from the tumor BM environment. IRESSA was active in four out of 14 patients (55% reduction in the number of viable myeloma cells, $P < 0.001$), shown in Table 2b. Again, the addition of an ErbB-inhibitor potentiated the apoptotic effect of DEX (69% reduction in the number of viable myeloma cells with DEX + IRESSA versus 31% reduction with DEX alone, $P = 0.001$, Table 2b). AREG protein was detected in the culture supernatants of BM cells of 12 patients with MM (median value, 26 pg/ml) and in the BM plasma of 15 patients with MM (median value, 15 pg/ml) (Figure 8b). High AREG concentrations (median value, 761 pg/ml) were also found in the myelomatous pleural effusions of four patients.

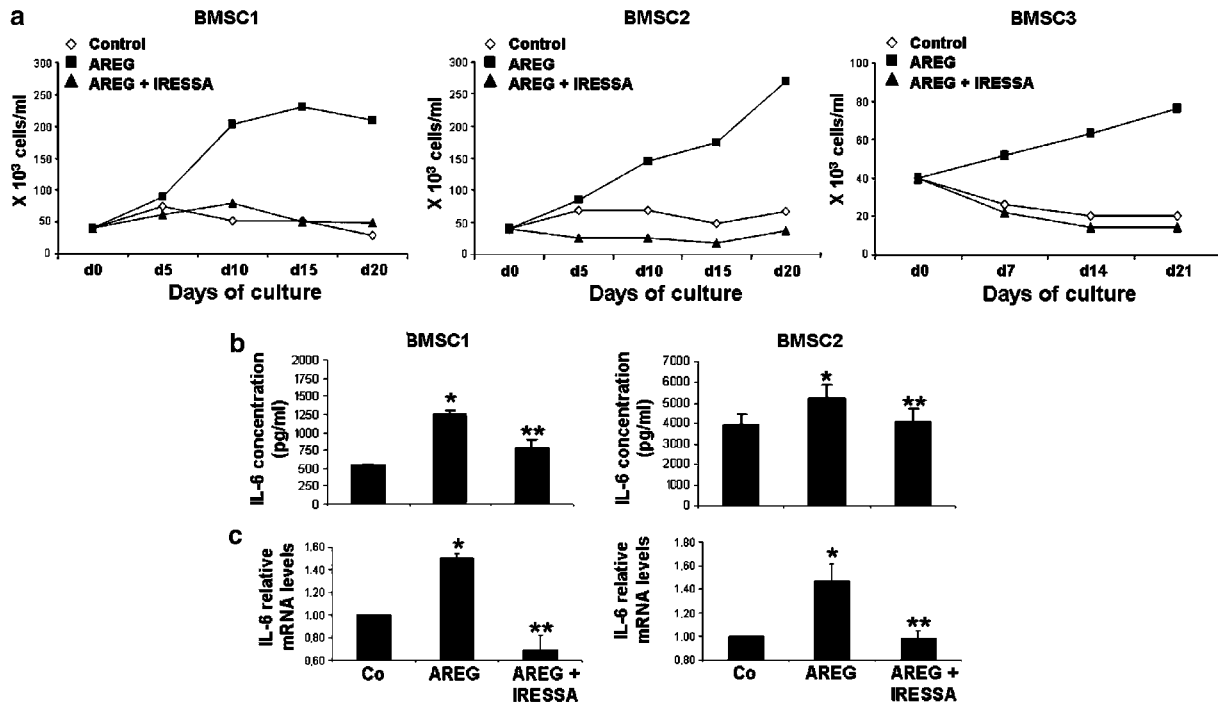


Figure 5 AREG promotes the growth and IL-6 production of bone marrow stromal cells (BMSCs). (a) BMSCs were cultured for 3 weeks in DMEM and 5% FCS in a six-well culture plate, with or without AREG (1 μ g/ml), with or without IRESSA (1 μ M), at a density of 4×10^4 cells/ml. At day 5 (BMSC1-2) or 7 (BMSC3), cells were trypsinized and replated at a concentration of 4×10^4 cells/ml. Results are the mean cell numbers of triplicate cultures and are of one experiment representative of three. (b) IL-6 concentrations were measured by ELISA in culture supernatants of BMSCs after stimulation with AREG (1 μ g/ml), \pm IRESSA (1 μ M) for 48 h. Concentrations of IL-6 are presented as pg/ml corrected for 4×10^4 cells. Data are means \pm s.d. of the IL-6 secretion determined on triplicate culture wells and are those of one experiment representative of five. (c) BMSCs were incubated with AREG, with or without IRESSA for 24 h and analysed for IL-6 expression using real-time RT-PCR. Each sample was normalized to β_2m and compared with the levels of *IL-6* expression in the control groups, which was assigned a relative expression value of 1. Data are the means \pm s.d. of the *IL-6* expression determined in five different experiments. *Indicates that the mean value is statistically significantly different from that obtained without AREG, using a Student's *t*-test for pairs ($P \leq 0.05$). **Indicates that the mean value is statistically significantly different from that obtained with AREG, without IRESSA, using a Student's *t*-test for pairs ($P \leq 0.05$).

Discussion

We show here that primary myeloma cells overexpressed the *AREG* gene compared to normal PPCs. Although *AREG* expression was very high in some MM samples, it was expressed in 65 of 65 patients' myeloma cells with a continuous log normal distribution. Thus, we did not confirm the observation of Zhan *et al.* (2002), who previously mentioned that *AREG* was a 'spike' gene, that is, a gene with a low to undetectable expression in the majority of patients or normal samples and highly overexpressed in a subset of MM samples. *AREG* was also highly expressed in normal BM plasma cells compared to normal PPCs and this suggests that *AREG* expression is likely a feature of mature plasma cell differentiation.

We found that the percentage of patients with $\beta_2m > 4$ mg/l or LDH > 190 IU/l was higher in the groups of patients with the lowest *AREG* expression level. Thus, patients with the lowest *AREG* expression might have a poor prognosis, but the clinical follow-up of the patients was too short at the time of this study to look for a difference in event-free or overall survival.

Primary myeloma cells did not express the *HB-EGF* gene, another EGF family member able to bind HSPGs (Iwamoto and Mekada, 2000), in agreement with our previous RT-PCR results (Mahtouk *et al.*, 2004). It should be noted that myeloma cell lines had a contrary pattern. Six out of 20 cell lines highly expressed *HB-EGF* but none of them expressed *AREG*. Several explanations can account for this striking difference. One is that myeloma cell lines are obtained from a minority of patients with extramedullary proliferation who behave differently regarding *AREG* and *HB-EGF* expression compared to the majority of patients with intramedullary MM. Another explanation is that there are components in the tumor milieu *in vivo* that are not produced *in vitro* that can trigger *AREG* expression. High *AREG* concentrations were detected in the myelomatous pleural effusions from four patients. According to our previous data with IL-6 (Lu *et al.*, 1995a), the diffusion of growth factors is more restricted in pleural effusion and is likely a good indication of their local concentration, close to the tumor environment *in vivo*. *AREG* was also detected in BM plasma of patients with MM at lower concentrations.

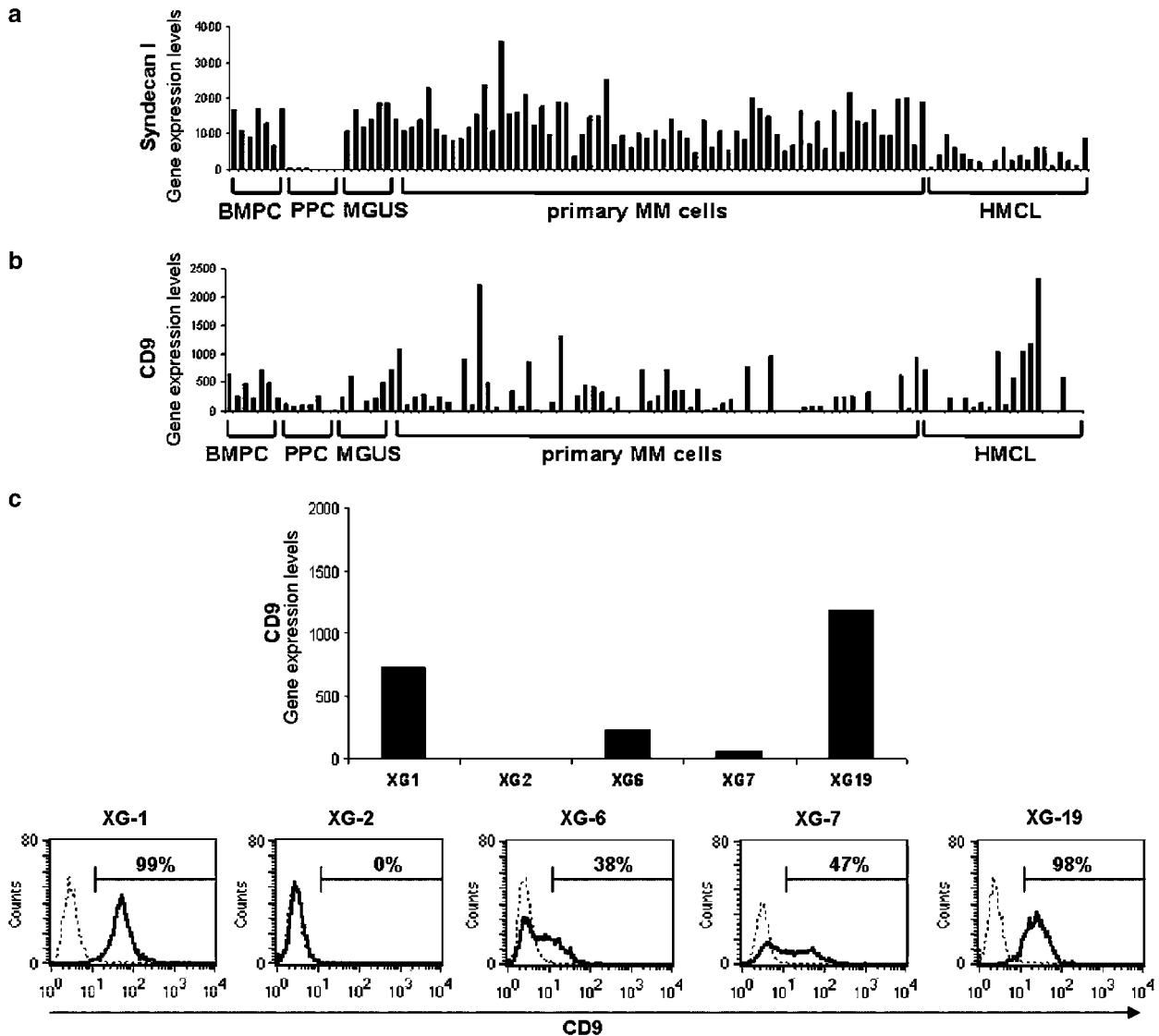


Figure 6 Gene expression profile of *syndecan 1* and *CD9*. Gene expression profiles of *syndecan 1* (a) and *CD9* (b) were determined with Affymetrix U133 A + B microarrays in seven normal BMPC samples, seven PPC samples, purified malignant plasma cells of seven patients with MGUS and 65 patients with MM, and 20 HMCLs. (c) Surface expression of CD9 was determined using FACS with an anti-CD9-PE antibody (solid line) and a PE-isotype control (dotted line)

We also determined the expression of ErbB receptors on myeloma cells and normal plasma cells. Our present data show that *ErbB* expression by myeloma cells is a frequent feature. In particular, we found *ErbB3* expression in 14 out of 17 HMCLs, which is greater than what Walters *et al.* (2003) reported in detecting *ErbB3* expression in one out of five HMCLs. Until now, the involvement of the receptors in normal plasma cells was unknown. We show here that BMPCs expressed *ErbB1* and *ErbB2*, unlike polyclonal early PPCs. These data indicate that *ErbB1* and *ErbB2* are induced throughout normal plasma cell differentiation. We were not able to detect *ErbB3/ErbB4* mRNA in BMPCs. One hypothesis is that they are not induced in late plasma cells. However, it is noteworthy that *ErbB3* is expressed in three of 21 patients only and *ErbB4* in

62% of patients (13/21), suggesting that it would be necessary to investigate additional normal BMPCs. This was not possible in the current study because plasma cells are rare BM cells (0.25%), and it was very difficult to obtain enough BM from healthy individuals to purify them.

Our findings suggest that AREG has a dual effect that sustains tumor growth *in vivo*. Firstly, it may be a paracrine mediator supporting myeloma cell growth through the stimulation of the stromal cell compartment, in particular BMSC proliferation and IL-6 production. Secondly, it may be an autocrine growth factor for myeloma cells. Surprisingly, we found that AREG was able to induce XG-1 myeloma cell growth through an ErbB1-independent mechanism, probably via ErbB2/ErbB3 or ErbB2/ErbB4 complexes. This is

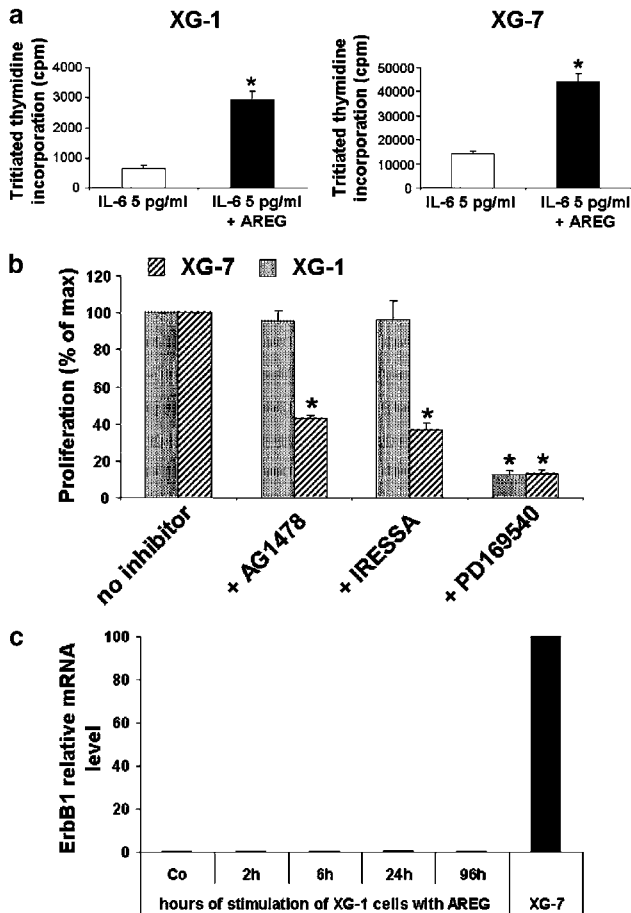


Figure 7 Sensitivity of myeloma cell lines to AREG. (a) XG-1 and XG-7 cells were IL-6-starved for 3 h and cultured in RPMI1640 culture medium and 5% FCS, with 5 pg/ml IL-6, with or without 1 μ g/ml AREG. Cells were cultured for 6 days and pulsed for 12 h with tritiated thymidine at the end of the culture. Data are means \pm s.d. of the tritiated thymidine incorporation determined on sixplicate culture wells and are those of one experiment representative of three. *Indicates that the mean value is statistically significantly different from that obtained without AREG, using a Student's *t*-test ($P \leq 0.05$). (b) XG-1 and XG-7 cells were IL-6-starved and cultured with 5 pg/ml IL-6 and 1 μ g/ml AREG with or without inhibitors (1 μ M). Mean tritiated thymidine incorporations were determined in sixplicate culture wells and results are expressed as percentages of the mean proliferation without ErbB inhibitor. *Indicates that the mean value is statistically significantly different from that obtained without inhibitor, using a Student's *t*-test ($P \leq 0.05$). (c) XG-1 cells were incubated with AREG (1 μ g/ml) for 2, 4, 6, 24 h and 4 days, and were analysed for *ErbB1* expression using real-time RT-PCR. Each sample was normalized to β_2m and compared with the level of *ErbB1* expression in XG-7 cells, which was assigned a relative expression value of 100

attractive because AREG is known to be an ErbB1 ligand and it is generally accepted that it does not bind other ErbB members. Accordingly, two studies reported that the ability of ErbB receptors to mediate signal transduction through EGF-like ligands is broader than has been assumed. In murine 32D cells transfected with ErbB4 and ErbB2, Wang *et al.* (1998) reported that EGF and TGF- α , known to specifically bind ErbB1, can also activate ErbB2/ErbB4 complexes and induce

proliferation and/or adhesion. Alimandi *et al.* (1997) showed that betacellulin (known to bind ErbB1 and ErbB4) was able to bind ErbB2/ErbB3 complexes. In a previous study, we showed that ErbB activation by HB-EGF in myeloma cells induced activation of the PI-3 kinase/AKT pathway, unlike MAP kinase or JAK/STAT pathways. HB-EGF-induced proliferation was blocked by a PI-3 kinase inhibitor (LY294002) (Mahtouk *et al.*, 2004). Likewise, AREG-induced proliferation was inhibited by LY294002 (unpublished results), indicating that the PI-3 kinase/AKT pathway is critical to triggering the myeloma cell growth activity of AREG.

We show that the PD169540 pan-ErbB inhibitor and the IRESSA ErbB1-inhibitor induced MM cell apoptosis in 71 and 29% of patients, respectively. We found dramatic myeloma cell apoptosis when PD169540 or IRESSA were used with DEX while nonmyeloma cells were unaffected. The ErbB inhibitors likely block ErbB activation on primary myeloma cells, as they did with HMCLs. They might also target the BM environment. We could not directly investigate this because purified primary myeloma cells cannot be maintained in short-term culture and apoptose as soon as they are removed from their BM environment (Gu *et al.*, 2000). These data indicate that ErbB activation is critical to triggering primary myeloma-cell survival in short-term culture. AREG was detected in culture supernatants of BM cells from patients with MM, indicating that AREG must be, at least in part, responsible for ErbB activation in primary myeloma cells. HB-EGF could also be involved since it is produced by the tumor environment (Mahtouk *et al.*, 2004). HB-EGF production in the culture supernatants could not be evaluated because no ELISA was commercially available. We tried to determine the relative contribution of HB-EGF and AREG with neutralizing antibodies against AREG or HB-EGF. However, these antibodies had no effect, either used alone or in combination (data not shown). One explanation is that it is difficult to neutralize the binding of an autocrine cytokine, as was already found for autocrine IL-6 in myeloma cells (Jourdan *et al.*, 1991). An inhibitor targeting the receptor directly, in particular the kinase activity, is far more efficient.

ErbB expression on myeloma cells is weak compared to those of BMSCs, and we previously reported that ErbB proteins could be detected only by immunoprecipitation in some cell lines (Mahtouk *et al.*, 2004). Thus ErbB receptors, even when expressed at low levels, can play an important role in the biology of myeloma and therefore be considered a potential therapeutic target. One explanation is that myeloma cells express two HB-EGF/AREG coreceptors – syndecan-1 and CD9 – which might increase the mitogenic actions of the EGF-ligands, stabilizing their interactions with the ErbB receptors. A second explanation could be a synergistic cooperation between ErbB receptors and gp130 IL-6 transducer signaling pathways. We have shown that HB-EGF and AREG both require

Table 2 Percentage of reduction of viable primary myeloma cell number by PD169540 and IRESSA, compared to the control group

<i>A</i>					
<i>Responder patients (to PD169540)</i>	<i>PD169540</i>	<i>DEX</i>	<i>DEX + PD169540</i>	<i>IRESSA</i>	<i>DEX + IRESSA</i>
Patient 1	90	23	97	0	23
Patient 2	38	59	92	0	62
Patient 3	48	95	95	8	92
Patient 4	65	40	93	64	75
Patient 5	58	0	44	95	37
Patient 6	90	22	93	45	87
Patient 7	19	4	22	1	0
Patient 8	21	13	31	0	13
Patient 9	19	93	93	0	92
Patient 10	21	40	41	21	62
Median value	43*	31*	92**	5	62
<i>Nonresponder patients</i>					
Patient 11	4	0	10	0	0
Patient 12	12	0	23	31	8
Patient 13	0	0	0	5	3
Patient 14	10	45	73	4	51
Median value	7	0	16	4	5
<i>B</i>					
<i>Responder patients (to PD169540 and IRESSA)</i>	<i>IRESSA</i>	<i>DEX</i>	<i>DEX + IRESSA</i>		
Patient 4	64	40	75		
Patient 5	95	0	37		
Patient 6	45	22	87		
Patient 10	21	40	62		
Median value	55*	31*	69**		

Bone marrow mononuclear cells from 14 patients with MM were cultured for 5 days in RPMI 1640 medium, 5% FCS, 1 ng/ml IL-6, with or without the PD169540 ErbB inhibitor (1 μ M), or the IRESSA ErbB1-inhibitor (1 μ M) or DEX (10⁻⁶ M). At day 5 of culture, the viability and cell counts were assessed and the percentage of CD138⁺ viable myeloma cells was determined by flow cytometry. Results are expressed as the percentage of reduction of the number of viable cells, compared to the control group cultured without inhibitors. All 14 patients are listed in (a). The median values were calculated separately for the 10 responder patients and the four nonresponder patients. The four patients responding to IRESSA are listed in (b). *Indicates that the percentage of reduction compared to the control group is statistically significantly different using a Wilcoxon test for pairs ($P \leq 0.05$). **Indicates that the percentage of reduction compared to the group cultured with DEX alone is statistically significantly different using a Wilcoxon test for pairs ($P \leq 0.05$).

low IL-6 concentrations to be able to trigger myeloma cell growth. In agreement with this observation, it is now becoming clear that ErbB receptors function as signal integrators crossregulating different classes of receptors (Hynes *et al.*, 2001), in particular the IL-6 transducer gp130 (Qiu *et al.*, 1998; Badache and Hynes, 2001). A last possible explanation might be the presence of mutations in the kinase domain of ErbB1 in responder patients, as it was recently demonstrated in non-small lung cancer cells (Lynch *et al.*, 2004; Paez *et al.*, 2004). These mutations enhanced tyrosine kinase activity in response to EGF and increased the sensitivity to inhibition by IRESSA. As we found a role of AREG or HB-EGF in triggering myeloma cell survival and a strong effect of ErbB inhibitors despite weak ErbB expression, it might be interesting to look for such mutations in ErbB1 or ErbB4 in myeloma cells of certain patients.

In conclusion, our data provide strong evidence for a major role of AREG in MM stimulating both plasma cells and BMSCs. These data emphasize the advantage of using ErbB inhibitors in myeloma, alone or in combination with DEX or anti-IL-6 monoclonal antibody (Tripathi *et al.*, 2003), to improve the patients'

outcome by targeting the MM cells as well as the BM microenvironment.

Materials and methods

Primary cells and polyclonal plasmablasts

Malignant plasma cells were purified from seven patients with MGUS and 65 myeloma patients at diagnosis (median age, 59 years) after informed consent was given. According to Durie-Salmon classification, 12 patients were at stage IA, 12 at stage IIA, 38 at stage IIIA, and three at stage IIIB. Normal BMPCs were obtained from healthy donors after informed consent was given. Plasma cells were purified with anti-CD138 MACS microbeads (Miltenyi-Biotec, Paris, France). Plasma from 15 patients with MM and myelomatous pleural effusions from patients were collected as described previously (Lu *et al.*, 1995b). Polyclonal plasmablasts (PPCs) were generated *in vitro* from purified CD19⁺ peripheral blood B cells as previously described (Tarte *et al.*, 2000). Bone marrow mononuclear cells from seven patients were obtained by removing myeloma cells with CD138 Miltenyi microbeads. BMSCs were obtained from patients with MM as previously described (Mahtouk *et al.*, 2004). Before use, BMSC populations were phenotyped by flow cytometry (cells were negative for CD34 and CD45 and positive for CD90, CD105 and CD73).

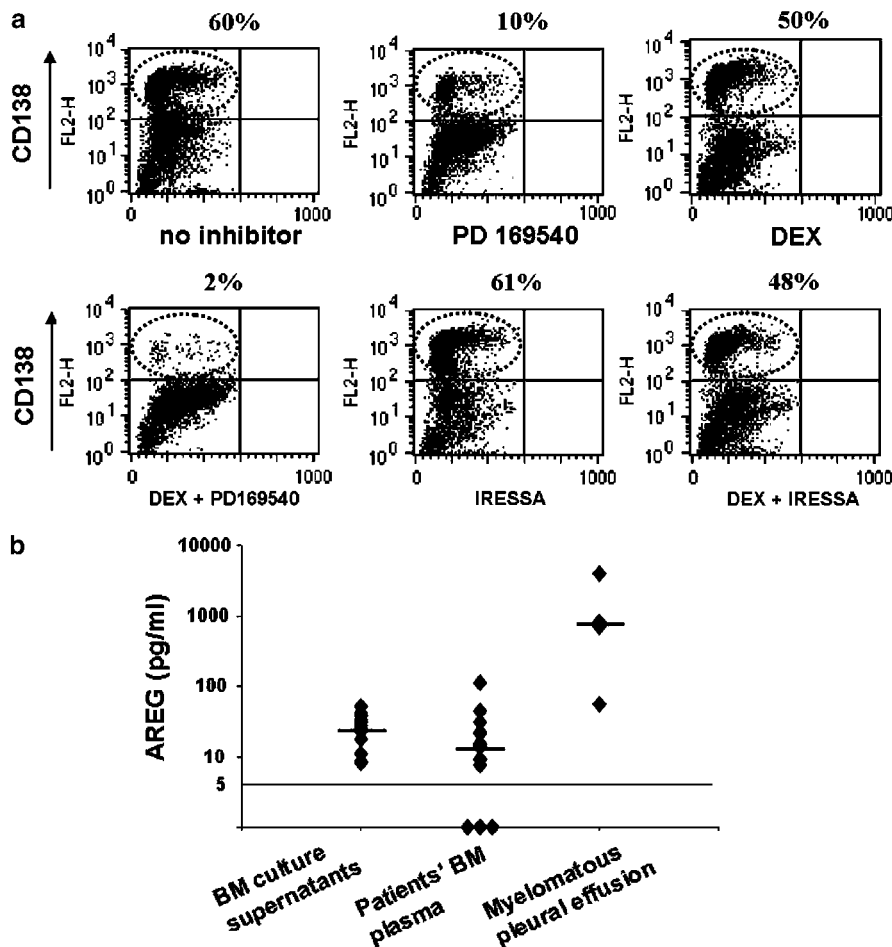


Figure 8 The PD169540 and IRESSA ErbB-inhibitors inhibit the survival of primary myeloma cells. **(a)** Mononuclear cells from patients with MM were cultured for 5 days at 5×10^5 cells/ml in RPMI 1640 medium and 5% FCS with either the PD169540 ErbB inhibitor ($1 \mu\text{M}$), or IRESSA ($1 \mu\text{M}$) or dexamethasone (DEX, 10^{-6}M), alone or in combination. As endogenous production of IL-6 is highly variable in short-term culture of patients' bone marrow, 0.5 ng/ml recombinant IL-6 was added to eliminate this source of variability. In each culture group, cells were stained with an anti-CD138-PE antibody. The percentages indicated are the percentages of CD138⁺ cells in the culture. **(b)** Concentrations of AREG in bone marrow culture supernatants from 12 patients with MM, bone marrow plasma from 15 patients with MM and myelomatous pleural effusions from four patients were determined by ELISA. The sensitivity of the ELISA was 5 pg/ml

Reagents

Recombinant amphiregulin and interleukins were purchased from R&D Systems (Minneapolis, MN, USA), IL-6 from Abcys SA (Paris, France) and AG1478 inhibitor from Alexis Biochemicals (San Diego, CA, USA). PD169540 (pan-ErbB inhibitor) was a generous gift from Pfizer Global Research and Development (Ann Arbor, MI, USA), IRESSA from AstraZeneca (Cheshire, UK) and the B-E8 anti-IL-6 MoAb from J Wijdenes (Diacclone, Besançon, France).

Myeloma cell lines

In total, 15 HMCLs were obtained in our laboratory (Zhang *et al.*, 1994; Rebouissou *et al.*, 1998). In total, 12 are IL-6-dependent myeloma cell lines (XG-1, XG-2, XG-3, XG-4, XG-6, XG-7, XG-10, XG-13, XG-14, XG-16, XG-19, XG-20). Upon removal of IL-6, myeloma cells progressively apoptose within 10–14 days (Gu *et al.*, 2000; Jourdan *et al.*, 2000). Three grow autonomously (XG-5, XG-11 and XG-12). L363, U266,

RPMI8226, LP1 and OPM2 cell lines were purchased from ATCC (Rockville, MD, USA). The HMCLs were routinely maintained in RPMI1640, 10% fetal calf serum (FCS), and for the IL-6-dependent cell lines, 2 ng/ml of IL-6.

Microarray hybridization

RNA was extracted using the RNeasy Kit (Quiagen, Hilden, Germany), the SV-total RNA extraction kit (Promega, Mannheim, Germany) and Trizol (Invitrogen, Karlsruhe, Germany) in accordance with the manufacturer's instructions. Biotinylated complementary RNA (cRNA) was amplified with a double *in vitro* transcription, according to the Affymetrix small sample labeling protocol. The biotinylated cRNA was fragmented and hybridized to the human U133 A and B GeneChip microarrays according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Fluorescence intensities were quantified and analysed using the GECOS software (Affymetrix). Arrays were scaled to an

average intensity of 100. A threshold of 1 was assigned to values under 1.

Real-time RT-PCR

RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA). We generated cDNA from 2 µg of total RNA using the Superscript II reverse transcriptase (Life Technologies) and oligo d(T)_{12–18} (Amersham Pharmacia Biotech, Orsay, France) as primer. For real-time RT-PCR, we used the assay-on-demand primers and probes and the TaqMan Universal Master Mix from Applied Biosystems (Courtaboeuf, France) according to the manufacturer's instructions. Gene expression was measured using the ABI Prism 7000 Sequence Detection System. For comparative analysis of gene expression, data were obtained by using the $\Delta\Delta C_t$ method derived from a mathematical elaboration previously described. For each sample, the C_T value for the gene of interest was determined, normalized to its respective C_T value for β_2m ($\Delta C_T = C_T - C_T \beta_2m$) and compared to A431 (for AREG and HB-EGF) or XG-7 cells (for ErbB receptor genes) used as positive control. The formula used was: $1/2^{\Delta\Delta C_T}$ sample- ΔC_T control cell line. C_T values were collected during the log phase of the cycle. The results were expressed as the relative mRNA levels to control cell line mRNA.

Myeloma cell proliferation assay

Cells were cultured for 5–7 days in 96-well flat-bottomed microtiter plates at 10^4 cells/well in 100 µl of RPMI 1640 culture medium and 5% FCS. Various concentrations of growth factors or inhibitors of growth factor were added at the beginning of the culture in six culture wells per group. At the end of the culture, cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech, Orsay, France) for 12 h, harvested and counted as reported previously (De Vos *et al.*, 2001).

Bone marrow stromal cell growth and IL-6 production

BMSCs from three different patients were cultured in six-well plates at 4×10^4 cells/ml, in 2 ml of DMEM culture medium at low serum concentration (5% FCS) to limit cell growth. Every 5–7 days, cells were counted in trypan blue and plated again at

4×10^4 cells/ml. The IL-6 production by BMSCs was assayed as described (Uchiyama *et al.*, 1993). Briefly, confluent BMSCs were starved in DMEM and 1% FCS for 24 h. Cells were washed and incubated for 48 h in DMEM and 1% FCS with AREG, with or without IRESSA. The culture supernatant was recovered at the end of the culture and assayed for IL-6 by ELISA (Beckman-Coulter, Marseilles, France).

Short-term culture of primary myeloma cells

Mononuclear cells from patients with MM were cultured for 5 days at 5×10^5 cells/ml in RPMI 1640 medium, 5% FCS, 0.5 ng/ml IL-6, without (control) or with the PD169540 pan-ErbB inhibitor (1 µM), or the IRESSA ErbB1 inhibitor (1 µM), or 10^{-6} M DEX used alone or in combination. In each culture group, the number of viable myeloma cells was determined by trypan blue staining, and myeloma cells were stained with anti-CD138-PE MoAb (Beckman-Coulter, Marseilles, France). At day 5 of culture, supernatants were collected to determine the amount of AREG by ELISA.

ELISA

AREG concentrations were determined with an ELISA purchased from R&D Systems (Minneapolis, MN, USA). The peroxidase reaction was performed with a tetramethylbenzidine (TMB) substrate kit (Sigma, St Louis, MO, USA). Light absorbance was measured at 450 nm. The sensitivity of the ELISA was 5 pg/ml.

Statistical analysis

GEPs were analysed with our bioinformatics platform (RAGE, remote analysis of microarray gene expression) designed by T Reme (INSERM U475, Montpellier, France). Statistical comparisons were made with the nonparametric Mann-Whitney test, the χ^2 test, the Student's *t*-test for pairs or the Wilcoxon test for pairs.

Acknowledgements

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SDF-1 α aussi nommé CXCL12 est le ligand du récepteur de chimiokine CXCR4 qui médie la migration des cellules souches hématopoïétiques (121). CXCR4 est exprimé par les lignées de MM et les cellules primaires de MM (122). Les cellules de MM et les BMSC produisent SDF-1 α et les taux circulants de SDF-1 α sont plus élevés chez les patients atteints de MM que chez les sujets sains (50, 122). Une étude rapporte que SDF-1 α active les voies de signalisation MAPK, PI3K/Akt et NF κ B dans le MM, induit une modeste prolifération des cellules tumorales, induit leur migration et les protège de l'apoptose induite par la dexaméthasone (122). SDF-1 α induit la sécrétion d'IL-6 et de VEGF par les BMSC (122). Plus récemment, l'équipe de *Zannettino et al* a montré que le taux circulant de SDF-1 α est corrélé avec la présence de lésions osseuses chez les patients atteints de MM, suggérant un rôle de SDF-1 α dans le recrutement et l'activation des précurseurs des ostéoclastes (50). De façon intéressante, un inhibiteur de CXCR4 bloque la formation d'ostéoclastes stimulée par les cellules de MM, confirmant que SDF-1 α peut jouer un rôle dans le recrutement et l'activité de résorption osseuse des ostéoclastes dans la moelle osseuse (50).

- Notch

La voie Notch joue un rôle clef dans le développement et la différenciation de nombreux types cellulaires hématopoïétiques (123). Dans le système hématopoïétique, les récepteurs Notch sont exprimés par les cellules souches hématopoïétiques alors que les ligands, incluant Jagged-1 et Delta-like-1 (DLL-1) sont exprimés par les BMSC, jouant un rôle pour la survie des cellules souches hématopoïétiques et leur différenciation. L'activation des récepteurs Notch par leurs ligands conduit à une cascade de clivages protéolytiques. Le premier clivage est

médié par l'enzyme TNF- α converting enzyme (TACE), suivi par un deuxième clivage médié l'activité γ -sécrétase de la PS (presenilins) qui libère le domaine intracellulaire des récepteurs de Notch (ICN). Cet ICN rentre dans le noyau et se fixe au facteur de transcription CSL (centromère binding factor 1) conduisant à l'activation de la transcription de ses gènes cibles (123).

La voie de signalisation Notch est impliquée dans les tumeurs hématologiques (123). Dans le MM, Notch-1, -2 et -3 sont exprimés à la surface des cellules de MM et des BMSC (124). L'interaction des cellules de MM avec les BMSC, qui expriment les ligands, active la voie de signalisation Notch dans les cellules de MM. L'activation de la voie Notch via Notch-1, à l'aide de Jagged-1 ou en transfectant les cellules de MM avec le gène Notch-1, inhibe l'apoptose induite par le melphalan et le mitoxantrone (124). Les ligands de Notch Jagged-1 et Jagged-2 sont exprimés par les cellules de MM mais pas par les cellules plasmocytaires normales (125, 126). L'interaction des cellules de MM avec les BMSC active aussi la voie de signalisation Notch chez les BMSC conduisant à la sécrétion d'IL-6, de VEGF et d'IGF-1 par les BMSC (125). De façon intéressante, la production d'IL-6 est bloquée par l'inhibition de la voie Notch par un anticorps anti-Notch-1. Cependant, une étude plus récente rapporte que l'activation de la voie Notch induit l'apoptose des cellules de MM (127). Dans cette étude, l'expression de la forme tronquée constitutivement active des 4 récepteurs Notch (ICN1-4) inhibe la croissance et induit l'apoptose de plusieurs lignées cellulaires, incluant des cellules B matures, des lignées de MM et des lignées de Hodgkin. Ces données démontrent que d'autres travaux sont nécessaires afin de conclure sur le rôle de la voie Notch dans le MM.

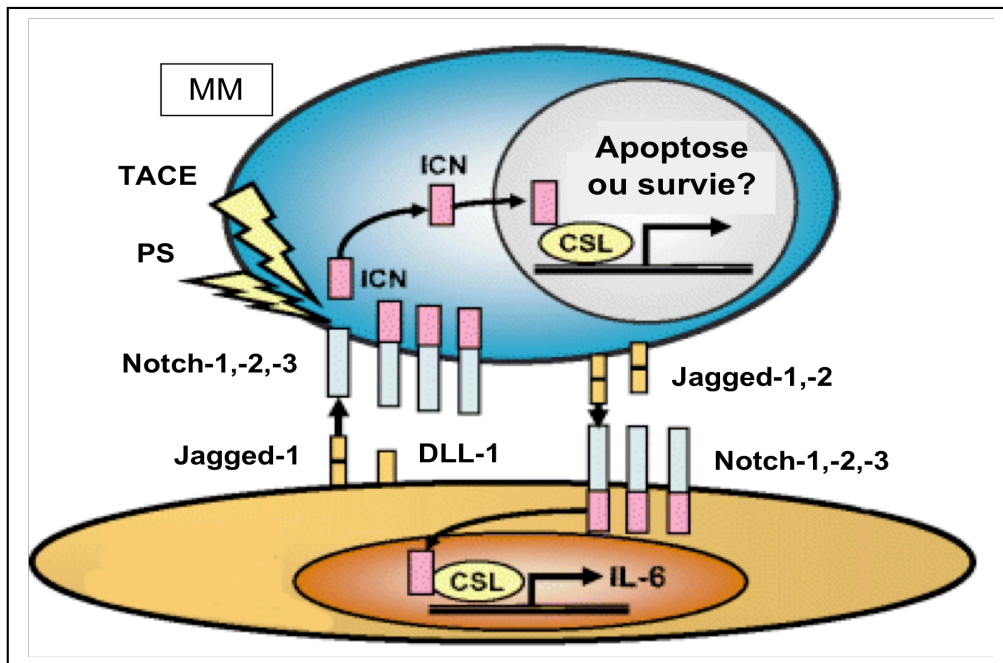


Figure 6 : Voie de signalisation Notch dans le MM. Notch-1, -2 et -3 sont exprimés par les cellules de MM et les BMSC. L'interaction des cellules de MM avec les BMSC qui expriment les ligands Jagged-1 et Delta-like (DLL)-1, active la voie de signalisation Notch dans les cellules de MM. Les ligands Jagged-1 et Jagged-2 exprimés par les cellules de MM activent la voie Notch dans les BMSC induisant la sécrétion d'IL-6, de VEGF et d'IGF-1 dans les BMSC.

- Wnt

Les Wnts sont une famille de glycoprotéines riches en cystéines qui se fixent aux récepteurs frizzled. Dans la cellule, la voie Wnt/ β -caténine inhibe l'activité de la GSK-3 β et bloque la phosphorylation de la β -caténine ainsi que sa dégradation par le protéasome, conduisant à une accumulation de β -caténine dans le cytoplasme. Dans le MM, *Derksen et al* ont montré que la voie Wnt est activée dans les cellules de MM (surexpression de la β -caténine active) et suggèrent que cette activation aberrante serait due à la production de diverses protéines de la famille Wnt par les cellules tumorales et/ou les cellules de l'environnement médullaire (128). Par ailleurs, les membres de la famille Wnt favorisent la migration/invasion des cellules de MM, en activant la voie de transduction Wnt/RhoA mais pas la voie de la β -caténine (129).

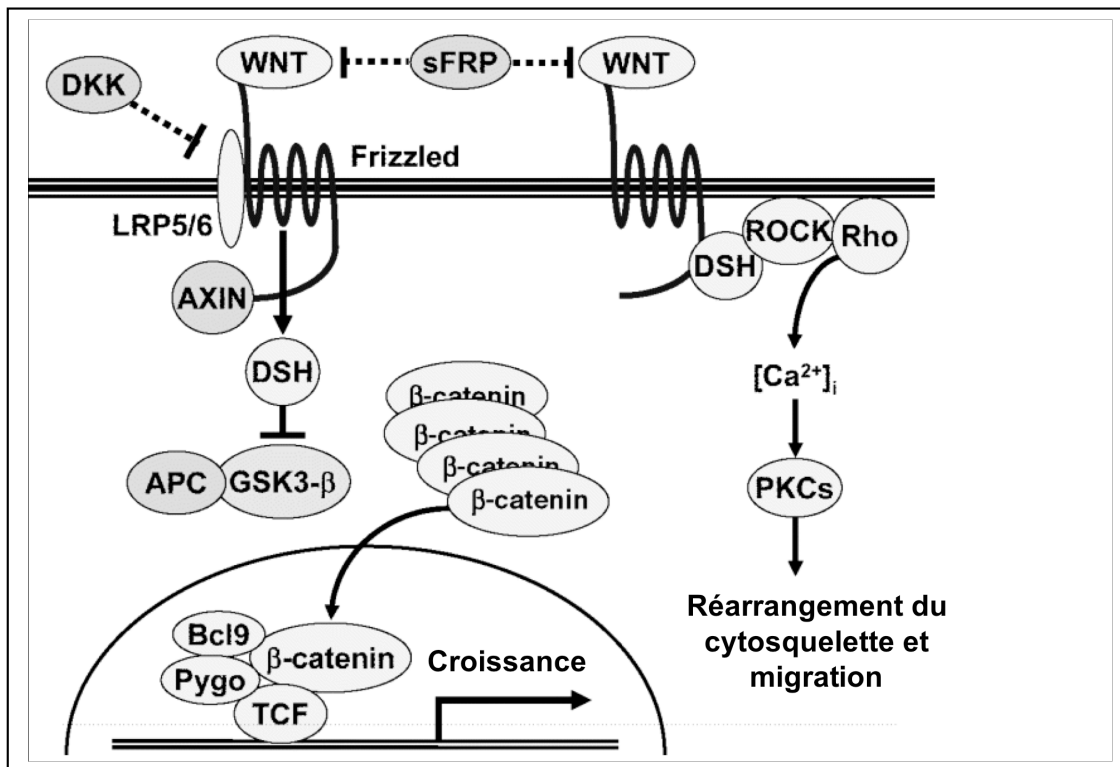


Figure 7 : Voie de signalisation Wnt dans le MM.

Dans le MM, la voie Wnt/ β -catenin est activée suite au traitement par Wnt-3a. Wnt-3a se fixe aux récepteurs membranaires frizzled induisant une inhibition de l'activité de GSK-3 β et bloquant ainsi la phosphorylation de la β -catenin et sa dégradation par le protéasome. Ceci entraîne une accumulation de β -catenin dans le cytoplasme, la translocation de la β -catenin dans le noyau et l'activation de la transcription des gènes cibles médiée par β -catenin/TCF (T-cell transcription factor). Wnt-3a stimule également la voie alternative Wnt/RhoA conduisant à des changements morphologiques et la migration des cellules via l'activation de PKC α , PKC β et PKC μ . sFRP : secreted frizzled-related protein ; AXIN : axis inhibitor ; DSH : disheveled ; Pygo : pygopus ; ROCK : Rho-associated coiled-coil-containing protein kinase.

- Autres facteurs

Au cours des dernières années, de plus en plus de travaux se sont concentrés sur l'identification de facteurs de croissance myélomateux. Une publication récente démontre que **BDNF**, un membre de la famille des neurotrophines exprimé par les cellules primaires de MM, induit la survie des cellules de MM qui expriment son récepteur TrkB, en activant les voies MAPK et PI3K/AKT (130).

Deux cytokines l'**IL-15** et l'**IL-21**, ont été décrites comme étant des facteurs de survie et de prolifération importants pour les cellules de MM. L'activation du récepteur de l'iL-15 (IL-15Ra) par l'IL-15 produite de façon autocrine ou paracrine, protège les cellules de MM de l'apoptose et diminue l'apoptose induite par les agents cytotoxiques (131). L'IL-21 induit la prolifération de lignées de MM IL-6 dépendantes, en synergie avec le TNF- α (132).

La chimiokine **MIP-1 α** est aussi un facteur de croissance pour les cellules de MM qui expriment son récepteur CCR5 (133).

Récemment, il a été démontré que les cellules de MM expriment les Toll-like récepteurs (TLR) (134, 135). Les TLR 1, 7 et 9 sont les plus fréquemment exprimés par les cellules de MM (135). L'ajout des ligands des TLR7 et TLR9 dans les cultures *in vitro* protège les cellules de MM de l'apoptose induite par la déprivation de sérum ou par la dexaméthasone. De plus, ces ligands induisent la croissance des cellules de MM. Cet effet est lié à une boucle autocrine impliquant l'IL-6 (134, 135).

Récemment, notre équipe a publié une revue sur les facteurs de croissance plasmocytaires (Article 2).

« Survival and proliferation factors of normal and malignant plasma cells. Int J Hematol. » 2003 Aug;78(2):106-13. Review.

H. Implications thérapeutiques

Les stratégies thérapeutiques visant à bloquer les facteurs de croissance ou les voies de signalisations activées par ces facteurs de croissance sont résumées dans le tableau suivant :

Tableau n°2 : Nouvelles thérapeutiques ciblées du MM.

Facteur de croissance ou voie de signalisation	Inhibiteur
IL-6	Superantagoniste de l'IL-6R : Sant7
VEGF	Anticorps anti-VEGF : Bevacizumab. Inhibiteur du VEGF : PTK787 et GW654652
CD40/CD40L	Anticorps anti-CD40 : SGN-40 et CHIR-12.12
IGF-1	Inhibiteur de l'IGF1R : NVP-ADW742
FGFR3	Inhibiteur du FGFR3 : SU5402, SU10991, PD173074 et PKC412
Farnesylation des protéines	Inhibiteur de farnesyltransférase : R115777, SCH66336, manumycin
mTOR	Inhibiteur de mTOR : Rapamycine et CCI-779
Voie NF- κ B	Inhibiteur d' κ B kinase : PS-1145
TGF β	Inhibiteur du TGF β 1R : SD-208
Voie MAPK	Inhibiteur de p38MAPK : SCIO-469

Inhibition des voies de signalisation importantes pour la survie et la prolifération des cellules de MM

L'IL-6, l'IGF-1 et le VEGF active la voie Ras/MEK/ERK. L'inhibition de la voie Ras/Raf/MEK/ERK en utilisant un inhibiteur de farnesyltransférase (FTI) R115777 bloque la croissance des cellules de MM (136, 137). De plus, la combinaison du FTI SCH66336 avec le Bortezomide/Velcade induit de façon synergique la mort des cellules de MM. Ceci est associé à une augmentation du clivage des caspases et une diminution de la phosphorylation d'Akt. Un essai clinique récent de phase II démontre que le FTI est bien toléré, induit une stabilisation de la maladie chez 64% des patients et inhibe la survie des cellules tumorales chez les patients avec un MM

avancé (138). Plus récemment, il a été démontré qu'un FTI (manumycin) lève la résistance des cellules de MM à l'apoptose induite par Fas (139).

La voie de signalisation PI3K/Akt joue également un rôle important dans la survie et la croissance des cellules de MM induite par les facteurs de croissance. L'activation d'Akt conduit à la phosphorylation de FKHR, GSK-3 β et mTOR. L'inhibition de mTOR par la rapamycine ou le CCI-779, un analogue de la rapamycine, a une activité anti-MM *in vitro*. Ces données ont été confirmées *in vivo* dans un modèle murin de xenogreffe de cellules de MM pour le CCI-779 (140, 141).

L'environnement médullaire comme cible thérapeutique

Comme décrit précédemment, l'environnement médullaire soutient la croissance, la migration et la résistance aux drogues des cellules de MM. L'inhibiteur de la kinase I κ B PS-1145 (142), l'inhibiteur du TGF β R1 SD-208 (143) et l'inhibiteur de p38MAPK SCIO-469 (144) inhibe la sécrétion d'IL-6 et de VEGF par les BMSC conduisant à une diminution de la prolifération des cellules de MM. De nouveaux inhibiteurs incluant le Bortezomid/Velcade et la lenalidomide/Revlimid inhibent la sécrétion des facteurs de croissance et des cytokines par l'environnement médullaire mais également l'activation des voies de signalisation induite par les facteurs de croissance dans les cellules de MM (142). L'inhibiteur du protéasome, le Bortezomid, peut lever l'effet de protection de l'IL-6 contre l'apoptose induite par le dexaméthasone en induisant l'activation des caspases -8/-9/-3 conduisant à un clivage caspase dépendant de la gp130 (32, 145). De nouveaux agents incluant le trioxide d'arsenic, le 2-méthoxyestradiol, les inhibiteurs d'histone deacetylase (HDAC) SAHA187 et NVP-LAQ824, l'inhibiteur de HDAC6 (146), l'azaspirane (146), le SDX-101 (147) et le FTY720 (analogie de la sphingosine) (148) induisent

l'apoptose des cellules de MM même si ces dernières sont cultivées en présence d'IL-6 exogène ou de l'environnement médullaire (35). Notre équipe a également démontré qu'un inhibiteur pan-ErbB entraîne une apoptose des plasmocytes tumoraux cultivés in vitro avec les cellules de l'environnement médullaire (120). De plus une combinaison inhibiteur pan-ErbB + dexaméthasone entraîne une élimination presque totale des cellules de MM présentes dans la culture (120). Au vue des résultats obtenus avec ces nouveaux inhibiteurs, leur association avec les agents de chimiothérapie conventionnels pourrait se révéler efficace pour le traitement du MM.

III- Etudes transcriptomiques et myélome multiple

III-1. Généralités sur les puces à ADN

La technologie des puces à ADN permet d'analyser l'expression simultanée de dizaines de milliers de gènes, dans des dizaines de conditions différentes aussi bien physiologiques que pathologiques. Cette technologie a donné un nouvel outil formidable pour l'étude de la biologie des cellules cancéreuses.

Les puces à ADN consistent en un support solide sur lequel des milliers ou des dizaines de milliers d'oligonucléotides ou de fragment d'ADNc sont déposés de façon géométrique, chacun des fragments étant représenté par un point sur le support. Le dépôt d'ADNc sur une membrane de nylon est appelé « macroarrays » tandis que le dépôt d'ADNc ou la synthèse in situ d'oligonucléotides (technologie Affymetrix) sur un support de verre de petite taille sont appelés « microarrays ». Les ADNc/oligonucléotides servent de sondes sur lesquelles vont s'hybrider de façon très spécifique les fragments de gènes complémentaires (cibles), présents dans les échantillons biologiques à tester. L'hybridation peut être mise en évidence par des techniques optiques sous éclairage fluorescent ou par détection de radioactivité, un système de marquage de l'échantillon au moyen de traceurs fluorescents ou radioactifs ayant été réalisé préalablement. La quantification des signaux obtenus et l'identification des fragments de gènes reconnus sont ensuite rendus possible au moyen d'un système d'acquisition d'image puis d'analyse des données faisant appel

à des logiciels informatiques spécialement conçus à cet effet. Les résultats obtenus sont ensuite validés sur le plan statistique et interprétés dans un contexte biologique. De nouvelles classifications des tumeurs et de nouveaux modèles pronostiques ont été développés en se basant sur les données des puces à ADN dans de nombreux cancers. Cette approche a conduit à de nombreux progrès dans la compréhension de la biologie des tumeurs lymphoïdes humaines (149).

III-2. Application au myélome multiple

Il existe au moins deux raisons qui font du MM un bon candidat pour des études transcriptomiques. Tout d'abord, le MM est une maladie cliniquement hétérogène. De plus, les anomalies génétiques dans le MM sont variables et complexes. L'étude du profil d'expression génique à l'aide des puces à ADN permet de définir des entités différentes de patients atteints de MM présentant des pronostics différents. Ceci peut permettre de stratifier les patients afin d'adapter le traitement. La première étude comparant les profils d'expression génique des cellules tumorales de patients atteints de MM au diagnostic, de lignées de MM et de plasmocytes médullaires normaux fut publiée en 2002 par le groupe américain de John Shaughnessy (150). Par la suite, notre équipe, en utilisant des macroarrays, a identifié des gènes de communications intercellulaires surexprimés dans les plasmocytes tumoraux (60). Les puces à ADN ont également été utilisées pour identifier les profils d'expression génique liés à la résistance des cellules de MM à la doxorubicin *in vitro*. Cette étude a mis en évidence que la sélection de cellules de MM résistantes à la doxorubicin s'accompagne d'une modification du profil d'expression génique traduisant une réduction de la réponse aux signaux apoptotiques (151). Une étude s'est également

intéressée aux mécanismes d'action de la dexaméthasone et de résistance des cellules de MM à la dexaméthasone (152). Lors du traitement des cellules de MM par la dexaméthasone, on note que l'activation transitoire des gènes impliqués dans la réparation de l'ADN est suivie par une induction de l'expression de gènes proapoptotiques et une diminution de l'expression de gènes impliqués dans la croissance et la survie des cellules de MM (152). Le groupe d'Anderson a démontré que le 2-méthoxyestradiol (2ME2) ayant une activité anti-myélomateuse est capable de moduler l'expression des gènes impliqués dans la résistance à la dexaméthasone ainsi que les gènes codant pour les protéines de la famille « heat shock protein » et pour des protéines impliquées dans l'ubiquitination (153). Une étude du groupe de Nantes utilisant des microarrays a mis en évidence que les programmes transcriptionnels associés à la commutation isotypique peuvent être maintenus pendant la différenciation plasmocytaire. Ces données ont été obtenues en comparant les profils d'expression génique de plasmocytes tumoraux IgA de patients avec des MM IgG. De plus, les auteurs ont également identifié des gènes différentiellement exprimés entre les MM à chaîne kappa et les myélomes lambda. De façon intéressante, MIP-1 α est surexprimé dans le sous-groupe kappa et cette forte expression de MIP-1 α est corrélée à une importante lyse osseuse chez les patients atteints de MM (154). Croonquist *et al* ont comparés les profils d'expression génique de cellules de MM cultivées sans IL-6, avec IL-6, en présence de BMSC ou transfectées par N-ras (155). Ils ont identifié des signatures d'expression génique distinguant une transformation génétique intrinsèque et des profils d'expression génique de la stimulation induite par l'IL-6 ou induite par les BMSC.

La progression multi-étapes du MM a été analysée à l'aide des puces à ADN en comparant les profils d'expression génique des plasmocytes normaux avec ceux de

plasmocytes de patients présentant un MGUS et ceux de plasmocytes tumoraux de patients atteints de MM (156). Cette étude a mis en évidence que les différences entre les plasmocytes myélomateux et les cellules de MGUS sont plus importantes que celles entre les plasmocytes normaux et les cellules de MGUS ou entre les plasmocytes normaux et les cellules de MM. Parmi les gènes différentiellement exprimés, les auteurs ont identifiés des oncogènes, des gènes codant pour des protéines impliquées dans la transduction du signal, des facteurs de transcription et des gènes de communication intercellulaire (156).

L'effet antiapoptotique de l'IL-6 sur la lignée IL-6 dépendante INA-6 a également été analysé à l'aide des puces à ADN. L'activation des gènes impliqués dans l'effet antiapoptotique de l'IL-6 sont dépendant de l'activation de STAT-3 (157). L'utilisation combinée du superantagoniste de l'IL-6 récepteur Sant7 et de la dexaméthasone, qui induit de façon synergique l'apoptose des cellules de MM *in vitro* et *in vivo*, induit l'expression de gènes proapoptotiques tout en engendrant une diminution de l'expression des gènes impliqués dans la prolifération et le contrôle et la maintenance du cycle cellulaire (74). Munshi et *al* ont comparé les profils d'expression génique de plasmocytes tumoraux et de plasmocytes myélomateux de deux vrais jumeaux, l'un des deux étant atteint de MM (158). Les plasmocytes myélomateux du jumeau atteint de MM présentent une surexpression de gènes impliqués dans la survie (MCL-1, dad-1 et FLIP), des oncogènes/facteurs de transcription (Jun-D, Xbp-1, Calmodulin, Calnexin et FGFR-3) et des gènes impliqués dans la voie d'ubiquitination et de dégradation des protéines par le protéasome. A l'opposé, les gènes proapoptotiques APAF-1 (apoptotic protease activating factor) et RAD51 (killer cell immunoglobulin-like receptor protein) sont plus faiblement exprimés par les plasmocytes du jumeau atteint de MM (158).

Notre équipe a pu mettre en évidence le rôle des membres de la famille EGF dans la biologie du MM à partir des données des puces à ADN (120, 159). Le groupe de Bergsagel a identifié la dérégulation de l'expression des gènes de la cycline D comme un événement précoce dans l'oncogenèse du MM.

Notre équipe a récemment publié une revue basée sur l'utilisation des données des puces à ADN pour progresser dans la compréhension de la biologie des cellules plasmocytaires normales et tumorales (Article 3).

**« Microarray-based understanding of normal and malignant plasma cells. »
Immunol Rev. 2006 Apr;210:86-104.**

Dernièrement, en nous intéressant aux gènes myélomateux, c'est-à-dire les gènes surexprimés dans les plasmocytes tumoraux par rapport aux plasmocytes normaux, nous avons identifié une surexpression de CD200 par les plasmocytes tumoraux. Le CD200 s'avère être un nouveau facteur pronostique du MM (Article 4).

« CD200 is a new prognostic factor in Multiple Myeloma. "Blood. 2006 Aug 31; [Epub ahead of print] ».

Notre équipe a analysé l'expression de CD200 par les plasmocytes purifiés de 112 patients au diagnostic. Le gène CD200 est soit « présent » soit « absent » (affymetrix call) chez 78% et 22% des patients respectivement. Le CD200 est une glycoprotéine membranaire qui transmet un signal immunorégulateur, via son récepteur le CD200R, conduisant à l'inhibition de la réponse immunitaire T. Les cellules de l'environnement médullaire n'expriment pas le CD200 excepté les BMSC et les cellules souches hématopoïétiques mais à un niveau plus faible que les cellules de

Brief report

CD200 is a new prognostic factor in multiple myeloma

Jerome Moreaux, Dirk Hose, Thierry Reme, Eric Jourdan, Michael Hundemer, Eric Legouffe, Philippe Moine, Philippe Bourin, Marion Moos, Jill Corre, Thomas Möhler, John De Vos, Jean Francois Rossi, Hartmut Goldschmidt, and Bernard Klein

Using Affymetrix microarrays, we identified the expression of the *CD200* gene in multiple myeloma cells (MMCs) of 112 patients with newly diagnosed multiple myeloma (MM). The *CD200* gene was either absent or present (Affymetrix call) in 22% and 78% of MMCs, respectively. The *CD200* gene is not expressed in cells of the patients' bone marrow (BM). CD200 is a membrane glyco-

protein that imparts an immunoregulatory signal through CD200R, leading to the suppression of T-cell-mediated immune responses. Patients with CD200^{absent} MMCs have an increased event-free survival (EFS; 24 months) compared with patients with CD200^{present} MMCs (14 months), after high-dose therapy and stem cell transplantation. In a Cox proportional-hazard model, the

absence or presence of *CD200* expression in MMCs is predictive for EFS for patients independently of ISS stage or β 2M serum levels. Thus, *CD200* is an independent prognostic factor for patients with MM that could represent a new therapeutic target in MM. (Blood. 2006;108:4194-4197)

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Introduction

Multiple myeloma (MM) is a plasma cell neoplasm characterized by the accumulation of malignant plasma cells within the bone marrow (BM). Several autocrine or paracrine factors can promote multiple myeloma cell (MMC) survival and proliferation.^{1,2} We anticipate that in the future, the inhibition of MMC growth factors may have clinical applications in combination with other drugs.³⁻⁶

In order to identify new molecules involved in the communication between MMCs and the BM environment, we compared gene expression profiles (GEPs) of MMCs with those of normal plasma cells, normal plasmablasts, and normal peripheral blood B cells. We identified that *CD200* was expressed in malignant plasma cells in 78% of newly diagnosed patients with MM. CD200, formerly known as OX-2, is a highly conserved type I transmembrane glycoprotein that is expressed by thymocytes, activated T cells, B cells, dendritic cells, endothelial cells, and neurons.⁷ The expression of the receptor for CD200 (CD200R1) is described as restricted to myeloid-derived antigen-presenting cells and certain populations of T cells.⁸ Three other genes, closely related to CD200R1 and termed CD200R2-4, have been identified, but the function of these encoded proteins, in particular their ability to bind CD200, is not fully elucidated.⁹ Several studies have shown that CD200 imparts an immunoregulatory signal through CD200R, leading to the suppression of T-cell-mediated immune responses. Increased survival of renal allografts following portal vein immunization with alloantigen correlates with an increase in *CD200* expression in both hepatic and splenic dendritic cells (DCs) in a murine model.¹⁰ Tolerance in this setting is reversed with a monoclonal antibody to CD200.¹¹ CD200-deficient mice have a compromised capacity to down-regulate the activation of antigen-

presenting cells. This results in chronic central nervous system inflammation, which causes an exaggerated inflammatory response to trauma and an increased susceptibility to develop both experimental autoimmune encephalitis and collagen-induced arthritis.¹² More recently, Gorczynski et al¹³ demonstrated that anti-CD200R²⁻⁴ monoclonal antibodies (MoAbs) promote the development of DCs and have the capacity to induce regulatory T cells (Tregs) and directly augment the production of Tregs in the thymus.

In this study, we demonstrate that MMCs of 78% of the patients with newly diagnosed MM express *CD200*. For patients included in protocols with high-dose chemotherapy (HDC) and autologous hematopoietic stem cell transplantation (ASCT), the presence or absence of CD200 expression is a prognostic factor independent of ISS stage or β 2M.

Materials and methods

Cell samples

XG human myeloma cell lines (HMCLs) were obtained as described.¹⁴⁻¹⁶ SKMM, OPM2, LP1, and RPMI8226 HMCLs were purchased from ATCC (LGC Promochem, Molsheim, France). MMCs were purified from 112 patients with newly diagnosed MM after written informed consent was given. The institutional review board approval was given by the local ethics committee (Comité Consultatif de Protection des Personnes dans la recherche biomédicale; CPPRB). These 112 patients were treated with HDC and ASCT. Seven of these 112 patients received allogeneic bone marrow transplantation after HDC and ASCT, and their event-free survival (EFS) was censored at the time of the allograft. In the series, according to the Durie-Salmon classification, 9 patients were of stage IA; 14, of stage

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IIA; 83, of stage IIIA; and 6, of stage IIIB. According to the International Staging System (ISS),¹⁷ 44 patients were of stage I; 53, of stage II; and 15, of stage III. Seventeen patients had IgAκ MM; 6, IgAλ MM; 38, IgGκ MM; 26, IgGλ MM; 14, Bence-Jones κ MM; 7, Bence-Jones λ MM; and 4, nonsecreting MM. The obtainment and purification of MMCs, normal bone marrow (BM) plasma cells (BMPCs), memory B cells, polyclonal plasmablasts, osteoclasts, BM stromal cell lines, BM CD3 T cells, BM monocytes, and BM polymorphonuclear neutrophils were performed as previously described.¹⁸

Preparation of complementary RNA (cRNA) and microarray hybridization

RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany). Biotinylated cRNA was amplified with a double in vitro transcription reaction and hybridized to the Affymetrix HG U133 set of Gene Chips, according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA).

CD200 expression by MMCs

CD200 expression by primary MMCs was determined using a double labeling of primary MMCs with an FITC-conjugated anti-CD138 MoAb and a PE-conjugated anti-CD200 MoAb (clone MRCOX-104; Becton Dickinson, San Jose, CA), or with FITC- or PE- conjugated isotype-matched control antibodies. The fluorescence intensity was determined with a FACScan device (Becton Dickinson), setting up the mean fluorescence intensity obtained with the control antibodies between 4 and 6.

Statistical analysis

Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platforms (RAGE, <http://rage.montp.inserm.fr/demo/demo.shtml?submit=Demo> and Amazonia, <http://amazonia.montp.inserm.fr/>). Statistical comparisons were done with R (<http://www.r-project.org/>) or SPSS10 (SPSS, Chicago, IL) software.

Results and discussion

Comparing the GEPs of MMCs in an initial series of 48 patients with newly diagnosed MM with the GEP of normal BM plasma cells, normal plasmablasts, and normal memory B cells using Affymetrix U133 A+B DNA microarrays, we found a clear-cut expression of *CD200* in MMCs. In 9 of 48 patients, *CD200* had an “absent” Affymetrix call (*CD200^{absent}*) in MMCs. In the remaining 39 patients, *CD200* had a “present” call (*CD200^{present}*) in MMCs and was overexpressed in MMCs compared with normal BMPCs (ratio = 6.2 and *P* < .01), plasmablasts (ratio = 26.5 and *P* < .01), or memory B cells (ratio = 10 and *P* < .01) (Figure 1A). These data were confirmed with another independent series of MMCs from 64 newly diagnosed patients using Affymetrix U133 2.0 plus microarrays (Figure 1A). Combining the 2 sets of data, *CD200* had a present call in MMCs in 87 (78%) of 112 patients. Microarray *CD200* expression was validated by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) for 20 HMCLs and 5 patients’ MMCs (*r* = .81, *P* < .001) (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Using fluorescence-activated cell sorting (FACS) analysis, the CD200 protein was detected on MMCs in 13 (86%) of 15 consecutive newly diagnosed patients, confirming the frequency of *CD200* expression determined by absent/present calls with Affymetrix microarrays (Table 1). If CD200 is

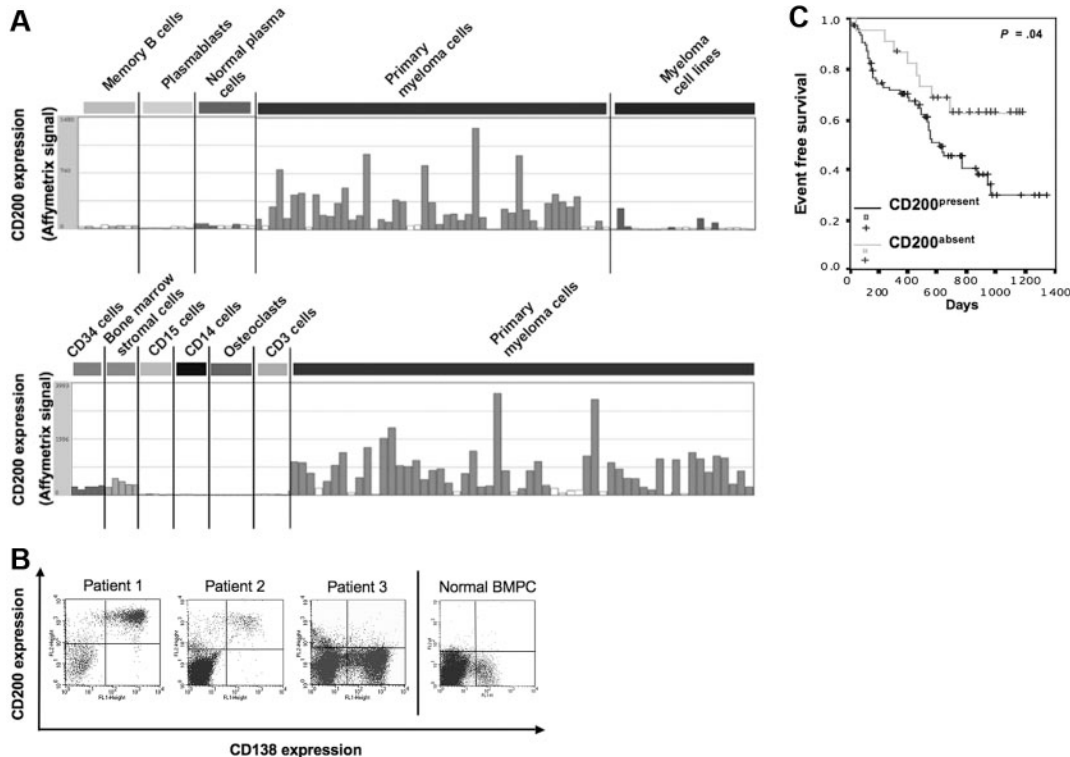


Figure 1. CD200 expression and association with event-free survival in patients with MM. (A) Affymetrix *CD200* gene expression in normal memory B cells; normal polyclonal plasmablasts; normal BM plasma cells (BMPCs); purified myeloma cells from patients with multiple myeloma (MM); human myeloma cell lines; BM stromal cells; BM CD34 cells; purified BM CD15, CD14, and CD3 cells; and osteoclasts. (B) CD200 expression by primary MMCs or normal BMPCs was determined using a double labeling of primary MMCs with an FITC-conjugated anti-CD138 MoAb and a PE-conjugated anti-CD200 MoAb, or with FITC- or PE-conjugated isotype-matched control antibodies. (C) Kaplan-Meier plot of the event-free survival in patients with *CD200^{present}* and *CD200^{absent}* MMCs.

Table 1. CD200 expression at the surface of myeloma cells

Patient no.	CD200 labeling	
	%	MFI
1	84.8	2273
2	90.4	4347
3	88.8	986
4	97.7	2023
5	80.0	1219
6	98.1	1155
7	< 5.0	—
8	99.6	8038
9	< 5.0	—
10	95.9	804
11	95.6	832
12	30.7	364
13	99.9	1259
14	62.0	601
15	97.8	442

CD200 expression by primary MMCs was determined using a double labeling of primary MMCs with an FITC-conjugated anti-CD138 MoAb and a PE-conjugated anti-CD200 MoAb, or with FITC- or PE-conjugated isotype-matched control antibodies. The fluorescence intensity was determined with a FACScan device, setting up the mean fluorescence intensity obtained with the control antibodies between 4 and 6.

— indicates no labeling of anti-CD20 antibody.

expressed in MMCs, the mean fluorescence intensity ranged from 364 to 8038 (Table 1) (Figure 1B). On the panel of 20 HMCLs used, only HMCLs with detectable *CD200* mRNA displayed a positive labeling with the anti-CD200 MoAb. Moreover, we found a very good correlation ($r = .92$; $P < .001$) between Affymetrix *CD200* expression and protein expression (mean fluorescence intensity) at the surface of our HMCLs (Figure S2). Subsequently, we investigated the *CD200* gene expression in the BM environment from patients with MM. *CD200* was not expressed by CD14 monocytes, CD15 polynuclear cells, and CD3 T cells that were purified from the BM of 5 newly diagnosed patients. It is also not expressed in 7 osteoclast samples (Figure 1A). BM stromal cells from 5 patients with MM expressed *CD200*, but at a 3.9-fold lower median signal compared with that in *CD200*^{present} MMCs ($P = .04$). CD34⁺ hematopoietic stem cells from 5 patients with MM expressed *CD200*, but at a 2.5-fold lower median signal compared with that in *CD200*^{present} MMCs ($P = .05$).

A significantly higher number of patients with *CD200*^{present} had a monoclonal protein containing lambda light chains, whereas the characteristic of age of 65 years or older appeared at a significantly higher frequency in patients with *CD200*^{absent} MMCs (Table S1). In our group of 112 newly diagnosed patients treated with HDT and ASCT, patients with *CD200*^{absent} MMCs

had a better event-free survival (24 months) compared with patients with *CD200*^{present} MMCs (14 months) (Figure 1C). In a Cox proportional-hazard model monitoring for the absence or presence of *CD200* ($P = .04$) and ISS stage ($P = .01$), both parameters are independently predictive for EFS ($P = .01$). If *CD200* expression is tested together with classical prognostic factors (ie, serum albumin and serum β 2M) *CD200* expression ($P = .04$) and β 2M ($P = .015$) remain independent prognostic factors, whereas serum albumin marginally fails to be significant ($P = .058$).

This better EFS of patients with MMCs lacking *CD200* could be linked to the role of CD200 in suppression of T-cell-mediated immune responses and in the development of DCs with a capacity to induce Tregs.^{9-11,13} A recent study has demonstrated that B-cell chronic lymphocytic leukemia expresses CD200 that leads to inhibition of the Th1 response in mixed lymphocyte reactions.¹⁹ In conclusion, we have identified that *CD200* expression by MMCs is an independent prognostic factor that could represent a new therapeutic target for patients with MM.

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Authorship

Contribution: J.M. performed the experiments and participated in the writing of the paper; D.H., M.H., and H.G. collected bone marrow samples and clinical data and participated in the writing of the paper; T.R. and J.D.V. performed the bioinformatic studies and participated in the writing of the paper; E.J., E.L., J.F.R., and T.M. collected bone marrow samples and clinical data; P.M. provided technical assistance; M.M. participated in the writing of the paper; P.B. and J.C. collected bone marrow samples; B.K. participated in the design of the research and the writing of the paper.

Conflict of interest disclosure: The authors declare no competing financial interests.

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MM. Les patients CD200⁻ ont une survie sans événement (24 mois) significativement plus importante que les patients CD200⁺ (14 mois). Le CD200 est un nouveau facteur pronostique du MM qui est indépendant de l'ISS et du taux de beta-2microglobuline circulant. Compte tenu de sa localisation membranaire et de son rôle dans l'immunité T, le CD200 représente une nouvelle cible thérapeutique intéressante dans le MM.

III-3. Objectif du travail de thèse

Au cours de nos analyses du transcriptome au laboratoire, nous avons comparé les profils d'expression génique des plasmocytes tumoraux de patients atteints de MM à ceux de plasmablastes normaux et de lymphocytes B normaux (Figure 8).

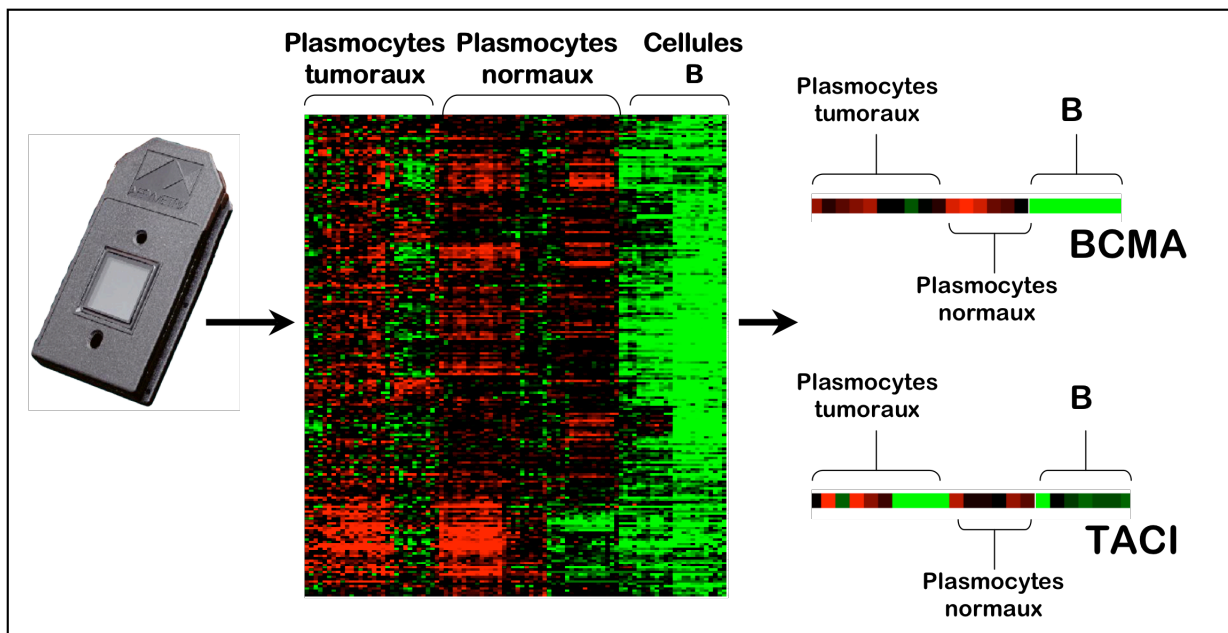


Figure 8 : Analyse non supervisée de plasmocytes tumoraux purifiés de patients, de plasmablastes normaux et de cellules B normales (logiciels Cluster et Treeview). Les résultats sont présentés sous forme de matrice où chaque ligne représente un gène et chaque colonne représente un échantillon. Le niveau d'expression de chaque gène est représenté selon le code couleur suivant : vert = expression inférieure à la valeur médiane de l'ensemble des échantillons ; rouge = expression

supérieure à la valeur médiane de l'ensemble des échantillons ; noir = expression médiane. Les échantillons sont regroupés en fonction des similitudes de leur profil d'expression de gènes.

Nous avons ainsi pu identifier une surexpression de **BCMA** et **TACI**, deux **récepteurs de BAFF** et **APRIL** par les plasmocytes tumoraux et les plasmocytes normaux par rapport aux cellules B normales. Cette observation a constitué le départ de mon travail de thèse. BAFF et APRIL sont deux membres de la famille TNF. Bien qu'à ce moment, le rôle de ces membres de la famille TNF soit inconnu dans le MM, leur rôle a été étudié dans la différenciation B normale ou d'autres cancers.

- Dans un premier temps, nous avons étudié l'expression, le rôle biologique et les mécanismes d'action de BAFF et APRIL dans la biologie du MM. Nous avons également recherché l'effet d'inhibiteurs spécifiques de la voie BAFF/APRIL, en vue d'une utilisation clinique pour le traitement de patients atteints de MM.

- Nous avons ensuite recherché les cellules étant la source de la production de BAFF et APRIL dans la moelle osseuse. Nous avons également étudié les profils d'expression génique associés à l'expression des récepteurs de BAFF/APRIL dans les cellules de MM. Cette étude du transcriptome présentant un intérêt certain afin de mettre en évidence une signature liée à la réponse au traitement par un inhibiteur de ces facteurs de croissance.

- Enfin, nous nous sommes intéressés aux profils d'expression génique associé à l'expression des récepteurs de BAFF et APRIL dans notre large panel de lignées de MM.

RESULTATS

DISCUSSION

**Rôle des membres de la famille
BAFF/APRIL dans le myélome multiple.**

II- Présentation du sujet : la famille BAFF/APRIL et ses récepteurs

I-1. Généralités

BAFF (B cell activating factor, aussi nommé Blys, TALL-1, THANK, zTNF-4 et TNFSF-13B) et APRIL (A proliferating-inducing ligand, aussi nommé TNFSF-13) sont deux membres de la superfamille TNF.

BAFF peut exister sous forme membranaire mais aussi sous forme soluble. En effet, BAFF a été détecté dans le sérum des souris transgéniques pour BAFF, dans les sérums de souris atteintes de maladies auto-immunes et également chez l'homme indiquant que le site de clivage est reconnu *in vivo* (160-164). APRIL apparaît être de façon prédominante sécrété puisque seule la forme soluble a pu être détectée chez les cellules transfectées par APRIL (165). Le clivage d'APRIL par une furine convertase a également été rapporté (165). BAFF et APRIL sont connus pour former des homotrimères (166, 167) mais il a été récemment suggéré que BAFF et APRIL peuvent s'associer pour former des hétéromères capables de stimuler la prolifération de lymphocytes B (168). L'hétéromère est composé de deux monomères BAFF et un monomère APRIL ou d'un monomère BAFF et deux monomères APRIL.

Trois groupes ont résolu la structure cristallographique de BAFF avec différents résultats. Une structure pour APRIL a été modelée sur la structure de BAFF (Figure 9). BAFF et APRIL ont 29% d'homologie de séquence. Il y a une bonne homologie de structure.

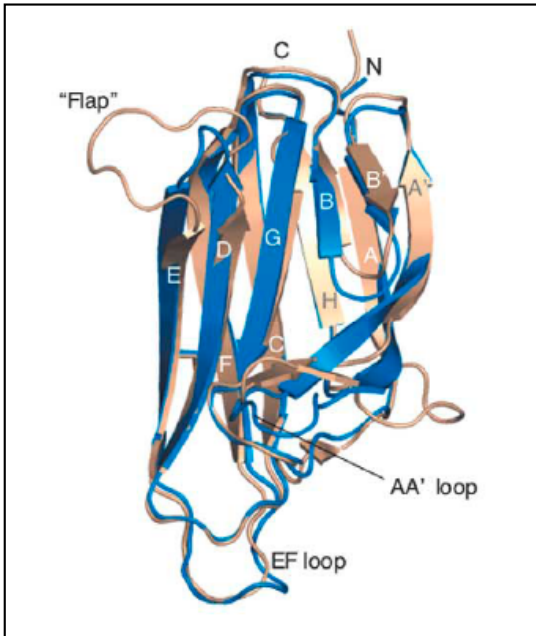


Figure 9 : Structure de BAFF et APRIL.

La plus grosse différence entre BAFF et APRIL est la boucle D-E aussi appelée Flap qui est beaucoup plus courte chez APRIL (il y a 6 résidus de moins) (voir figure 9). Cette boucle n'est pas impliquée dans des interactions pour le trimère d'APRIL. Pour BAFF, cette boucle est requise pour la formation de trimère. De plus cette boucle est la seule impliquée dans les contacts trimère/trimère.

A. Expression des ligands

BAFF est exprimé par les cellules mononuclées du sang périphérique, la rate et les ganglions alors que de faibles niveaux d'expression ont été observés pour les autres tissus (163, 164, 169-171). BAFF est exprimés par différents types cellulaires incluant les monocytes, les macrophages, les cellules dendritiques et les lymphocytes T (163, 164, 171). Normalement, BAFF n'est pas exprimé par les lymphocytes B mais il est exprimé par les cellules B de leucémie lymphocytaire chronique (LLC) (172). Le manque de cellules B matures dans le sang périphérique chez les souris « knock-out » (KO) pour BAFF ou chez les souris traitées par des antagonistes de BAFF indique que le taux basal de BAFF est requis pour la maturation et la survie des lymphocytes B matures (173-175). Des études ont

démontré que l'expression de BAFF est régulée par des cytokines (176). L'expression membranaire de BAFF à la surface des monocytes est faible mais un traitement par l'interferon (IFN) α , IFN β , LPS ou l'IL-10 induit une surexpression de BAFF (163, 176, 177). L'expression de BAFF à la surface des macrophages et des cellules dendritiques est aussi régulée de façon positive par l'IFN α (seulement pour les cellules dendritiques) et l'IFN γ (163, 176, 177). La sécrétion de BAFF par les monocytes n'est pas affectée par l'IL-10 ou l'IFN γ alors que les cellules dendritiques et les macrophages sécrètent plus de BAFF en réponse à ces cytokines (176). Cependant une étude séparée rapporte que les monocytes sécrètent BAFF en réponse à l'IFN α , IFN γ et au LPS (177). Des données plus récentes démontrent que d'autres membres de la famille TNF peuvent contrôler l'expression de BAFF. L'activation du récepteur à la lymphotoxine (LT) β requise pour l'organogenèse des ganglions lymphatiques et l'organisation des tissus lymphoïdes, ou le CD40 requis dans la réponse immunitaire adaptative, stimulent respectivement l'expression de BAFF dans les splénocytes murins ou dans les cellules dendritiques (177, 178). L'IL-4 et la PMA/ionomycine sont des régulateurs négatifs de l'expression de BAFF (171, 176).

Tout comme BAFF, APRIL est exprimé par les monocytes, les macrophages, les cellules dendritiques et les lymphocytes T (171, 177, 179, 180). Tout comme BAFF, l'expression d'APRIL par les cellules dendritiques est augmentée en réponse à l'IFN α , l'IFN γ et le CD40L (177). Le site de synthèse de l'hétéromère BAFF/APRIL n'est pas connu. BAFF et APRIL sont exprimés par les mêmes types cellulaires. APRIL est clivé à l'intérieur de la cellule et ensuite sécrété (165) alors que BAFF est exprimé à la surface et ensuite clivé (164, 176). APRIL est également exprimé par des cellules non lymphoïdes comme les lignées tumorales SW480 et Hela mais

aussi par les tissus tumoraux (179, 181, 182). Plus récemment, une protéine TWE-PRIL, provenant d'un ARNm hybride d'APRIL et de TWEAK (TNF-related weak inducer of apoptosis), a été identifié (182, 183). La protéine TWE-PRIL est composée des parties cytoplasmiques et transmembranaires de TWEAK et de la partie TNF carboxy-terminale d'APRIL. Cette protéine peut ainsi représenter une forme membranaire d'APRIL.

B. Les récepteurs de BAFF et APRIL

Les récepteurs de la famille TNF sont généralement des protéines transmembranaires de type I, comprenant une partie extracellulaire avec de multiples domaines riches en cystéine (DRC) et une partie intracellulaire comprenant des sites de fixation aux protéines TRAF et dans certains cas un domaine de mort cellulaire (184). Généralement, chaque domaine riche en cystéine est composé de deux modules contenant trois ponts disulfures (185). L'analyse de la structure cristallographique des complexes TNF ligand/récepteurs connus ont démontré que les points de contact critiques sont formés entre le ligand et trois modules du récepteur (184). BAFF et APRIL peuvent fixer deux récepteurs de la superfamille TNF, B cell maturation antigen (BCMA) et CAML interactor (TACI) (161, 167, 175, 186-190). Il apparaît que l'hétéromère BAFF/APRIL fixe uniquement TACI, bien que cela ne soit pas démontré directement (168). Les récepteurs qui interagissent avec TWE-PRIL ne sont pas connus. Cependant TWE-PRIL comprend le domaine complet de fixation d'APRIL, suggérant qu'il peut se fixer à BCMA et TACI. Un troisième récepteur, spécifique de BAFF, existe : BAFF-R (191, 192). BAFF-R, BCMA et TACI sont exprimés par les cellules B. BAFF-R, BCMA et TACI sont tous des protéines transmembranaires de type III (193, 194). Ces récepteurs ne

contiennent pas de domaine de mort. Ceci va de pair avec les fonctions de survie et de prolifération de BAFF et APRIL. La Figure 10 représente les interactions récepteur/ligand identifiées jusqu'à aujourd'hui.

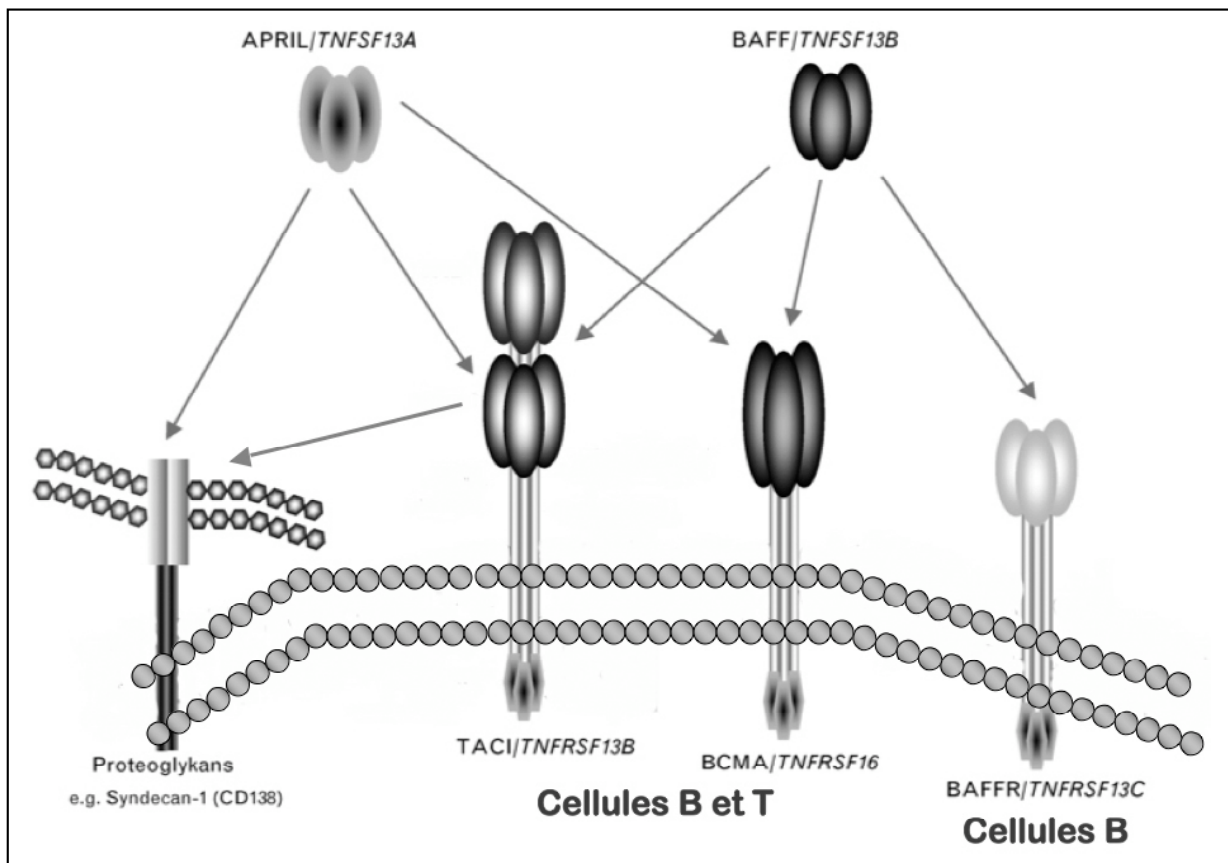


Figure 10 : BAFF, APRIL et leurs récepteurs. Les interactions de BAFF et APRIL avec leurs récepteurs BCMA, TACI et BAFF-R sont présentées ci-dessus. Contrairement à BAFF, APRIL est capable de fixer les protéoglycanes.

Plusieurs études ont posé la question de l'existence d'un troisième récepteur spécifique d'APRIL. APRIL stimule modestement la croissance de la lignée T Jurkat, la lignée fibroblastique NIH3T3, la lignée de carcinome du colon HT-29 et de la lignée épithéliale du poumon A549 bien qu'aucune des ces lignées n'expriment ni TACI ni BCMA (179, 187). Une différence structurale importante entre BAFF et APRIL explique la récente identification des protéoglycanes à chaînes héparane

sulfate (HSPG) comme le nouveau partenaire d'APRIL (195, 196). APRIL se fixe à aux HSPG via une séquence basique (QKQKKQ) dans la région N terminale. La fixation de ce motif avec les HSPG négativement chargés peut être inhibé par l'héparine. APRIL peut se fixer aux HSPG et à BCMA simultanément suggérant que des régions différentes d'APRIL sont cruciales pour chaque interaction (195).

TACI qui fût identifié par son interaction avec la molécule intracellulaire CAML contient 2 DRC et est le plus long des récepteurs de BAFF (194). Les DRC de TACI et BCMA ont une forte homologie. La partie intracellulaire de TACI fixe TRAF 2, 5 et 6 (189).

BCMA a été identifié à partir d'une translocation chromosomique chez un patient présentant un lymphome T (197). Le domaine extracellulaire de BCMA est très court, avec seulement 50 acides aminés inclus dans un DRC (193). BCMA est exprimé par les lymphocytes B matures et les plasmocytes, mais l'expression de l'ARN et de la protéine en surface n'est pas corrélée. La protéine est localisée dans le golgi dans la lignée de myélome U266 (198). Le TNFR1 a également été premièrement identifié comme localisé dans le golgi chez les cellules endothéliales. Dans ce cas, le TNFR1 ne forme pas de complexe avec les molécules de signalisation intracellulaires excepté si le TNFR1 est également exprimé à la membrane, indiquant que la transduction du signal ne peut pas intervenir dans le golgi (199, 200). Il peut en être de même pour BCMA. BCMA peut interagir avec les TRAF 1, 2 et 3 et activer différentes voies de signalisation (201).

BAFF-R est un récepteur spécifique de BAFF. BAFF-R présente un domaine extracellulaire de 70 acides aminés formant un DRC partiel (191, 192). C'est le plus petit DRC identifié à l'heure actuelle. BAFF-R est exprimé par tous les lymphocytes B de la rate chez la souris et par les lymphocytes B du sang périphérique chez

l'homme. Le domaine intracellulaire de BAFF-R s'associe uniquement avec TRAF 3 (202).

C. Affinité de BAFF et APRIL pour leurs récepteurs

Bien que les premières études ont indiquées que TACI-Ig a une affinité significativement plus faible pour APRIL que pour BAFF (167, 186, 190), d'autres études ont démontrées que les affinités sont comparables (203). TACI-Ig fixe APRIL avec un $K_D = 0,45-1,5$ nM et BAFF avec un $K_D = 0,16-0,79$ nM. La protéine de fusion BCMA-Ig soluble fixe également APRIL et BAFF avec des affinités similaires ($K_D = 0,2-1,31$ nM) (203). APRIL contrairement à BAFF se fixe à des lignées cellulaires n'exprimant pas TACI et BCMA de façon héparine dépendante. Cette interaction met en jeu des HSPG. Le K_D de cette interaction varie entre 20 et 80 μ M (203). D'autres équipes ont rapporté des affinités plus divergentes entre la fixation de BAFF et d'APRIL et leurs récepteurs. Ces différences peuvent être dues à une hétérogénéité des sources de protéines testées ou à des différences dans les méthodologies utilisées (167, 204, 205). L'affinité de BAFF pour BAFF-R est d'environ 3nM indiquant une interaction de forte affinité bien que BAFF-R présente un petit DRC (191).

D. Les fonctions biologiques de BAFF et APRIL

De nombreuses expériences *in vitro* ont démontré que l'ajout de BAFF recombinant dans la culture prolonge la survie des lymphocytes B (206-208). De plus, l'injection de BCMA-Ig chez la souris induit rapidement une disparition des lymphocytes B de tous les organes lymphoïdes (175). Le nombre de lymphocytes B est beaucoup plus élevé chez les souris transgéniques pour BAFF et on note une surexpression de la

protéine antiapoptotique Bcl-2 dans les cellules B de ces souris (161, 162, 209). Ces résultats démontrent que BAFF est un facteur de survie important pour les lymphocytes B (169, 210). Ces données ont conduit les investigateurs à suspecter un rôle majeur de BAFF dans la différenciation B. Ceci fût confirmé avec les souris déficientes pour BAFF qui présentent une perte des lymphocytes B T2 ainsi que des lymphocytes B matures et des lymphocytes B de la zone marginale (173, 174). Les souris A/WySnJ, ayant une mutation de BAFF-R, ont un phénotype similaire à celui des souris KO pour BAFF, indiquant que l'axe BAFF/BAFF-R contrôle la survie et la maturation des cellules B (191, 192). Le rôle de BCMA est plus obscur car les souris KO pour BCMA ne présentent pas de phénotype caractéristique (174, 211). Les souris déficientes pour TACI ont un nombre de cellules B élevé, suggérant un rôle négatif de TACI dans le contrôle de l'homéostasie des cellules B (212, 213). Chez les souris KO pour TACI, seule l'immunité T-indépendante est touchée mais pas l'immunité T-dépendante (212, 213). Le rôle de TACI pourrait se révéler plus complexe car c'est le seul récepteur capable de lier l'hétérotrimère BAFF/APRIL (168).

Les fonctions d'APRIL *in vivo* ne sont pour le moment pas aussi bien établies que celles de BAFF. Un des problèmes fût de produire une protéine recombinante APRIL active (214). APRIL stimule la prolifération des lymphocytes B *in vitro* et l'injection d'APRIL augmente le nombre total de lymphocytes B chez la souris (190). L'étude réalisée avec des souris transgéniques surexprimant APRIL, sous le contrôle d'un promoteur cellules T spécifique, ne montre pas de rôle de ce facteur sur la survie et la différenciation des lymphocytes B (180). Par contre, la survie des lymphocytes T est augmentée chez les souris transgéniques pour APRIL et la réponse humorale T-indépendante de type II est induite (180). Récemment, il a été démontré qu'APRIL

peut induire une surexpression des molécules de costimulation chez les lymphocytes B et augmenter de façon significative leur fonction de présentation de l'antigène (215). Le rôle de BAFF sur la biologie des lymphocytes T reste encore ouvert au débat. Des études *in vitro* ont démontrées que BAFF peut jouer un rôle dans la costimulation des cellules T humaines et induit la sécrétion d'IL-2 (216). De plus, un grand nombre de lymphocytes T activés est détecté chez les souris transgéniques pour BAFF. Cependant, certaines équipes ne rapportent pas de fixation convaincante de BAFF à la surface des cellules T (164).

En plus de son rôle dans la survie et la différenciation des lymphocytes B, BAFF est également impliqué dans la réponse immunitaire B. *In vitro*, BAFF peut stimuler la réponse des cellules B liée à l'activation via le BCR (B cell receptor) (163, 164). Cet effet est le résultat d'une augmentation de la survie des cellules B dans la culture, permettant à un nombre plus important de cellules B d'être activées plutôt qu'un effet direct de BAFF sur la prolifération (207). Cependant, le traitement de lignées B par BAFF induit l'expression de PIM-2 et de l'IL-10 (217). Ces deux facteurs induisent la prolifération des cellules B (218) et un effet de costimulation indirect de BAFF ne peut donc pas être exclu. De plus, BAFF et APRIL induisent la commutation isotypique (switch) en présence d'IL-10 ou de TGF- β (177). BAFF supporte la génération de cellules sécrétrices d'anticorps à partir de cellules B mémoires activées en supportant leur survie (219). Une étude démontre que la survie de plasmablastes dérivant de cellules B de la zone marginale est inhibée par TACI-Ig (220). Ce travail met en évidence le rôle de BAFF et/ou d'APRIL dans la maintenance des cellules sécrétant des anticorps.

Plus récemment, il a été démontré que BAFF-R était aussi présent à la surface des cellules T. L'expression de BAFF-R est augmentée au cours de l'activation des

lymphocytes T et le signal BAFF/BAFF-R agit comme un signal de costimulation des lymphocytes T (221). BAFF induit l'expression de Bcl-2 supportant la survie des cellules T. Suite à l'activation du TCR et au signal BAFF/BAFF-R, les cellules T se différencient en cellules effectrices sécrétant préférentiellement des cytokines de type Th1 (221).

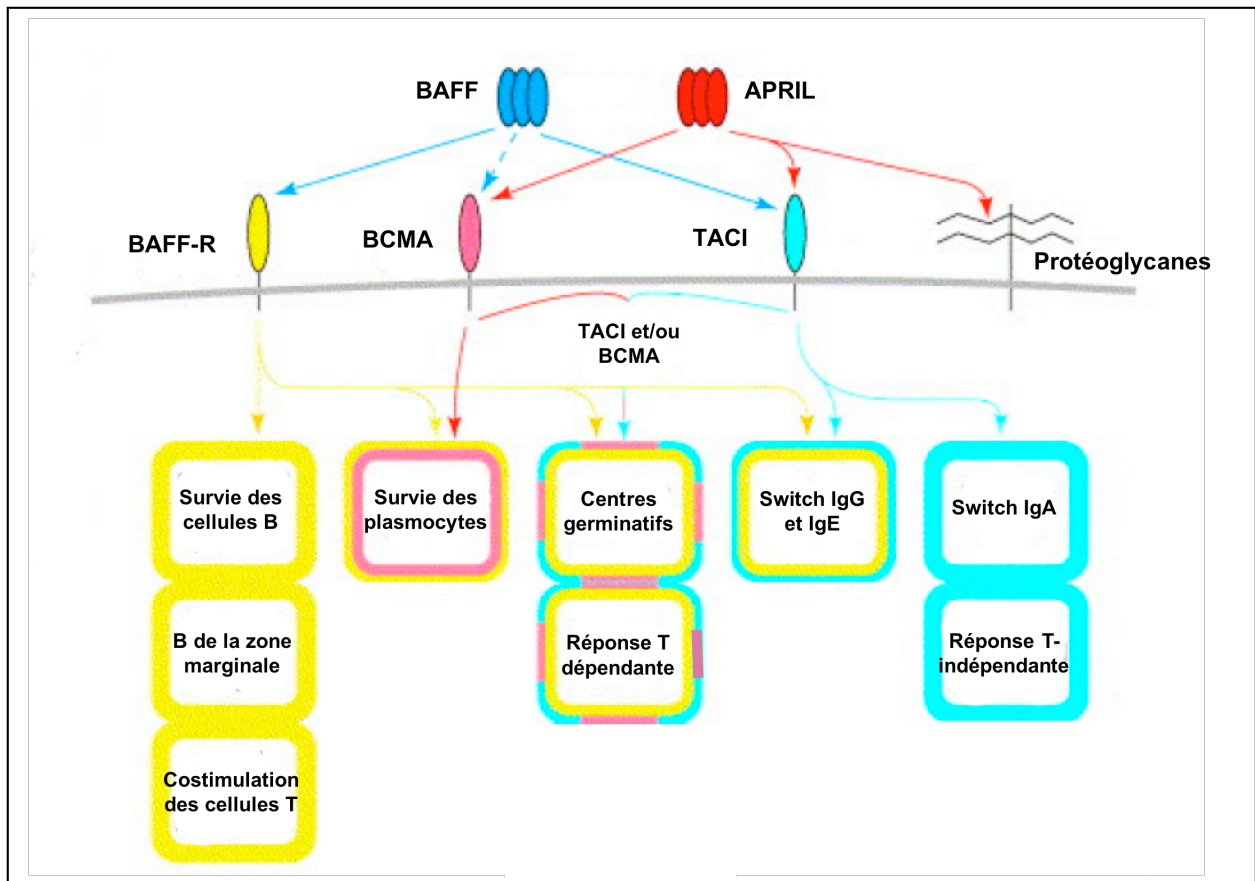


Figure 11 : Fonctions immunes de BAFF/APRIL et de leurs récepteurs.

Tableau 3 : Fonctions de BAFF et APRIL identifiées d'après les souris transgéniques ou KO.

Souris	Phénotype	Références
Souris transgénique pour APRIL (APRIL humain exprimé par les cellules T)	<ul style="list-style-type: none"> - Induit la survie des lymphocytes T transgéniques <i>in vitro</i>. - Induit la survie des cellules T réactives aux superantigènes <i>in vivo</i> (corrélé avec des taux élevés de Bcl2). - Augmente la sécrétion d'IgM en réponse aux antigènes T dépendant. - Induction de la réponse humorale T-indépendante de type II (IgM et IgG). 	(180)
	<ul style="list-style-type: none"> - Développement de néoplasies B chez les souris transgéniques âgées. 	(222)
KO APRIL	<ul style="list-style-type: none"> - Diminution du taux d'IgA circulant. - Blocage de la réponse IgA. - Augmentation du ratio cellules T effectrices/ cellules T mémoires. - Populations de lymphocytes B périphériques normales. 	(223)
	<ul style="list-style-type: none"> - Pas de phénotype discernable. - Développement normal des lymphocytes B et T. - Réponses humorales T dépendantes et T indépendantes normales. 	(224)
KO BAFF		
Souris transgénique pour TACI-Ig	<ul style="list-style-type: none"> - Perte des cellules B-1. - Blocage des cellules B-2 au stade T1 de leur développement. 	(173)
	<ul style="list-style-type: none"> - Déplétion sévère des cellules B-2 de la zone marginale et de la zone folliculaire. - Effet modéré sur les cellules B-1 du péritoine. 	(225)
Souris transgénique pour BCMA-Ig (BCMA murin qui fixe bien APRIL mais faiblement BAFF)	<ul style="list-style-type: none"> - Pas de phénotype discernable. 	(225)
KO TACI	<ul style="list-style-type: none"> - Augmentation du nombre de lymphocytes B matures. - Blocage de la réponse aux antigènes T indépendants. - Hyperréactivité des cellules B. 	(212)
	<ul style="list-style-type: none"> - Lupus érythémateux systémique (SLE). - Mort prématurée due aux maladies autoimmunes et/ou lymphomes. - Lymphoadenopathie. 	(192, 226)
KO BCMA	<ul style="list-style-type: none"> - Développement des lymphocytes B et T normal. - Blocage de la survie des plasmocytes matures. 	(174, 211)
	<ul style="list-style-type: none"> - Blocage de la survie des plasmocytes 	(227)

	matures.	
Double KO TACI x BCMA	- Similaire au souris KO pour TACI.	(228)

E. Transduction du signal de BAFF et APRIL

BAFF

L'étude de la transduction du signal de BAFF n'est pas aisée car BAFF a trois récepteurs et que les cellules B peuvent exprimer un ou plusieurs de ces récepteurs. Une étude de l'expression des récepteurs de BAFF à la surface des différents types des cellules B n'a pas été publiée. Les cellules B des souris transgéniques pour BAFF surexpriment Bcl-2 suggérant un rôle de BAFF dans la survie des lymphocytes B (162). De plus, une surexpression de A1 et Bcl-xL a été mise en évidence chez des lymphocytes B murins de rate traités par BAFF. L'ajout de BAFF en combinaison avec le CD40L induit une diminution de Bax et une surexpression de Bcl-2 (207, 229). Compte tenu de ces résultats, il avait été supposé que BAFF devait activer la voie NF- κ B (207, 230, 231). BAFF active la voie NF- κ B chez les lymphocytes B murins de la rate (207), les cellules B des ganglions (166) et la lignée B murine A20 (189). Les souris KO pour des protéines majeures de la voie de signalisation NF- κ B (les souris déficiente pour la kinase NF- κ B inducing kinase (NIK), les souris KO pour IKK α ou les doubles KO c-Rel/RelA) présentent des anomalies dans la différenciation des cellules B (231). Des études de cellules transfectées surexprimant TACI ou BCMA ont démontrées que ces deux récepteurs pouvaient activer la voie NF- κ B ainsi que la voie c-Jun N-terminal kinase (JNK), NF-AT, Elk-1, AP-1 et p38 kinase (167, 171, 194, 201). Cependant, le phénotype des souris KO pour BCMA et TACI indiquent que ces récepteurs ne médient pas le signal de survie et de

différenciation de BAFF dans les cellules B (211, 212). L'identification de BAFF-R et la mise en évidence de son rôle majeur dans la survie des lymphocytes B (191, 192), ont conduit à étudier en détail la signalisation médiée par ce récepteur. Il a été démontré que BAFF-R active la voie alternative de NF- κ B (232, 233). Cette voie de signalisation conduit à la phosphorylation de NF- κ B-2 (p100) suivi de l'ubiquitination et de la libération de p52 qui va être transloquée dans le noyau, sous forme de complexe avec RelB, où il va activer l'expression des gènes cibles (234). L'injection d'antagonistes de BAFF tels que les protéines de fusion TACI-Ig ou BAFF-R-Ig chez la souris inhibe la libération de p52 indiquant que BAFF est nécessaire pour l'activation de cette voie de signalisation dans les lymphocytes B spléniques de souris (233). BAFF-R active bien NF- κ B-2 car les cellules B spléniques de souris A/WySnJ, un mutant naturel pour BAFF-R (191, 213), n'expriment pas p52 et que l'activation d'un récepteur chimérique BAFF-R active NF- κ B-2 (232, 233). L'activation de la voie alternative de NF- κ B par BAFF conduit à l'induction de l'expression des protéines anti-apoptotiques Bcl-2 et Bcl-xL dans des cultures de moelle osseuse dérivées de souris sauvages contrairement à celles dérivées de moelle osseuse de souris déficientes pour NF- κ B-2 (232). Il a été démontré que la voie alternative de NF- κ B n'est pas uniquement nécessaire à la survie des lymphocytes B, mais également à leur maturation (232). La voie alternative de NF- κ B joue un rôle important dans la signalisation de BAFF/BAFF-R induisant la survie et la maturation des lymphocytes B *in vivo*.

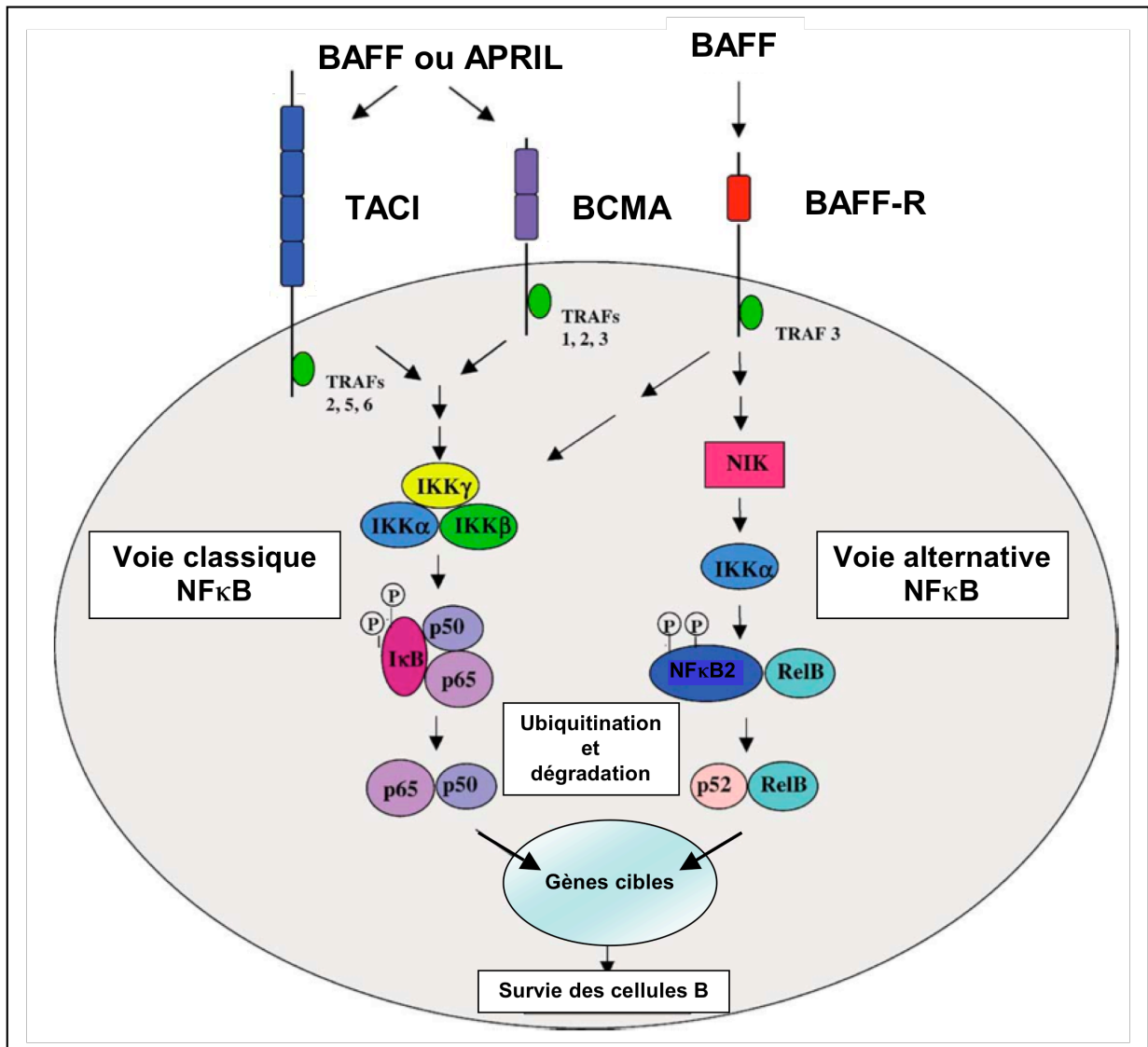


Figure 12 : Activation de la voie de signalisation NFκB par BAFF et APRIL.

APRIL

La connaissance de la signalisation d'APRIL est moins grande que celle de BAFF. APRIL, qui induit modestement la prolifération de la lignée NIH-3T3, active les voies de signalisation NF-κB et p38MAPK dans ces cellules. APRIL induit une surexpression de la protéine anti-apoptotique X-IAP chez des cellules de gliomes (235). *In vitro*, APRIL stimule la prolifération des cellules B et T (190), et la surexpression d'APRIL conduit de la survie des cellules T et induit une réponse

humorale de type II aux antigènes T indépendants chez les souris transgéniques (180). Le récepteur impliqué n'a pas été décrit. APRIL pourrait médier son signal via TACI chez les cellules B car les souris KO pour TACI ne produisent pas d'anticorps en réponse aux antigènes T indépendant de type II (212) alors que cette réponse immunitaire est induite chez les souris transgéniques pour APRIL (180).

Les conséquences biologiques de la fixation d'APRIL aux HSPG ne sont pas encore parfaitement claires. *Ingold et al* ont émis l'hypothèse que les cellules hématopoïétiques exprimant des HSPG pouvaient accumuler APRIL à leur surface et ainsi faciliter la transduction du signal via TACI et/ou BCMA (196). De plus, cette équipe suggère que la fixation d'APRIL aux HSPG peut médier la migration des plasmocytes dans la moelle osseuse, soit de façon direct, soit en induisant l'expression de récepteurs de chimiokines (196). Il demeure encore à élucider comment APRIL induit modestement la prolifération des cellules tumorales en se liant uniquement aux HSPG. Certains syndecans, une fois liés à un ligand, peuvent délivrer un signal grâce à leur domaine intracellulaire (236). Néanmoins, la transduction d'un signal médié par un HSPG se fixant à APRIL n'a pas encore été rapportée. Il demeure l'hypothèse de l'existence d'un autre récepteur spécifique d'APRIL, qui pourrait être présent à une très faible densité à la surface de ces cellules tumorales. Le signal médié par les hétéromères BAFF/APRIL pourrait être médié par TACI puisque seul TACI-Ig bloque la prolifération des cellules B spléniques induite par l'hétéromère BAFF/APRIL (168). BCMA-Ig et BAFF-R-Ig n'ont aucun effet. Cependant, des analyses biochimiques de l'hétérotrimère BAFF/APRIL et de son interaction avec les différents récepteurs sont encore nécessaires.

I-2. Rôle de BAFF/APRIL dans les pathologies humaines

Les souris transgéniques pour BAFF développent des symptômes typiques de maladies autoimmunes humaines telles que le lupus érythémateux systémique (SLE) et le syndrome de Sjögren (SjS) (160-162, 209). De plus, le taux de BAFF circulant est élevé dans le sérum de souris développant des pathologies autoimmunes (161) et certaines souris transgéniques pour BAFF développent des lymphomes (237). Ces observations ont conduit à penser qu'une dérégulation de l'expression ou de la fonction de BAFF/APRIL ou de leurs récepteurs pouvait jouer un rôle dans l'établissement de différentes pathologies humaines. Des travaux ont depuis été réalisés impliquant BAFF/APRIL dans le développement, la progression et la biologie de maladies aussi diverses que l'autoimmunité, les tumeurs B et l'immunodéficience.

A. Autoimmunité

Les taux de BAFF, APRIL et de l'hétérotrimère BAFF/APRIL sont élevés dans le sérum et le tissu synovial de patients atteints de SLE, de polyarthrite rhumatoïde (PR) et de SjS (160, 168, 238). Les taux sériques de BAFF sont également élevés chez des patients atteints de granulomatose de Wegener (239) et dans les lésions neurologiques de patients atteints de sclérose multiple (240). Les hétérotrimères BAFF/APRIL détectés chez les patients atteints d'autoimmunité sont biologiquement actifs (168, 241) et, en général, les taux sériques élevés de BAFF sont associés à un taux plus élevé d'autoanticorps (238, 241-244). Le type cellulaire responsable de la production de BAFF a été identifié dans certains cas. BAFF est produit par les lymphocytes T et les macrophages qui infiltrent les glandes salivaires dans le SjS (245) et par les astrocytes et les monocytes dans la sclérose multiple (240, 246, 247). Dans la PR, uniquement les macrophages produisent BAFF alors que les

cellules dendritiques sécrètent APRIL (244). Les neutrophiles peuvent également contribuer au taux élevé de BAFF circulant dans la PR (244). D'autres cytokines dont le CD40L, l'IL-4, l'IL-6, l'IFN- α et l'IL-10 présentent des taux élevés dans les maladies autoimmunes (248-251). De plus il y a une forte augmentation de l'expression de CD40L par les lymphocytes T et B des patients atteints de SLE (252). De façon intéressante, la plupart de ces facteurs peuvent induire une augmentation de la production de BAFF par les macrophages, les monocytes et les cellules dendritiques *in vitro* (176, 177, 240, 253).

Dans les maladies autoimmunes, les tissus affectés sont généralement non lymphoïdes et dépourvus de cellules immunitaires. Cependant, le tissu synovial ou les glandes salivaires des patients atteints de PR et de SjS contiennent un grand nombre de lymphocytes infiltrants (254). Chez 30% des patients, les cellules infiltrantes forment des structures proches de centres germinatifs comprenant des cellules B, des cellules T, des cellules dendritiques et des cellules dendritiques folliculaires (254, 255). La formation de ces structures ectopiques semblables à des centres germinatifs pourrait résulter d'une expression aberrante de facteurs solubles tels que le TNF, la lymphotoxine (LT), CXCL13, CCL19, et CCL21 qui sont requis pour la néogénèse lymphoïde normale et la formation des centres germinatifs (255-257). Il a été récemment démontré que l'incidence des structures proches des centres germinatifs dans les tissus enflammés de patients atteints de PR et de SjS est corrélée avec le niveau de LT et de CCL19 mais aussi avec les taux sériques de BAFF et APRIL (243, 244, 250, 258). En utilisant un modèle murin de greffe de tissu synovial de patients atteints de PR chez la souris SCID, il a été démontré que le développement de centres germinatifs ectopiques dans ces lésions est inhibé par le récepteur soluble TACI-Ig (244). Ceci s'accompagne d'une réduction des taux d'IgG

humaines, d'IFN- γ , de CCL19, de TNF- α et du nombre de cellules B, T et de cellules dendritiques folliculaires infiltrant le tissu greffé (244). Ces résultats suggèrent que BAFF et APRIL jouent un rôle critique dans la néogénèse lymphoïde et la maintenance de structures ectopiques proches des centres germinatifs chez les patients atteints d'autoimmunité médiant leur effet sur les cellules B, T et les cellules dendritiques folliculaires. Ces données sont en accord avec le rôle de BAFF dans la costimulation des lymphocytes T (216, 259, 260), avec le fait que le nombre de cellules T effectrices soit plus élevé dans la rate des souris transgéniques pour BAFF (162) et avec la dissolution accélérée des centres germinatifs dans la rate des souris dont la signalisation BAFF/BAFF-R est inhibée comparativement aux souris contrôles (257, 261).

B. Tumeurs lymphoïdes B

Les résultats montrant que les taux de BAFF et APRIL sont élevés dans certaines maladies impliquant les cellules B et le rôle majeur de BAFF dans la survie des cellules B a conduit à émettre l'hypothèse suggérant que BAFF, APRIL et leurs récepteurs pourraient jouer un rôle dans la biologie des tumeurs lymphoïdes B. Les cellules B tumorales de lymphomes non Hodgkinien (NHL) (262-265), lymphomes Hodgkinien (HL) (266), leucémie lymphocytaire chronique (LLC) (172, 267) et de Macroglobulinémie de Waldenström (MW) (268) sont capables de fixer BAFF et dans certain cas APRIL. Cependant le profil d'expression des récepteurs diffère car les cellules de NHL (262), de LLC (172, 267), de WM (268) expriment BAFF-R et TACI alors que les cellules de HL expriment plutôt BCMA et TACI. La fixation de BAFF et APRIL à la surface des cellules tumorales est fonctionnelle car l'ajout de BAFF et

APRIL exogènes induit la prolifération et/ou la survie des cellules de CLL (172, 267, 269), NHL (262, 263, 265), HL (266) et WM (268) *in vitro*.

L'expression de BAFF-R et de TACI par les cellules de NHL et de LLC et les effets de BAFF et APRIL sur la croissance et la survie de ces cellules sont les mêmes que pour les cellules B normales (219, 270, 271). Une explication de l'implication de BAFF et APRIL dans la pathologie des tumeurs lymphoïdes B pourrait être leur expression et leur production aberrantes dans ces pathologies. BAFF et APRIL sont plus fortement exprimés par les cellules B de LLC (172, 222, 267) et de NHL (264, 265) que les cellules B normales. De plus les taux sériques circulants de BAFF et APRIL sont plus élevés chez les patients atteints de NHL (262, 263), LLC (222, 267) et MW (268) comparativement aux sujets sains. Le taux sérique de BAFF est un facteur de mauvais pronostic dans les NHL (262). De façon intéressante, les souris transgéniques pour APRIL développent des tumeurs lymphoïdes des cellules B B1 (CD5⁺) (222) correspondant aux cellules malignes de LLC (272). Une autre observation pouvant expliquer une participation de BAFF/APRIL dans les tumeurs lymphoïdes B provient du fait que l'infection des cellules B par le virus de l'EBV induit l'expression de BAFF et APRIL (273). Les patients atteints de SjS ont une prédisposition à développer des tumeurs lymphoïdes B et particulièrement les NHL (243). Comme les patients atteints de NHL ont des taux sériques circulants de BAFF élevés (262, 263), il est possible que les taux sériques importants de BAFF observés chez les patients atteints de SjS pourraient contribuer au développement des manifestations autoimmunes de ce syndrome mais aussi au développement de tumeurs lymphoïdes B fréquemment observées chez ces patients (243). L'environnement des tumeurs lymphoïdes B joue également un rôle. Dans les LLC, les cellules de l'environnement nommées « nurse-like cells » expriment fortement

BAFF et APRIL. La production de BAFF et APRIL par les « nurse-like cells » supporte la survie des cellules de LLC (269). Dans le cas des lymphomes B, les macrophages infiltrant la tumeur sécrètent de forts taux de BAFF qui supportent la survie des cellules de lymphomes (274).

BAFF protège les cellules B normales de l'apoptose en activant la voie NF κ B et en modulant l'expression des gènes de la famille Bcl-2 (207, 227, 229, 275-277). C'est certainement le mécanisme par lequel BAFF et APRIL supportent la survie des cellules B tumorales. *In vitro*, l'ajout de BAFF ou APRIL dans la culture de cellules de NHL, de LLC et HL active la voie NF κ B et induit l'expression de Bcl-2, Bcl-xL et diminue l'expression de la protéine pro-apoptotique Bax (172, 265, 266). De façon similaire, la survie, la prolifération et l'expression des protéines anti-apoptotiques sont diminuées dans les cellules B tumorales cultivées *in vitro* en présence d'antagonistes de BAFF (172, 264). Ces données mettent en évidence qu'une production aberrante et/ou excessive de BAFF et/ou APRIL par les cellules B tumorales (boucles autocrines) ou par les cellules présentes dans le microenvironnement des cellules tumorales (paracrines) pourrait faciliter leur croissance et leur survie. Ainsi, l'inhibition de l'interaction de BAFF et APRIL et de leurs récepteurs pourrait représenter une approche thérapeutique intéressante dans le traitement des tumeurs lymphoïdes B.

C. Immunodéficiences

De nombreuses études ont mis en évidence le rôle majeur de BAFF et BAFF-R dans le développement et l'homéostasie des cellules B (174, 175, 225, 228). Pour ces raisons, il a été émis l'hypothèse que des mutations de BAFF-R pourraient être retrouvées chez des patients atteints d'immunodéficiences caractérisées par un déficit

en cellules B. Cette étude a été réalisée chez une cohorte de 48 patients atteints d'immunodéficience commune variable (CVID) (278). L'immunodéficience commune variable se caractérise par une hypogammablobulinémie malgré un nombre normal (ou presque) de lymphocytes B. Des variants hétérozygotes de BAFF-R ont été observés dans 20% des cas, mais la même fréquence a été observée chez les sujets sains suggérant qu'il s'agit plutôt d'un polymorphisme que des mutations impliquées dans la maladie (278). A l'opposé, des mutations de TACI sont associées à des CVID familiales ou sporadiques et à des déficiences IgA (279, 280). Six mutations différentes ont été identifiées chez 8 familles avec CVID et chez 10 patients indépendants sans histoire familiale de CVID (soit une cohorte de 24 patients) (279-281). Ces mutations de TACI sont localisées à différentes positions : dans la partie extracellulaire (C104R et S144X), dans la partie transmembranaire (A181E) et dans le domaine intracellulaire (S194X et R202H). Seule la mutation S144X affecte l'expression membranaire de TACI alors que le mutant C104R présente une altération de la capacité à fixer BAFF et APRIL (279-281). Le développement des lymphocytes B est normal dans la majorité de ces patients. La plupart des patients sont hétérozygotes pour leur mutation respective de TACI. Actuellement, il reste à comprendre comment une mutation d'un allèle de TACI pourrait induire une immunodéficience. TACI étant exprimé sous forme trimérique, il est possible que la stoechiométrie du complexe soit instable en présence de mutants de TACI. Des mutations de la partie intracellulaire de TACI pourraient diminuer ses capacités à recruter les médiateurs des voies de signalisation et ainsi compromettre la qualité du signal délivré par le récepteur TACI muté.

Des patients présentant les mêmes mutations hétérozygotes de TACI développent des maladies variables comme des déficiences IgA, des CVID et des CVID

associées à des lymphoproliférations tumorales ou autoimmunes. Malgré la variabilité des pathologies associées, l'identification et la caractérisation de ces patients renforcent l'importance de BAFF et/ou APRIL dans la biologie des cellules B *in vivo*. En résumé, une amplification de la signalisation médiée par BAFF/APRIL cause des pathologies immunes impliquant les cellules B et une insuffisance de la signalisation médiée par BAFF/APRIL cause des immunodéficiences B.

BAFF et/ou APRIL pourraient être des candidats thérapeutiques pour le traitement des patients atteints d'immunodéficiência.

II- Résultats, discussion

II-1 Article 5

« **BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood. 2004 Apr 15;103(8):3148-57. Epub 2003 Dec 4. »**

Nous avons observé à l'aide des puces à ADN affymetrix, une surexpression de BCMA et TACI, deux récepteurs de BAFF et APRIL, chez les plasmocytes tumoraux et normaux par rapport aux cellules B normales. L'objectif de notre travail était de déterminer un rôle éventuel de BAFF et d'APRIL dans le myélome multiple et d'identifier ses mécanismes d'action.

Nous avons démontré que :

- Toutes les lignées de MM et les plasmocytes tumoraux de tous les patients expriment *BCMA*. L'expression de *BAFF-R* et *TACI* est plus hétérogène.
- L'ARNm de BAFF est exprimé par 12/13 lignées de MM et par les plasmocytes de 11/11 patients atteints de MM. Les lignées exprimant l'ARNm de BAFF expriment également la protéine à leur surface. L'ARNm d'APRIL est exprimé chez les cellules de MM de 10/11 patients et chez 6/13 lignées de MM. APRIL étant sécrété directement, nous avons confirmé la présence de la protéine dans le surnageant de culture des lignées exprimant l'ARNm.

- BAFF et APRIL protègent les lignées de MM de l'apoptose induite par déprivation d'IL-6.
- Nous avons identifié une boucle autocrine impliquant BAFF/APRIL et leurs récepteurs chez deux lignées de MM dont la croissance est indépendante de cytokines exogènes.
- BAFF et APRIL protègent les cellules de MM de l'apoptose induite par la dexaméthasone.
- Un inhibiteur de BAFF/APRIL entraîne une réduction de 48% de la viabilité des cellules de MM cultivées *in vitro* pendant 5 jours avec leur environnement. En présence de dexaméthasone ou d'un anticorps anti-IL-6, la viabilité des cellules tumorales est diminuée de 71% et 60% respectivement. Cet inhibiteur ne touche pas les autres populations cellulaires de la moelle osseuse.
- Les taux sériques circulants de BAFF et APRIL sont plus élevés chez les patients atteints de MM que chez les sujets sains.
- BAFF et APRIL activent les voies de signalisation NF- κ B, PI3K/AKT et MAPK.

En conclusion, BAFF et APRIL sont deux facteurs de croissance pour les cellules de MM. L'inhibition de la voie BAFF/APRIL apparaît donc être une cible thérapeutique intéressante pour le traitement du myélome multiple.

BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone

Jérôme Moreaux, Eric Legouffe, Eric Jourdan, Philippe Quittet, Thierry Rème, Cécile Lugagne, Philippe Moine, Jean-François Rossi, Bernard Klein, and Karin Tarte

Identification of growth factors in neoplasias may be a target for future therapies by blocking either growth factor receptor interaction or the induced pathway. Using gene expression profiling, we identified overexpression of 2 receptors for a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF) in malignant plasma cells compared with normal plasma cells. APRIL and BAFF are involved in a variety of tumor and autoimmune diseases, including B-cell malignancies. We confirmed the expression of BAFF and APRIL receptors (B-cell maturation antigen [BCMA], transmembrane ac-

tivator and calcium modulator and cyclophilin ligand interactor [TACI], and BAFF-R) in a majority of 13 myeloma cell lines and in the purified primary myeloma cells of 11 patients. APRIL and BAFF were potent survival factors for exogenous cytokine-dependent myeloma cell lines and were autocrine growth factors for the RPMI8226 and L363 autonomously growing cell lines. These factors activated nuclear factor (NF)- κ B, phosphatidylinositol-3 (PI-3) kinase/AKT, and mitogen-activated protein kinase (MAPK) kinase pathways and induced a strong up-regulation of the Mcl-1 and Bcl-2 antiapoptotic

proteins in myeloma cells. BAFF or APRIL was also involved in the survival of primary myeloma cells cultured with their bone-marrow environment, and protected them from dexamethasone (DEX)-induced apoptosis. Finally, the serum levels of BAFF and APRIL were increased about 5-fold in patients with multiple myeloma (MM) as compared with healthy donors. Altogether, these data suggest that APRIL/BAFF inhibitors may be of clinical value in MM. (Blood. 2004;103:3148-3157)

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Introduction

Multiple myeloma (MM) is a clonal B-cell neoplasia characterized by the accumulation of malignant plasma cells within the bone marrow, in close contact with stromal cells. Several autocrine or paracrine soluble factors can promote myeloma cell survival and proliferation.¹ Interleukin 6 (IL-6), which is mainly produced by cells of the tumor microenvironment, is a major myeloma growth factor.² Interferon alpha (IFN- α), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), and heparin-binding epidermal growth factor-like growth factor (HB-EGF) can also promote the survival or proliferation of myeloma cells.³⁻⁷ The inhibition of myeloma cell growth factors may have clinical applications, eventually in combination with other drugs. For example, anti-IL-6 monoclonal antibody (MoAb) may lead to tumor regression in some advanced myeloma patients.^{8,9}

In order to identify new myeloma cell growth factors, we recently compared gene expression profiles of myeloma cells with those of normal plasmablasts and peripheral blood B cells.¹⁰⁻¹² Interestingly, the *TACI* (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and *BCMA* (B-cell maturation antigen) genes coding for 2 receptors of B-cell activating factor (BAFF, also called BLys)^{13,14} were highly expressed in malignant plasma cells.^{10,11} BAFF is a tumor necrosis factor (TNF) family member essentially expressed by monocytes, macrophages, dendritic cells, and some T cells.¹⁵ It is produced as both a membrane-bound and a proteolytically cleaved soluble pro-

tein.^{13,14} A third receptor for BAFF, called BAFF-R, was recently identified.¹⁶ The expression of BCMA and BAFF-R is B-cell-specific, whereas TACI is also found on a subset of activated T cells.¹⁵ Finally, BAFF shares significant homology with a proliferation-inducing ligand (APRIL), which is expressed at a low level by normal lymphoid and myeloid cells, and at a high level by a variety of human cancers.^{17,18} APRIL, which is directly secreted without cell-surface expression, binds to BCMA and TACI but not to BAFF-R.¹⁵

Several studies have indicated that BAFF is a survival factor for immature, naive, and activated B cells.¹⁵ The production of BAFF by myeloid dendritic cells in response to innate immune signals was shown to promote T-cell-independent immunoglobulin class switching and to sustain survival of extrafollicular plasmablasts.¹⁹ BAFF-transgenic mice develop mature B-cell hyperplasia with autoimmune manifestations, especially production of autoantibodies.¹⁵ Moreover, dysregulation of the BAFF pathway seems to be involved in autoimmunity in humans.¹⁵ On the contrary, BAFF- or BAFF-R-deficient mice and mice treated with TACI-Fc or BCMA-Fc display severe loss of mature B cells.¹⁶

The role of APRIL is less well characterized. Recent reports have shown that APRIL provides survival and activation signals to normal B and T cells.²⁰⁻²² In addition, APRIL is highly expressed in several tumor tissues and stimulates growth of tumor cells in vitro and in vivo.¹⁷

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The signal transduction pathways driven by BAFF and APRIL are not fully characterized. The activation of nuclear factor (NF)- κ B by TACI, BCMA, and BAFF-R²³ is consistent with the antiapoptotic role of BAFF, since NF- κ B enhances the transcription of several cell survival genes.^{24,25} Depending on the B-cell maturation stage, BAFF was reported to induce the antiapoptotic proteins Bcl-2, A1, and Bcl-XL and to reduce the proapoptotic protein Bak.^{23,26,27} BAFF also activates Jun kinase (JNK), Elk-1, p38 kinase, activating protein 1 (AP-1), and NF-AT in various models.¹⁵

The striking roles of BAFF, APRIL, and their receptors in normal B-cell homeostasis and in several tumor models raise the possibility that they may be involved in the pathogenesis of B-cell malignancies. Recent studies reported the aberrant expression of BAFF and APRIL by tumor B cells isolated from a subset of patients with chronic lymphoid leukemia, suggesting the existence of an autocrine survival loop in this disease.^{28,29} In vitro, a BCMA-Fc fusion protein is able to enhance apoptosis of B-cell chronic lymphocytic leukemia (B-CLL) cells.²⁹ In addition, patients with follicular non-Hodgkin lymphomas have increased levels of soluble BAFF in their serum, and BAFF seems to favor B-lymphoma cell survival.³⁰

In this study, we show that myeloma cell lines and primary myeloma cells express BAFF, APRIL, and their receptors and that BAFF and APRIL are myeloma cell growth factors and rescue myeloma cells from apoptosis induced by dexamethasone. BAFF and APRIL activated nuclear factor (NF)- κ B, phosphatidylinositol-3 (PI-3) kinase/AKT, and mitogen-activated protein kinase (MAPK) kinase pathways in myeloma cells and induced a strong up-regulation of the Mcl-1 and Bcl-2 antiapoptotic proteins. Finally, we demonstrate a 5-fold increase in the serum levels of BAFF or APRIL in patients with MM compared with age-related healthy individuals.

Materials and methods

Myeloma cell lines and primary samples

XG-1, XG-2, XG-5, XG-6, XG-7, XG-11, XG-13, XG-14, and XG-20 are IL-6-dependent human myeloma cell lines (HMCLs) obtained in our laboratory.³¹ Upon removal of IL-6, these cells progressively apoptose within 10 to 14 days. These HMCLs were routinely maintained in RPMI 1640 and 10% fetal calf serum (FCS; Biowittaker, Walkersville, MD), except XG-14, which was maintained in X-VIVO 20 (Biowittaker) supplemented with 3 ng/mL IL-6 (Peprotech, Rocky Hill, NJ). The human myeloma cell lines RPMI8226, U266, LP1, and L363 (ATCC, Rockville, MD) grew autonomously in RPMI-10% FCS. All cell lines were free of *Mycoplasma*, as assayed by an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer, Mannheim, Germany). Peripheral blood B cells (PBBs) were purified using CD19 microbeads (Miltenyi Biotech, Paris, France), and dendritic cells (DCs) were generated from adherent monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-13.

Bone marrow or peripheral blood samples were collected from 5 patients with plasma cell leukemia (PCL) and 14 patients with intramedullary myeloma after informed consent was obtained. Mononuclear cells were obtained by centrifugation on Ficoll-hypaque medium. For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, myeloma cells were purified (> 95% purity) using CD138 microbeads (Miltenyi Biotech, Paris, France), whereas phenotype and apoptosis were analyzed on whole mononuclear cells. Polyclonal plasmablastic cells (PPCs) were generated from purified CD19⁺ PBBs in vitro.¹⁰ Briefly, PBBs were cultured in RPMI 1640 and 10% FCS in the presence of mitomycin-treated CD40L transfectant, IL-2 (20 U/mL), IL-4 (50 ng/mL), IL-10 (50 ng/mL), and IL-12 (2

ng/mL; R&D Systems, Abington, United Kingdom). After 4 days of culture, B cells were harvested and cultured without CD40 ligand (CD40L) transfectant and with IL-2, IL-10, IL-12, and IL-6 (5 ng/mL). On day 6 of culture, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD20 (Beckman-Coulter, Marseille, France) and phycoerythrin (PE)-conjugated anti-CD38 (Becton Dickinson, San Jose, CA) and CD20⁺CD38⁺ PPCs were sorted with a FACSvantage (Becton Dickinson).

Sera from 26 patients with myeloma at diagnosis, 10 patients with PCL, and 9 age-related healthy individuals were collected as described previously.³²

mRNA analysis

We generated cDNA with 2 μ g total RNA using the Superscript II reverse transcriptase (Life Technologies) and oligo d(T) (Amersham Pharmacia Biotech, Orsay, France). Each 25- μ L PCR reaction contained 1 μ L of the first-strand cDNA, 1 μ M of each primer (sense and antisense), 0.2 mM each of dNTP (2'-deoxynucleoside 5'-triphosphate), 1.5 mM MgCl₂, 1 \times polymerase buffer, and 2 units of *Taq* polymerase (Life Technologies). The following primers were used: BAFF, 5'-GGA GAA GGC AAC TCC AGT CAG AAC (sense) and 5'-CAA TTC ATC CCC AAA GAC ATG GAC (antisense); APRIL, 5'-CCT TGC TAC CCC ACT CTT G (sense) and 5'-ACA CTC AGA ATA TCC CCT TGG (antisense); BCMA, 5'-TTA CTT GTC CTT CCA GGC TGT TCT (sense) and 5'-CAT AGA AAC CAA GGA AGT TTC TAC C (antisense); TACI, 5'-CAC CCT AAG CAA TGT GC (sense) and 5'-TGG GAC TCA GAG TGC C (antisense); BAFF-R, 5'-GGA GAA GGC AGG AAC CAC (sense) and 5'-AAG GCA AGC ACA CCA AA (antisense); β 2-microglobulin (β 2M), 5'-CCA GCA GAG AAT GGA AAG TC (sense) and 5'-GAT GCT GCT TAC ATG TCT CG (antisense). The sizes of the PCR products were as follows: BAFF, 311 bp; APRIL, 729 bp; BCMA, 806 bp; TACI, 931 bp; BAFF-R, 300 bp; and β 2M, 269 bp. The amplification profile was 1 minute at 94°C, 1 minute at 62°C (BAFF), 67°C (APRIL), 58°C (BCMA), 60°C (TACI), 61°C (BAFF-R), and 60°C (β 2M), 1 minute at 72°C, followed by a final extension of 10 minutes at 72°C. Reaction products were electrophoresed on a 1.5% agarose gel.

Flow cytometry analysis

The overall expression of receptors for BAFF on HMCLs was evaluated by incubating 5×10^5 cells with 10 μ g/mL of a human BAFF-murine CD8 (BAFF-muCD8) biotinylated fusion protein (Ancell, Bayport, MN) in phosphate-buffered saline (PBS) containing 30% human AB serum at 4°C for 30 minutes followed by incubation with PE-conjugated streptavidin (Beckman-Coulter). For primary samples, cells were double stained with BAFF-muCD8 fusion protein and FITC-conjugated anti-CD138 (Beckman-Coulter). The expression of BAFF was evaluated using an anti-BAFF antibody (Buffy-1; Alexis Biotechnology, Lausen, Switzerland). Flow cytometry analysis was done on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Study of apoptosis

IL-6-dependent HMCLs were starved of IL-6 for 3 hours and cultured in 24-well, flat-bottomed microtiter plates at 10^5 cells per well in RPMI 1640-10% FCS or X-VIVO 20 culture medium with or without IL-6 (3 ng/mL), BAFF (200 ng/mL; Peprotech), or APRIL (200 ng/mL; R&D Systems). After 3 days of culture, cells were washed twice in PBS and apoptosis was assayed with FITC-conjugated annexin V labeling (Boehringer). Fluorescence was analyzed on a FACScan flow cytometer. In order to study the dexamethasone (DEX)-induced apoptosis, autonomously growing HMCLs were cultured for 3 days in 24-well, flat-bottomed microtiter plates at 10^5 cells per well in RPMI 1640-10% FCS with or without DEX (10^{-6} M), IL-6 (3 ng/mL), BAFF (200 ng/mL), or APRIL (200 ng/mL) and apoptosis was assayed with annexin V labeling.

Proliferation assay

HMCLs were IL-6 starved for 3 hours and cultured for 5 days in 96-well, flat-bottomed microtiter plates at 10^4 cells per well in RPMI 1640-10% FCS or X-VIVO 20 with or without IL-6 (3 ng/mL), BAFF (200 ng/mL),

APRIL (200 ng/mL), the B-E8 anti-IL-6 antibody (10 μ g/mL) (Diaclone, Besancon, France), an inhibitor of PI-3K/AKT pathway (Ly 294002; 25 μ M), an inhibitory peptide of NF- κ B pathway (SN50), or the corresponding inactive peptide (100 μ g/mL) (BIOMOL, Plymouth Meeting, PA), or a fusion protein of TACI and the human Fc fragment of immunoglobulin (TACI-Fc; 10 μ g/mL; R&D Systems). Cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech) for the last 12 hours of culture, harvested, and counted on a liquid scintillation analyzer.

Mononuclear cell culture

Mononuclear cells from tumor samples of 8 patients with MM were cultured for 4 days at 5×10^5 cells/mL in RPMI 1640 medium, 5% FCS, 1 ng/mL IL-6, with or without 10^{-6} M dexamethasone (DEX), BAFF (200 ng/mL), or APRIL (200 ng/mL). In each culture group, viability and cell counts were assayed and myeloma cells were stained with an anti-CD138-PE MoAb (Immunotech).

ELISA

ELISA microplates (Nunc MaxiSorp; Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 100 μ L mouse anti-human BAFF antibody (RDI, Flanders, NJ) or mouse anti-human APRIL antibody (R&D Systems) (10 μ g/mL in PBS). Plates were washed 5 times with PBS, 0.1% Tween 20, and blocked with PBS, 1% BSA for 2 hours at room temperature. Patients' or healthy donors' sera were added and plates were incubated for 2.5 hours at 37°C and washed. Rabbit anti-human BAFF antibody (Upstate, Lake Placid, NY) or goat anti-human APRIL antibody (R&D Systems) (2 μ g/mL in PBS, 1% BSA, 0.05% Tween 20) were added for 2 hours at room temperature and the bound antibodies were detected with goat anti-rabbit (Sigma, 1:15 000) or rabbit anti-goat (Dako, Copenhagen, Denmark; 1:1000) peroxidase-conjugated antibodies. The peroxidase reaction was developed with a tetramethylbenzidine (TMB) substrate kit (Sigma, St Louis, MO). Light absorbance was measured at 450 nm and standard curves were generated using known concentrations of recombinant human BAFF or APRIL. The sensitivity of the ELISA was 1.5 ng/mL for BAFF and 3 ng/mL for APRIL. The intra-assay variability of the ELISA was determined by measuring serum samples from 7 patients in 2 separate experiments and was less than 14% for BAFF and less than 17% for APRIL.

Western blot analysis

HMCLs were starved overnight in RPMI 1640–1% bovine serum albumin (BSA) without IL-6. Cells were lysed in 10 mM tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate (NaPPi), 1% Triton X-100, 5 μ M ZnCl₂, 100 μ M Na₃VO₄, 1 mM dithiothreitol (DTT), 20 mM β -glycerophosphate, 20 mM *P*-nitrophenolphosphate (PNPP), 2.5 μ g/mL aprotinin, 2.5 μ g/mL leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM benzamide, 5 μ g/mL pepstatin, and 50 nM okadaic acid. Lysates were cleared by centrifugation at 10 000g for 10 minutes and resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Membranes were blocked for 1 hour at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM tris-HCl (pH 7.4), 0.1% Tween 20 (TBS-T), 5% BSA, then incubated for 1 hour at room temperature with primary antibodies (phospho-specific antibodies anti-ERK1/2, anti-signal transducer and activator of transcription 3 (STAT3) and anti-AKT; New England Biolabs, Beverly, MA) at a 1:1000 dilution in 1% BSA TBS-T. The primary antibodies were visualized with goat anti-rabbit (Sigma) or goat anti-mouse (Bio-Rad, Hercules, CA) peroxidase-conjugated antibodies using an enhanced chemiluminescence detection system. As a control for protein loading, we used anti-STAT3 (1:2000; Transduction Laboratories, Lexington, KY), anti-ERK1/2 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-AKT (New England Biolabs) antibodies. Rabbit polyclonal antibodies specific for Bcl-x and Mcl-1 were obtained from Santa Cruz Biotechnology and Bcl-2 antibody from Dako.

Blots were quantified by densitometry using acquisition into Adobe Photoshop (Apple, Cupertino, CA) and analyzing with the NIH Image software (National Institutes of Health, Bethesda, MD).

Nuclear transcription factor- κ B assay

NF- κ B activation was determined with a Trans-AM NF- κ B p50 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. This ELISA used a 96-well plate coated with an oligonucleotide containing the NF- κ B consensus binding site (5'-GGGACTTCC-3'). Following overnight starvation, cells were seeded on a 24-well plate (10^6 cells/well) and were stimulated for 1 hour with IL-6 (3 ng/mL), BAFF (200 ng/mL), APRIL (200 ng/mL), or TNF- α (20 ng/mL). Cell lysates were diluted (1:10) and added to the ELISA plate. NF- κ B binding to the target oligonucleotide was detected by incubation with primary antibody specific for the activated form of p50, visualized by anti-IgG horseradish peroxidase conjugate, and quantified at 450 nm. Each condition was run in triplicate.

Statistical analysis

Statistical significance was tested using a nonparametric Wilcoxon test for pairs or a Student *t* test for pairs.

Results

BCMA, TACI, and BAFF-R expression in malignant and normal plasma cells

RT-PCR analysis indicated that 13 of 13 HMCLs expressed *BCMA* according to our microarray results (Figure 1A).^{10,11} The expression pattern of *TACI* and *BAFF-R* was more heterogeneous. As shown in Figure 1A, *TACI* and *BAFF-R* were expressed, respectively, by 8 of 13 and 9 of 13 HMCLs. Unlike DCs, purified B cells expressed *BCMA*, *TACI*, and *BAFF-R* as reported.³³ We next looked for the expression in primary myeloma cells of 6 patients with intramedullary myeloma (patients 1-6) and of 5 patients with plasma cell leukemia (PCL) (patients 7-11). *BCMA* RNA was detected in 11 of 11 samples (Figure 1A). *TACI* and *BAFF-R* were simultaneously expressed by 8 of 11 primary myeloma samples.

Expression of BAFF and APRIL receptors was found in 5 of 5 in vitro-generated normal plasmablasts. In particular, *BCMA* and *TACI* were detected at a high level whereas *BAFF-R* was less expressed. These results are in agreement with our Affymetrix data^{10,11} and with the recent study of Avery et al³⁴ (Figure 1A).

To confirm the membrane expression of receptors for BAFF, we used a biotinylated human BAFF–murine CD8 fusion protein, which binds to TACI, BCMA, and BAFF-R. In agreement with previous studies, this BAFF–murine CD8 did not label monocyte-derived DCs but efficiently bound purified B cells (Figure 1B). BAFF–murine CD8 fusion protein bound to 8 of 13 HMCLs. All of them expressed high levels of *TACI* or *BAFF-R* (XG-2, XG-13, XG-14, XG-20, LP1, L363, U266, and RPMI 8226). The other 5 HMCLs that were not labeled by BAFF–murine CD8 expressed *BCMA* alone or *BCMA* and a low level of *BAFF-R* (XG-1, XG-5, XG-6, XG-7, and XG-11; Figure 1A). In addition, we confirmed the presence of membrane receptors on primary myeloma cells and normal plasmablasts (Figure 1C).

BAFF and APRIL expression in malignant and normal plasma cells

Since an autocrine production of APRIL and BAFF was previously reported in several tumor models,^{17,28,29} we looked for their expression in HMCLs and in primary myeloma cells. BAFF RNA was detected in 12 of 13 HMCLs and in 11 of 11 primary myeloma samples. An anti-BAFF antibody stained 10 of 12 HMCLs that

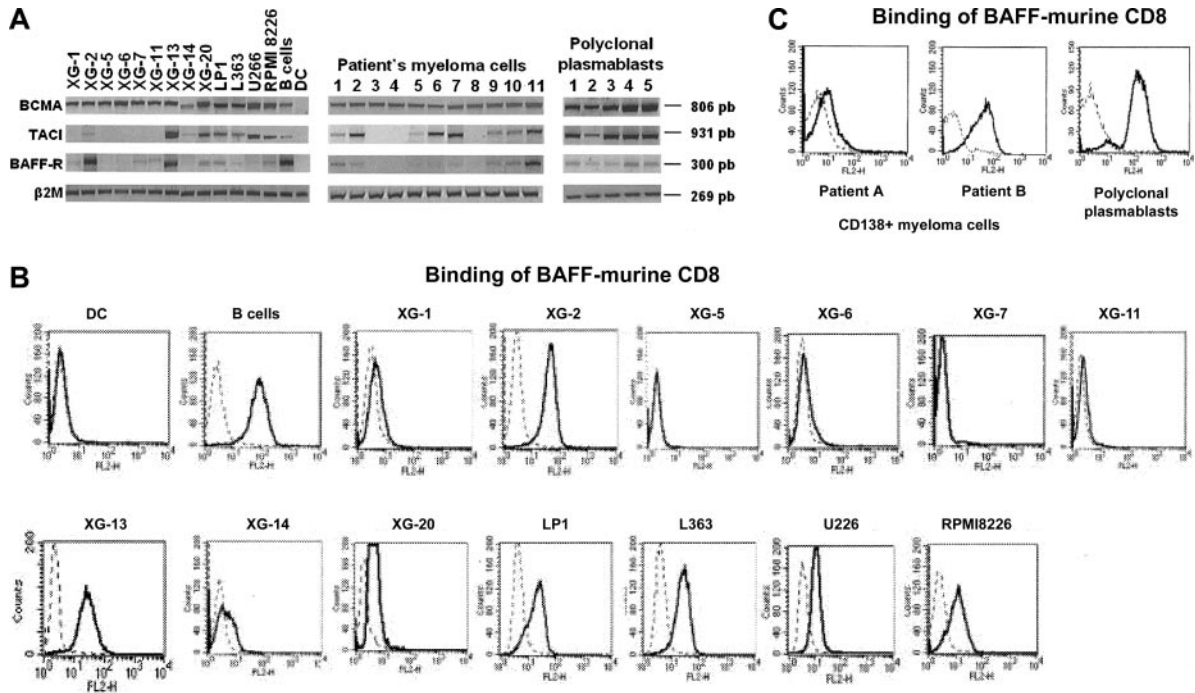


Figure 1. Expression of BCMA, TACI, and BAFF-R in myeloma cells and normal plasmablasts. (A) Expression of BCMA, TACI, and BAFF-R mRNA was analyzed by RT-PCR in 13 HMCLs, in CD138⁺ purified primary myeloma cells from 6 patients with intramedullary MM (patients 1-6), and 5 patients with plasma cell leukemia (patients 7-11), and in 5 normal in vitro-generated polyclonal plasmablastic cells (PPCs). B cells and dendritic cells (DCs) were used as positive and negative controls, respectively. (B) Cell-surface expression of receptors for BAFF by HMCLs was determined by flow cytometry using a biotinylated human BAFF–murine CD8 fusion protein and phycoerythrin-conjugated streptavidin. (C) Cell-surface expression of receptors for BAFF by primary myeloma cells and PPCs was determined by flow cytometry using a biotinylated human BAFF–murine CD8 fusion protein and phycoerythrin-conjugated streptavidin. Broken lines indicate Ig control; and solid lines, BAFF-mu CD8.

expressed BAFF mRNA, showing the presence of the membrane-bound form of BAFF. Data for 4 cell lines are shown in Figure 2B. XG-6 showed no cell-surface expression of BAFF, in agreement with the absence of detectable BAFF RNA in these cells (Figure 2B). APRIL RNA was expressed in most primary samples (10 of 11) and in 6 of 13 HMCLs (Figure 2A). As APRIL is a secreted protein, we looked for APRIL protein in myeloma cell culture supernatants. Using ELISA, soluble APRIL levels were 30.9 ng/mL and 33.3 ng/mL in culture supernatants of RPMI8226 and XG-20, respectively, that expressed APRIL mRNA. APRIL was not detectable in culture supernatant of XG-6 which did not express the gene. Polyclonal plasmablasts expressed BAFF and APRIL RNA and were labeled by the anti-BAFF antibody (Figure 2A-B).

BAFF and APRIL rescue IL-6-dependent HMCLs from apoptosis induced by IL-6 deprivation

To investigate the effect of BAFF and APRIL on myeloma cell survival and proliferation, we first used 3 cell lines whose growth is dependent on addition of IL-6: XG-13 and XG-20 HMCLs that expressed *TACI* and *BAFF-R* and XG-14 that expressed mainly *TACI*. In the absence of exogenous cytokines, the 3 HMCLs did not proliferate and a strong proliferation was induced by recombinant IL-6 (Figure 3A).³¹ BAFF and APRIL were also potent proliferation factors for XG-13 and XG-20 cells, whereas XG-14 cells responded to APRIL only (Figure 3A). Using annexin V as an indicator of apoptosis, we looked for the effect of BAFF and APRIL on myeloma cell survival. BAFF

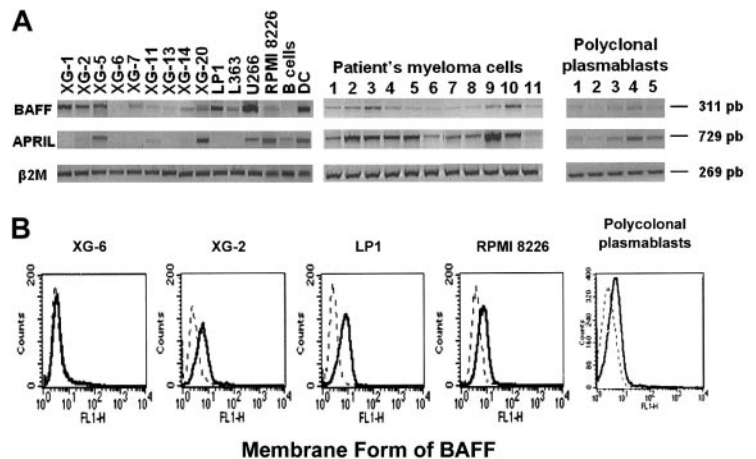


Figure 2. Expression of BAFF and APRIL in myeloma cells and normal plasmablasts. (A) Expression of BAFF and APRIL mRNA was analyzed by RT-PCR in 13 HMCLs, in CD138⁺ purified primary myeloma cells from 6 patients with intramedullary MM (patients 1-6), and 5 patients with plasma cell leukemia (patients 7-11), and in 5 normal in vitro-generated polyclonal plasmablastic cells (PPCs). B cells and dendritic cells (DCs) were used as negative and positive controls, respectively. (B) Cell-surface expression of BAFF was determined by flow cytometry using an anti-BAFF antibody. Broken lines indicate Ig control; and solid lines, anti-BAFF Ab.

Membrane Form of BAFF

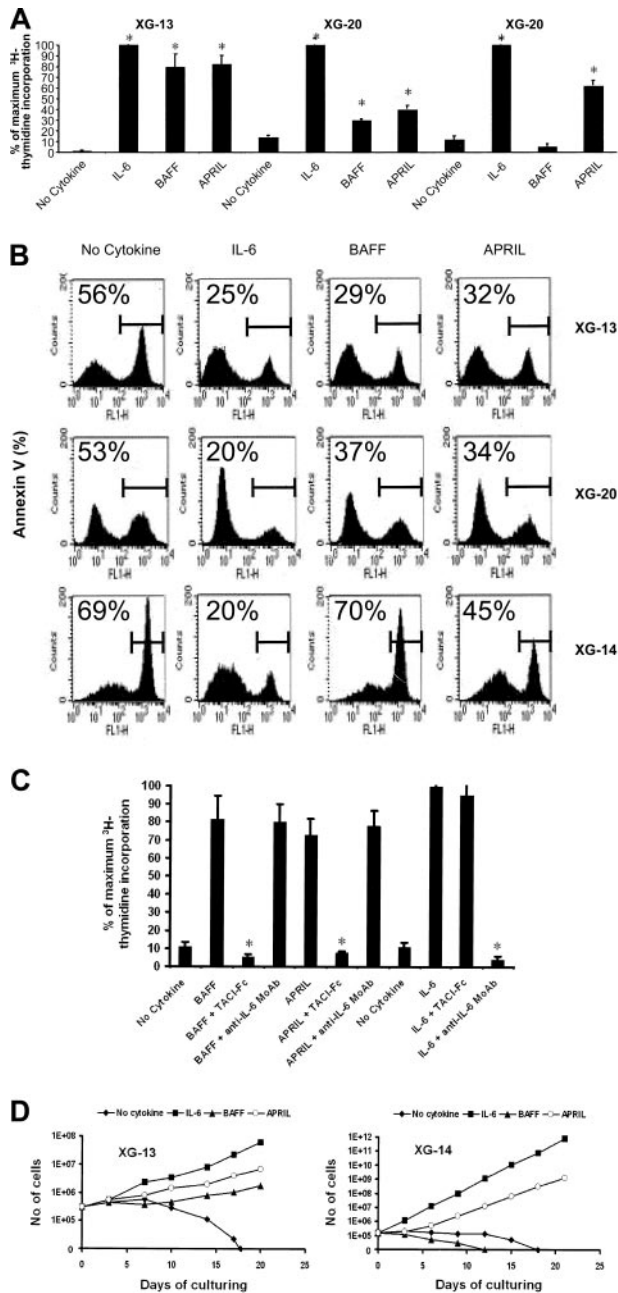


Figure 3. BAFF and APRIL protect HMCLs from IL-6 deprivation-induced apoptosis. (A) XG-13, XG-14, and XG-20 were IL-6 starved for 3 hours and cultured without cytokine, or in the presence of BAFF (200 ng/mL), APRIL (200 ng/mL), or IL-6 (3 ng/mL). Results are the mean values plus or minus standard deviation (SD) of the tritiated thymidine incorporation determined on sextuplet culture wells and are expressed as the percentage of the proliferation obtained with IL-6. Results are those of one experiment representative of 5. *Mean value is significantly different from that obtained without adding cytokine using a Student *t* test ($P \leq .05$). (B) XG-13 and XG-14 HMCLs were cultured at 10^5 cells/mL without cytokine or in the presence of IL-6 (3 ng/mL), BAFF (200 ng/mL), or APRIL (200 ng/mL). Cells were recovered after 3 days of culture and apoptotic cells were detected by annexin V staining. Results are those of one experiment representative of 5. The percentage of apoptotic cells is indicated in each panel. (C) XG-13 cells were IL-6 starved for 3 hours and cultured without cytokine, or in the presence of IL-6 (3 ng/mL), BAFF (200 ng/mL), or APRIL (200 ng/mL). When indicated, TACI-Fc (10 μ g/mL) or anti-IL-6 MoAb (10 μ g/mL) was added. Results are the mean values plus or minus SD of the tritiated thymidine incorporation determined on sextuplet culture wells and are expressed as the percentage of the proliferation obtained with IL-6. Results are for one experiment representative of 3. * Mean value is statistically significantly different from that obtained with either BAFF, APRIL, or IL-6 using a Student *t* test ($P \leq .05$). (D) XG-13 and XG-14 cells were cultured, respectively, at 2.5×10^5 and at 1.5×10^5 cells/mL without cytokine or in the presence of IL-6 (3 ng/mL), BAFF (200 ng/mL), or APRIL (200 ng/mL). Every 3 or 4 days, cells were counted and diluted with fresh culture medium containing the initial cytokine concentration. Results are the cumulative cell numbers obtained within 20 days of culture and are those of one experiment representative of 2.

and APRIL efficiently protected XG-13 cells (respectively, $P = .01$ and $P = .001$; $n = 5$) and XG-20 cells (respectively, $P = .0003$ and $P = .0002$; $n = 5$) from IL-6 deprivation-induced apoptosis. Only APRIL protected XG-14 ($P = .003$; $n = 5$) cells from apoptosis, in agreement with the above-mentioned proliferation data (Figure 3B). A TACI-Fc fusion protein abrogated specifically the myeloma cell proliferation induced by BAFF or APRIL, whereas an anti-IL-6 MoAb did not affect it (Figure 3C). Conversely, TACI-Fc had no effect on IL-6-induced proliferation that was completely inhibited by an anti-IL-6 MoAb (Figure 3C).

Finally, we looked for the ability of BAFF and APRIL to support the long-term growth of XG-13 and XG-14 HMCLs. As shown in Figure 3D, XG-13 and XG-14 cells died within 17 to 18 days upon removal of IL-6. IL-6 induced an exponential growth of the 2 HMCLs, with a doubling time of 48 hours for XG-13 and 20 hours for XG-14. APRIL and BAFF were both able to support the long-term growth of XG-13 cells with a doubling time, respectively, 2.2-fold and 1.75-fold higher than that obtained with IL-6. In agreement with the survival and proliferation data shown above, only APRIL supported long-term growth of XG-14 cells with a doubling time 1.5-fold higher than that obtained with IL-6.

These data indicate that BAFF and APRIL myeloma cell growth factors are able to support the long-term growth of cytokine-dependent HMCLs.

Autocrine BAFF and/or APRIL are involved in the autonomous growth of cytokine-independent HMCLs

As BAFF and/or APRIL are growth factors for IL-6-dependent HMCLs and are produced by some autonomously growing HMCLs, we investigated whether BAFF/APRIL could be autocrine myeloma growth factors. We used RPMI8226 and L363 HMCLs that expressed BAFF and/or APRIL together with their receptors (Figures 1 and 2). The autonomous proliferation of L363 and RPMI8226 cells was blocked by TACI-Fc, which neutralizes both BAFF and APRIL (Figure 4). Adding an excess of recombinant BAFF and APRIL abrogated the inhibitory effect of TACI-Fc. These data indicated that a BAFF/APRIL autocrine loop is involved in the autonomous growth of some HMCLs.

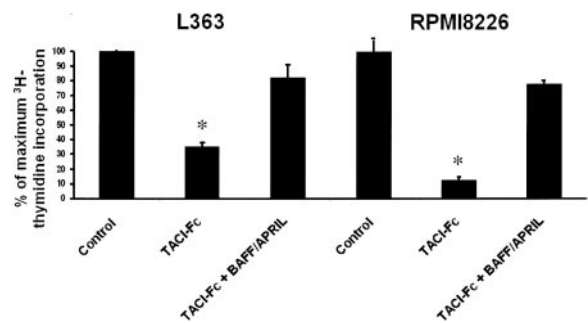


Figure 4. Autocrine BAFF or APRIL are involved in the growth of autonomously growing HMCLs. L363 and RPMI8226 cells were starved for 3 hours and cultured without cytokine, or in the presence of TACI-Fc (10 μ g/mL) or TACI-Fc (10 μ g/mL) and BAFF/APRIL (200 ng/mL BAFF and 200 ng/mL APRIL). Results are the mean values plus or minus SD of the tritiated thymidine incorporation determined on sextuplet culture wells and are expressed as the percentage of the proliferation obtained without cytokine. * Mean value is statistically significantly different from that obtained without adding TACI-Fc using a Student *t* test ($P \leq .05$).

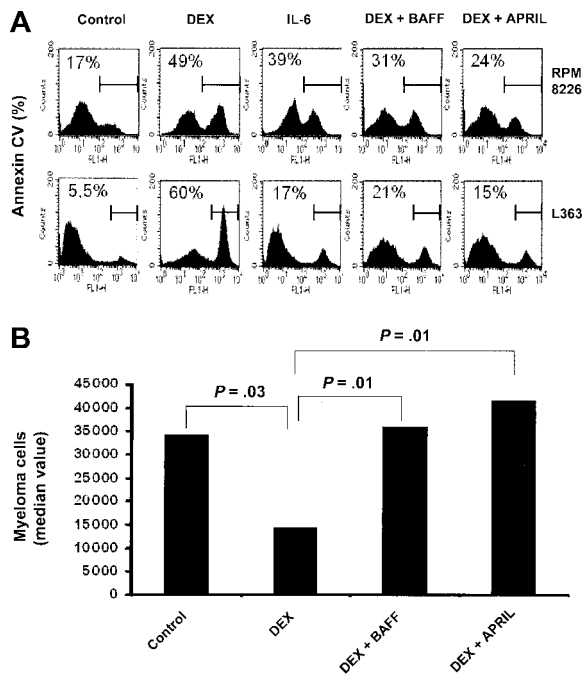


Figure 5. BAFF and APRIL rescue myeloma cells from dexamethasone-induced apoptosis. (A) RPMI 8226 and L363 myeloma cells were cultured in the presence of DEX (10⁻⁶ M) with or without IL-6 (3 ng/mL), BAFF (200 ng/mL), or APRIL (200 ng/mL). Cells were recovered after 3 days of culture and apoptotic cells were detected by annexin V staining. Results are those of one experiment of 5. The percentage of apoptotic cells is indicated in each panel. (B) Mononuclear cells from 8 patients with MM were cultured for 4 days in the presence of IL-6 (1 ng/mL) with or without DEX (10⁻⁶ M), BAFF (200 ng/mL), or APRIL (200 ng/mL). At day 4 of culture, the viability and total cell counts were assessed and the percentage of CD138⁺ viable plasma cells was determined by flow cytometry. Results are median values of the numbers of myeloma cells in the culture wells. The values were compared with a Wilcoxon test for pairs.

BAFF and APRIL rescue myeloma cells from dexamethasone-induced apoptosis

We next sought to determine whether BAFF or APRIL could protect myeloma cells from the apoptosis induced by DEX, a potent drug for MM treatment. As indicated in Figure 5A, DEX induced apoptosis in RPMI 8226 and L363 HMCLs. Both BAFF and APRIL significantly protected the RPMI 8226 HMCL from DEX-induced apoptosis (respectively, $P = .001$ and $P = .0002$; $n = 5$). The same results were obtained with L363 (respectively, $P = .0007$ and $P = .001$; $n = 5$). In fact, both BAFF and APRIL were as potent as IL-6 in protecting myeloma cells from DEX-induced apoptosis (Figure 5A).

We next investigated whether BAFF and APRIL could protect primary myeloma cells from DEX-induced apoptosis. Since puri-

fied myeloma cells are highly susceptible to spontaneous apoptosis in vitro, myeloma cells were cultured in the presence of their bone marrow environment. In addition, recombinant IL-6 was added to reduce the variability resulting from the heterogeneous endogenous IL-6 production in cultured tumor samples.³⁵ As shown in Figure 5B, DEX reduced the median number of viable myeloma cells of 8 patients by 58% ($P = .03$; $n = 8$). BAFF and APRIL enhanced survival of myeloma cells in the presence of DEX (respectively, $P = .01$ and $P = .01$; $n = 8$) yielding a number of malignant plasma cells that was not statistically different between DEX and BAFF, DEX and APRIL, and the control group (Figure 5B).

BAFF/APRIL inhibitor induces apoptosis of primary myeloma cells

Our data showing that BAFF and APRIL are survival factors for malignant plasma cells suggest that new therapeutic agents inhibiting BAFF/APRIL may be promising for myeloma treatment. We thus investigated the effect of the TACI-Fc fusion protein, able to block BAFF and APRIL, on primary myeloma cell survival and on drug sensitization. Primary myeloma cells were cultured with their bone marrow environment and recombinant IL-6. Detailed results obtained with 6 patients are shown in Table 1. TACI-Fc significantly reduced the median number of viable myeloma cells by 48% ($P = .028$; $n = 6$). TACI-Fc also potentiated the inhibitory effect of DEX or B-E8 anti-IL-6 MoAb (respectively, $P = .028$, $P = .046$; $n = 6$). When the 3 inhibitors were used together, a 90% reduction of viable myeloma cells was observed within 4 days of culture (Figure 6A). Of interest, the nonmyeloma cells present in the culture wells were unaffected by these 3 inhibitors (results not shown).

Signal transduction and antiapoptotic protein regulation by BAFF or APRIL

As shown in Figure 7A, BAFF and APRIL induced a rapid phosphorylation of AKT and a late phosphorylation of MAPK in 3 myeloma cell lines (XG-13, XG-14, and RPMI8226), whereas no phosphorylation of STAT3 was detected. IL-6 induced the phosphorylation of STAT3, MAPK, and AKT, in agreement with previous data.^{36,37}

We also looked for NF- κ B signaling, as there is accumulating evidence that BAFF and APRIL activate NF- κ B transcription factors in B cells.^{33,38-40} We found that BAFF and APRIL, like TNF- α , enhanced NF- κ B binding activity in XG-13 and RPMI8226 cells, whereas IL-6 induced a weak and transient activation of NF- κ B, in agreement with previous studies⁴¹ (Figure 7B). For XG-14 cells that biologically responded only to APRIL, we found that APRIL, unlike BAFF, enhanced NF- κ B binding activity.

Table 1. TACI-Fc induces apoptosis of primary myeloma cells

Patient no.	Myeloma cell number/culture well							
	Control	TACI-Fc	DEX	DEX + TACI-Fc	Anti-IL-6 MoAb	Anti-IL-6 MoAb + TACI-Fc	Anti-IL-6 MoAb + DEX	Anti-IL-6 MoAb + DEX + TACI-Fc
1	113 774	97 474	81 280	56 940	120 250	98 610	37 180	23 800
2	69 960	37 884	28 980	16 168	42 560	31 740	15 752	9 550
3	18 864	10 494	19 800	8 400	20 692	12 096	19 008	4 344
4	34 768	30 380	30 608	14 084	16 226	16 434	7 872	5 265
5	99 540	53 508	34 914	28 457	59 280	30 576	15 228	7 912
6	171 000	154 400	158 420	107 100	160 056	130 764	112 624	52 920
Median value	84 750	45 696	32 761	22 312	50 920	31 158	17 380	8 731

Mononuclear cells from tumor samples of 6 patients with MM were cultured for 4 days in the presence of IL-6 (1 ng/mL) with or without DEX (10⁻⁶ M), TACI-Fc (10 μ g/mL), or B-E8 anti-IL-6 MoAb (10 μ g/mL). At day 4 of culture, the cell count and viability were determined and the percentage of CD138⁺ viable plasma cells was determined by flow cytometry.

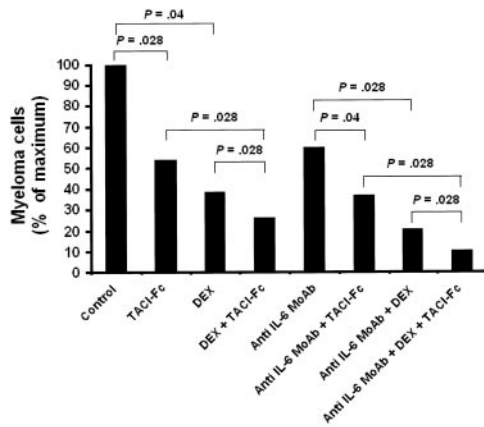


Figure 6. TACI-Fc induces apoptosis of primary myeloma cells. Mononuclear cells from tumor samples of 6 patients with MM were cultured for 4 days in the presence of IL-6 (1 ng/mL) with or without DEX (10⁻⁶ M), TACI-Fc (10 ng/mL), or B-E8 (10 ng/mL). At day 4 of culture, the cell count and viability were determined and the percentage of CD138⁺ viable plasma cells was determined by flow cytometry. The power of an inhibitor is given as the percentage of reduction of the median value of viable myeloma cell count with the inhibitor compared with the median value of viable myeloma cell count without inhibitor.

Interestingly, an inhibitor of PI3K/AKT (Ly 294002) abrogated the proliferation of XG-13 cells induced by BAFF or APRIL. A peptide inhibitor of the NF-κB pathway (SN50) also inhibited BAFF- or APRIL-induced myeloma cell proliferation, unlike the corresponding inactive peptide (Figure 7C).

We then studied the regulation by BAFF or APRIL of 3 Bcl-2 family antiapoptotic members (Mcl-1, Bcl-2, and Bcl-xL) known to be involved in growth factor-mediated myeloma cell survival or in BAFF-mediated survival in B cells.⁴²⁻⁴⁷ BAFF and APRIL induced an up-regulation of Mcl-1 and Bcl-2 in XG-13 and RPMI8226 cells, whereas only APRIL increased Mcl-1 and Bcl-2

levels in XG-14 cells. In contrast, no change was noted in Bcl-xL protein expression (Figure 8A-B). IL-6 increased Mcl-1 but neither Bcl-2 nor Bcl-xL levels according to our previous studies.⁴⁵

Levels of circulating BAFF and APRIL in sera of patients with MM

To further assess the biologic relevance of our data, we looked for levels of soluble BAFF and APRIL in the sera of 36 patients with MM and 9 age-related healthy individuals. Results shown in Figure 9 demonstrated that BAFF or APRIL median serum levels were increased, respectively, 4.2-fold (P = .02) and 5.9-fold in patients (P = 6.10⁻⁹) compared with healthy individuals.

Discussion

Accumulating experimental evidence supports the notion that BAFF is essential for the survival of normal immature and mature B cells¹⁵ as well as normal plasmablastic cells.³⁴ BAFF plays a key role in the survival of B-CLL tumor cells.^{28,29} In addition, APRIL stimulates the growth of some human and murine tumor cell lines in vitro and in vivo.¹⁷ As BAFF/APRIL receptor genes are overexpressed in malignant plasma cells,^{10,11} our aim was to look for a role played by BAFF and APRIL in MM.

We demonstrate here that BAFF and APRIL are growth factors for 2 myeloma cell lines that highly expressed TACI and BAFF-R and whose survival is completely dependent on addition of exogenous growth factors. APRIL is also a growth factor for a third cell line, XG-14, which expressed only TACI, unlike BAFF-R. We also show that an autocrine loop involving BAFF, APRIL, and their receptors is involved in the autonomous growth of 2 well-known HMCLs, L363 and RPMI8226. BAFF and APRIL contribute to the

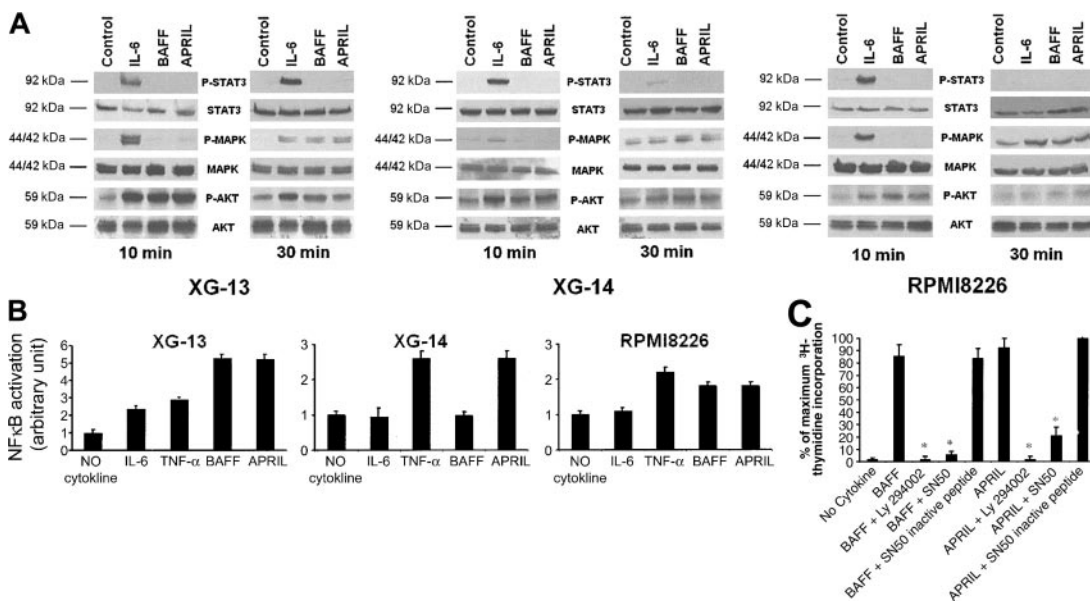


Figure 7. Signal transduction induced by BAFF or APRIL in myeloma cells. (A) XG-13, XG-14, and RPMI8226 cells were starved overnight and cultured without cytokine, or with either IL-6 (30 ng/mL), BAFF (800 ng/mL), or APRIL (800 ng/mL) for 10 and 30 minutes at 37°C. Cell lysates were analyzed by Western blotting with antisera against phospho-STAT3 (pSTAT3), phospho-ERK1/2 (pMAPK), and phospho-AKT (pAKT). Immunoblotting for STAT3, MAPK, and AKT confirmed equal protein loading. Western blots are of one representative experiment of 3. (B) XG-13, XG-14, and RPMI8226 cells were starved overnight and cultured without cytokine, or with either IL-6 (3 ng/mL), TNF-α (20 ng/mL), BAFF (200 ng/mL), or APRIL (200 ng/mL) for 30 minutes at 37°C. NF-κB activity was detected by ELISA according to the manufacturer's instructions. (C) XG-13 cells were IL-6 starved for 3 hours and cultured without cytokine or in the presence of BAFF (200 ng/mL) or APRIL (200 ng/mL). When indicated, Ly 294002 (25 μM), SN50 (100 μg/mL), or the SN50 inactive peptide (100 μg/mL) was added. Results are the mean values ± SD of the tritiated thymidine incorporation determined on sextuplet culture wells and are expressed as the percentage of the proliferation obtained with APRIL and SN50 inactive peptide. *Mean value is statistically significantly different from that obtained with BAFF or APRIL using a Student t test (P ≤ .05).

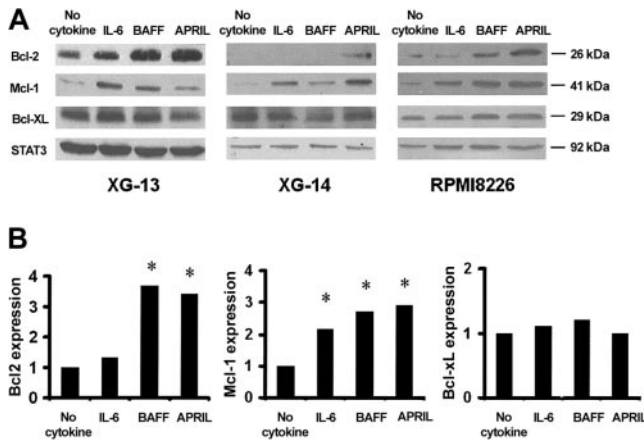


Figure 8. Regulation of Bcl-2 family antiapoptotic proteins by BAFF and APRIL. (A) XG-13, XG-14, and RPMI8226 cells were starved overnight before culture with no cytokine, or with IL-6 (30 ng/mL), BAFF (800 ng/mL), or APRIL (800 ng/mL) for 6 hours in RPMI-1% BSA. At the end of the culture, cells were immediately lysed and assayed for Bcl-2 family antiapoptotic protein expression using Western blot analysis. In this experiment, STAT3 expression was used as loading protein control. (B) Blots of 3 independent experiments were scanned and the values were normalized using STAT3-band intensities as internal standards. Results are the median values for the 3 main Bcl-2 family members expressed in the XG-13 HMCLs starved of IL-6 and cultured for 6 hours with no cytokine, or with IL-6 (30 ng/mL), BAFF (800 ng/mL), or APRIL (800 ng/mL). *Significant increase in expression with a Student *t* test for pairs (*P* < .05).

survival of primary myeloma cells cultured together with their bone marrow environment and these 2 factors prevent DEX-induced apoptosis in primary myeloma cells. Since the initial submission of this manuscript, another paper also indicated that BAFF and APRIL are myeloma cell growth factors.⁴⁸ Altogether, the current data and this paper extend previous reports indicating that BAFF/APRIL are involved in various B-cell neoplasias, in particular B-CLL and follicular lymphoma,²⁸⁻³⁰ and in autoimmune diseases such as Sjögren syndrome and systemic lupus erythematosus.¹⁵

An interesting question is the nature of the receptors involved in the BAFF and APRIL activity on myeloma cells. BAFF and APRIL bind, respectively, 3 (BAFF-R, TACI, and BCMA) and 2 (TACI and BCMA) distinct receptors. In addition, another receptor for APRIL probably exists, as reported for adenocarcinoma cells.⁴⁹ We show here that, although *BCMA* was expressed by 5 of 5 normal plasmablasts, 13 of 13 HMCLs, and 11 of 11 primary myeloma cells, *TACI* and *BAFF-R* expression was heterogeneous. In some HMCLs expressing only *BCMA* (XG-5 and XG-6 HMCLs, for example), *BCMA* RNA was not associated with a functional membrane receptor since these HMCLs were unable to bind BAFF-murine CD8 fusion protein. This is in agreement with the described retention of *BCMA* in the Golgi complex in HMCLs⁵⁰ and with the lack of B-cell deficiency in *BCMA*^{-/-} mice.⁵¹ On the contrary, HMCLs that highly expressed *TACI* or *BAFF-R* bound BAFF-murine CD8 complex, confirming the presence of functional membrane receptors for BAFF. BAFF- and BAFF-R-deficient mice present a similar loss of follicular and marginal zone B cells in secondary lymphoid organs, suggesting that BAFF-R is the predominant stimulatory receptor for BAFF.⁵²⁻⁵⁵ The expression of TACI and BAFF-R is tightly regulated during the B-cell maturation process, and one can hypothesize that these 2 molecules could have different biologic activities depending on the cell type. Interestingly, XG-14 cells expressing a low level of *TACI* and no *BAFF-R* were sensitive to APRIL only. One hypothesis is that XG-14 expresses a receptor specific for APRIL that has not yet

been identified, as reported for adenocarcinoma cells.⁴⁹ Novak et al reported that all myeloma cells they tested bound soluble BAFF.⁴⁸ In that study, BAFF-R was not detectable on the cell surface of HMCLs using an anti-BAFF-R antibody, whereas purified primary myeloma cells expressed BAFF-R. Thus, these data and our results indicate that we cannot yet draw firm conclusions on the respective role of TACI and BAFF-R in myeloma cells that express the 2 receptor genes, that is, the majority of purified primary myeloma cells.

In order to better understand the effect of BAFF and APRIL on myeloma cells, we examined intracellular signaling pathways. BAFF was reported to induce NF- κ B activation in B cells²³ and an overexpression of *BCMA* in human 293 cells activates the Rel/NF- κ B, JNK, Elk-1, and p38 kinase transcription factors.^{56,57} Activation of TACI in Jurkat T cells also results in activation of AP-1, NF- κ B, and nuclear factor of activated T cells (NF-AT).⁵⁸ In myeloma cells, we and others have shown that IL-6 activates 3 essential pathways: the JAK/STAT, MAPK, and PI3K/Akt cascades, whereas IGF-1 activates MAPK and PI3K/Akt.^{36,41,59-62} IGF-1 also activates NF- κ B.⁴¹ We show here that neither BAFF nor APRIL was able to induce STAT3 phosphorylation but did activate ERK1/2 and PI3K/Akt pathways. An inhibitor of PI3K/AKT abrogated the growth of myeloma cells induced by BAFF and APRIL. Of interest, BAFF and APRIL were also able to efficiently activate NF- κ B, as was TNF- α . Finally, we found that NF- κ B activation was critical for the myeloma cell proliferation activity of BAFF or APRIL since it was dramatically blocked by an NF- κ B peptide inhibitor.

BAFF and APRIL induced an up-regulation of Bcl-2 and Mcl-1 antiapoptotic proteins in myeloma cells, whereas the level of Bcl-xL remained unchanged. In murine B cells, the survival effect of BAFF was associated with induction of A1, Bcl-2, and Bcl-xL.^{23,26,27} This is in agreement with the activation of NF- κ B, which enhances the transcription of a number of antiapoptotic genes, including A1, Bcl-2, Bcl-xL, cIAP2, and cFLIP. Previously, we demonstrated that among 10 antiapoptotic and proapoptotic proteins, including A1, Bcl-xL, and Bcl-2, only Mcl-1 was regulated by IL-6 or IFN- α in myeloma cells.⁴⁵ Furthermore, oligonucleotide antisenses to Mcl-1, but not to Bcl-2 or Bcl-xL, were able to induce apoptosis of myeloma cell lines,⁴⁷ and constitutive Mcl-1 expression strongly reduced apoptosis induced by IL-6 withdrawal.⁴⁶ Collectively, these data suggest that Mcl-1 is the major antiapoptotic protein involved in IL-6-mediated survival in myeloma. An overexpression of Bcl-2 in myeloma cell lines could also confer protection from apoptosis induced by dexamethasone,⁶³ etoposide, or doxorubicin⁶⁴ and may therefore contribute to tumor cell survival and a multidrug-resistant phenotype. Thus, BAFF and APRIL up-regulate in myeloma cells the expression of the 2 major antiapoptotic proteins, Mcl-1 and Bcl-2, known to be involved in

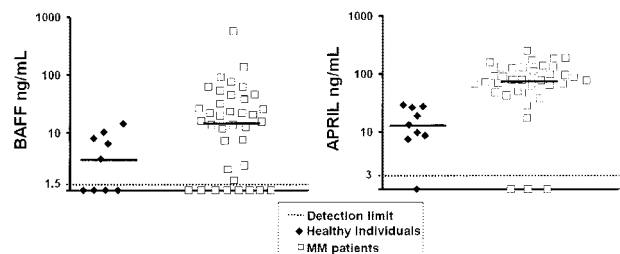


Figure 9. Serum level of circulating BAFF and APRIL in myeloma patients. Serum levels of BAFF and APRIL were determined by ELISA in the sera from 36 patients with myeloma and 9 age-related healthy individuals.

the rescue from apoptosis induced by growth-factor removal or DEX treatment.

The data mentioned previously in this paper that were obtained with myeloma cell lines could be extended to primary myeloma cells that expressed *BAFF-R* and/or *TACI*. As primary myeloma cells rapidly apoptosed as soon as they were purified,⁶⁵ they were cultured together with their bone marrow environment. The TACI-Fc fusion protein, able to block both BAFF and APRIL, reduced the survival of primary myeloma cells, and increased inhibition was obtained when TACI-Fc was used together with DEX or anti-IL-6 MoAb. Interestingly, when the 3 inhibitors were combined, virtually all primary myeloma cells died, whereas the cocultured nonmyeloma cells were unaffected. These *in vitro* data suggest that inhibitors of BAFF and TACI could be very useful to induce apoptosis of myeloma cells when used alone or in combination with DEX and/or anti-IL-6 MoAb. The advantage of using BAFF/APRIL inhibitors in MM is emphasized by the current finding that serum levels of BAFF

and APRIL were increased roughly 5-fold in patients with MM as compared with age-related healthy individuals. These serum concentrations were in the range of those able to promote myeloma cell growth *in vitro*. The presence of circulating APRIL has not been reported in humans. The circulating serum levels of BAFF reported here were close to those found in autoimmune diseases, where it was correlated with the autoantibody level.^{66,67} Further studies are necessary to determine whether BAFF or APRIL serum levels are prognostic factors in patients with multiple myeloma.

New therapeutic agents have now been developed to inhibit BAFF/APRIL in B-cell neoplasia and autoimmune diseases, such as anti-BAFF MoAb and the TACI-Fc, or the BAFF/APRIL signaling pathway, such as the PS-1145 I κ B kinase inhibitor.^{55,68-70} Thus, the present report suggests that these novel inhibitors may be promising elements in the treatment of patients with MM, possibly in association with DEX and/or anti-IL-6 MoAb.

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II-2 Article 6

« The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature. Blood. 2005 Aug 1;106(3):1021-30. Epub 2005 Apr 12 »

Nous avons recherché à identifier si la production de BAFF et APRIL dans le MM est autocrine ou paracrine et les populations cellulaires responsables de cette production. L'objectif de notre travail était de déterminer une signature d'expression génique liée à la voie BAFF/APRIL dans le MM.

Nous avons démontré que :

- BAFF et APRIL sont produits principalement par les cellules de l'environnement médullaire. BAFF est produit par les monocytes et les polynucléaires neutrophiles. APRIL est sécrété majoritairement par les ostéoclastes mais aussi par les monocytes et les polynucléaires neutrophiles.
- BAFF et APRIL présentent trois récepteurs identifiés : BCMA, TACI et BAFF-R. Par RT-PCR classique, nous avons démontré précédemment que BCMA est exprimé par les 17 lignées de myélome testées, TACI par 9/17 et BAFF-R par 10/17 . De façon intéressante, TACI est un bon indicateur de la présence d'un récepteur fonctionnel à la surface des cellules tumorales car uniquement les lignées exprimant TACI par RT-PCR sont capables de lier la protéine de fusion BAFF-murineCD8. Les lignées

exprimant uniquement BCMA sont incapables de fixer BAFF-murineCD8 (282). Nous avons étendu cette observation aux plasmocytes tumoraux frais de 6 patients. Seules les cellules myélomateuses de patients exprimant fortement TACI par RT-PCR sont capables de fixer BAFF-murineCD8. Ces données démontrent que l'expression génique de TACI est un très bon indicateur de la présence d'un récepteur fonctionnel de BAFF/APRIL à la surface des cellules de myélome.

- Nous disposons du transcriptome (puces affymetrix 33000 gènes U133 A+B) des plasmocytes tumoraux purifiés, au diagnostic, de 65 patients atteints de MM. Le gène codant pour TACI est présent chez les 65 patients de l'étude. Afin d'identifier une signature génique associée à l'expression de TACI, nous avons comparé les profils d'expression génique des cellules myélomateuses ayant une forte expression de TACI (TACI^{high}) avec celles ayant une faible expression (TACI^{low}). 659 gènes sur les 33000 sont différenciellement exprimés entre les deux groupes. Ce set de gènes permet de classer parfaitement les cellules de MM TACI^{high} et TACI^{low} dans deux groupes indépendants de patients atteints de MM.
- Les plasmocytes tumoraux TACI^{high} expriment un plus grand pourcentage de gènes codant pour des protéines impliquées dans la communication intercellulaire (chimiokines/récepteurs de chimiokines, facteurs de croissance/récepteurs de facteurs de croissance, intégrines), le cytosquelette et la transduction du signal. A l'opposé, les cellules TACI^{low} surexpriment des gènes codant pour des protéines nucléaires et impliquées dans le cycle cellulaire.

- Les cellules de myélomes TACI^{high} ont une signature génique de plasmocytes médullaires matures liée à une dépendance au microenvironnement médullaire alors que les plasmocytes tumoraux TACI^{low} ont une signature génique de plasmablastes proliférants peu dépendants de l'environnement médullaire.

En conclusion, nos données encouragent le développement d'inhibiteurs à usage thérapeutique bloquant l'interaction entre BAFF/APRIL et TACI dans le MM comme la protéine de fusion TACI-Ig et suggère l'utilisation des puces à ADN pour identifier le groupe de patients pouvant bénéficier de ce type de traitement.

The level of *TACI* gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature

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B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) have been shown to promote multiple myeloma (MM) cell growth. We show that the main site of production for BAFF and APRIL is the bone marrow (BM) environment, and that production is mainly by monocytes and neutrophils. In addition, osteoclasts produce very high levels of APRIL, unlike BM stromal cells. Myeloma cells (MMCs) express TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), the receptor of BAFF/APRIL, at varying levels. *TACI* expres-

sion is a good indicator of a BAFF-binding receptor. Expression data of purified MMCs from 65 newly diagnosed patients have been generated using Affymetrix microarrays and were analyzed by supervised clustering of groups with higher (*TACI*^{hi}) versus lower (*TACI*^{lo}) *TACI* expression levels. Patients in the *TACI*^{lo} group had clinical parameters associated with bad prognosis. A set of 659 genes was differentially expressed between *TACI*^{hi} and *TACI*^{lo} MMCs. This set makes it possible to efficiently classify *TACI*^{hi} and *TACI*^{lo} MMCs in an independent cohort of 40 patients. *TACI*^{hi}

MMCs displayed a mature plasma cell gene signature, indicating dependence on the BM environment. In contrast, the *TACI*^{lo} group had a gene signature of plasmablasts, suggesting an attenuated dependence on the BM environment. Taken together, our findings suggest using gene expression profiling to identify the group of patients who might benefit most from treatment with BAFF/APRIL inhibitors. (Blood. 2005;106:1021-1030)

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Introduction

Multiple myeloma (MM) is a universally fatal neoplasia characterized by the accumulation of malignant plasma cells (MMCs) in the bone marrow (BM).¹ There is increasing evidence for a major role of interactions between MMCs and BM stroma for the support of survival and proliferation of the MMCs.²

Patients with MM can be grouped in accordance to cytogenetic aberrations identified by conventional metaphase cytogenetics or (interphase) fluorescence in situ hybridization (iFISH) in MMCs.^{3,4} Patients can also be grouped in accordance to gene expression profiling (GEP) with a link between cytogenetic and GEP data.^{4,5} A good example is the correlation between the translocation t(4;14) and the overexpression of the multiple myeloma set domain (MMSET), or between the translocation t(11;14) and a high CCND1 overexpression.^{6,7} GEP has the potential to identify new pathways involved in the biology of MMCs, in particular regarding communication with the tumor environment.⁸⁻¹⁰ DNA arrays permitted to show that some human myeloma cell lines (HMCLs) expressed a gene coding for heparin-binding epidermal growth factor-like (HB-EGF) growth factor, a member of the epidermal growth factor family able to bind syndecan-1.¹¹ Further studies demonstrated that HB-EGF is a potent myeloma cell growth

factor.^{12,13} Using Affymetrix microarrays, we also found that *TACI* (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and *BCMA* (B-cell maturation antigen), each gene coding for a receptor of B-cell activating factor (BAFF)^{14,15} and a proliferation-inducing ligand (APRIL),¹⁶⁻¹⁸ were overexpressed in malignant plasma cells compared with their normal counterparts.^{8,9} BAFF is a tumor necrosis factor (TNF) family member involved in the survival of normal and malignant B cells as well as normal plasmablasts.^{18,19} APRIL is highly expressed in several tumor tissues and stimulates the growth of tumor cells.¹⁶ A third receptor specific for BAFF, BAFF-receptor (BAFF-R), was also identified.²⁰ We and others showed that BAFF and APRIL serum levels are elevated in patients with MM and that BAFF and APRIL contribute to the survival of some HMCLs and of primary MMCs.^{21,22}

In this study, we have shown that the tumor environment is the main source of BAFF and APRIL in patients with MM and that *TACI* expression yields a functional receptor. Using Affymetrix microarrays, we demonstrated that *TACI*^{hi} MMCs displayed a mature plasma cell gene signature, indicating dependence on the BM environment. In contrast, the *TACI*^{lo} group had a gene

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signature of plasmablasts, suggesting an attenuated dependence on interactions with cells in the BM environment.

Materials and methods

Cell samples

XG-1, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-12, XG-13, XG-16, XG-19, and XG-20 HMCLs were obtained as described.²³ SKMM, OPM2, LP1, and RPMI8226 HMCLs were purchased from ATTC (Rockville, MD). MMCs were purified from a first series of 65 patients with myeloma at diagnosis (median age, 59 years) and a second series of 40 newly diagnosed patients after written informed consent was given according to the Declaration of Helsinki. In the first series, according to Durie-Salmon classification, 12 patients were of stage IA, 12 of stage IIA, 38 of stage IIIA, and 3 of stage IIIB. There were 11 patients who had IgA κ MM, 7 IgA λ MM, 26 IgG κ MM, 9 IgG λ MM, 7 Bence-Jones κ MM, 3 Bence-Jones λ MM, and 2 nonsecreting MM. In the second series, 6 patients were of stage IA, 5 of stage IIA, 27 of stage IIIA, and 2 of stage IIIB. There were 4 patients who had IgA κ MM, 5 IgA λ MM, 18 IgG κ MM, 7 IgG λ MM, 4 Bence-Jones κ MM, 1 Bence-Jones λ MM, and 1 nonsecreting MM. Normal BM plasma cells (BMPCs) and whole BM cells (WBMCs) were obtained from healthy donors after informed consent was given. WBMCs were collected after lysis of red blood cells with NH₄Cl. After Ficoll-density gradient centrifugation, plasma cells were purified using anti-CD138 MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). BM environment cells from 7 newly diagnosed patients were obtained after depletion of MMCs with anti-CD138 MACS microbeads (Miltenyi Biotec). BM culture supernatants were prepared culturing 5×10^5 BM cells depleted of MMCs for 3 days in RPMI1640 and 10% fetal calf serum (FCS). Supernatants were frozen at -20°C until use. For 3 newly diagnosed patients, BM T cells, monocytes, and polymorphonuclear neutrophils (PMNs) were purified. BM cells were labeled with a phycoerythrin (PE)-conjugated anti-CD3 monoclonal antibody (MoAb), allophycocyanin (APC)-conjugated anti-CD14 MoAb, and a fluorescein isothiocyanate (FITC)-conjugated anti-CD15 MoAb (all from Becton Dickinson, San Jose, CA). CD3⁺, CD14⁺, and CD15⁺ cells were sorted with a FACSAria cell sorter (Becton Dickinson). Memory B cells, polyclonal plasmablasts (PPCs), immature dendritic cells (DCs), and BM stromal cell lines (BMSCs) were generated as described previously.^{9,24,25} BMSC culture supernatants were prepared from 3-day culture before reaching confluence and were stored at -20°C until use.

Osteoclasts

Peripheral blood mononuclear cells were obtained from 7 patients with MM after informed consent. Cells were cultured at 2.5×10^6 cells/mL in α minimum essential medium (α MEM)-10% FCS. After 12 hours of culture, nonadherent cells were eliminated and adherent cells were cultured in α MEM-10% FCS, receptor activator of NF- κ B ligand (RANKL) (50 ng/mL; PeproTech, EC Ltd, London, United Kingdom), macrophage-colony-stimulating factor (M-CSF; 25 ng/mL; Peprotech), and 10 nM dexamethasone for 14 days. Before use, osteoclasts were phenotyped by reverse transcriptase-polymerase chain reaction (RT-PCR; TRAP and cathepsin K expression), by cytometry (integrin α v β 3 expression), and by bone resorbing activity (OsteoLyse assay kit; Cambrex, Emerainville, France). Culture supernatants were prepared from mature osteoclasts cultured for 3 days with M-CSF, RANKL, and dexamethasone and were frozen at -20°C until use.

BAFF or APRIL ELISA

The concentration of BAFF was assayed using the enzyme-linked immunosorbent assay (ELISA) described previously,²¹ and APRIL was assayed with an ELISA purchased from Bender MedSystems (Burlingame, CA) in accordance with the manufacturer's instructions.

Modulation of the gene expression profile by addition or deprivation of BAFF/APRIL in MMCs

The modulation of gene expression by addition of BAFF and APRIL was investigated with the XG-13 HMCL.²¹ XG-13 cells were starved of interleukin 6 (IL-6) for 2 hours, washed, and cultured for 12 hours in RPMI1640-10% FCS. Then BAFF and APRIL (200 ng/mL each) were added in one culture group for 10 hours. We also investigated the modulation of gene expression profile in primary MMCs by BAFF and APRIL.²⁶ BM cells of a newly diagnosed patient with TACI^{hi} MMCs were harvested and WBMCs (5×10^5 cells/mL) were cultured for 24 hours in RPMI1640-10% FCS. Then, 20 μ g/mL TACI-Fc (R&D Systems, Abington, United Kingdom) was added for 10 hours in one culture group. Cells were harvested, labeled with CD138 MACS microbeads at 4°C , and primary MMCs were purified. RNA was extracted for gene expression profiling.

Preparation of complementary RNA and microarray hybridization

RNA was extracted using the RNeasy Kit (Quiagen, Hilden, Germany). Biotinylated complementary RNA was amplified with a double in vitro transcription and hybridized to the human HG U133 A and B or U133 2:0 plus GeneChips, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Fluorescence intensities were quantified and analyzed using the GECOS software (Affymetrix).

Conventional and real-time RT-PCR

BCMA, TACI, and BAFF-R PCR analysis was done as previously described.²¹ The assays-on-demand primers and probes and the *TaqMan* Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). Real-time RT-PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample.

Binding of BAFF to MMCs

The binding of BAFF to HMCLs and primary MMCs was determined using a human BAFF-murine CD8 biotinylated fusion protein (Ansell, Bayport, MN) and fluorescence-activated cell sorting (FACS) analysis as previously described.²¹

Fluorescence in situ hybridization (FISH)

Interphase FISH was performed according to our previously reported standard protocol.²⁷ Metaphase spreads and interphase cells were evaluated using a DM RXA2 fluorescence microscope (Leica, Bensheim, Germany).

Statistical analysis and TACI^{lo} and TACI^{hi} group definition

Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platform (RAGE [remote analysis of microarray gene expression]) designed by Dr T Reme (INSERM U475, Montpellier, France). Hierarchical clustering was performed with Cluster and Treeview software from Eisen et al.²⁸ We performed a 2-sided supervised clustering between a group of patients with a high (TACI^{hi}) and one with low (TACI^{lo}) expression of *TACI*. To account for the log-normal distribution of *TACI* expression, we performed this analysis with decreasing proportions of patients in the TACI^{hi} and TACI^{lo} group: 40%, 25%, and 15% of patients with the highest versus lowest *TACI* expression. Genes were interpreted as differentially expressed if the *P* value (Mann-Whitney test) was .01 and the ratio 1.5 or 0.67. A permutation test²⁹ was performed to generate 100 repetitions of 2 arbitrary groups comprising 40%, 25%, or 15% of patients in order to get an evaluation for the number of genes differentially expressed between 2 arbitrary groups (*P* = .01 and ratio 1.5 or 0.67) by chance. Statistical comparisons were done with Mann-Whitney, chi-square, or Student *t* tests.

Results

BAFF and APRIL genes are mainly expressed by the BM environment

BAFF and APRIL gene expression levels were analyzed by real-time RT-PCR in 4 samples of memory B cells, 7 of normal plasmablasts, 10 of HMCLs, 7 of primary MMCs, 7 of BM environment cells depleted from MMCs, and 5 of BM cells of healthy individuals. These data were compared with those of dendritic cells, to which an arbitrary value of 100 was assigned (Figure 1A). BAFF and APRIL were highly expressed in the MM BM environment as well as in normal BM. No significant differences were found between MM and normal BM samples ($P = .26$ for BAFF expression and $P = .88$ for APRIL). Concerning MM BM environment samples, median expression values were 66 for BAFF and 46 for APRIL, about half of the expression in dendritic cells known to highly produce BAFF or APRIL.³⁰ Primary MMCs weakly expressed BAFF or APRIL; the median expressions were 102-fold and 45-fold lower, respectively, than those in the tumor environment ($P = .005$). The majority of HMCLs and normal plasmablasts also expressed BAFF or APRIL but at a rather low level as compared with the BM environment. The 2 cell lines (XG-5 and XG-20) with the highest APRIL expression have been previously identified with conventional RT-PCR.²¹ BAFF and APRIL expressions in plasmablasts were 29- and 31-fold lower, respectively, than those in memory B cells from which they arose ($P = .002$ and $P = .007$).⁹ Thus, BAFF and APRIL genes are mainly expressed by the BM environment and only occasionally by MMCs.

In order to identify the cell type expressing BAFF or APRIL in the MM environment, BM CD3, CD14, and CD15 cells from 3 patients were purified. BAFF was mainly expressed by BM CD14 and CD15 cells and APRIL by CD14 cells. CD3 cells expressed BAFF and APRIL only at a low level (Figure 1B). To assess a broad

range of populations present in the bone microenvironment, BMSCs and osteoclasts were also studied. BMSCs from 7 patients with MM weakly expressed BAFF and APRIL (Figure 1C). Of major interest, osteoclasts largely expressed the APRIL gene. Median APRIL expression was 80-fold higher than that in BM environment ($P = .001$). Osteoclasts also express the BAFF gene but in the same extent as the BM environment.

BAFF and APRIL production by BM cells

The production of BAFF and APRIL was investigated in 3-day culture supernatants of BM cells of 10 newly diagnosed patients, 7 osteoclasts, and 7 BMSCs. In agreement with real-time RT-PCR data, a low amount of BAFF and no APRIL could be found in BMSC supernatants. Median concentrations of 12.5 ng/mL BAFF and 24.6 ng/mL APRIL were measured in supernatants of BM cells. Again, in agreement with RT-PCR data, osteoclasts produced large amounts of APRIL (a concentration of 206 ng/mL), ie, 8-fold higher than BM cells ($P = .001$). The median production of BAFF by osteoclasts was 3-fold higher than that of BM cells ($P = .001$).

TACI gene expression yields a functional BAFF-binding receptor in HMCLs and primary MMCs

Gene expression profiling (GEP) was performed on 17 HMCLs using Affymetrix U133 A+B microarrays. On these, no probeset for BAFF-R was available. The Affymetrix data for TACI expression perfectly matched the real-time RT-PCR and previous RT-PCR data,²¹ and were consistent with the HMCL ability to bind BAFF-murine CD8. Indeed, TACI had an Affymetrix “absent” call (as decided by the GECOS software) in the HMCLs that showed a lack of TACI mRNA expression by real-time or conventional RT-PCR and a lack of binding of BAFF-murine CD8. TACI had an Affymetrix “present” call in the TACI RT-PCR⁺ HMCLs, which also bound BAFF-murine CD8 (Table 1). The BCMA gene had a “present” call in 17 of 17 HMCLs, as had been assumed after

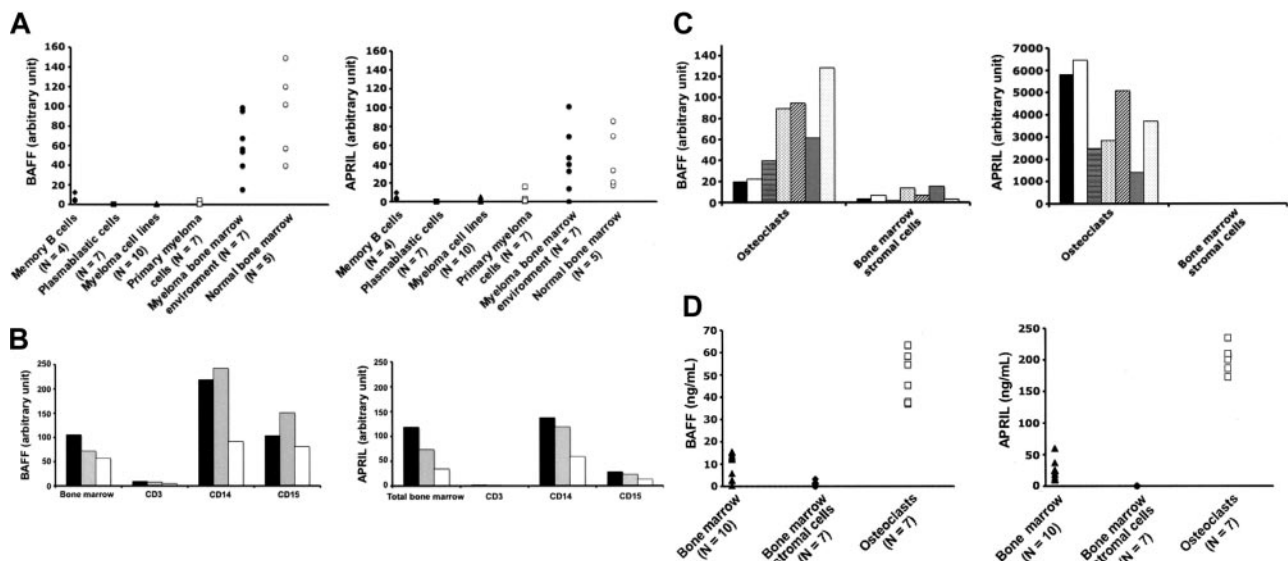


Figure 1. Expression of BAFF and APRIL in the BM environment cells and MMCs. (A) BAFF and APRIL expressions were determined by quantitative PCR in 4 samples of memory B cells, 7 samples of normal plasmablasts, 10 samples of HMCLs, 7 samples of primary MMCs, 7 samples of BM environment cells depleted from MMCs, and 5 samples of BM cells of healthy individuals. (B) BAFF and APRIL expressions were determined by quantitative PCR in purified BM CD3⁺, CD14⁺, or CD15⁺ cells. These cells were obtained from 3 newly diagnosed patients with MM (represented by differently shaded bars). (C) BAFF and APRIL expressions were determined by quantitative PCR in 7 samples of BM stromal cell lines and 7 samples of in vitro-generated osteoclasts. Primary cells were obtained from newly diagnosed patients with MM (represented by differently shaded bars). (D) BAFF and APRIL concentrations in culture supernatants of BM cells of 10 patients with MM, 7 different BM stromal cell line samples from patients with MM, and 7 different samples of osteoclasts were determined by ELISA.

Table 1. *TACI* gene expression yields a BAFF/APRIL receptor with functional binding capacity in HMCLs

HMCLs	PCR			Binding of BAFF-murine CD8	Real-time RT-PCR <i>TACI</i>	Affymetrix <i>TACI</i>	Affymetrix <i>BCMA</i>
	<i>BCMA</i>	<i>BAFF-R</i>	<i>TACI</i>				
XG-12	+	-	+	+	+	P	P
XG-13	+	+	+	+	+	P	P
XG-19	+	+	+	+	+	P	P
XG-20	+	+	+	+	+	P	P
RPM18226	+	+	+	+	+	P	P
LP1	+	+	+	+	+	P	P
XG-1	+	+	-	-	-	A	P
XG-3	+	-	-	-	-	A	P
XG-4	+	+	-	-	-	A	P
XG-5	+	-	-	-	-	A	P
XG-6	+	-	-	-	-	A	P
XG-7	+	+	-	-	-	A	P
XG-10	+	+	-	-	-	A	P
XG-11	+	-	-	-	-	A	P
XG-16	+	-	-	-	-	A	P
OPM2	+	+	-	-	-	A	P
SKMM	+	-	-	-	-	A	P

Expression of *BCMA*, *BAFF-R*, and *TACI* mRNA was determined by RT-PCR in 17 HMCLs. *TACI* and *BCMA* expressions were also determined with Affymetrix microarrays. The probeset for *BAFF-R* was not available on the Affymetrix HG U133 A + B DNA microarrays. The expression of BAFF binding was determined by flow cytometry using biotinylated human BAFF-murine CD8 fusion protein and phycoerythrin-conjugated streptavidin.

+ indicates the presence of the receptor; -, the receptor's absence; P, present cell; and A, absent cell.

*These data are from our previously published data.²¹

BCMA was found in RT-PCR experiments (Table 1). These data suggest that although *BCMA* is expressed, the *BCMA* protein is not functional, possibly due to retention in the Golgi apparatus as shown for plasma cells.³¹ This observation holds true for purified primary MMCs. MMCs (no. 1-5) that highly expressed *BCMA* but weakly expressed *TACI* according to RT-PCR results, failed to bind BAFF-murine CD8 (Figure 2). In contrast, primary MMCs (no. 6-10) that highly expressed *TACI* bound human BAFF-murine CD8 (Figure 2). Thus, *TACI* gene expression yields a BAFF-binding receptor in HMCLs or primary MMCs.

Clinical and genetic data of the *TACI*^{lo} and *TACI*^{hi} patients

Gene expression profiles of the 65 primary myeloma cell samples were determined with Affymetrix microarrays. *TACI* had a "present" Affymetrix call in all primary MMCs investigated, displaying a lognormal distribution. We did not find the clear-cut difference (absent or present Affymetrix call) for primary MMCs that was observed for HMCLs. One possible explanation is the presence of

MMC subclones with different levels of *TACI* expression in the same patient. We looked for differences in the major clinical, biologic, or genetic markers in patients with a low or high *TACI* expression in MMCs (*TACI*^{lo} or *TACI*^{hi} patients). To find the best way to delimit *TACI*^{lo} and *TACI*^{hi} patients, we considered subgroups defined by 50%, 40%, 25%, or 15% of the patients with the highest or lowest *TACI* expression in MMCs. The clinical and biologic differences observed showed the clearest distinction when the 15% group was considered. Here, the *TACI*^{hi} group included a higher frequency of patients within stage I MM, lambda light-chain isotype, t(4;14) translocations, and hemoglobin levels more than 10 g/dL, and a lower frequency of patients with bone lesions (Table 2). The percentage of patients with MMCs harboring a t(4;14) translocation was 14%, in agreement with published data.^{3,32} Most of these differences were kept when considering *TACI* groups representing 25% and 40% of the patients (distribution of bone lesions, t(4;14) translocations, lambda light-chain isotype). However, they were lost when considering *TACI* subgroups delimited

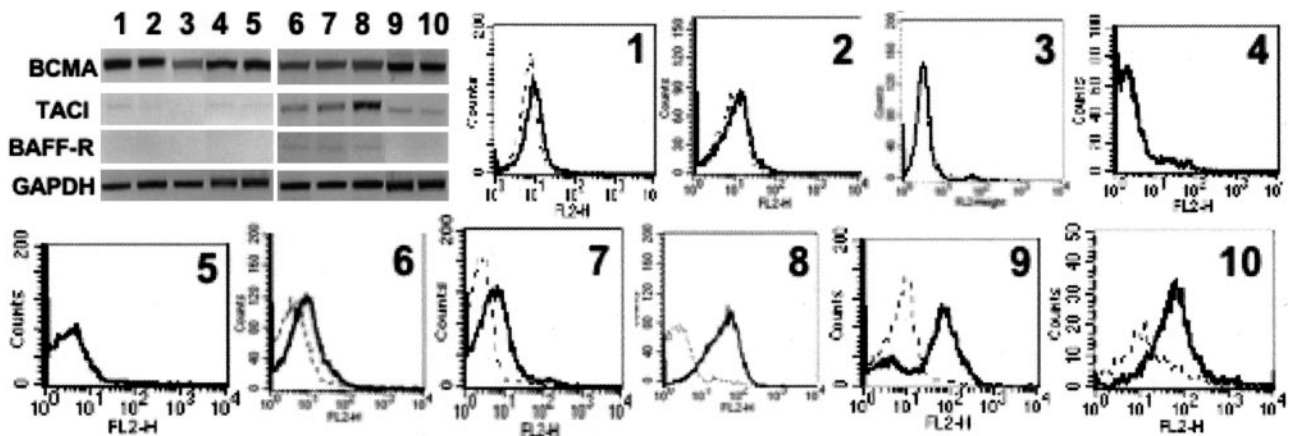


Figure 2. Expression of the receptors for BAFF and APRIL in MMCs. (Top) Expression of *BCMA*, *BAFF-R*, *TACI*, and *GAPDH* mRNA were determined by RT-PCR in CD138⁺ purified primary MMCs from 10 patients with intramedullary MM. (Bottom) The ability of MMCs to bind BAFF was determined by flow cytometry using a biotinylated human BAFF-murine CD8 fusion protein and phycoerythrin-conjugated streptavidin. Dotted lines indicate Ig control; solid lines, BAFF-mu CD8.

Table 2. Clinical data of the TACI^{hi} and TACI^{lo} patients

Categories	Definition of TACI ^{lo} and TACI ^{hi} subgroups, % of all patients							
	50/50		40/40		25/25		15/15	
	TACI ^{hi}	TACI ^{lo}	TACI ^{hi}	TACI ^{lo}	TACI ^{hi}	TACI ^{lo}	TACI ^{hi}	TACI ^{lo}
Age of at least 65 y	30	24	28	28	20	40	20	40
Kappa light chain	65	70	64	80	60	66	50	70
Lambda light chain	35	26	36	16	40	16	50	20
Nonsecreting	0	4	0	4	0	18	0	10
IgA subtype	25	31	24	24	33	27	20	30
β2-microglobulin at least 4 mg/L	44	37	40	40	40	60	30	60
C-reactive protein at least 4 mg/L	44	53	40	56	47	67	50	70
Lactate dehydrogenase at least 190 IU/L	28	16	28	20	27	20	40	30
Albumin less than 35 g/L	37	37	40	36	47	33	60	40
Hemoglobin less than 10 g/dL	28	35	36	44	20	47	20	60
Bone lesions								
0 lesions	42	20	45	10	43	8	44	0
At least 1 lesion	58	80	55	90	57	92	56	100
Chromosomal abnormalities								
Chromosome 13 deletion	22	31	24	32	27	33	30	40
t(4;14) translocation	22	6	20	8	27	6	30	0
t(11;14) translocation	9	9	12	12	20	6	20	10
Staging								
I	9	3	8	3	6	0	4	0
II	5	5	5	4	2	3	1	2
III	18	24	12	18	7	12	5	8
<i>P</i> *	.1		.02		.02		.08	

We separated 65 newly diagnosed patients with MM into 2 subgroups according to TACI gene expression in MMCs assayed with Affymetrix microarrays. The 2 subgroups represent either 50% (n = 32), 40% (n = 25), 25% (n = 15), or 15% (n = 10) of the patients with highest (TACI^{hi}) or lowest (TACI^{lo}) TACI expression. Data are the percentages of patients within each subgroup with the indicated clinical or biologic parameters. When the percentages were different with the chi-square test (*P* = .05), the data are shown in italics.

**P* value compares high and low subgroups in each category.

by the median. Whatever method was used to define TACI^{lo} and TACI^{hi} subgroups, no differences in age, β2 microglobulin, C-reactive protein (CRP), albumin, lactate dehydrogenase (LDH) levels, IgG subtypes, or chromosome 13 deletion were found (Table 2).

Gene expression signature of microenvironment dependence in TACI^{hi} MMCs and plasmablastic signature in TACI^{lo} MMCs with Affymetrix microarrays

In order to investigate if different gene signatures could be identified comparing 2 groups with different TACI expression, we performed a supervised clustering. We considered the 3 groups with significant clinical differences (40%, 25%, and 15% of the patients). Of 49 000 probesets present on the U133 A+B DNA microarrays, we found 1612, 2709, and 1805 probesets, respectively, to be differentially expressed between the 2 groups (*P* = .01 with Mann-Whitney test and ratio of the mean expressions of 1.5 or 0.67). To get an impression about the number of genes differentially expressed by chance between the groups, we performed 100 permutations with 2 subgroups comprising 40%, 25%, or 15% of the patients with MM randomly sorted out of the 65 samples. Only 5% of the genes appeared to be differentially expressed, if the groups were selected by chance. A common set of 548 distinct genes and 111 distinct expressed sequence tags (ESTs) were differentially expressed between TACI^{hi} and TACI^{lo} MMCs independently of the definition of TACI^{hi} and TACI^{lo} groups. This list of 659 genes/ESTs is available on the *Blood* website as Supplemental Tables S1 and S2 (see the Supplemental Tables link at the top of the online article).

We performed an unsupervised hierarchical clustering using these 659 genes/ESTs (Figure 3A). TACI^{hi} and TACI^{lo} MMCs appear in different branches of the cluster analysis. The TACI₄₀^{lo},

TACI₂₅^{lo}, and TACI₁₅^{lo} groups were represented by graded blue color (more intense in TACI₁₅^{lo}) and were classified in one branch with limited misclassifications in each category. For example, the 40% groups were significantly classified (*P* = .01), with 5 misclassifications. The TACI₄₀^{hi}, TACI₂₅^{hi}, and TACI₁₅^{hi} groups were represented with graded red color (more intense in TACI₁₅^{hi}) and classified in another branch. Patients outside the subgroups (white color) were interdispersed in the 2 clusters. The ability of this 659 gene/EST list to classify TACI^{hi} and TACI^{lo} MMCs was validated with another series of GEP from 40 purified MMCs, unrelated to the 65 previous ones, that was obtained with Affymetrix U133 2.0 plus microarrays (Figure 3B). Although this new series of primary MMCs was smaller and a different microarray was used, the U133 2.0 plus probesets corresponding to the 659 U133 A+B genes/ESTs were able to efficiently classify TACI^{hi} and TACI^{lo} MMCs. In particular, the 40% groups were significantly classified (*P* = .01), with 6 misclassifications. This differential gene expression in TACI^{hi} and TACI^{lo} MMCs observed with Affymetrix microarrays was validated for TACI and 3 other genes (*CDC2*, *TYMS*, and *c-met*) with real-time RT-PCR.^{33,34} Real-time RT-PCR and Affymetrix data showed a good correlation (*r* = .85, *P* = .01; Figure 4).

Of the 659 genes and ESTs mentioned above, 339 (52% of the 479 genes/ESTs overexpressed in TACI^{hi} patients and 50% of the 180 genes/ESTs overexpressed in TACI^{lo} patients) could be assigned to 8 functional categories listed in Table 3 using gene ontology terms. TACI^{hi} MMCs expressed a higher percentage of genes coding for intercellular communication signals, cytoskeleton-associated proteins, and signal transduction (Table 3). Conversely, TACI^{lo} MMCs overexpressed genes coding for proteins involved in cell cycle and nuclear functions (Table 3).

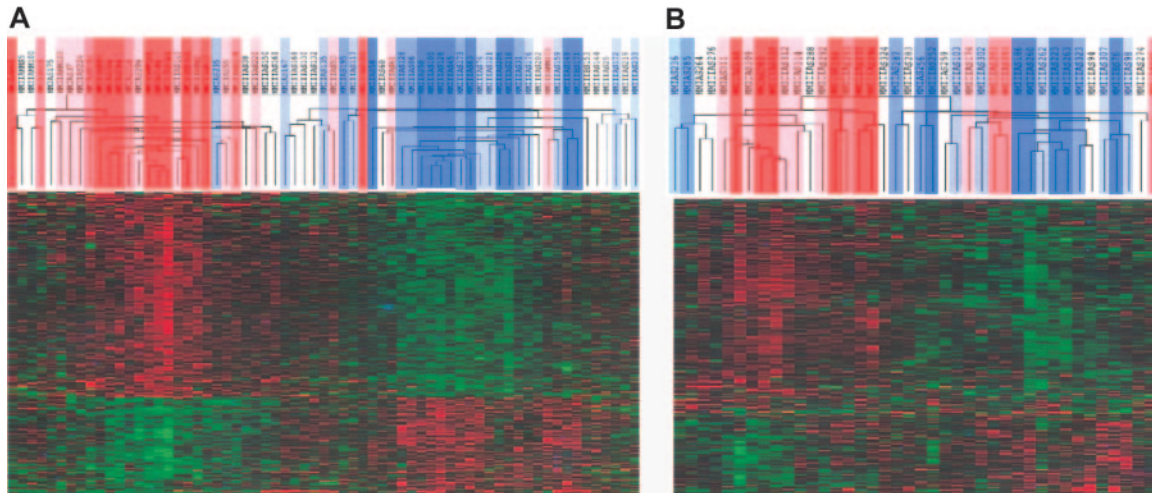


Figure 3. Unsupervised hierarchical clustering of TACI^{hi} and TACI^{lo} MMCs. The 659 genes that statistically ($P = .01$) distinguished (ratio 1.5 or 0.67) TACI^{hi} patients and TACI^{lo} patients are presented graphically using hierarchical clustering. The color of each cell in the tabular image represents the expression level of each gene (red, expression higher than the mean; green, expression lower than the mean; increasing color intensity represents a higher magnitude of deviation from the mean). The TACI₄₀^{lo}, TACI₂₅^{lo}, and TACI₁₅^{lo} subgroups were represented by graded blue color (more intense in TACI₁₅^{lo}) and the TACI₄₀^{hi}, TACI₂₅^{hi}, and TACI₁₅^{hi} subgroups were represented with graded red color (more intense in TACI₁₅^{hi}). (A) Initial set of 65 patients. (B) Validation set of 40 patients.

TACI^{hi} MMCs have a BMPC signature and TACI^{lo} MMCs have a plasmablastic signature

We investigated the association of plasmablasts and normal BMPCs with MMCs in the TACI^{hi} and TACI^{lo} groups. Using an unsupervised clustering with the above-mentioned 659 genes, the samples of patients in the TACI^{lo} group clustered together with plasmablasts (correlation coefficient, 0.33; $P = .001$) whereas TACI^{hi} samples clustered with BMPCs (correlation coefficient, 0.35; $P = .001$; Figure 5). When comparing plasmablasts with normal BMPCs, of the 659 genes/ESTs differentially expressed between the TACI^{hi} and TACI^{lo} groups, 195 also appeared as differentially expressed between the plasmablasts and BMPCs. Of these, 115 were overexpressed in BMPCs compared with plasmablasts and 80 were overexpressed in plasmablasts compared with BMPCs (ratio of the mean expressions 1.5 and 0.67; $P = .01$). Of 115 BMPC genes, 111 were also upregulated in TACI^{hi} compared with TACI^{lo} MMCs. Conversely, 77 of the 80 PPC genes were overexpressed in TACI^{lo} MMCs ($P = .01$). BMPC and plasmablast genes are indicated in Tables S3 and S4.

Taken together, these results suggest that grouping of patients in accordance with “high” and “low” *TACI* expression delineates a group of patients whose MMCs share gene expression characteristics with plasmablasts (TACI^{lo}) and a group in which the gene expression profiling resembles normal mature BM plasma cells (TACI^{hi}). TACI^{hi} MMCs overexpress many genes coding for potentially interesting intercellular communication or transduction signals whose biology is discussed below. In contrast, TACI^{lo} MMCs mainly overexpress cell cycle genes.

Modulation of gene expression profile by addition or deprivation of BAFF and APRIL in MMCs

In order to determine whether the TACI^{hi} and TACI^{lo} gene signature could be induced by BAFF/APRIL, we first used the XG-13 HMCL, which is stimulated by BAFF and APRIL.²¹ After growth factor starvation, addition of BAFF and APRIL for 10 hours

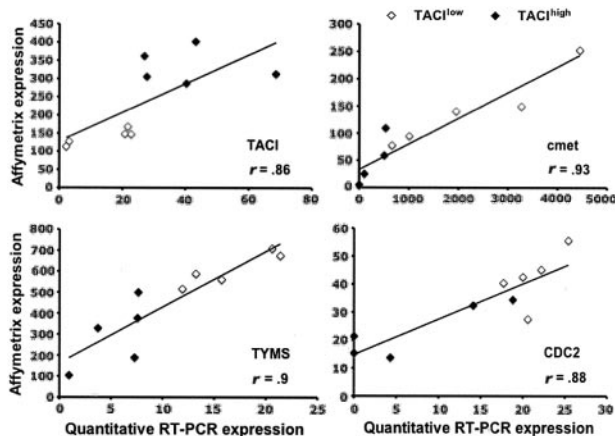


Figure 4. Validation of Affymetrix data. Gene expressions of *TACI*, *MET*, *TYMS*, and *CDC2* in TACI^{lo} and TACI^{hi} patients were assayed with real-time RT-PCR and normalized with GAPDH. The XG-13 HMCL was used as the standard. The coefficient of correlations between Affymetrix and real-time RT-PCR values were determined.

Table 3. Intercellular communication signature in TACI^{hi} MMCs and plasmablastic signature in TACI^{lo} MMCs

Genes coding for protein implicated in different functions	No. genes*	TACI ^{lo} , %	TACI ^{hi} , %	P†
Intercellular communication signals	69	2.1	26.3	< .001
Cytoskeleton	20	2.1	7.2	.04
Transduction signals	52	11.2	17	.05
Protein synthesis and regulation	19	7.7	4.8	NS
Cell cycle	11	10	0.8	< .001
Metabolism	85	22.1	26.6	NS
Apoptosis	10	4.2	2.8	NS
Nuclear functions	73	40.6	15.2	< .001
Total of classified genes	339	100‡	100§	NS

Of the 659 genes differentially expressed between the TACI^{hi} and TACI^{lo} groups, 339 could be assigned to 8 functional categories using gene ontology terms. Data are the percentages of genes of a given category compared with the total number of TACI^{lo} (91 genes) or TACI^{hi} (248 genes) genes.

NS indicates not significant.

*n indicates the number of genes out of the 339 in one category.

†The statistical comparisons of the percentages of genes belonging to one gene ontology category between the TACI^{lo} and TACI^{hi} group genes were performed with the chi-square test.

‡n = 91.

§n = 248.

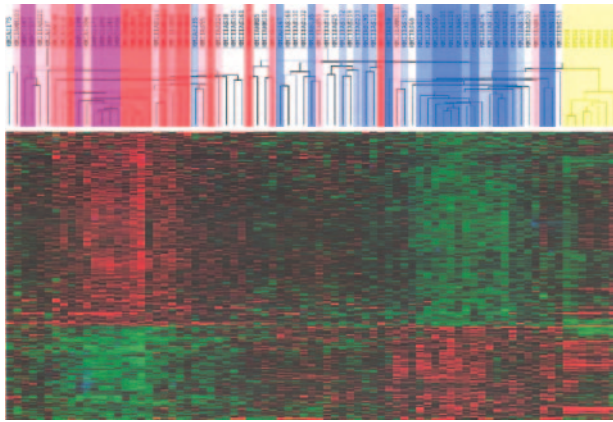


Figure 5. Hierarchical clustering of MMCs, BMPCs, and PPCs identifies a signature of BM stroma interaction for TACI^{hi} patients and a plasmablastic signature for TACI^{lo} patients. Unsupervised hierarchical clustering analysis of the expression profile of MMCs of 65 patients at diagnosis, 7 PPC samples, and 7 BMPC samples show that TACI^{lo} MMCs cluster (blue) together with PPCs (yellow) whereas TACI^{hi} MMCs cluster (red) with normal BMPCs (purple). The clustering was performed on the 659 genes differentially expressed between TACI^{hi} and TACI^{lo} MMCs. The TACI₄₀^{lo}, TACI₂₅^{lo}, and TACI₁₅^{lo} subgroups were represented by graded blue color (more intense in TACI₁₅^{lo}) and the TACI₄₀^{hi}, TACI₂₅^{hi}, and TACI₁₅^{hi} subgroups with graded red color (more intense in TACI₁₅^{hi}).

induced an increase of 196 genes/ESTs and a decrease of 348 genes/ESTs ($P = .01$ and ratio 1.5 or 0.67). However, only 19 genes were common with the TACI-related gene signature (supplemental data in Table S5). We then decided to investigate the effect of BAFF/APRIL deprivation using a whole BM culture system in which BAFF and APRIL were blocked by TACI-Fc inhibitor for 10 hours. Subsequently, MMCs were purified for gene expression profiling with Affymetrix U133 2.0 plus microarrays. There were 188 genes and ESTs that were downregulated in BAFF/APRIL-deprived MMCs (treated with TACI-Fc) and 259 that were upregulated ($P = .01$ and ratio 1.5 or 0.67). However, only some of these genes were common with the TACI^{hi} or TACI^{lo} gene signature. This list is available as supplemental data in Table S6.

Discussion

Two recent studies have shown that BAFF and APRIL are growth factors for human MMCs and that serum levels of BAFF and APRIL are increased in these patients.^{21,22} Using real-time PCR, we show that *BAFF* and *APRIL* genes are poorly expressed by MMCs and are expressed mainly by the BM environment of patients with MM. BM cells from healthy individuals exhibit a similar expression. The MM bone environment comprises mainly of PMNs (45%), monocytes (5%), and T cells (10%). We show here that *BAFF* was largely expressed by PMNs and monocytes, whereas *APRIL* was expressed by monocytes only. A majority of the studies about MM use BM mononuclear cells after removal of PMNs with centrifugation on a Ficoll hypaque cushion. Thus, the contribution of PMNs is in many cases ignored, whereas they are the main BM cell components in vivo and are here shown to be the main source of BAFF. Another cell component in the BM environment important for myeloma cell biology is bone cells. Primary MMCs can survive in contact with BMSCs³⁵ or osteoclasts.³⁶ In addition, fetal bone is critical to grow MMCs in severe combined immunodeficient (SCID) mice.^{37,38} As these bone cells cannot be harvested by BM aspiration, they are usually produced in vitro. We show here that cultured osteoclasts display a huge *APRIL* expression, about

80-fold higher than those found in the MM BM environment or in dendritic cells. The BAFF expression was in the same order of magnitude in both cases. Using ELISA, we confirmed that osteoclasts produced very large amounts of APRIL. Thus, APRIL could likely play an important role in the MMC growth stimulation by osteoclasts.³⁶ BM stromal cells produced only a low amount of BAFF and no APRIL.

Purified MMCs also express *BAFF* or *APRIL* but in the median about 100-fold weaker than the BM environment. This BAFF or APRIL expression pattern and distribution resembles that of IL-6. A current consensus is that IL-6 is a paracrine growth factor in MM and that some MMCs may produce autocrine IL-6 and use it to escape from their dependency on the tumor environment.^{39,40} In particular, several HMCLs use IL-6 as an autocrine growth factor.^{41,42} Similarly, we have previously found that 2 HMCLs (L363 and RPMI 8226) use BAFF or APRIL as autocrine growth factors, whereas we found here that BAFF and APRIL are mainly paracrine growth factors if assessing primary MM cells. The autocrine production may have contributed to the escape of these MMCs from the tumor environment.

Given that BAFF and APRIL are paracrine growth factors, our aim was to look for a difference in the GEP of patients' MMCs expressing a high or a low level of BAFF and APRIL receptors. An important point is that *TACI* expression (assayed with Affymetrix microarrays or RT-PCR) is a good indicator for the presence of a receptor, able to bind BAFF. There are 3 receptors for BAFF and APRIL: BCMA, BAFF-R, and TACI. Probesets for *BCMA* and *TACI* were available on the Affymetrix U133 A+B chips, unlike BAFF-R. We could not identify an EST with *BAFF-R* sequence homology in any Affymetrix probeset. BCMA was probably not functional in MMCs and *TACI* expression was a good indicator of a functional BAFF receptor. Indeed, only 6 of the 17 HMCLs with a "present" call for *TACI* expression with Affymetrix microarrays were able to bind a BAFF-murine fusion protein, independent of *BCMA* expression. BCMA was already shown to be not functional in several cell types, in particular in human plasma cells and the U266 myeloma cell line due to retention in Golgi apparatus.³¹ However, it is not likely the case for murine plasma cells since *BCMA*^{-/-} mice have normal B-cell function but fail to develop mature plasma cells.⁴³

When grouping the patients in accordance with the *TACI* expression levels as described above, the MMCs in the TACI^{hi} group show a signature resembling mature BMPCs with an emphasis on the expression of genes with products functioning in intercellular communication and signal transduction. In contrast, the TACI^{lo} MMCs showed a signature sharing similarities with plasmablasts, especially with regard to the expression of cell cycle genes. The consistency of this signature was validated on an independent group of 40 MMCs using Affymetrix U133 2.0 plus microarrays. We looked for the possibility that this gene signature could be due in part to signal transduction induced by TACI activation. We used first the XG-13 HMCL, whose growth is stimulated by BAFF or APRIL.²¹ We also used primary MMCs from a newly diagnosed patient. In this case, as primary MMCs rapidly apoptosed as soon as they are purified,²⁶ we looked for the effect of BAFF/APRIL deprivation on the gene expression profile of MMCs cultured together with their BM environment. In the 2 cases investigated, a large number of genes were up- or down-regulated but only a few genes belonged to the TACI signature.

This indicated that the gene signature is not linked to TACI stimulation but to the degree of dependency of myeloma cell growth on the interaction with the BM environment. As BAFF or APRIL are mainly produced by the BM environment and are potent

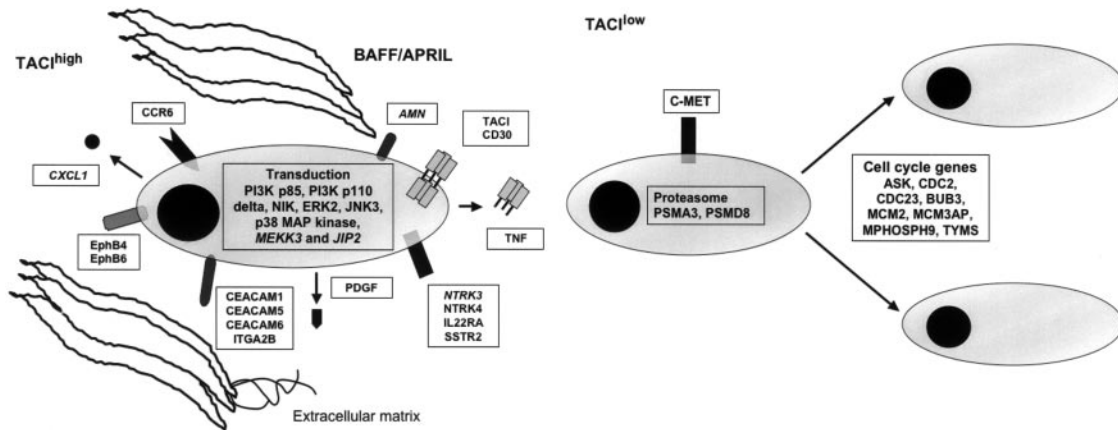


Figure 6. BM environment dependence signature of TACI^{hi} patients and plasmablastic signature of TACI^{lo} patients. (A) TACI^{hi} MMCs had a BM environment dependence signature with overexpression of intercellular communication and transduction genes. (B) TACI^{lo} MMCs have a plasmablastic signature with an overexpression of genes involved in the cell cycle. ITGA2B indicates integrin, alpha 2b; PDGF, platelet-derived growth factor beta polypeptide; IL22RA, interleukin 22 receptor alpha 1; AMN, amnionless homolog; PI3K, phosphoinositide-3-kinase; C-MET, met proto-oncogene (hepatocyte growth factor receptor); PSMA3, proteasome subunit, alpha type 3; PSMD8, proteasome subunit, non-ATPase, 8; ASK, activator of S phase kinase; CDC2, cell division cycle 2; CDC23, cell division cycle 23; BUB3, budding uninhibited by benzimidazoles 3; MCM2, minichromosome maintenance deficient 2; MCM3AP, minichromosome maintenance deficient 3 associated protein; and MPHOSPH9, M phase phosphoprotein 9.

growth factors for normal and malignant plasma cells, it seems logical that MMCs that depend more on BAFF/APRIL for their growth overexpress TACI and molecules involved in the interaction with their environment.

Of interest, clinical and genetic differences were found between patients with TACI^{lo} or TACI^{hi} MMCs. TACI^{lo} patients had a plasmablast signature, an increase in the percentage of stage III MM, a decrease in hemoglobin level, and an increase in the percentage of bone lesions. It is of note that clinical parameters and risk factors (β 2 microglobulin, CRP, LDH) were not different between TACI groups, suggesting that TACI might be an independent prognostic factor that has to be evaluated in further studies. No significant difference in event-free and overall survivals were found between TACI^{lo} and TACI^{hi} patients. This is understandable because the median follow-up of this series of patients was short at the time of the study (18 months). Recently, *DKK* gene upregulation was associated with increased bone lesions.¹⁰ Tian et al pointed out that *DKK1* was not expressed in plasmablastic MMCs,¹⁰ one of the major characteristic features of TACI^{lo} MMCs. This may explain why we found no upregulation of *DKK1* in TACI^{lo} MMCs although increased bone lesions are found in TACI^{lo} patients. Regarding genetic alterations, we found a higher percentage of patients with a t(4;14) translocation within the TACI^{hi} group and no increase of chromosome 13 deletion in the TACI^{hi} group although t(4;14) was strongly associated with deletion 13.³ Actually, about half of the MMCs have deletion 13 and only 14% have a t(4;14) translocation, which may explain this discrepancy.

It was not in the scope of our work to comment on all the genes differentially expressed between TACI^{lo} and TACI^{hi} MMCs. Therefore, in Figure 6, we have selected for genes that appeared to us the most promising for myeloma biology understanding. First, we found an increased proportion of MMCs secreting lambda light chain in TACI^{hi} MMCs. Given that immunoglobulin light chain rearrangement occurs in pre-B cells, we have no explanation for this observation. Focusing on growth factor-related molecules, we found that MMCs in the TACI^{hi} group overexpress 2 neurotrophic tyrosine kinase receptors, NTRK3 which binds neurotrophin 3, and NTRK4. They also overexpress platelet-derived growth factor, CCR6, TNF, interleukin-22 receptor, 2 ephrins (B4 and B6), and the somatostatin receptor 2 (SSTR2). The IL-22 receptor complex consists of IL-10R β and IL-22R.⁴⁴ The IL-10R β serves as a

common receptor chain for both IL-10 and IL-22. As we previously demonstrated IL-10 to be a growth factor for human MMCs,⁴⁵ the role of IL-22 in MM biology needs to be investigated. The somatostatin receptor family comprises several members and SSTR2 is expressed in different cancers.^{46,47} Of interest, the somatostatin analog octeotide binds SSRT2 and has been shown to induce apoptosis in MMCs.⁴⁸ MMCs in the TACI^{hi} group overexpress the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), CEACAM5, and CEACAM6 adhesion molecules. CEACAM1 is necessary for VEGF activity.⁴⁹ Given the likely role of VEGF in multiple myeloma, a role of CEACAM1 in MM demands further investigation. Furthermore, we found an overexpression of 2 ephrin receptors: EphB4 and EphB6. Ephrin receptors are overexpressed in various tumor types, suggesting that they play a role in cancer progression.⁵⁰ Recently, a study demonstrated that EphB4 acts proangiogenic in breast cancer.⁵¹ It proposes that the interplay between the EphB4 ectodomain on tumor cells and the ephrin-B2 in the vasculature promotes blood vessel formation and remodeling. TACI^{hi} MMCs overexpress the chemokine CXCL1. This is of interest because CXCL1 was recently found to bind soluble syndecan-1,⁵² a proteoglycan that is a hallmark of plasma cell differentiation. Actually, MMP7-deficient mice have a major defect in neutrophil attraction due to a defect in cleavage of syndecan-1 by MMP7, of binding of CXCL-1 to syndecan-1, and thus of a chemoattracting role of CXCL-1.⁵² MMCs produce soluble syndecan-1, the serum level of which is increased in patients with MM.⁵³ MMP7 is expressed in all myeloma cell samples investigated by Affymetrix microarrays. CXCL1 produced by MMCs in the TACI^{hi} group could play an important role in the biology of MMCs by recruiting cells of the microenvironment, in particular PMNs that largely express *BAFF*.

Regarding adhesion molecules, MMCs in the TACI^{hi} group overexpress several of these, in particular integrin alpha 2B, also overexpressed in BMPCs compared with plasmablasts.⁵⁴

Regarding the transduction elements that may be associated with TACI activation, we found an overexpression of mitogen-activated kinase kinase 3 (*MKK3*), mitogen-activated protein kinase (ERK2), mitogen-activated protein kinase 10 (JNK3), mitogen-activated protein kinase 14 (P38 MAP kinase), and mitogen-activated protein kinase 8-interacting protein 2 (*JIP2*) in MMCs belonging to the TACI^{hi} group. The substrate of MKK3 is

P38 MAP kinase, which is known to be induced by BAFF/APRIL in various cells.⁵⁵ JIP2 forms complexes with MAP kinases, in particular with MKK3 and P38.^{56,57} The overexpression of ERK2 in MMCs of the TACI^{hi} group is in good agreement with our previous results demonstrating that the MAPK pathway is activated, in MMCs, by BAFF and APRIL. P38 kinase is also activated in MMCs and an inhibitor of P38 induces apoptosis in MMCs.⁵⁸ BAFF/APRIL trigger PI-3 kinase in MMCs,²¹ and in good agreement, the *P85 subunit* of PI-3K is overexpressed in TACI^{hi} MMCs. *PIK3CD (P110 delta polypeptide)* is also overexpressed in TACI^{hi} MMCs. P110 delta is expressed at high levels in lymphocytes of lymphoid tissues and plays a role in phosphatidylinositol 3-kinase-mediated signaling in the immune system.⁵⁹ Promotion of survival of BAFF and APRIL in B cells and MMCs are consistent with NF-kappaB activation. In agreement with these data, the NF-kappaB-inducing kinase (NIK) is overexpressed in TACI^{hi} patients. In T1 B cells, BAFF stimulation promotes B-cell survival, causing the processing of the p100 form of NFkB2 to p52 which required BAFF-R and NIK.⁶⁰ Previously, we demonstrated that BAFF and APRIL induce the upregulation of the antiapoptotic protein Bcl-2.²¹ Confirming these results, we show here that TACI^{hi} patients overexpress *Bcl-2*.

MMCs from patients belonging to the TACI^{lo} group overexpress genes coding for proteins implicated in cell cycle and in

nuclear functions. These cells overexpress genes coding for protein involved in G1/S transition and mitosis and contributing to plasmablastic signature. Among them, we validated 2 genes: thymidilate synthetase (TYMS) and cell cycle controller CDC2.⁶¹ Their overexpression is associated with chemotherapy resistance in cancer^{62,63} and these 2 genes are also differentially expressed between patients with or without chromosomal abnormalities.^{33,64} MMCs in the TACI^{lo} group overexpress 2 genes coding for proteasome subunits and could also be an indicator of increased sensitivity to proteasome inhibitors.⁶⁵ MMCs from patients belonging to the TACI^{lo} group overexpress *c-met*, the receptor of HGF. HGF is a myeloma growth factor.⁶⁶⁻⁶⁸ These results suggest that *c-met* could be a therapeutic target in TACI^{lo} MMCs.

In conclusion, TACI is overexpressed in patients whose MMCs have a gene signature indicative of a dependence on the tumor environment. This fits well with the production of the TACI ligands, BAFF and APRIL, by the tumor environment. TACI^{lo} MMCs overexpress, in turn, cell cycle genes indicative of a plasmablastic signature. Taken together, our findings foster a clinical use of inhibitors interfering with the BAFF/APRIL-TACI interaction like the TACI-Fc fusion protein, and at the same time suggest using gene expression profiling to identify the group of patients that might benefit most from this kind of treatment.

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II-3 Article 7

« TACI expression is associated with a mature bone marrow plasma cell signature and C-MAF overexpression in human myeloma cell lines. Haematologica. En révision. »

Nous avons recherché les profils d'expression génique lié au récepteur TACI un large panel de lignées de MM.

- Contrairement aux cellules primaires de MM, l'expression du récepteur TACI est dichotomique chez les lignées de MM, avec des lignées exprimant TACI (TACI⁺) et des lignées n'exprimant pas TACI (TACI⁻). Nous avons comparé les profils d'expression génique des lignées TACI⁻ (7 lignées) à celui des lignées TACI⁺ (11 lignées).
- Nous avons ainsi identifié 80 gènes différentiellement exprimés entre les lignées TACI⁺ et TACI⁻. Lors de notre précédente étude (article 6), nous avons montré que les cellules primaires de myélomes TACI^{high} ont une signature génique de plasmocytes médullaires matures liée à une dépendance au microenvironnement médullaire alors que les plasmocytes tumoraux TACI^{low} ont une signature génique de plasmablastes proliférants peu dépendants de l'environnement médullaire. Ces signatures sont conservées chez les lignées de MM TACI⁺ et TACI⁻.
- Parmi les gènes différentiellement exprimés entre les lignées TACI⁺ et TACI⁻, c-maf, la cycline D2 et l'intégrine beta 7 sont surexprimés par les lignées TACI⁺.

- En partant de la corrélation existant entre l'expression de c-maf et de TACI chez les lignées de MM, nous avons démontré que (1) l'activation de TACI par BAFF ou APRIL induit l'expression de c-maf, de la cycline D2 et de l'intégrine beta 7 chez les lignées TACI⁺, (2) le blocage de la boucle autocrine BAFF/APRIL/TACI par la protéine de fusion TACI-Ig réduit l'expression de c-maf, de la cycline D2 et de l'intégrine beta 7, (3) un siRNA c-maf réduit l'expression de c-maf, de la cycline D2 et de l'intégrine beta 7 sans affecter l'expression de TACI.

En conclusion, l'activation de TACI par BAFF/APRIL, dans le MM, peut induire l'expression de c-maf qui va induire l'expression de la cycline D2 et de l'intégrine beta 7. Ces données permettent de progresser un peu plus dans la compréhension des mécanismes d'action de BAFF/APRIL dans le MM.

TACI expression is associated with a mature bone marrow plasma cell signature and C-MAF overexpression in human myeloma cell lines

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Key words: myeloma, TACI, gene expression profiles, c-maf.

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Abstract.

BAFF and APRIL stimulate the growth of multiple myeloma cells (MMC). BAFF and APRIL share two receptors – TACI and BCMA – and BAFF binds to a third receptor, BAFF-R. We previously reported that *TACI* gene expression is bimodal in 18 human MM cell lines (HMCL), being either present or absent, unlike BCMA that is expressed on all HMCL. BAFF-R is lacking. *TACI* expression is a good indicator of a BAFF-binding receptor in HMCL. In primary MMC, the level of TACI expression correlates with a characteristic phenotypic pattern: TACI^{high} MMC resemble bone marrow plasma cells and TACI^{low} resemble plasmablasts.

Using gene expression profiling, we show here that these patterns are kept in TACI⁺ or TACI⁻ HMCL. 80 genes/EST interrogated by Affymetrix microarrays were differentially expressed between TACI⁺ and TACI⁻ HMCL, particularly *c-maf*, *cyclin D2*, and *integrin beta7*.

Triggered by the finding that *TACI* and *c-maf* expressions correlate in TACI⁺ HMCL, we demonstrate in this study that TACI activation influences *c-maf* expression: (1) activation of TACI by BAFF or APRIL increases *c-maf*, *cyclin D2*, and *integrin beta7* gene expressions in TACI⁺ HMCL, (2) blocking of autocrine BAFF/APRIL stimulation in some TACI⁺ HMCL by the TACI-Fc fusion protein reduces *c-maf*, *cyclin D2*, and *integrin beta7* gene expression, (3) nucleofection of siRNA to *c-maf* decreases *c-maf* mRNA levels and reduces the expression of *cyclin D2* and *integrin beta7* gene expressions, without affecting *TACI* expression. Thus we conclude that TACI activation can upregulate *c-maf* expression, that in turn controls *cyclin D2*, and *integrin beta7* gene expression.

Introduction

Multiple myeloma (MM) is an incurable plasma cell (PC) neoplasm characterized by the displacement of physiological hematopoiesis, the presence of osteolytic bone lesions and impairment of renal function due to the accumulation of malignant PC in the bone marrow and the production of monoclonal protein.

Almost all MM cells (MMC) show an aberrant or overexpression of a D-type cyclin, *i.e.* cyclin D1 (CCND1) in the case of a t(11;14) translocation or gain of 11q13, cyclin D3 (CCND3) overexpression in case of the rare t(6;14) translocation, or an overexpression of cyclin D2 (CCND2) on the background of a translocation involving *c-maf* (t(14;16)) or *FGFR3* (t(4;14)) (1-3). During the course of the disease, further cytogenetic aberrations accumulate(4). Still, survival of MMC depend on the autocrine and paracrine stimulation by growth factors, like interleukin-6 (IL-6) (5), interferon alpha (6), insulin-like growth factor (7), hepatocyte growth factor (8, 9), members of the EGF family (10-12) and members of the TNF-family (13, 14) From the latter, we and others have recently shown that BAFF (B-cell activating factor, also called BLys) and APRIL (A Proliferation-Inducing Ligand) are potent MMC growth factors (15, 16). BAFF binds to 3 receptors - BAFF-R, BCMA and TACI -, and APRIL binds to BCMA and TACI (17).

The activation of nuclear factor (NF)- κ B by TACI, BCMA and BAFF-R(18) is consistent with the antiapoptotic role of BAFF since NF κ B enhances the expression of several cell survival genes (19, 20). Depending on the B-cell maturation stage, BAFF was reported to induce the anti-apoptotic proteins Bcl-2, A1, and Bcl-XL and to reduce the pro-apoptotic protein Bak (18, 21, 22). BAFF also activates JNK, Elk-1, p38 kinase, AP-1 and NF-AT in various models (23). We recently identified that BAFF and APRIL activate MAPK, PI3K/AKT and NF κ B pathways in MMC leading to an

upregulation of Mcl-1 and Bcl-2 antiapoptotic proteins (16). Recently Tai *et al.* shown that MMC express BCMA and TACI but very low level of BAFF-R (24). They demonstrated that BAFF induces activation of NF κ B and PI3K/AKT pathways confirming our previous results. Furthermore, they showed BAFF could activate the canonical and the non canonical NF κ B pathways in MMC.

Using gene expression profiling (GEP) with Affymetrix microarrays, we found that all primary MMC as well as HMCL express *BCMA* (25). *TACI* is also expressed on almost all MMC as well as normal bone marrow plasma cells (BMPC), plasmablasts and CD27-positive B-cells, but only on about one third (8/18) of HMCL. We have shown *TACI* expression to be necessary for BAFF binding on HMCL and that primary MMC with high expression of TACI (*TACI*^{high}) have a gene expression signature resembling bone marrow plasma cells (BMPC) dependant on the interaction with the bone marrow environment (25). In contrast, primary MMC with low TACI expression (*TACI*^{low}) have a signature resembling proliferating polyclonal plasmablasts (25). The TACI ligands are produced by the bone marrow microenvironment, and in particular, APRIL by osteoclasts (25). Some HMCL, e.g. RMPI8226, L363 and LP1, are rendered independent of this paracrine stimulation and have acquired the property of autocrine BAFF and/or APRIL production (16).

Here, we show that HMCL have kept the signature seen in primary MMC, namely a signature resembling BMPC in TACI-expressing HMCL (*TACI*⁺), and a signature resembling plasmablasts in *TACI*⁻ HMCL. We found 80 genes/EST to be differentially expressed between *TACI*⁺ and *TACI*⁻ HMCL, in particular *c-maf*, *cyclin D2* and *integrin beta7*. As *TACI* and *c-maf* expression are tightly correlated, we have provided evidence that *c-maf* is a target of the TACI/BAFF/APRIL signaling pathway.

Materials and methods

Cell samples

XG-1, XG-2, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-12, XG-13, XG-16, XG-19, and XG-20 human myeloma cell lines (HMCL) were obtained and characterized in our laboratory (26-29). SKMM, OPM2, LP1 and RPMI8226 were purchased from ATTC (Rockville, MD). Normal bone marrows were obtained from healthy donors after informed consent was given and BMPC were purified using anti-CD138 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) as described (25). Polyclonal plasmablasts were generated by differentiating peripheral blood CD19⁺ B cells *in vitro* (30).

Flow cytometry analysis

The expression of TACI on HMCLs was evaluated by incubating 5 x 10⁵ cells with an anti-TACI monoclonal antibody (MoAb) biotinylated in PBS containing 30% human AB serum at 4°C for 30 min followed by incubation with PE-conjugated streptavidin (Beckman-Coulter, Marseille, France). Flow cytometry analysis was done on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Modulation of the gene expression profile by addition or deprivation of BAFF/APRIL in MMC

The modulation of gene expression by addition of BAFF and APRIL was investigated with the XG-7, XG-13 and XG-20 HMCL. XG-7, XG-13 and XG-20 cells were starved of IL-6 for 3 hours and washed. Then BAFF (Peprotech, Rocky Hill, NJ) and APRIL (R&D Systems, Abington, UK) (200 ng/mL each) were added in one culture group for 12 hours in RPMI1640/10% FCS. The modulation of gene expression by deprivation of BAFF/APRIL in RPMI8226 and LP1 HMCL was also investigated. RPMI8226 and LP1 HMCL were starved for 3 hours and washed. Then TACI-Fc (R&D Systems) (10

µg/mL) was added in one culture group for 12 hours in RPMI1640/10% FCS. RNA were extracted for gene expression profiling or real-time PCR analysis.

Modulation of the gene expression profile after NF-kappaB pathway inhibition

To investigate the modulation of gene expression by NF-kappaB pathway inhibition, RPMI8226 and LP1 cells were cultured for 12 hours with an inhibitory peptide of NF-kappaB pathway (100 µg/ml, SN50) or the corresponding inactive peptide (BIOMOL, Plymouth meeting, PA), or TACI-Fc (R&D Systems, 10 µg/mL) in RPMI1640/10% FCS. RNA was extracted and gene expression assayed by real-time PCR.

Preparation of complementary RNA (cRNA) and microarray hybridization

RNA was extracted using the RNeasy Kit (Quiagen, Hilden, Germany). Biotinylated cRNA was amplified with a double *in vitro* transcription and hybridized to the Affymetrix HG U133 set microarrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Fluorescence intensities were quantified and analyzed using the GCOS 1.2 software (Affymetrix). Gene expression data were normalized with the MAS5 algorithm by scaling each array to a target value of 100 using the "global scaling" method.

Western blot analysis

Cells were lysed in 10 mM tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate (NaPPi), 1% Triton X-100, 5 µM ZnCl₂, 100 µM Na₃VO₄, 1 mM Dithiothreitol (DTT), 20 mM β-glycerophosphate, 20 mM p-nitrophenolphosphate (PNPP), 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM benzamidine, 5 µg/ml pepstatin and 50 nM okadaic acid.

Lysates were cleared by centrifugation at 10,000 g for 10 min and resolved by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany).

Membranes were blocked for 1 hour at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM tris-HCl (pH 7.4), 0.1% Tween 20 (TBS-T), 5% BSA, then incubated for 3 hours at room temperature with anti-c-maf MoAb (Abnova, Taiwan) at a 1:1000 dilution in 1% BSA TBS-T. The primary antibodies were visualized with goat anti-rabbit (Sigma) or goat anti-mouse (Bio-Rad, Hercules, CA) peroxidase-conjugated antibodies using an enhanced chemiluminescence detection system. As a control for protein loading, we used anti-Beta actin (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA) antibody.

siRNA transduction

The *c-maf* siRNA duplex construct ACGGCUCGAGCAGCGACAA (Dharmacon Inc, IL, USA) was transduced by electroporation (Amaxa, Köln, Germany) using nucleofaction. We also used Dharmacon's negative control siRNA (ON-TARGETplus siCONTROL Non-Targeting siRNA) as control. RPMI8226 and LP1 HMCL were electroporated using respectively the programs T-001 or A-023 and the T solution according to the manufacturer's instructions.

Real-time RT-PCR

Total RNA was converted to cDNA using the Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). The assays-on-demand primers and probes and the *TaqMan* Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). The measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System and analyzed using the ABI PRISM 7000 SDS Software. For each primer, serial dilutions of a standard cDNA were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve in order to assess the PCR efficiency. Ct values were obtained for GAPDH and the respective genes of

interest during log phase of the cycle. Gene of interest levels were normalized to GAPDH for each sample ($\delta Ct = Ct \text{ gene of interest} - Ct \text{ GAPDH}$) and compared with the values obtained for a known positive control using the following formula $100/2^{\delta\delta Ct}$ where $\delta\delta Ct = \delta Ct \text{ unknown} - \delta Ct \text{ positive control}$.

Statistical analysis

Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platform (RAGE, <http://rage.montp.inserm.fr/>) or the SAM (Significance Analysis of Microarrays) software (31). Statistical comparisons were done with Mann-Whitney, Chi-square, or Student t-tests. Probesets differentially expressed between TACI⁺ and TACI⁻ HMCL were picked by 2 methods. First, we selected 109 probesets that were differentially expressed between TACI⁺ and TACI⁻ HMCL with a Mann-Whitney test ($P \leq .01$) and with a ratio of the mean expression in TACI⁺ and TACI⁻ HMCL that was ≥ 2 or ≤ 0.5 . Secondly, we used the SAM software based on a Wilcoxon test, filtering the probesets with the 3-presence and 2-ratio filters. This SAM selection yielded 330 probesets with a false discovery rate of 25.5% using 100 permutations. Crossing the 2 gene lists yielded 86 probesets, corresponding to 80 genes/EST, that were differentially expressed between TACI⁺ and TACI⁻ HMCL.

Results

Gene expression profile associated with TACI expression in HMCL

As *TACI* expression yields a functional BAFF-binding receptor in our 18 HMCL (25), we compared the gene expression profiles of 7 TACI⁺ HMCL and of 11 TACI⁻ HMCL. 109 probesets out of the 49000 interrogated with U133 set Affymetrix microarrays were differentially expressed between TACI⁺ and TACI⁻ HMCL ($P \leq .01$ with a Mann Whitney test and ratio of the mean expressions ≥ 2 or ≤ 0.5). The analysis performed on the same samples using the SAM software with a 3-presence and a 2-ratio filters on probesets and 1000 permutations led to a larger 330 probeset list with a higher false discovery rate (FDR) of 25.5 %. This high FDR is due to the number of samples. For the further analysis, we considered the probesets picked up by the two methods, *i.e.* 86 probesets corresponding to 80 genes/EST. This gene/EST list is available in supplementary Tables A (TACI⁺ probesets) and B (TACI⁻ probesets).

Some genes are noteworthy, particularly *c-maf*, *cyclin D2*, *integrin beta7*, *MAGE-A3*, *kappa and lambda immunoglobulin (Ig)-light chains*. The differential expression of these genes was validated with real-time RT-PCR for *TACI*, *c-maf*, *cyclin D2* and *integrin beta7* (Figure 1) and with flow-cytometry for kappa/lambda Ig (data not shown). Interestingly, 7/7 TACI⁺ HMCL expressed lambda Ig light chains, whereas among the 11 TACI⁻ HMCL, 6 expressed kappa chains and 5 expressed lambda chains. 46 of the 80 genes /EST (58%) mentioned above (28 genes overexpressed in TACI⁺ HMCL and 18 genes overexpressed in TACI⁻ HMCL) could be assigned to 8 functional categories using Gene Ontology terms (Table 1). TACI⁺ HMCL express a higher percentage of genes coding for cell communication signals or signal transduction ($P < .05$, Table 1). Conversely, TACI⁻ HMCL overexpressed genes coding for proteins involved in nuclear functions (Table 1).

TACI⁺ HMCL have a gene signature of mature bone marrow plasma cells and TACI⁻ HMCL of plasmablasts

Based on our recent finding that TACI^{high} primary MMC have a gene signature resembling normal mature BM plasma cells, whereas TACI^{low} MMC have a plasmablastic gene signature, we investigated whether TACI⁺/TACI⁻ HMCL keep these properties. The GEP of 7 normal BMPC and 7 normal plasmablasts were determined with U133 Affymetrix microarrays. Using an unsupervised clustering with the above-mentioned 80 genes/EST, the TACI⁺ HMCL clustered together with BMPC whereas 6 out of 7 TACI⁻ HMCL clustered with plasmablasts (Figure 2A). In addition, out of 80 genes/EST differentially expressed between TACI⁺ and TACI⁻ HMCLs, 19 are upregulated in BMPC compared to plasmablasts, and 15 in plasmablasts versus BMPC. 19 out of the 19 BMPC genes were upregulated in TACI⁺ HMCL compared to TACI⁻ HMCL and conversely, 11 of the 15 plasmablast-genes were overexpressed in TACI⁻ HMCL, confirming that TACI⁺ HMCL have a BMPC gene signature and TACI⁻ one of plasmablast. These “BMPC” and “plasmablast-genes” are indicated in supplementary Tables C and D. In Figure 2B, we show the expression of some remarkable TACI⁺ HMCL or TACI⁻ HMCL genes in BMPC and PPC. TACI⁺ HMCL overexpressed integrin beta8 that is expressed by BMPC only in normal B cell differentiation (32) (Figure 2B). In the TACI⁺ gene signature, CX3CR1 and CD74 are also overexpressed in BMPC compared to plasmablasts (Figure 2B).

TACI expression is correlated with *c-maf* expression in HMCL

TACI⁺ HMCL showed a significantly higher mean expression of *c-maf* compared to TACI⁻ HMCL (mean expression in TACI⁺ of 209.7 versus 25.6 TACI⁻ HMCL, ratio = 8.4, $P < .01$). In the TACI⁺ HMCL, *TACI* and *c-maf* expressions correlated well ($r = .94$, $P < .01$) (Figure 3A). This correlation was found with expression signals

determined by Affymetrix microarrays and by real-time RT-PCR as well. We looked further for c-maf protein in 3 TACI⁺ HMCLs and 3 TACI⁻ HMCLs (Figure 3b). C-maf Affymetrix expression was significantly correlated with c-maf protein ($r = .8, P < .05$). TACI⁺ HMCLs also showed higher expressions of *cyclin D2* (mean expression in TACI⁺ of 2059.4 versus 588.2 TACI⁻ HMCL, ratio = 3.5, $P < .01$) and *integrin beta7* (mean expression in TACI⁺ of 1842.4 versus 458.5 TACI⁻ HMCL ratio = 4, $P < .01$) (Figure 3B).

TACI influences c-maf expression

In order to determine whether signaling via TACI could induce *c-maf* expression, we exposed the XG-13 and XG-20 TACI⁺ HMCL, whose growth can be stimulated by BAFF and APRIL (16), for 12-hours with BAFF and APRIL. For XG-13 and XG-20 HMCL, BAFF/APRIL stimulation induced a significant upregulation of *c-maf*, *cyclin D2* and *integrin beta7* expressions in 5 separate experiments (Figure 4A, $P = .01$, $P = .03$ and $P = .02$, respectively). In the TACI⁻ XG-7 HMCL, in which growth or proliferation cannot be stimulated by BAFF/APRIL, no increased expression of these genes by BAFF/APRIL stimulation was found (Figure 4A). The effect of IL-6 was also investigated. For XG-7, XG-13 and XG-20, IL-6 stimulation did not modify *c-maf* and *integrin beta7* gene expressions in 5 separate experiments (Figure 4B). *Cyclin D2* expression was upregulated by IL-6 stimulation in XG-13 and XG-20 cells, unlike XG-7 cells (Figure 4B). Next we investigated the RPMI8226 and LP1 cells, which produces BAFF/APRIL as autocrine growth factors (16). A blocking of the BAFF/APRIL autocrine loop by a TACI-Fc fusion protein that acts as decoy-receptor for BAFF and APRIL resulted in a reduction of *c-maf* expression by 32% in RPMI8226 cells ($P = .01$) and by 40% in LP1 cells ($P = .005$). The expression of *cyclin D2* was also reduced by 35% ($P = .005$) and 28% ($P = .01$) in these 2 HMCL

as well as that of *integrin beta7* (39% of inhibition in RPMI8226, $P = .006$ and 27% of inhibition in LP1, $P = .01$) (Figure 4C).

As RPMI8226 and LP1 could be nucleotransfected with siRNA, unlike XG HMCL, we used these two cell lines to investigate further the link between TACI activation and *c-maf* expression. The nucleofection with *c-maf* siRNA significantly ($P = .001$) decreases the expression of *c-maf* (55 and 45% in RPMI8226 and LP1 HMCL, respectively) as well as the expression of *cyclin D2* (41 and 47% in RPMI8226 and LP1, respectively) and *integrin beta7* (40 and 35% in RPMI8226 and LP1, respectively) ($P \leq .05$) (Figure 4D). The *c-maf* siRNA nucleofection did not affect *TACI* expression in these HMCL. Addition of BAFF/APRIL could not reverse the downregulation of *cyclin D2* and *integrin beta 7* expression induced by the *c-maf* siRNA (Figure 4E).

NF-kappaB pathway is activated by BAFF/APRIL stimulation in MMC (16). We found here that the expression of *c-maf* was not affected by a peptide inhibitor of the NF-kappaB pathway (SN50), unlike TACI-Fc (Figure 5). This SN50 peptide inhibitor efficiently inhibited NF-kappaB activation in MMC (Figure S1 in supplementary data).

Discussion

The aim of this work was to further characterize the role of TACI-expression in MM. We have previously shown that BAFF and APRIL are important growth factors for MMC, and that the respective receptors, namely TACI, BCMA and BAFF-R, show a characteristic expression pattern in MMC. BAFF-R is not expressed (33) and BCMA is expressed by all primary MMC and HMCL(25). MMC expressing only BCMA seems not to be able to bind BAFF/APRIL. Indeed, the ability of HMCL to bind BAFF is strictly restricted to TACI⁺ HMCL (16). Interestingly, the level of TACI expression in primary MMC correlated with a characteristic phenotypic pattern, namely, TACI^{high} MMC with an expression pattern resembling BMPC, and TACI^{low} MMC with a plasmablastic expression pattern (25).

First we showed that these expression patterns are kept in HMCL. Using GEP determined with Affymetrix microarrays, TACI⁺ HMCL have a gene signature of BMPC, indicative of a dependence on the microenvironment whereas TACI⁻ HMCL have a plasmablastic gene signature. Indeed, an unsupervised clustering shows that TACI⁺ HMCL clustered together with BMPC whereas 6 out of 7 TACI⁻ HMCL clustered with plasmablasts. Secondly, TACI⁺ HMCL overexpressed genes coding for cell communication, noteworthy the adhesion molecules (integrin alpha8, integrin beta2 and integrin beta7), the CX3CR1 chemokine receptor and CD74. Integrin alpha8 is an adhesion protein characteristic of terminally differentiated BMPC (32). TACI⁻ HMCL overexpressed cancer testis antigens *MAGE-A1*, *MAGE-A3* and *MAGE-A6*. The tyrosine phosphatase CD45 is a marker of normal plasmablasts (34) and of proliferating plasmablastic myeloma cells (35). CD45 gene was not picked up in this study because there is only a trend ($P = .01$) of higher CD45 expression in TACI⁻

HMCL (7 of 11, 64%) compared to TACI⁺ HMCL (2 of 7, 28%) using Affymetrix data or FACS analysis.

Of note, comparing the gene lists making it possible to distinguish TACI⁺ and TACI⁻ HMCLs and TACI^{high} and TACI^{low} primary MMC - see our previous report (25)) - only 4 genes/EST were common: TACI, lambda Ig light chain, a gene coding for a cell cycle protein and one EST. In particular, *c-maf* gene was not significantly overexpressed in TACI^{high} MMC and no correlation between *c-maf* and *TACI* expression in 65 primary MMC could be found (data not shown). Thus the patterns of cell communication and signaling of TACI^{high} MMC and of plasmablast of TACI^{low} MMC are conserved in TACI⁺ and TACI⁻ HMCL but not the individual genes making it possible to define these patterns. This might be explained by the fact that the clear cut expression of *TACI* found in HMCL (absent or present using real time RT-PCR or Affymetrix microarrays) is not found in primary MMC, in which *TACI* expression is always present. Using labeling of primary MMC with an anti-TACI antibody, we looked for TACI expression by primary MMC of five consecutive newly-diagnosed patients (Table S1 in supplementary data). TACI expression was heterogeneous in primary MMC patients ranging from 1.1% to 87.1% of MMC. These data suggest that there are likely MMC at different stages of dependency on the microenvironment in a given patient. This may be due to a differentiation of the MM tumor *in vivo*, eventually as the counterpart of the normal plasma cell differentiation that is poorly known. This might be also due to a proceeding oncogenic process, rendering MMC less dependent on their dependency on the microenvironment for their survival, proliferation and differentiation. When obtaining an HMCL, which is almost only possible in patients with extramedullary proliferation, only one clone of MMC, frozen at a specific stage of dependency on the BM environment, might be selected.

Driven by the observation that *TACI* and *c-maf* expressions correlated in TACI⁺ HMCL, we have shown that TACI can signal via c-maf. Indeed, we have shown that addition or capturing of BAFF/APRIL yields an up- or a downregulation of *c-maf* expression whereas IL-6 did not affect the expression of *c-maf*. It also yields a concomitant increase or decrease of *cyclin D2* and *integrin beta7* expressions. A recent study has shown that these two genes are upregulated in response to c-maf (36). It suggested that c-maf could promote malignant transformation of plasma cells by enhanced proliferation and adhesion with BM stromal cells known to provide survival signals to plasma cells (36, 37). A regulation of *cyclin D2* and *integrin beta7* genes by c-maf was also shown in a model of murine lymphoma (38). Blocking *c-maf* RNA we confirmed that a decrease of *c-maf* mRNA levels reduce the expression of *cyclin D2* and *integrin beta7*. Blocking *c-maf* RNA did not affect *TACI* expression and addition of BAFF/APRIL could not reverse the downregulation of *cyclin D2* and *integrin beta 7* expression induced by the *c-maf* siRNA. These results indicate that TACI activation can upregulate *c-maf* expression, that in turn controls *cyclin D2*, and *integrin beta7* gene expressions as reported (36).

The mechanisms of regulation of *c-maf* expression are poorly known. TACI activates several transduction pathways in human myeloma cells, the ERK, PI-3-Kinase and NF-kappa B pathways (16). We show here that an inhibitor of the canonical NFκB pathway did not influence *c-maf* expression. BAFF/APRIL could also activate the non canonical NFκB pathway that could participate to the regulation of *c-maf* expression driven by TACI, in MMC. Furthermore, it was recently identified that MMC with a dysregulated expression of TACI showed increased NFκB2 p52/p100 ratios, consistent with activation of the non-canonical NFκB pathway (39). This regulation of *c-maf* expression by TACI could be explained in part by its activation of ERK that

triggers *c-maf* expression (40). But this is not the only mechanism since *c-maf* expression is not activated in some TACI⁻ HMCL that are stimulated by IL-6, which also triggers ERK pathway (41).

Given the importance of the TACI/BAFF/APRIL pathway, we recently initiated a clinical trial with the TACI receptor fused with Ig-Fc fragment (Ares Serono, TACI-Fc5), a BAFF and APRIL inhibitor. Preliminary results indicate that TACI-Fc5 treatment decreases the level of polyclonal Ig in patients with MM (42), fostering a role of TACI/BAFF/APRIL signaling in BMPC survival. It is of interest to investigate whether the different level of TACI-expression together with the associated patterns of gene expression that we have shown to be present in MMC (25) and HMCL will transmit into differences in responsiveness to TACI-Fc5 treatment. In particular, it will be important to investigate whether in some patients the TACI-Fc treatment may select for TACI⁻ MMC subclones with a plasmablastic gene signature.

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Figure legends

Figure 1. Validation of Affymetrix data.

Gene expressions of *TACI*, *c-maf*, *cyclin D2* and *integrin beta7* in $TACI^+$ HMCL were assayed with real time RT-PCR and normalized with *GAPDH* expression. The correlation between Affymetrix and real-time RT-PCR values was determined with a spearman test and the coefficient correlations and *P* value are provided in the panels.

Figure 2. $TACI^+$ HMCL have a gene signature of BMPC and $TACI^-$ HMCL a plasmablastic gene signature.

(A) Hierarchical clustering of HMCL, BMPC, and PPC identifies a signature of BMPC for $TACI^+$ HMCL and a plasmablastic signature for $TACI^-$ HMCL.

Unsupervised hierarchical clustering analysis of the gene expression profiles of 18 HMCL, 7 PPC samples and 7 BMPC samples show that $TACI^-$ HMCL cluster (green) together with PPC (blue) whereas $TACI^+$ cluster (red) with normal BMPC (purple). The clustering was performed on the 80 genes/EST differentially expressed between $TACI^+$ and $TACI^-$ HMCL.

(B) Histograms show the expression of 3 $TACI^+$ HMCL and 3 $TACI^-$ HMCL genes in PPC and BMPC.

Figure 3. *TACI* and *c-maf* expressions are correlated in HMCL.

(A) Correlation between *TACI* and *c-maf* expressions in $TACI^+$ HMCL using Affymetrix microarrays or real time RT-PCR.

(B) Expression level of *c-maf*, *cyclin D2* and *integrin beta7* in $TACI^+$ and $TACI^-$ HMCL using Affymetrix microarrays.

(C) Expression level of *TACI* and *c-maf* in HMCL using Affymetrix microarrays, western blot and flow cytometry. For each cell line, the ratios of *c-maf* and beta actin

proteins were determined in order to compare c-maf protein expression between cell lines.

Figure 4. BAFF and APRIL regulate the expression of c-maf, cyclin D2 and integrin beta7 in TACI⁺ HMCL.

(A) BAFF/APRIL upregulate *c-maf*, *cyclin D2* and *integrin beta7* expressions in the XG-20 and XG-13 TACI⁺ HMCL unlike the XG-7 TACI⁻ HMCL. *Cyclin D2* and *integrin beta7* expressions were determined by real-time RT-PCR and normalized with *GAPDH* expression. For each experiment, the expression of the studied gene in BAFF/APRIL stimulated myeloma cells was compared to that of untreated myeloma cells that was assigned the 1 arbitrary value. Data are mean values of 5 independent experiments. * The mean value is statistically significantly different from that obtained without BAFF/APRIL stimulation (control) using a Student *t* test ($P \leq .05$).

(B) *Cyclin D2* and *integrin beta7* expressions were determined by real-time RT-PCR with or without IL-6 stimulation for XG-13, XG-20 and XG-7 HMCL. Expression values were normalized using those of *GAPDH*. For each experiment, the expression of the studied gene in IL-6 stimulated myeloma cells was compared to that of untreated myeloma cells that was assigned the 1 arbitrary value. Data are mean values of 5 independent experiments. * The mean value is statistically significantly different from that obtained without IL-6 stimulation (control) using a Student *t* test ($P \leq .05$).

(C) BAFF/APRIL deprivation using TACI-Fc inhibitor induces a downregulation of c-maf, cyclin D2 and integrin beta7 expressions in RPMI 8226 and LP1 HMCL. C-maf, cyclin D2 and integrin beta7 expressions were determined by real-time RT-PCR and normalized with GAPDH expression. For each experiment, the expression of the studied gene in TACI-Fc treated myeloma cells was compared to that of untreated

myeloma cells that was assigned the 1 arbitrary value.. Data are mean values of 5 independent experiments. * The mean value is statistically significantly different from that obtained without TACI-Fc inhibitor (control) using a Student *t* test ($P \leq .05$).

(D) Real time RT-PCR assay for *c-maf*, *cyclin D*, *integrin beta7* and *TACI* expressions in RPMI8226 and LP1 HMCL 24 hours after being transduced with a *c-maf* siRNA oligonucleotide. Data are mean values of 5 independent experiments. * Mean value is statistically significantly different from that obtained without siRNA *c-maf* using a Student *t* test ($P \leq .05$).

(E) Real time RT-PCR assay for *c-maf*, *cyclin D2* and *integrin beta7* expressions in LP1 HMCL 24 hours after being transduced with a *c-maf* siRNA oligonucleotide and cultured with or without BAFF/APRIL. Data are mean values of 5 independent experiments. * Mean value is statistically significantly different from that obtained without siRNA *c-maf* using a Student *t* test ($P \leq .05$).

Figure 5. *C-maf* regulation by TACI is nor linked to NF-kappaB pathway.

Real time RT-PCR assay for *c-maf* expression in RPMI8226 and LP1 cells cultured with the TACI-Fc inhibitor, the SN50 NF-kappaB inhibitor (100 µg/ml), or the SN50 inactive peptide control (IC) (100 µg/ml). Expression values were normalized using those of *GAPDH*. For each experiment, the expression of the studied gene in IL-6 stimulated myeloma cells was compared to that of untreated myeloma cells that was assigned the 1 arbitrary value. Data are mean values of 5 independent experiments. * Mean value is statistically significantly different from that obtained without inhibitor using a Student *t* test ($P \leq .05$).

Table 1 : Cell communication signature in TACI⁺ HMCL and plasmablastic signature in TACI⁻ HMCL

	TACI ⁺	TACI ⁻	
<u>Genes coding for protein implicated in:</u>			
Cell communication signals (N = 8)	28.6 %	0 %	<i>P</i> < .01
Cytoskeleton (N = 3)	10.6 %	0 %	NS
Transduction signals (N = 10)	35.7 %	0 %	<i>P</i> < .01
Protein synthesis and regulation (N =2)	3.6 %	5.5 %	NS
Cell cycle (N = 2)	3.6 %	5.5 %	NS
Metabolism (N = 6)	14.3 %	11 %	NS
Cancer-testis antigens (N = 3)	0 %	16.5 %	NS
Nuclear functions (N = 12)	3.6 %	61.1 %	<i>P</i> < .01
Total of classified genes (N= 46)	100% (n = 28)	100% (n = 18)	

Of the 80 genes/EST differentially expressed between the TACI⁺ and TACI⁻ HMCL, 46 could be assigned to 8 functional categories using Gene Ontology terms. Data are the percentage of genes of a given category compared with the total number of TACI⁺ (28 genes) or TACI⁻ (18 genes) genes.

NS indicates not significant.

Figures

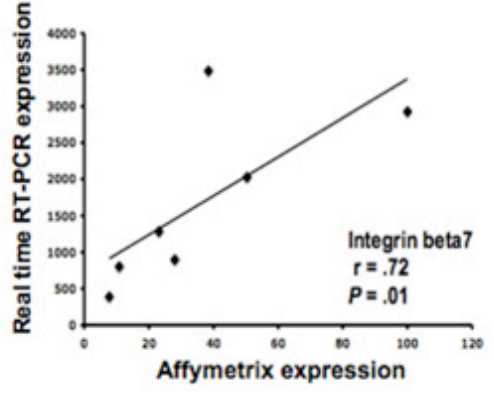
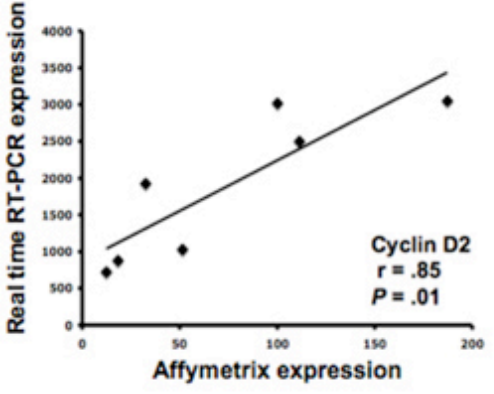
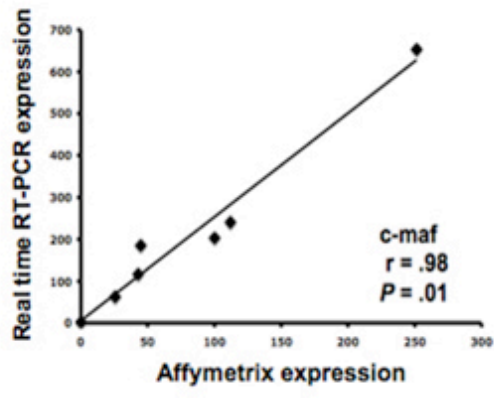
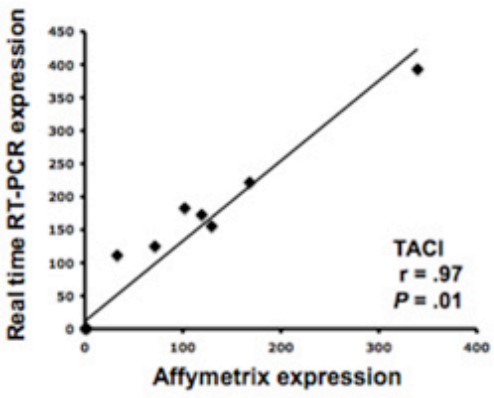


Figure 1A

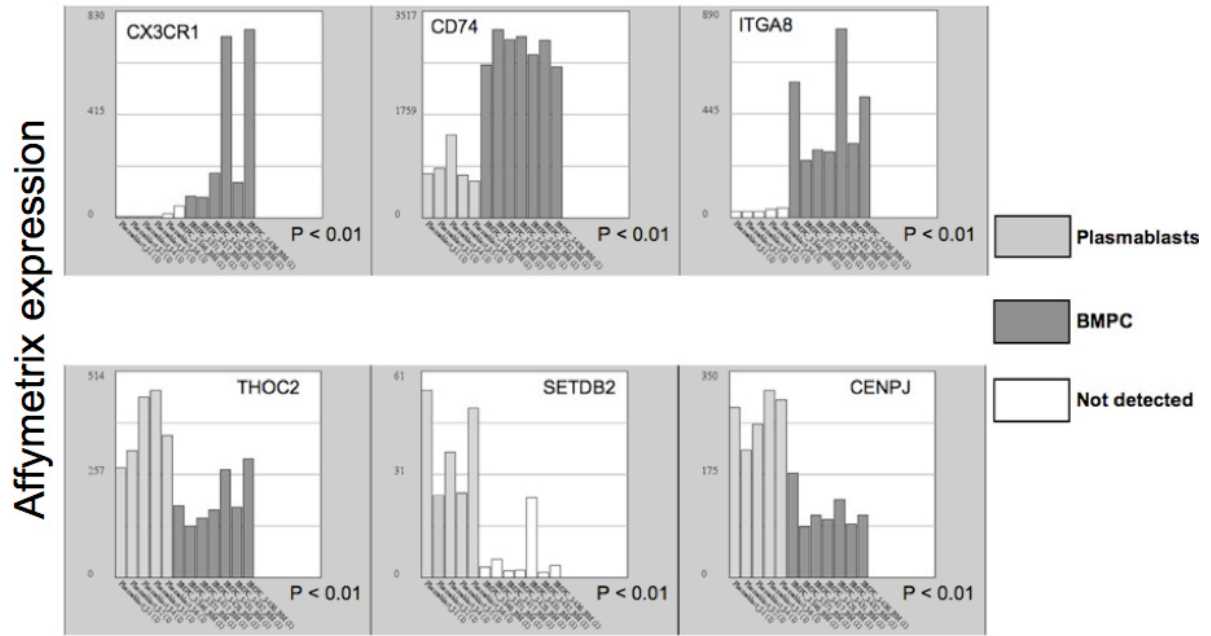


Figure 2B

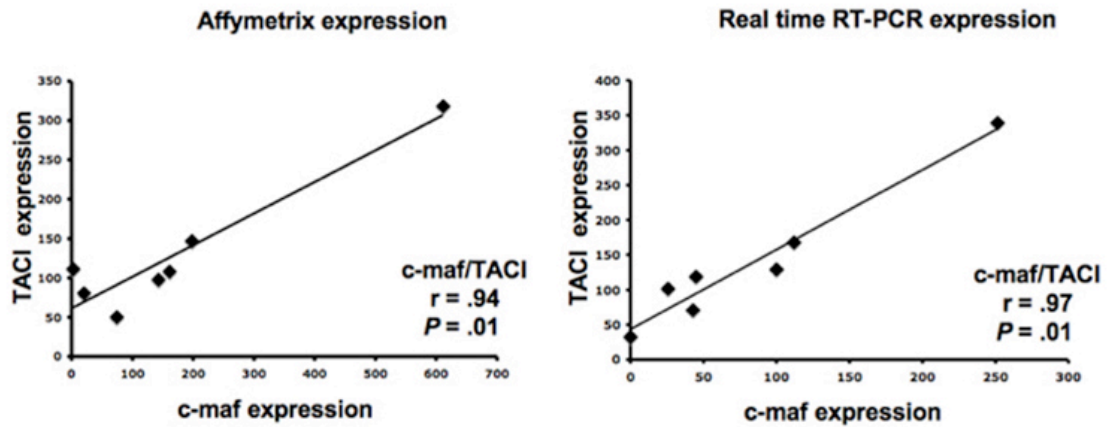


Figure 3A

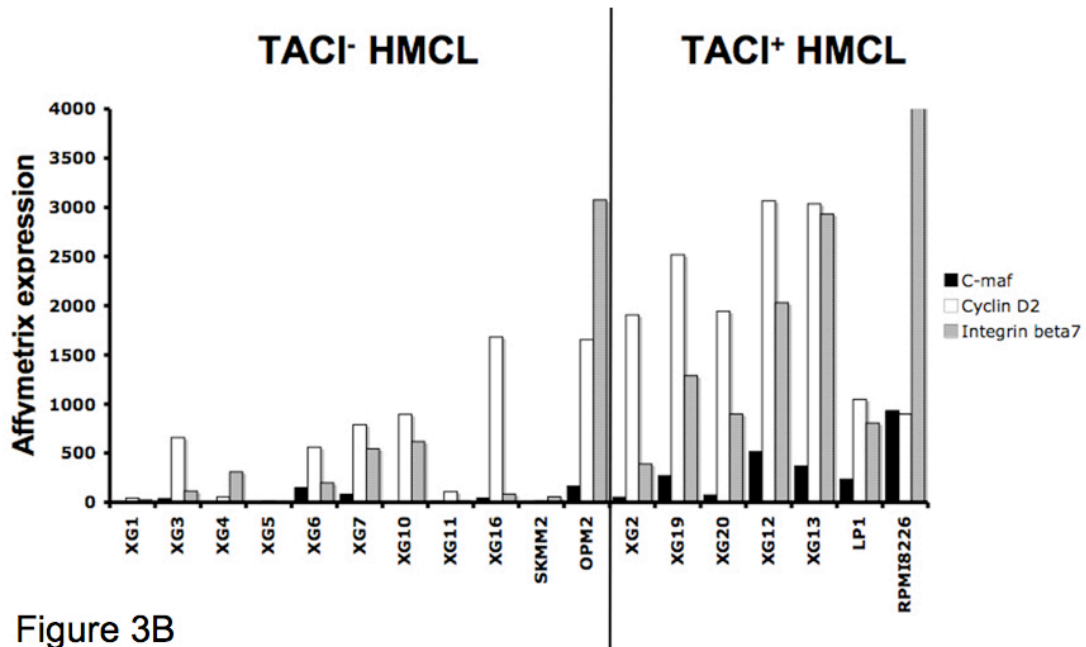


Figure 3B

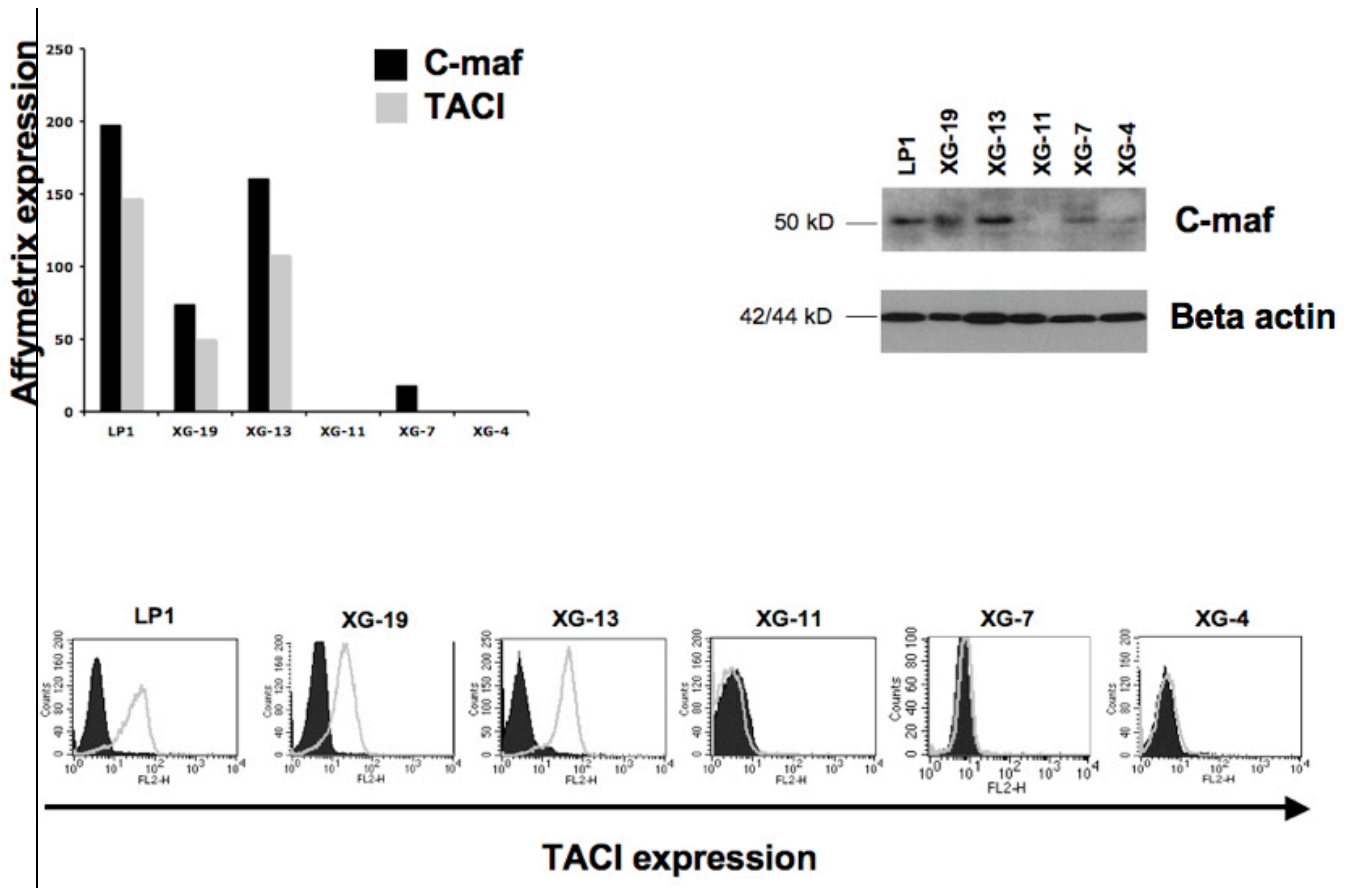
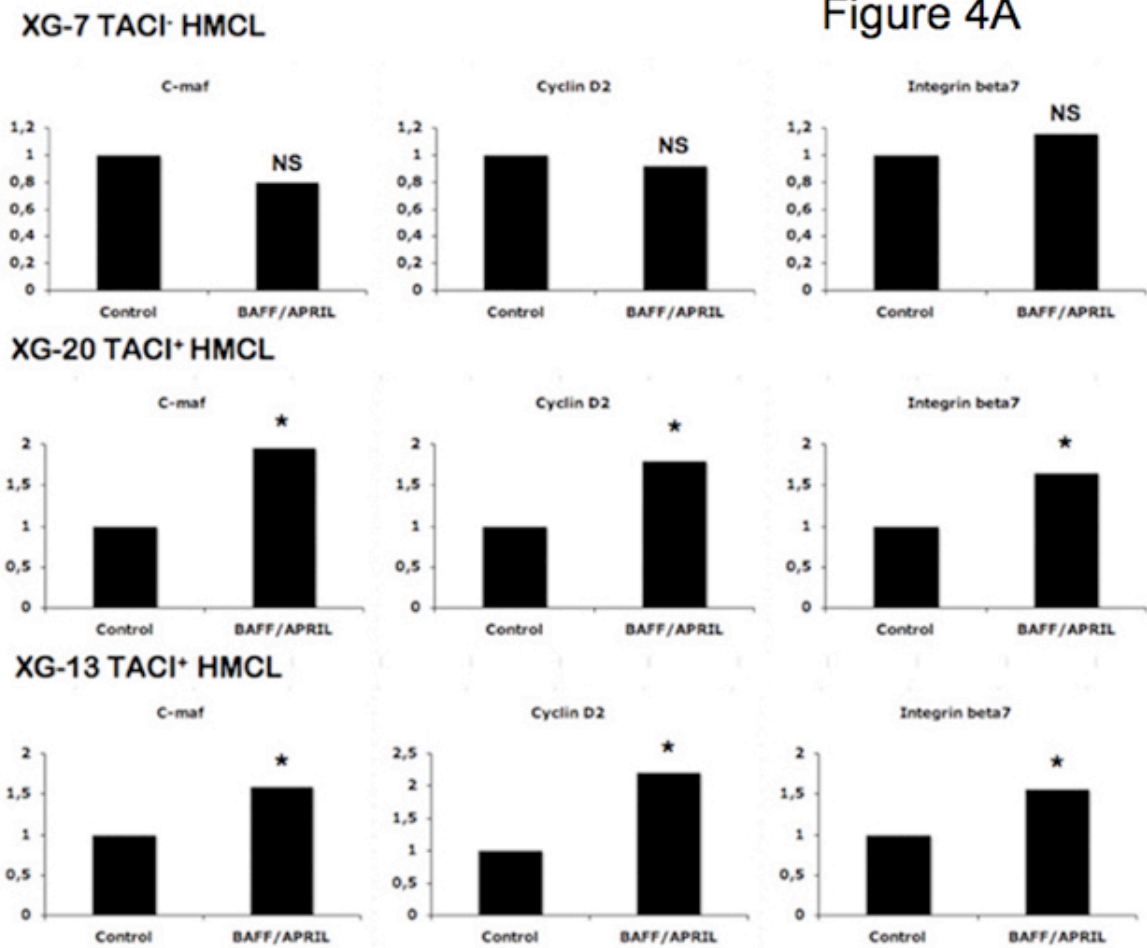


Figure 3C

Ratio of gene expression in treated cells compared to control cells

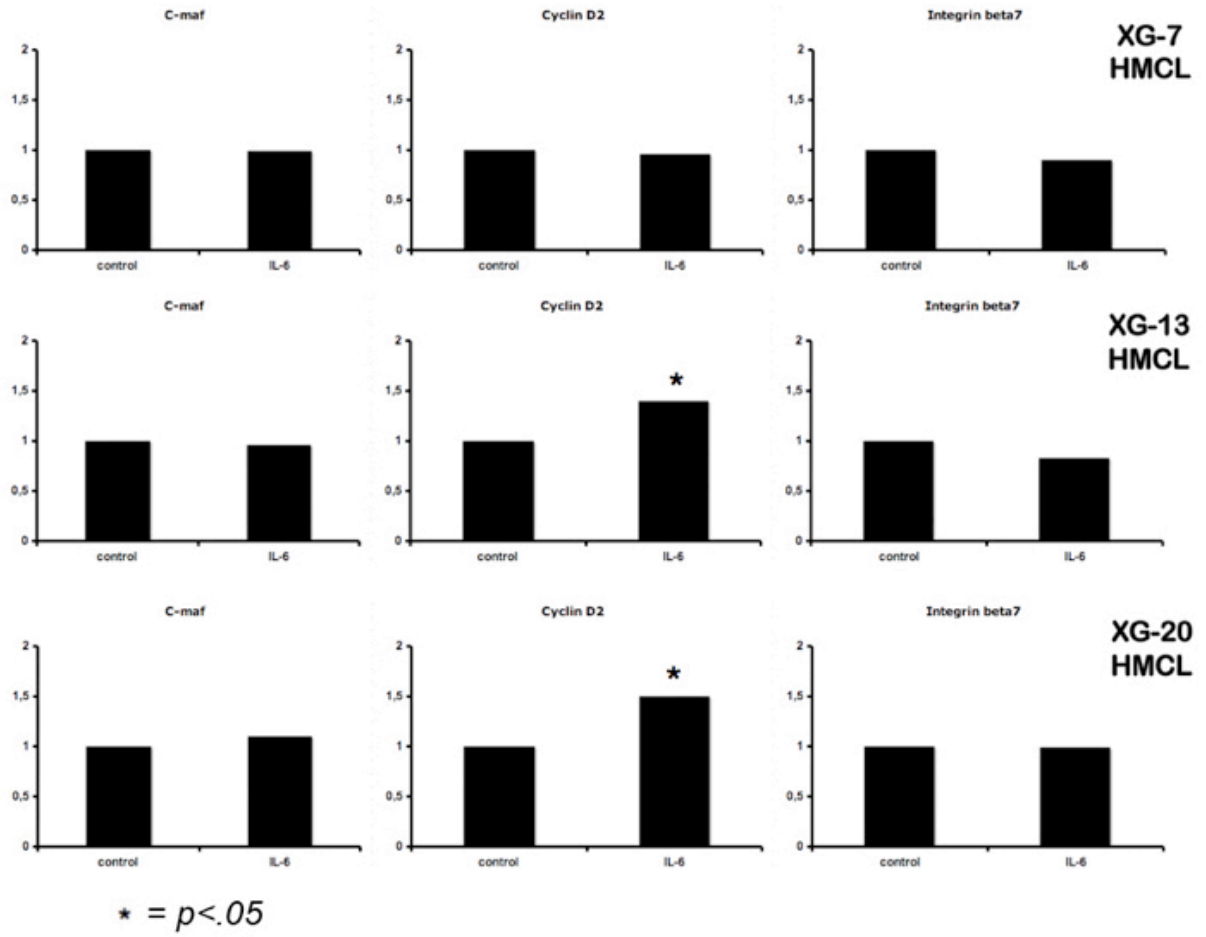
Figure 4A



* = $p < .05$

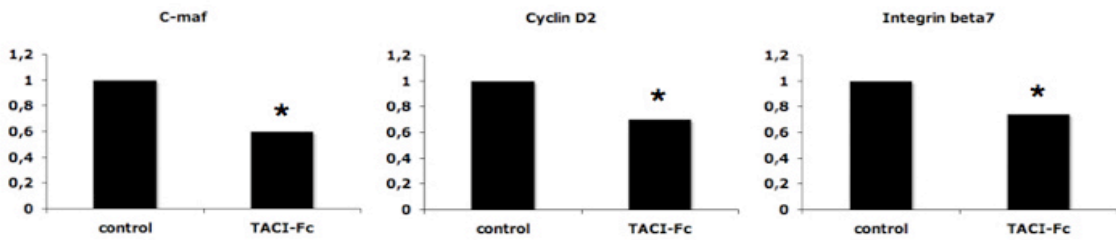
Ratio of gene expression in treated cells compared to control cells

Figure 4B

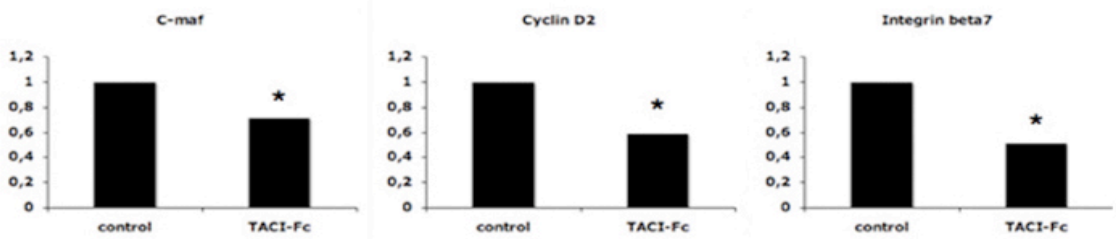


Ratio of gene expression in treated cells compared to control cells

LP1 TACI⁺ HMCL



RPMI8226 TACI⁺ HMCL

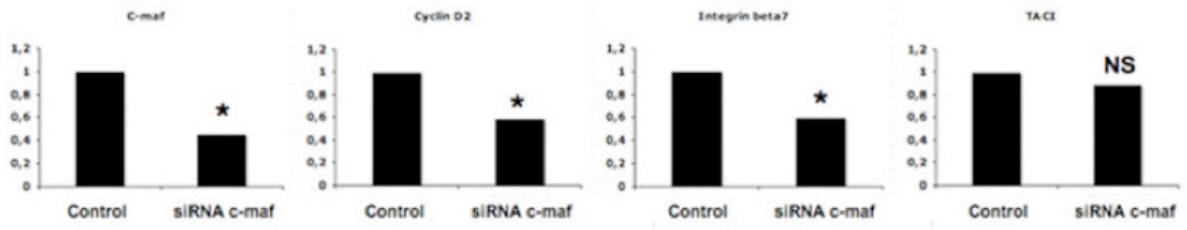


* = $p < .05$

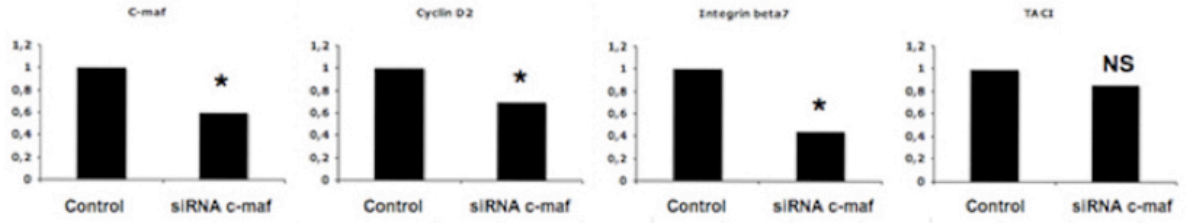
Figure 4C

Ratio of gene expression in treated cells compared to control cells

RPMI8226



LP1

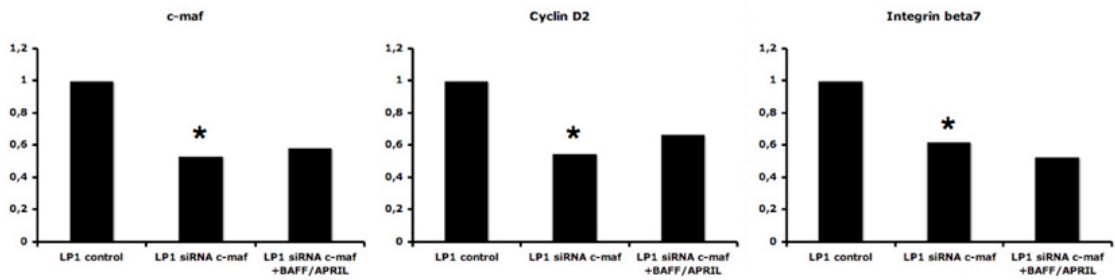


* = $p < .05$

Figure 4D

Ratio of gene expression in treated cells compared to control cells

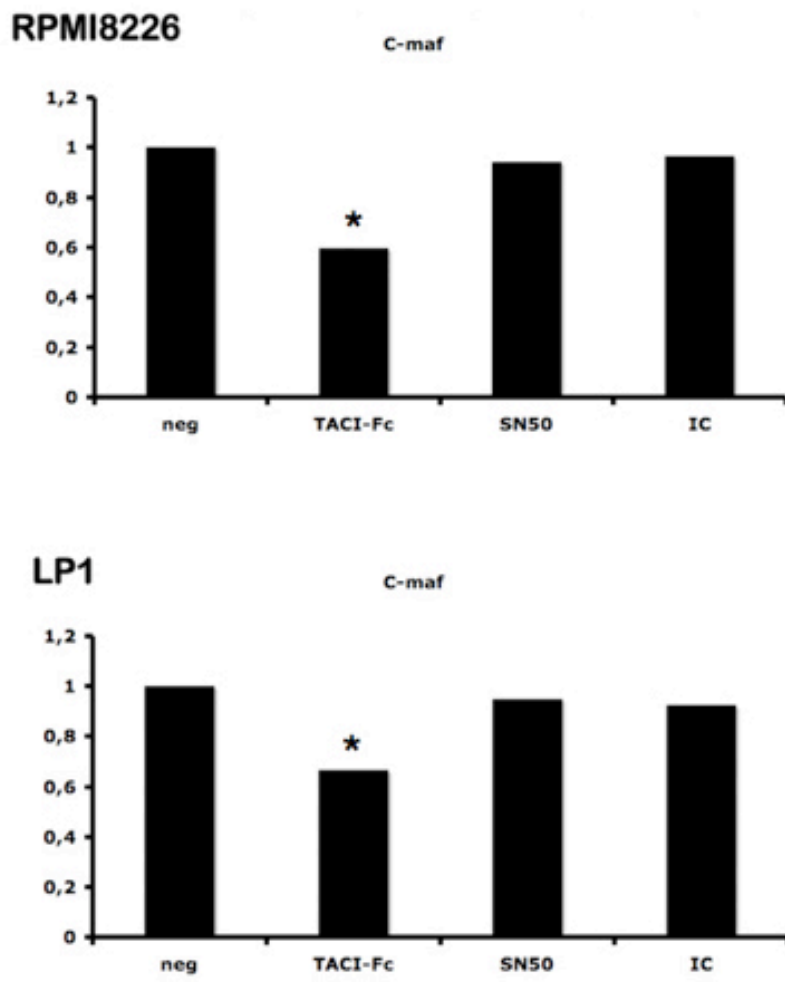
LP1



* = $P < .05$

Figure 4E

Ratio of gene expression in treated cells compared to control cells



* = $p < .05$

Figure 5

III- Discussion

Nous démontrons pour la première fois une implication des membres de la famille BAFF/APRIL dans le MM.

Dans le MM, les récepteurs BCMA et TACI sont exprimés par les cellules tumorales et sont activés par BAFF et APRIL, produits principalement par les cellules de l'environnement médullaire. Dans la moelle osseuse, les plasmocytes tumoraux prolifèrent au contact de l'os. Nous avons montré que les ostéoclastes produisent des taux élevés d'APRIL. Seules les lignées exprimant TACI à leur surface répondent à BAFF et APRIL. Les lignées n'exprimant que BCMA ne sont pas capables de fixer BAFF et ne sont pas sensibles à la stimulation par BAFF ou APRIL *in vitro*. Ces données mettent en évidence que TACI est un marqueur de la présence d'un récepteur fonctionnel de BAFF/APRIL à la surface des cellules de MM. L'absence de fonctionnalité de BCMA dans le MM est intrigant puisque les souris KO pour BCMA présentent un déficit en plasmocytes matures (174, 211, 227). Une étude démontre que l'activation de BCMA par BAFF/APRIL induit l'expression de protéines de costimulation à la surface des cellules B (215). Après stimulation de lignées de MM exprimant BCMA avec BAFF/APRIL, nous n'avons pas observé d'induction de ces protéines de costimulation. Néanmoins d'autres investigations sont nécessaires pour conclure à une absence de fonctionnalité de BCMA dans le MM. Il est important de déterminer si des voies de signalisation peuvent être activées en réponse à BAFF/APRIL chez des lignées de MM n'exprimant que BCMA. Des mutations de TACI et de BAFF-R ont déjà été identifiées (278-280) chez des patients

immunodéficientes. Il serait intéressant de rechercher si des mutations de BCMA sont présentes dans le MM.

Le lien existant entre TACI et c-maf est un mécanisme d'action intéressant de la voie BAFF/APRIL dans la physiopathologie du MM. C-maf contrôle l'expression de la cycline D2 et de l'intégrine beta 7 dans le MM (283). TACI pourrait donc agir sur la survie des cellules de MM en activant NF κ B, sur le cycle cellulaire en dérégulant l'expression de la cycline D2 via c-maf. De plus, l'intégrine beta 7 est impliquée dans l'adhésion entre les cellules MM et les cellules stromales induisant une augmentation de la production de VEGF jouant un rôle majeur dans l'angiogénèse.

Les travaux récents mettant en évidence la fixation d'APRIL et de TACI aux protéoglycanes à chaînes héparane sulfate s'annoncent importants pour la compréhension du rôle de la famille BAFF/APRIL dans le MM. Syndecan-1 est un protéoglycane à chaînes héparane sulfate. Il est exprimé essentiellement par deux types cellulaires : les cellules endothéliales et les plasmocytes tumoraux et normaux. Syndecan-1 est à l'heure actuelle le marqueur standard utilisé pour l'identification et la purification de cellules myélomateuses (150, 284). De plus, le taux sérique élevé de syndecan-1 soluble est un facteur de mauvais pronostic du MM.

Les protéoglycanes se composent d'une partie protéique sur laquelle sont fixées une ou plusieurs chaînes de glycosaminoglycanes (GAG) au niveau de sites d'ancrages spécifiques. On trouve les protéoglycanes à la membrane extracellulaire ou ancrés à la membrane plasmique des cellules. La fonction des protéoglycanes est l'adhésion et la fixation de facteurs de croissance dont ils régulent l'activité. Les protéoglycanes membranaires contiennent généralement des chaînes héparane sulfate. Les HSPG comprennent 3 grandes familles : les syndecans, les glypicans et les perlecans. Les syndecans sont des protéines transmembranaires de type I de taille moyenne (20-45

kDa) pouvant avoir jusqu'à 5 sites d'attachement pour les chaînes héparane sulfate. La famille des syndecans comporte 4 membres, syndecan-1/-3 et syndecan-2/-4 formant deux sous-familles sur la base des homologues de séquences protéiques. Du point de vue de la structure, on peut distinguer les fonctions conférées par la partie protéique et les fonctions conférées par les chaînes héparane sulfate. Les fonctions de la partie protéique concernent la liaison à des composants du cytosquelette au niveau des microdomaines membranaires spécialisés tels que les complexes d'adhérence focale. Les chaînes héparane sulfate confèrent aux protéoglycanes leurs fonctions essentielles de par leur capacité à fixer différents ligands, solubles (facteurs de croissance) ou insolubles (protéine de la membrane cellulaire ou protéines de la matrice extracellulaire).

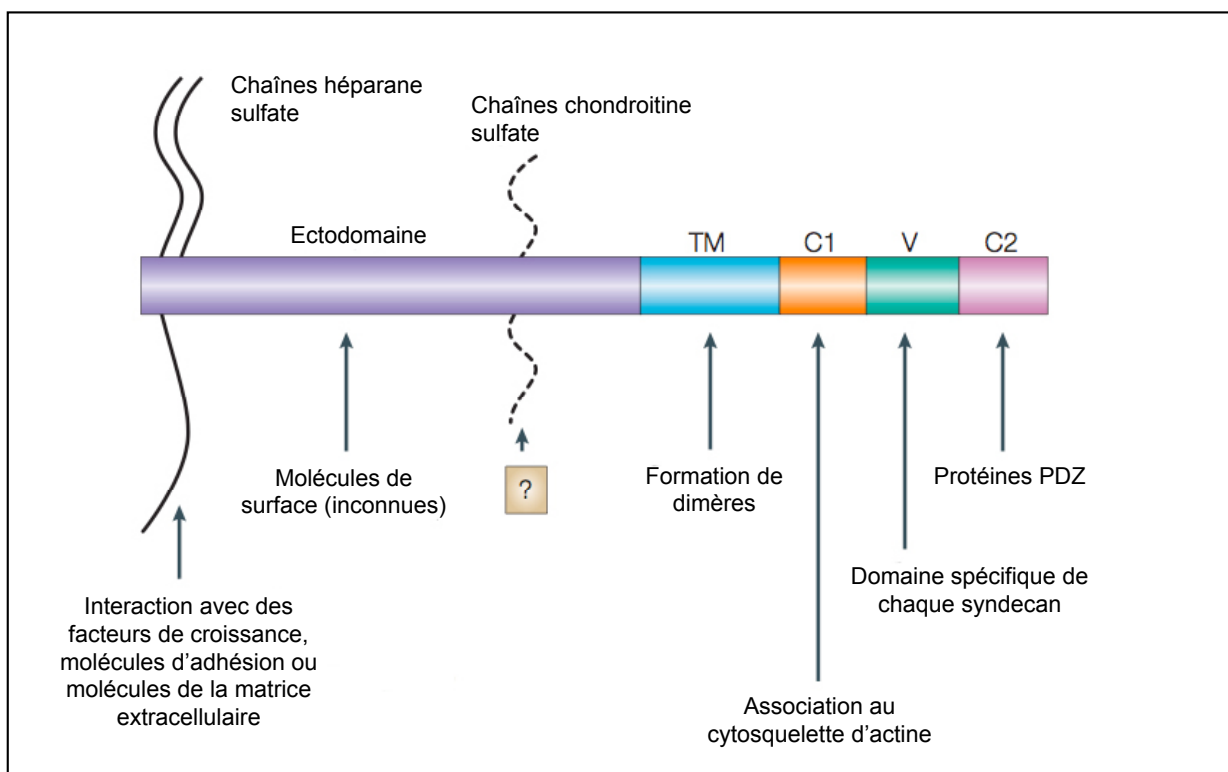


Figure 13 : Structure des syndecans et vision schématique des interactions possibles. TM= domaine transmembranaire, C1 et C2 sont les domaines conservés et V la région variable du domaine intracytoplasmique. Les protéines ayant des domaines PDZ sont souvent

localisées dans des structures spécialisées de la membrane plasmique, où elles peuvent interagir avec divers ligands.

La partie cytoplasmique des syndecans est courte (30 aa) et présente un haut degré de conservation. Elle peut être divisée en trois régions : les régions conservées C1 et C2 qui encadrent une région variable (V) différente d'un syndecan à l'autre. Le domaine transmembranaire et une petite séquence juste externe ont une forte tendance à former des dimères (236) avec une très forte affinité. Il semble que les syndecans ne puissent pas former d'hétérodimères malgré les homologies de séquences. La très forte affinité pour l'association indique que la forme en homodimère pourrait être l'unité fonctionnelle des syndecans (285). Les fonctions biologiques exercées par le domaine extracellulaire (ectodomaine) sont pratiquement inconnues. Une des fonctions est de porter les chaînes glycosaminoglycanes mais il a également été démontré que l'ectodomaine est impliqué dans des liaisons protéine-protéine pour syndecan-1 et -4 (286, 287). Les chaînes héparane sulfate confèrent l'essentiel de leurs propriétés aux syndecans, de par leur capacité à lier différents ligands qui possèdent un domaine « heparin binding » régulant ainsi l'activité de ces facteurs.

III-1. Les HSPG dans le MM

Comme décrit précédemment, les cellules de MM expriment très fortement et spécifiquement syndecan-1 (150, 284). L'expression de syndecan-1 est perdue rapidement lorsque les cellules de MM rentrent en apoptose (288). Le variant d'épissage v3 de la glycoprotéine CD44 (CD44v3) contenant des sites d'attachement

pour les chaînes de type héparane sulfate, est faiblement exprimé à la surface de certaines lignées de MM (115, 289). Plus récemment, notre équipe a étudié l'expression des syndecans (-1 à -4) et des glypicans (-1 à -6) dans le MM à l'aide des puces à ADN (290). A part syndecan-1, les autres HSPG sont peu ou pas exprimés par les cellules de MM.

Syndecan-1 membranaire peut être clivé en une forme soluble qui est présente à des taux élevés dans le sérum des patients atteints de MM (291). Le taux sérique de syndecan-1 est un facteur de mauvais pronostic dans le MM (292). La forme soluble de syndecan-1 étant biologiquement active, comprend des chaînes héparane sulfate biologiquement active (291). Syndecan-1 soluble étant un facteur de mauvais pronostic dans le MM, il peut soit représenter un reflet de la masse tumorale importante soit contribuer directement à la progression de la maladie. Il a été démontré que syndecan-1 soluble augmente la croissance de tumeurs myélomateuses *in vivo* chez la souris (293) suggérant un rôle de syndecan-1 soluble dans le progression de la maladie. D'autres études mettent en évidence l'implication de syndecan-1 dans la progression et la diffusion des tumeurs de myélome (293, 294). Cependant, contrairement aux données obtenues *in vivo*, la croissance des cellules de MM exprimant syndecan-1 soluble est similaire à celle des cellules contrôle *in vitro*. L'effet de syndecan-1 soluble est donc dépendant de l'environnement médullaire où il est présent en grande quantité (295). Dans la moelle osseuse, la cellule tumorale est ainsi baignée au sein de grandes quantités d'héparane sulfate biologiquement actif. L'effet de syndecan-1 sur la croissance des cellules tumorales semble donc lié aux interactions existant entre syndecan-1 et les facteurs de croissance capables de fixer les chaînes héparane sulfate.

Dans le MM, il a été montré que l'interaction syndecan-1/HGF favorise l'activation de c-met (115). HGF est présent à de fortes concentrations dans la moelle osseuse de patients et forme des complexes avec syndecan-1 soluble (296). Le taux sérique d'HGF est augmenté chez les patients traités par l'héparine (297). Ceci s'explique par le fait que l'héparine déplace la fixation d'HGF à syndecan-1. De la même façon, syndecan-1 soluble est également capable de déplacer la fixation d'HGF à syndecan-1. Dans ce cas, on est en droit de se demander si syndecan-1 soluble agit comme un agoniste facilitant l'action des facteurs de croissance ou comme un antagoniste agissant comme l'héparine. Dans la moelle osseuse, syndecan-1 soluble est présent à des concentrations de l'ordre du microgramme par millilitre (296). A cette concentration, syndecan-1 potentialise l'effet d'HGF alors qu'il l'inhibe à des concentrations plus faibles (296). Des études montrent que syndecan-1 soluble peut inhiber la croissance de lignées de MM et de carcinome *in vitro*. Ceci conforte le fait que syndecan-1 peut être activateur ou inhibiteur selon sa concentration. Syndecan-1 soluble peut également contribuer à garder les facteurs de croissance dans l'environnement tumoral. En effet, une fois fixé à l'héparine, la demi-vie d'HGF est considérablement augmentée (298). Kato et al ont montré que l'héparanase plaquettaire pouvait convertir syndecan-1 d'un antagoniste en un agoniste pour FGF2 (299). Enfin, les sulfatases qui modulent le degré de sulfatation des chaînes héparane sulfate, sulf1 et sulf2, inhibent la croissance tumorale *in vivo* chez la souris (300).

Récemment, nous avons démontré un rôle de 2 des 10 membres de la famille EGF ans la physiopathologie du MM. Par la suite, nous avons cherché à réaliser une analyse exhaustive, en termes d'expression et de fonctionnalité, de l'ensemble de cette famille de facteurs de croissance (Article 8).

« Heparan sulphate proteoglycans are essential for the myeloma cell growth activity of EGF-family ligands in multiple myeloma. » Oncogene. 2006 May 29; [Epub ahead of print].

Dans un premier temps, nous avons décrit de manière générale le profil d'expression de 10 membres de la famille EGF, au cours de la différenciation plasmocytaire, par les cellules de MM et par les cellules de l'environnement médullaire. Nous avons observé que :

- 5 facteurs sont fortement exprimés par les cellules de MM. Parmi ceux ci, AREG, NRG1 et TGF- α sont des gènes « plasmocytaires », c'est à dire exprimés par les plasmocytes normaux et tumoraux, alors que NRG2 et NRG3 sont des gènes « myélomateux », c'est à dire exprimés exclusivement par les cellules tumorales.
- 4 ligands sont produits par l'environnement, dont HB-EGF, ce qui confirme nos précédentes données.

7/10 membres de la famille EGF sont donc exprimés dans le MM.

Nous avons ensuite cherché l'effet des différents facteurs de croissance de la famille EGF sur des lignées de MM exprimant les récepteurs ErbB. Nous avons été surpris d'observer que la capacité de stimuler la croissance de cellules de MM n'est pas une propriété commune à tous les membres de la famille EGF. Seuls AREG, HB-EGF et NRG1 sont des facteurs de croissance myélomateux.

Ces trois facteurs sont les seuls à posséder un domaine heparin-binding capable de fixer l'héparine et les protéoglycanes à chaînes héparane sulfate, ce qui suggère que

l'interaction de ces ligands avec des HSPG pourrait induire leur activité de facteur de croissance. Nous avons montré que :

- Les cellules de MM fixes une grande quantité de ces facteurs à leur surface. Cette fixation est dépendante des chaînes héparane sulfate car elle est bloquée par l'héparatinase.
- Cette fixation est indispensable pour l'activité de facteur de croissance des ligands de la famille EGF. En effet, la croissance induite par HB-EGF, AREG ou NRG1 est complètement inhibée par l'héparine. De plus, une forme tronquée de NRG1 (qui n'a pas de domaine heparin binding) ne possède pas les mêmes fonctions que la forme entière de NRG1.

Enfin, nous avons montré que syndecan-1 joue un rôle de corécepteur. D'une part, une lignée qui ne l'exprime pas ne fixe pas et ne répond pas à HB-EGF. D'autre part, les autres HSPG sont peu ou pas exprimés par les cellules de MM.

En conclusion, syndecan-1 est absolument nécessaire pour l'activité des membres de la famille EGF, en concentrant de grandes quantités de facteurs de croissance à la surface des cellules.

Les travaux récents mettant en évidence la fixation d'APRIL et de TACI aux HSPG nous pousse à étudier leurs interactions dans le MM.

Nous recherchons actuellement à identifier si syndecan-1 joue également un rôle important dans l'activité de la voie APRIL/TACI dans le MM.

ORIGINAL ARTICLE

Heparan sulphate proteoglycans are essential for the myeloma cell growth activity of EGF-family ligands in multiple myeloma

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The epidermal growth factor (EGF)/EGF-receptor (ErbB1-4) family is involved in the biology of multiple myeloma (MM). In particular, ErbB-specific inhibitors induce strong apoptosis of myeloma cells (MMC) *in vitro*. To delineate the contribution of the 10 EGF-family ligands to the pathogenesis of MM, we have assessed their expression and biological activity. Comparing Affymetrix DNA-microarray-expression-profiles of CD138-purified plasma-cells from 65 MM-patients and 7 normal individuals to those of plasmablasts and B-cells, we found 5/10 EGF-family genes to be expressed in MMC. *Neuregulin-2* and *neuregulin-3* were expressed by MMC only, while *neuregulin-1*, *amphiregulin* and *transforming growth factor- α* were expressed by both MMC and normal plasma-cells. Using real-time polymerase chain reaction, we found *HB-EGF*, *amphiregulin*, *neuregulin-1* and *epiregulin* to be expressed by cells from the bone marrow-environment. Only the EGF-members able to bind heparan-sulphate proteoglycans (HSPGs) – *neuregulin-1*, *amphiregulin*, *HB-EGF* – promote the growth of MMC. Those ligands strongly bind MMC through HSPGs. The binding and the MMC growth activity was abrogated by heparitinase, heparin or deletion of the HS-binding domain. The number of HS-binding EGF ligand molecules bound to MMC was higher than 10^5 molecules/cell and paralleled that of syndecan-1. Syndecan-1, the main HSPG present on MM cells, likely concentrates high levels of HS-binding-EGF-ligands at the cell membrane and facilitates ErbB-activation. Altogether, our data further identify EGF-signalling as promising target for MM-therapy.

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Keywords: myeloma; EGF-family; neuregulin; syndecan-1; heparin

Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by the accumulation of malignant plasma cells in the bone marrow (BM). Despite recent therapeutic advances, the disease remains incurable with a median survival of approximately 3–4 years (Kumar *et al.*, 2003). MM cells (MMC) are dependent on several growth factors and cytokines, produced by the MMC themselves or by the BM microenvironment. Besides the well-known MMC factors, interleukin-6 (IL-6) (Kawano *et al.*, 1988; Klein *et al.*, 1989) and insulin-like growth factor-1 (IGF-1) (Georgii-Hemming *et al.*, 1996; Jelinek *et al.*, 1997; Ferlin *et al.*, 2000), an increasing number of additional factors are being identified, providing novel therapeutic targets for myeloma (Klein *et al.*, 2003).

The epidermal growth factor (EGF) receptor family comprises 4 members – ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 – that are involved in the development of numerous types of human cancers. Expression and/or activation of ErbB receptors are altered in many epithelial tumors and are involved in tumor progression (Holbro *et al.*, 2003). This has led to the development of ErbB-specific inhibitors that are now at various stages of clinical development (Hynes and Lane, 2005). In MM, we have previously demonstrated that MMC express ErbB receptors, and that their activation is required for *in vitro* survival of MMC in a majority of patients. Indeed, a pan-ErbB inhibitor induced strong apoptosis of MMC cultured for 5 days with their BM environment in 71% of the patients (Mahtouk *et al.*, 2004). When the ErbB-specific inhibitor was combined with dexamethasone or anti-IL-6 antibody, apoptosis was increased leading to an almost complete elimination of viable MMC while non-MMC were unaffected (Mahtouk *et al.*, 2004). For two of the 10 EGF-ligands – HB-EGF and AREG – we have shown that they support the growth of MMC in cooperation with IL-6 (Mahtouk *et al.*, 2004, 2005). However, the significance of other EGF-family ligands in MM has yet not been elucidated.

Ten ligands have been described for ErbB receptors. They can be subdivided in three groups according to their specificity: the first one includes EGF, amphiregulin (AREG), and transforming growth factor- α

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(TGF- α) which bind to ErbB1/EGFR exclusively. The second one includes heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC) and epiregulin (EPR) which bind to both ErbB1/EGFR and ErbB4. The last one includes the four neuregulins (NRG1, NRG2, NRG3 and NRG4) which bind to ErbB3 and/or ErbB4 (Harris *et al.*, 2003). ErbB2 has no ligand but is the preferred heterodimerization partner for other ErbBs (Citri *et al.*, 2003). A common feature to 4 out of the 10 EGF-family members (HB-EGF, AREG, NRG1 and NRG2) is their ability to bind heparin and heparan sulfate proteoglycans (HSPGs) (Higashiyama *et al.*, 1993; Johnson and Wong, 1994; Paria *et al.*, 1999). NRG1 and NRG2 bind HSPGs through an immunoglobulin (Ig)-like domain, located terminally to the EGF-like domain (Loeb and Fischbach, 1995; Carraway *et al.*, 1997).

A hallmark of plasma cell differentiation is the expression of the syndecan-1 HSPG at a high density. Syndecan-1 is expressed on normal and malignant plasma cells (PC) (Wijdenes *et al.*, 1996) and is now widely used to identify and purify PC (Sun *et al.*, 1997; Costes *et al.*, 1999). Syndecan-1 is found predominantly on epithelial cells. It is involved in several cellular processes that rely on interactions with extracellular matrix proteins, growth factors, chemokines and adhesion molecules (Rapraeger, 2000; Couchman, 2003). In myeloma, syndecan-1 has been shown to colocalize with growth factors in the uropods of MMC (Borset *et al.*, 2000), and to promote hepatocyte growth factor (HGF) activity on MMC (Derksen *et al.*, 2002).

To delineate the contribution of all 10 EGF-family members to the pathogenesis of MM, we studied here their expression and biological activity in MMC. We found 5/10 EGF-family genes to be expressed in MMC compared to normal BMPC, plasmablastic cells (PPC) and B cells; 5/10 genes to be expressed in some human myeloma cell lines (HMCLs); and 4/10 genes to be expressed by cells from the BM-environment. Among all EGF-ligands, only those able to bind HSPGs can promote the growth of MMC. In particular, we show for the first time that NRG1 is a growth factor for MMC. We show evidence that HSPGs, mainly syndecan-1, concentrate high levels of HS-binding-EGF-ligands at the MMC membrane, in the proximity of ErbB-receptors. This HSPG-dependent accumulation of EGF-family members is required for their MMC growth activity.

Results

Gene expression profile of EGF-family members in purified myeloma cells

Gene expression profile of the 10 EGF-family members was evaluated with U133A + B Affymetrix microarrays. Two genes (*NRG2* and *NRG3*) were overexpressed in MMC compared to B cells, PPC and BMPC (Figure 1a). The median *NRG2* expression in myeloma cells was 3-, 6.5- and 10-fold higher than that in BMPC ($P = 0.031$),

PPC ($P = 0.002$), or B cells ($P < 10^{-4}$), respectively (Figure 1a). The median *NRG3* expression in myeloma cells was 5-, 40- and 80-fold higher than that in BMPC ($P = 0.016$), PPC ($P < 10^{-4}$) or B cells ($P < 10^{-4}$), respectively (Figure 1a). Furthermore, *NRG2* and *NRG3* had an Affymetrix 'present call' in MMC exclusively. The call ('present' or 'absent') is determined by the GCOS software and indicates whether a gene is reliably expressed or not (Liu *et al.*, 2002). *NRG2* was 'present' in 26% (16/65) of the myeloma samples and *NRG3* in 58% (39/65) of them, and they were both 'absent' in all B cell, BMPC and PPC (Figure 1a). Two other genes (*NRG1* and *TGF- α*) were overexpressed in MMC and BMPC compared to B cells and PPC (Figure 1a). Although the median *NRG1* expression in MMC was not statistically significantly different to that found in B cells, PPC or BMPC, *NRG1* was present in 28% (18/65) of the myeloma samples and 43% (3/7) of the BMPC samples but was 'absent' in all B cells and PPC. *NRG1* expression in MMC + BMPC was statistically significantly higher than that found in B cells + PPC ($P = 0.05$). *TGF- α* was detected in 72% of myeloma samples, and was statistically significantly overexpressed in MMC (median value = 67) compared to B cells ($P = 0.007$) and PPC ($P < 10^{-4}$). *TGF- α* was also detected in 6/7 BMPC samples with a median value similar to that of MMC. Regarding HMCLs ($n = 20$), the median expression levels of *NRG2*, *NRG3*, *NRG1* and *TGF- α* were lower than those found in MMC (Figure 1a). As for the MMC, their expression was heterogeneous: *NRG2*, *NRG3*, *NRG1* and *TGF- α* had a 'present' call in 15, 15, 10 and 60% of the HMCLs, respectively (Figure 1a). In a previous study (Mahtouk *et al.*, 2005), we also found that *AREG* was overexpressed in MMC compared to B cells, PPC and BMPC. *AREG* was expressed in BMPC but at a significant lower level than in MMC and was not expressed in HMCLs (data not shown). Of note, 65/65 patients expressed at least one of the five EGF-family ligands – *AREG*, *NRG1*, *NRG2*, *NRG3* and *TGF- α* – in MMC. Five of 65 patients expressed only one of the 5 ligands, 16/65 two of them, 32/65 three of them, 10/65 four of them, and 2/65 all the 5 ligands. There was no significant difference in *NRG1*, *NRG2*, *NRG3* or *TGF- α* expression between MMC of patients with stage I, II or III MM (Figure 1a). The expression of *NRG1*, *NRG2*, *NRG3* and *TGF- α* was validated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) on 4 B cell samples, 7 PPC samples, 3 BMPC samples, 7 MMC samples and the 20 HMCLs (Figure 1b).

Other five EGF family members – *HB-EGF*, *EGF*, *EPR*, *BTC* and *NRG4* – displayed a very weak expression level associated with an 'absent' Affymetrix call in almost all primary MMC samples included in the study (percentage of presence <10%) (Figure 2a). Again, real-time RT-PCR confirmed the Affymetrix data (Figure 2b). According to our previous data (Mahtouk *et al.*, 2004), the *HB-EGF* gene showed a different expression pattern in HMCLs compared to MMC and was highly expressed in 8/20 HMCLs (Figure 2b). Of note, 19/20 HMCLs expressed at least

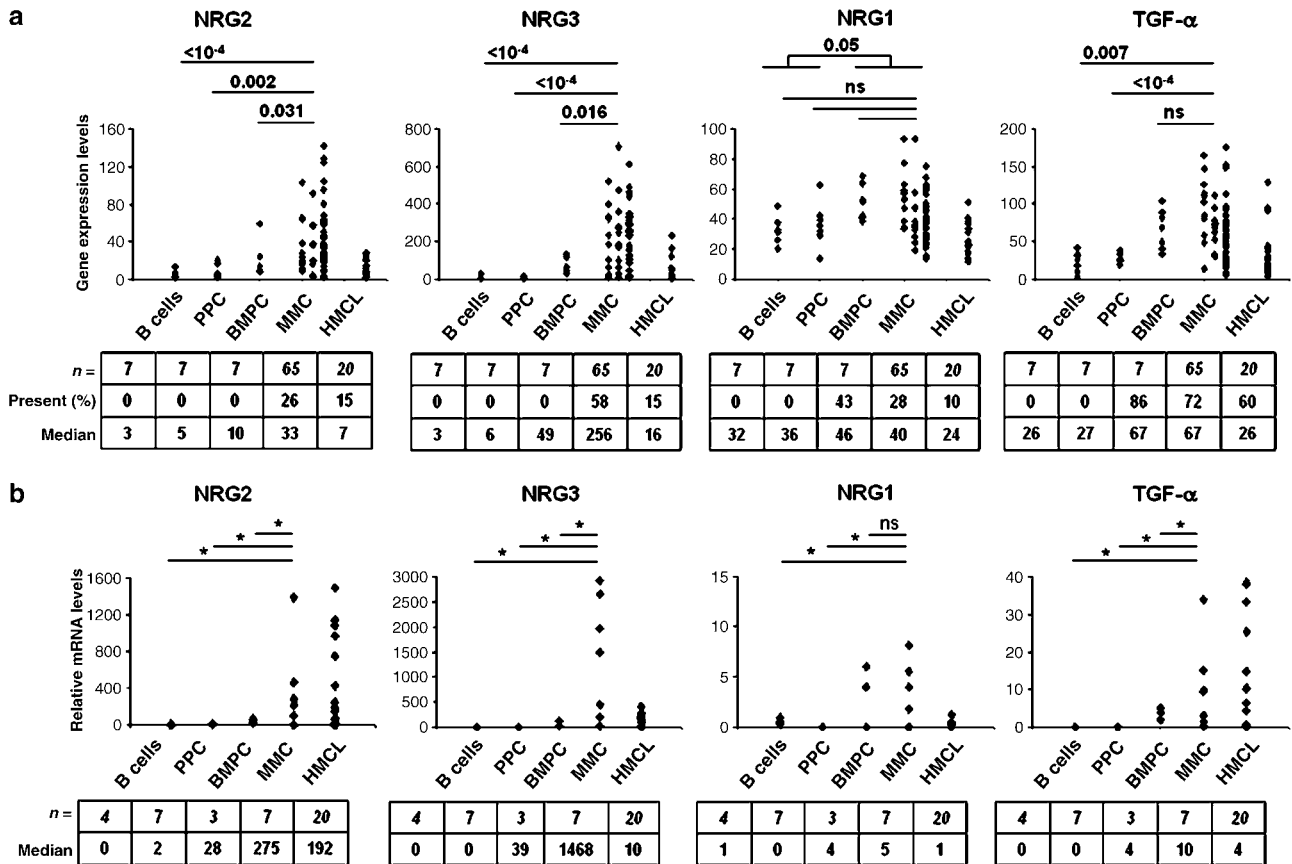


Figure 1 Overexpression of *NRG1*, *NRG2*, *NRG3* and *TGF-α* in multiple myeloma. (a) Gene expression profiles were determined in B cells, PPC, BMPC, MMC of 65 patients and 20 HMCLs. Data from MM patients are presented in three columns. The left, middle and right columns correspond to MMC from patients at stage I, II or III, respectively. Statistical comparisons were made with a Mann-Whitney test. *P*-values are indicated, 'NS' indicates that differences between samples are 'not significant' ($P > 0.05$). Percentages of presence and median values are indicated under each graphic. (b) Real-time RT-PCR analysis was completed as described in Materials and methods. *indicates that differences between samples are significant ($P \leq 0.05$). Median values are indicated under each graphic.

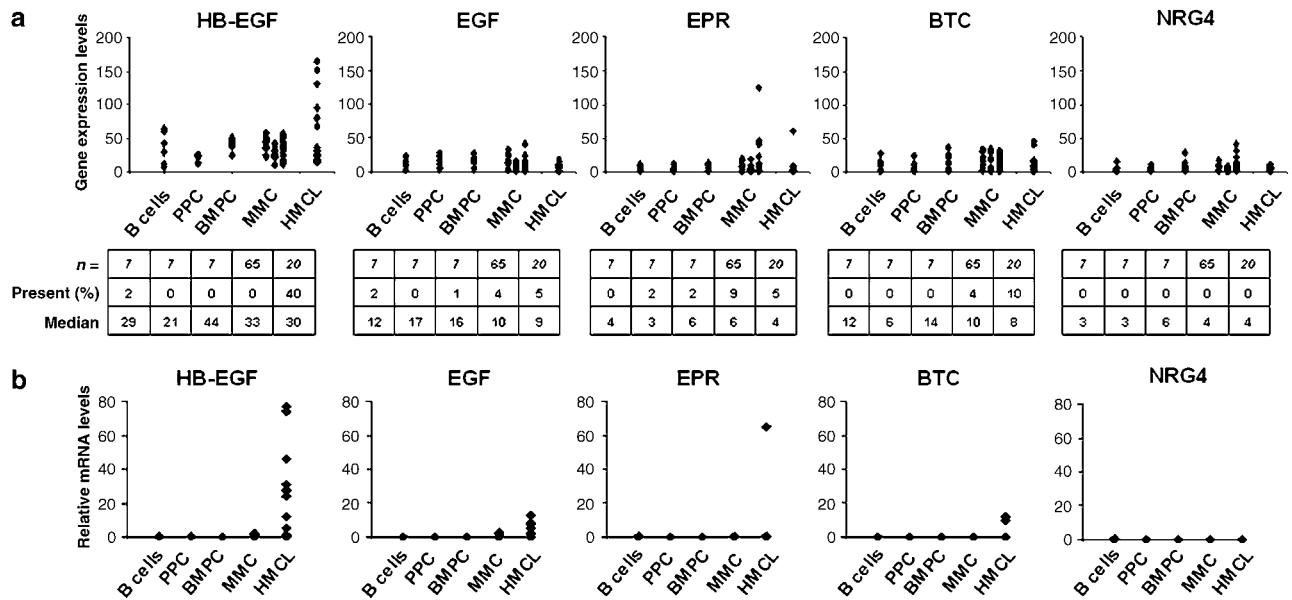


Figure 2 Gene expression profile of EGF family members. (a) Gene expression profiles were determined in B cells, PPC, BMPC, MMC of 65 patients and 20 HMCLs. Data from MM patients are presented in three columns. The left, middle and right columns correspond to MMC from patients at stage I, II or III, respectively. Statistical comparisons were made with a Mann-Whitney test. Percentages of presence and median values calculated over all samples are indicated under each graphic. (b) Real-time RT-PCR analysis was completed as described in Material and methods.

one of the 10 EGF-family ligands, 9/20 HMCLs expressed one of the 10 ligands, 5/20 expressed 2 of them, 3/20 expressed 3 of them and 2/20 expressed 4 of them.

Expression of EGF family members by the bone-marrow environment compared to myeloma cells

We investigated whether – besides MMC themselves – the BM microenvironment could be a source of EGF family members, thus delivering a paracrine growth signal to MMC. Using real-time RT-PCR, we looked for their relative expression in 7 samples of BM environment cells depleted from MMC (<1% MMC, see Materials and methods section) compared to purified MMC. Among the 5 EGF-family members overexpressed in MMC, *AREG* and *NRG1* were also expressed by the BM environment with a median value of 21 and 8, respectively (Figure 3a). *NRG2*, *NRG3* and *TGF- α* were weakly expressed by the BM environment compared to MMC. Regarding the other 5 EGF family members, *EPR* and *HB-EGF* were highly expressed by the BM environment compared to MMC (Figure 3b). *EGF*, *BTC* and *NRG4* were weakly or not expressed both in the BM environment and in MMC (Figure 3c).

Only HS-binding EGF-family members have a myeloma cell growth activity

To clarify the involvement of EGF family members in myeloma, we looked for their effect on MMC growth. We used two IL-6-dependent HMCLs (XG-7, XG-12) that express all four ErbB receptors, and the XG-5 HMCL that lacks ErbB4 (Mahtouk *et al.*, 2005). HMCLs were cultured at a low cell density to limit the

biological activity of autocrine EGF-ligands produced by the HMCLs themselves. This is the case for XG-7 cells that express HB-EGF, and whose spontaneous proliferation when cultured at a concentration of 5×10^5 cells/ml is inhibited by a pan-ErbB inhibitor (data not shown). Regarding ErbB1-specific ligands, AREG increased the growth of the three HMCLs ($P < 0.05$), which confirms our previous data (Mahtouk *et al.*, 2005). No stimulatory effect was found with EGF or TGF- α , in spite of the expression of their specific receptor (Figure 4a). Regarding ErbB1/4-specific ligands, HB-EGF was also a potent myeloma cell growth factor ($P < 0.05$) in agreement with our previous observations (Mahtouk *et al.*, 2004). In contrast, neither BTC nor EPR could stimulate myeloma cell growth (Figure 4a). Regarding ErbB3/4-specific ligands, NRG1 increased the growth of the XG-5, XG-7 and XG-12 HMCLs 18-, 5.2- and 4.7-fold, respectively ($P < 0.05$). The effect of NRG2-4 could not be investigated because the recombinant growth factors are not available commercially. AREG, HB-EGF and NRG1 also protected myeloma cells from apoptosis induced by IL-6 deprivation (Figure 4b). Again, no effect was found with EGF (Figure 4b), BTC, EPR and TGF- α (data not shown). Finally, AREG, HB-EGF and NRG1 dramatically promoted the proliferation of myeloma cells, 26-, 28- and 22-fold, respectively (Figure 4c). As expected, a pan-ErbB inhibitor (PD169540) reversed the HS-binding EGF-ligands-induced proliferation of XG-7 cells (Figure 4c). The inhibitor was used at 1- μ M concentration, which is known to inhibit specifically ErbB1, ErbB2, and ErbB4 kinase activities, without affecting a large panel of other kinases (Mahtouk *et al.*, 2004). EGF (Figure 4c), BTC, EPR and TGF- α (data

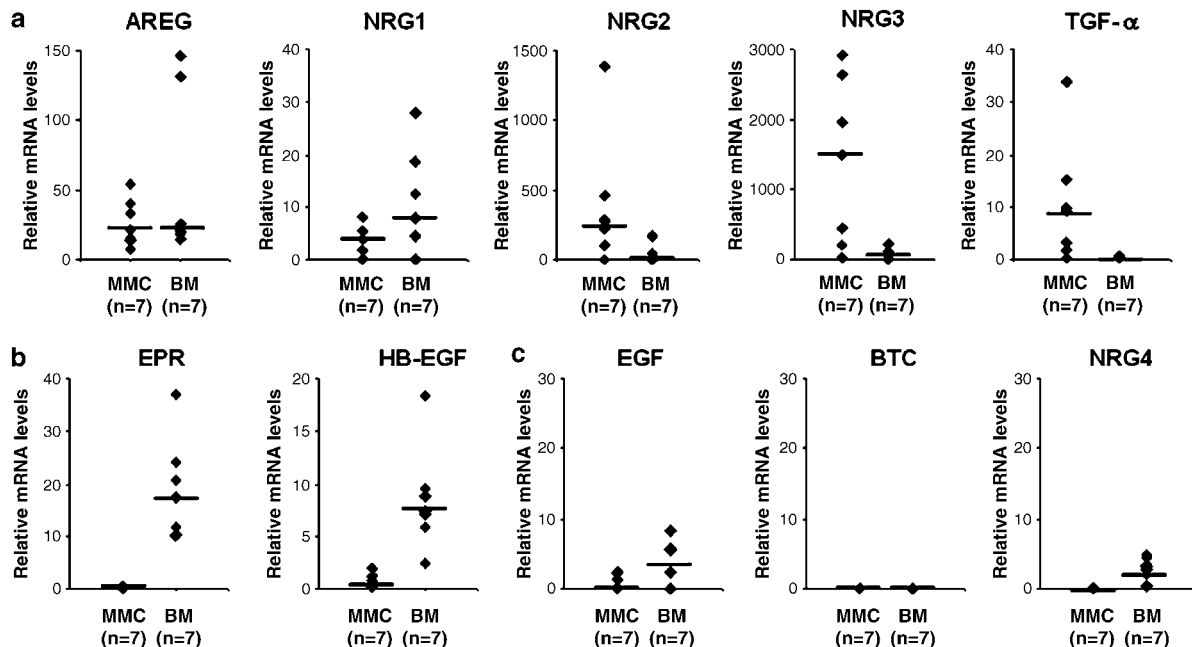


Figure 3 EGF-family members mRNA quantification in the tumor environment compared to myeloma cells. (a) Real-time RT-PCR was performed on RNA samples isolated from primary MMC and BM mononuclear cells depleted of myeloma cells (BM) from seven patients. (b) Real-time RT-PCR analysis was made as described in Materials and methods. Bars indicate median values.

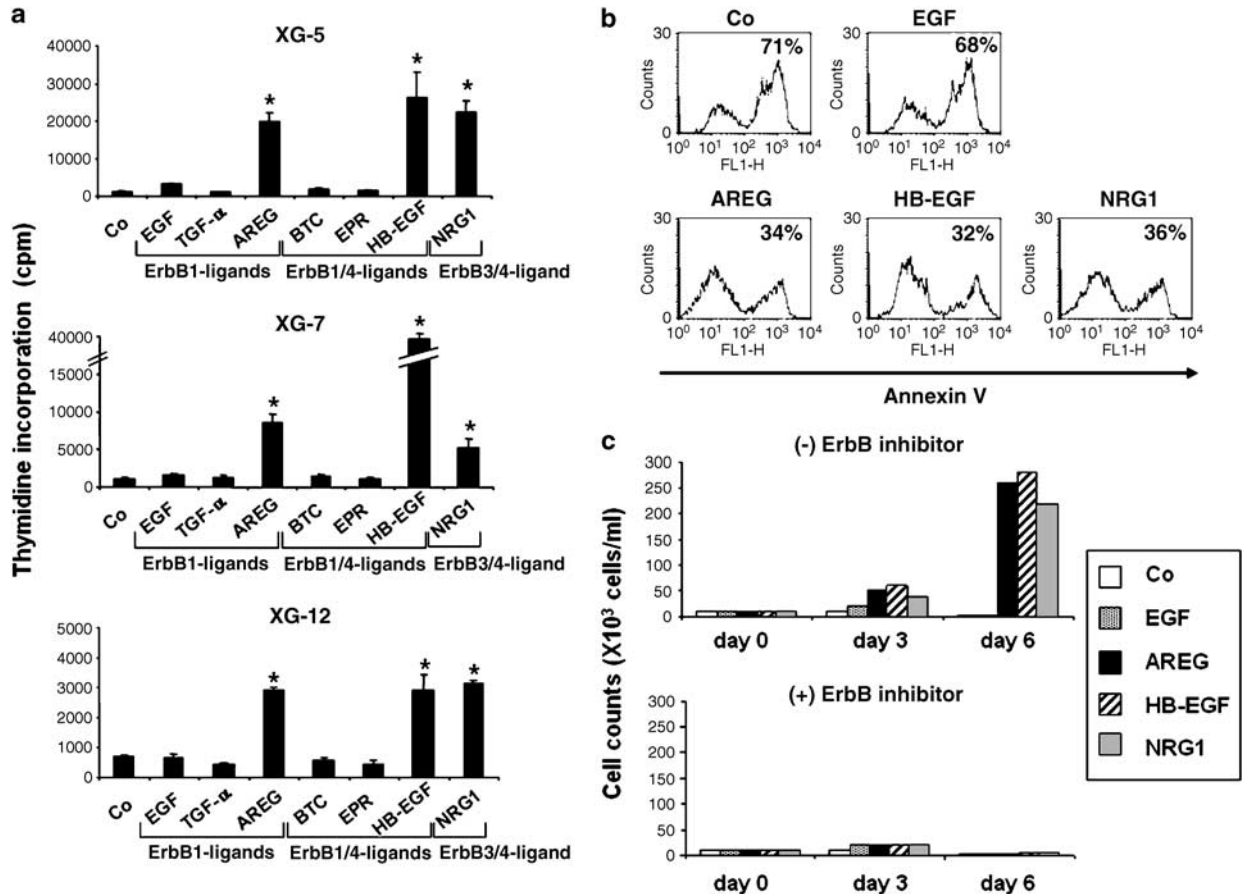


Figure 4 Only heparan-sulfate-binding EGF-family members have a myeloma cell growth factor activity. (a) XG-5, XG-7 and XG-12 cells were IL-6-starved for 3 h and cultured in RPMI1640 culture medium and 5% FCS with 5 pg/ml IL-6, with or without 1 μ g/ml of recombinant EGF, TGF- α , AREG, BTC, EPR, HB-EGF or NRG1. Cells were cultured for 6 days and pulsed for 12 h with tritiated thymidine at the end of the culture. Data are means \pm s.d. of the tritiated thymidine incorporation determined on sixplicate culture wells and are those of one experiment representative of three. * indicates that the mean value is statistically significantly different from that obtained without EGF family proteins (Co), using a Student's *t*-test ($P \leq 0.05$). (b) XG-7 cells were IL-6-starved for 3 h and cultured in RPMI1640 culture medium and 5% FCS with 5 pg/ml IL-6, with or without 1 μ g/ml of recombinant EGF, AREG, HB-EGF or NRG1. Cells were cultured for 6 days and stained with FITC-annexin V to determine the percentage of apoptotic cells. Results are of one experiment representative of three. (c) Cells were cultured for 6 days, with or without a pan-ErbB inhibitor (PD169540), and were counted at day 3 and day 6 of the culture. Data are of one experiment representative of three.

not shown) had no effect on myeloma cell proliferation. These data indicate that the ability to promote myeloma cell growth is not a common feature of all members of the EGF family. Only HS-binding EGF-ligands can enhance survival and proliferation of IL-6-dependent HMCLs.

Myeloma cells bind high levels of HS-binding EGF-family members through membrane HSPG

HB-EGF, AREG and NRG1 have a heparan sulphate (HS)-binding domain in contrast to EGF, TGF- α , BTC and EPR. This suggests that interaction of EGF-family members with cell surface HSPGs might be required to promote their MMC growth activity. To explore this hypothesis, we looked for the ability of MMC to bind EGF-ligands. XG-7 cells were preincubated with saturating concentrations of recombinant AREG, HB-EGF, NRG1 or EGF, washed, and stained with corresponding

specific antibodies. As shown in Figure 5a, a strong labelling of MMC was found with anti-AREG, anti-HB-EGF or anti-NRG1 antibodies when MMC were preincubated with either AREG or HB-EGF or NRG1 – mean fluorescence intensities (MFI) of, respectively, 1530, 1840 and 560 – suggesting that MMC could bind high amounts of these EGF-family ligands. No staining of MMC was detected with an anti-EGF antibody when MMC were preincubated with EGF (data not shown). AREG, HB-EGF, or NRG1 binding to MMC was dependant on HS chains since it was abrogated by pretreatment with heparitinase, an enzyme that cleaves heparan-sulfate chains of HSPGs (Figure 5a). Heparitinase did not affect syndecan-1 protein expression (Figure 5b). The binding of the three HS-binding EGF-family ligands to MMC was also abrogated by heparin (Figure 5c). These data indicate that a HSPG present on MMC membrane can bind large amounts of HS-binding EGF family ligands.

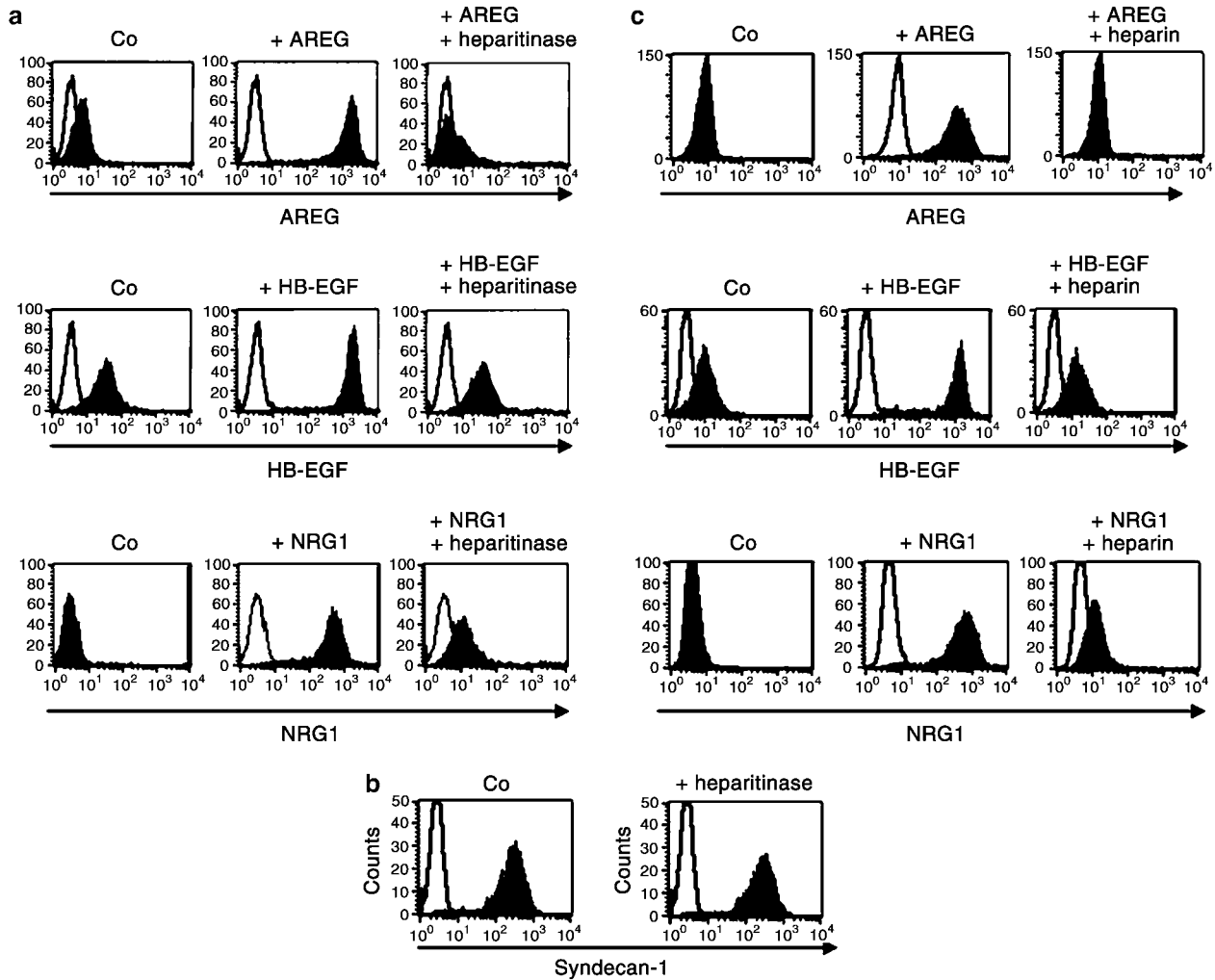


Figure 5 Myeloma cells bind HS-binding EGF-ligands through heparan-sulfate chains. (a) Cells were incubated with or without (Co) 1 μ g/ml of AREG or HB-EGF or NRG1, washed, and stained with corresponding antibodies (black histogram) or isotype control (open histogram), followed by PE-conjugated secondary antibody. When indicated, cells were pretreated with 10mU/ml of heparitinase for 1h. The fluorescence was determined using a FACSCalibur cytometer. (b) Surface expression of syndecan-1 was determined on XG-7 cells with a PE-conjugated anti-CD138 antibody (black histogram) and a PE-isotype control (open histogram), before and after treatment with 10mU/ml heparitinase for 1h. (c) Cells were incubated with or without (Co) 1 μ g/ml of AREG or HB-EGF or NRG1, preincubated with 4IU/ml heparin for 1h when indicated, washed, and stained with corresponding antibodies (black histogram) or isotype control (open histogram), followed by PE-conjugated secondary antibody. All results are those of one experiment representative of five.

Syndecan-1 is the main membrane HSPG on MMC able to bind HS-binding EGF members

Ten membrane HSPGs have been identified up to now: 4 members of the syndecan family and 6 members of the glypican one. We assessed their gene expression profile using Affymetrix U133A + B microarrays. The data are summarized in Table 1. *Syndecan-1* was highly expressed and displayed a 'present' Affymetrix call in all MMC and BMPC samples and in 19/20 (95%) HMCLs but was 'absent' in PPC and B cells, according to our previous data (Tarte *et al.*, 2002). Other HSPG genes, including *syndecan-2*, *-3* and *-4* and the six *glypican* genes, were not expressed ('absent' call) by most myeloma cell samples, and displayed a very weak signal compared to *syndecan-1*. This lack of expression cannot

be attributed to a failed detection by a non-functional Affymetrix probeset, as a high expression of the 10 HSPG genes could be detected on various normal tissues according to the data available (Su *et al.*, 2004) (data not shown). A further HSPG, the variant v3 of CD44, was very weakly expressed on HMCLs (MFI ranging from 5–15, data not shown), in agreement with previous reports (Derksen *et al.*, 2002; Van Driel *et al.*, 2002). Thus, *syndecan-1* is the major HSPG present on the surface of plasma cells.

To further investigate the role of syndecan-1 for the binding of EGF members, we quantified the number of recombinant AREG molecules that could be bound by MMC. Data are shown in Table 2. As expected from the high MFI found in Figure 5a, very large numbers of

Table 1 Gene expression profile of heparan-sulfate proteoglycans

Gene	B cells (n=7)		PPCs (n=7)		BMPCs (n=7)		MMC (n=65)		HMCLs (n=20)	
	Median	P (%)	Median	P (%)	Median	P (%)	Median	P (%)	Median	P (%)
<i>Syndecan-1</i>	19	0	29	0	1126	100	1286	100	347	95
<i>Syndecan-2</i>	19	0	4	0	4	0	22	0	2	1
<i>Syndecan-3</i>	3	0	3	0	3	0	10	0	3	0
<i>Syndecan-4</i>	69	29	54	14	54	3	55	14	39	2
<i>Glypican-1</i>	10	0	13	0	18	0	17	0	11	0
<i>Glypican-2</i>	36	0	32	0	32	0	67	0	28	4
<i>Glypican-3</i>	30	0	22	0	22	8	37	0	16	0
<i>Glypican-4</i>	11	0	14	0	14	2	26	0	15	2
<i>Glypican-5</i>	2	0	4	0	4	0	3	0	2	0
<i>Glypican-6</i>	7	0	4	0	4	9	7	0	7	3

Gene expression profile of *syndecan-1-4* and *glypican-1-6* was determined with Affymetrix U133A + B DNA microarrays in seven B cell samples, seven PPC samples, seven BMPC samples, purified malignant plasma cells of 65 patients with MM (MMC) and 20 HMCLs. The median expression level and the percentage of samples that display a 'present' call (P (%)) according to the Affymetrix definition are indicated for each gene.

Table 2 Number of syndecan-1 molecules expressed and AREG molecules bound on myeloma cells

	<i>Syndecan-1</i> ^a (molecules/cell)	<i>AREG</i> ^b (molecules/cell)	<i>AREG + heparin</i> ^b (molecules/cell)
XG-5	158 097 (± 9660)	213 902 (± 13 679)	76 (± 50)
XG-7	168 479 (± 17 713)	172 241 (± 20 814)	229 (± 224)
XG-12	592 758 (± 13 505)	431 487 (± 30 418)	511 (± 338)
XG-10	100 (± 29)	94 (± 16)	74 (± 21)

^aCells were labelled with the MI15 anti-syndecan-1 MoAb. ^bCells were incubated with 1 µg/ml of AREG (pre-incubated without or with 4 IU/ml heparin), washed and labelled with an AREG-specific mouse MoAb. The quantification of molecule numbers bound to cell membrane was done with QIFIKIT (DakoCytomation), using a set of calibrating beads precoated with an average of 0, 3400, 15000, 69000, 206000 and 591000 mouse IgG molecules/bead. Data are means (±s.d.) of the number of molecules determined on three independent experiments.

AREG molecules ($\geq 10^5$ molecules/cell) were bound to the membrane of the 3 HMCLs. In addition, the numbers of bound AREG molecules paralleled those of syndecan-1 present on the surface of MMC. In agreement with the data presented in Figure 5b, binding was abrogated by pretreatment with heparin. As we failed to downregulate syndecan-1 expression and the binding of EGF-ligands to MMC using syndecan-1-specific siRNA (this can be explained by the very high syndecan-1 expression level), we used XG-10, a HMCL that does not express syndecan-1, as a control (see Table 1 and Figure 6a). As shown in Figure 6b and Table 2, XG-10 cells were unable to bind large levels of AREG, HB-EGF or NRG1. In addition, XG-10 cells were not stimulated by HB-EGF or NRG1 although they expressed ErbB4 (Mahtouk *et al.*, 2005) (results not shown). Altogether, these data indicate that syndecan-1 is the main membrane HSPG on MMC able to bind HS-binding EGF members.

The binding of EGF-family members to myeloma cell HSPG is required for their myeloma cell growth activity
To demonstrate that interaction of HS-binding EGF-family members with HSPGs is a prerequisite for their

myeloma cell growth activity, we used two proof elements. First, heparin, used as a competitor of membrane syndecan-1, completely abolished the MMC growth promoted by AREG, HB-EGF or NRG (Figure 7a), as well as it abolished the binding of the growth factor to the MMC membrane (Figure 5c). The inhibitory effect of heparin was dose-dependant. A significant 40% inhibition was found with 0.1 IU/ml ($P \leq 0.05$), a 88% inhibition with 1 IU/ml, and a complete inhibition (99% inhibition) was obtained with 10–100 IU/ml (Figure 7b). Secondly, we used a feature of NRG1 that distinguishes it from HB-EGF and AREG. The NRG1 HS-binding domain (Ig-like domain) is separated from the ErbB-binding domain (EGF-like domain) by a spacer region (Loeb and Fischbach, 1995). This makes it possible to obtain two forms of recombinant NRG1, one including the Ig-like domain (NRG1), and one without it (Ig⁻-NRG1). Of interest, Ig⁻-NRG1 had no effect on XG-5, XG-7, or XG-12 HMCL contrary to NRG1 (Figure 7c). As expected, no binding of recombinant NRG1 devoid of the HS-binding Ig domain (Ig⁻-NRG1) could be found on XG-7 cells, whereas a strong labelling was obtained with the HS-binding NRG1 (Figure 7d).

Discussion

We have previously shown that EGF-family receptors are frequently expressed on MMC (Mahtouk *et al.*, 2005) and that their activation is required for survival of MMC cultured *in vitro* with their BM environment (Mahtouk *et al.*, 2004). We have shown that 2 members of the EGF family – AREG and HB-EGF – can support the growth of myeloma cells (Mahtouk *et al.*, 2004, 2005). The aim of the current study was to examine the global significance of all EGF-family members in MM. Using Affymetrix microarrays and real-time RT-PCR validations, we first provide a global picture of the expression of the 10 EGF-family members in MMC and throughout PC differentiation. Data are summarized in

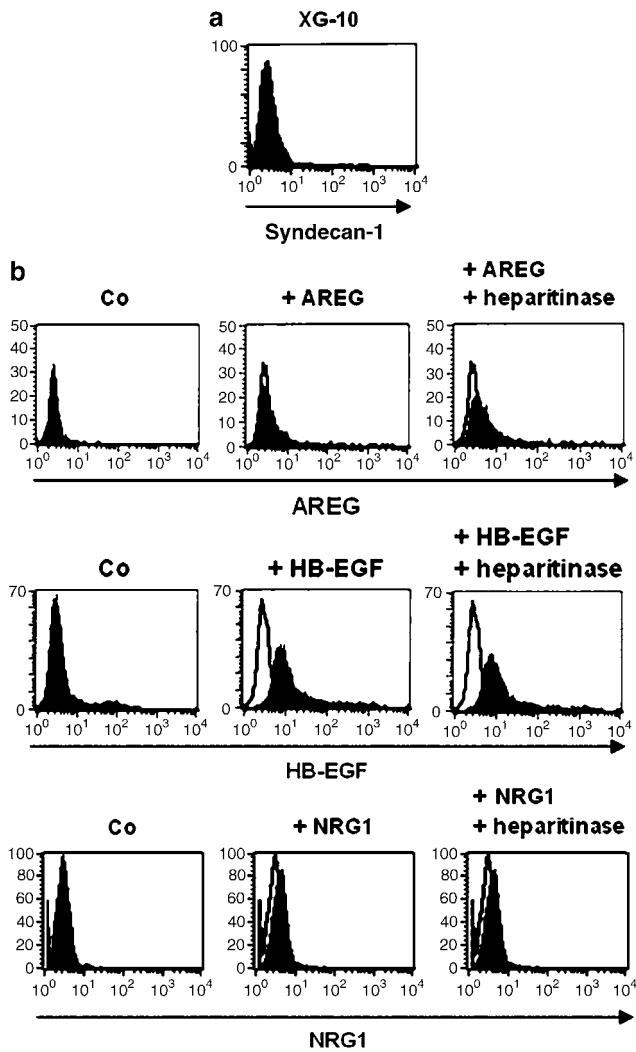


Figure 6 Syndecan-1 negative myeloma cells are not able to bind EGF-family members. (a) Surface expression of syndecan-1 was determined on the XG-10 myeloma cell line with a PE-conjugated anti-CD138 antibody (black histogram) and a PE-isotype control (open histogram). (b) XG-10 cells were incubated with or without (Co) 1 μ g/ml of AREG or HB-EGF or NRG1, washed, and stained with the corresponding antibodies (black histogram) or isotype control (open histogram), followed by PE-conjugated secondary antibody. When indicated, cells were pretreated with 10 mU/ml of heparitinase for 1 h. The fluorescence was determined using a FACSCalibur cytometer. Results are those of one experiment representative of five.

Figure 8. We show here that 7 of 10 EGF-family members are expressed by MMC and/or by cells from the BM environment. Five genes – *AREG*, *TGF- α* , *NRG1*, *NRG2* and *NRG3* – are expressed by primary MMC. Among them, two – *NRG2* and *NRG3* – are ‘myeloma genes’, that is, they are significantly over-expressed in MMC compared to B cells, PPC and BMPC, and display an Affymetrix ‘present call’ in MMC exclusively. Three other genes – *AREG*, *TGF- α* and *NRG1* – are ‘plasma cell genes’, that is, they are expressed both in normal and malignant PC but not in B cells and PPC. Thus they are induced during late plasma

cell differentiation, and their expression on MMC is – at least in part – the reflection of their normal counterpart. In addition, 4 EGF-family member genes (*HB-EGF*, *AREG*, *NRG1* and *EPR*) are expressed by the BM environment of MM patients. *HB-EGF* expression by the BM environment confirms our previously published data (Mahtouk *et al.*, 2004). Five of 10 EGF-family members – *TGF- α* , *NRG1*, *NRG2*, *NRG3* and *HB-EGF* – are expressed by a large panel of 20 HMCLs, indicating that autocrine activation loops may also be present in myeloma cell lines. Four of those five genes – *TGF- α* , *NRG1*, *NRG2* and *NRG3* – are common to MMC and HMCLs (see Figure 1). Regarding *AREG* and *HB-EGF*, there is an opposite pattern between HMCLs and MMC. None of the HMCLs express *AREG* contrary to MMC (Mahtouk *et al.*, 2005). *HB-EGF* is not expressed by MMC but is expressed by the BM microenvironment and by some HMCLs. Thus, HMCLs have acquired the ability to express growth factors, initially produced by the BM environment, and to use them as growth factors.

The high expression of several EGF-family members by MMC and/or the microenvironment provided the rationale to investigate their involvement in myeloma biology. We first observed that the ability to stimulate MMC growth was not a common feature to all EGF-ligands. EGF, BTC, EPER, *TGF- α* had no effect on MMC proliferation in spite of the expression of their specific receptors, ErbB1-4. Only the EGF family members able to bind HS chains – *AREG*, *HB-EGF* and *NRG1* – could stimulate the growth of MMC, which suggested a major contribution of HSPGs for the growth factors activity of EGF-family ligands. In agreement with our previous data (Mahtouk *et al.*, 2004), this effect was abrogated by an pan-ErbB inhibitor, indicating that the myeloma cell growth activity of HS-binding EGF-members is mediated by ErbB receptors. In the current experiments, cells were cultured at a low concentration to limit the biological activity of autocrine loops involving EGF-ligands. Indeed, we have previously shown that autocrine *HB-EGF* contributes to the IL-6-induced growth of some IL-6-dependent HMCLs cultured at a high cell density (De Vos *et al.*, 2001; Wang *et al.*, 2002; Mahtouk *et al.*, 2004). Compiling our previous data about ErbB expression in HMCLs with those of EGF-ligand expression, we provide here a comprehensive description of autocrine loops in HMCLs. Those data are summarized in Table 3 and indicate that functional autocrine loops can be found in 10/20 HMCLs. In those 10 HMCLs, at least one HS-binding EGF-ligand (that is, *AREG*, *HB-EGF*, *NRG1*–3) and its corresponding specific receptor are both expressed. We could not examine the function of *NRG2* and *NRG3* as those two growth factors have been little studied and recombinant proteins are not commercially available. *NRG2* and *NRG3* are myeloma genes and the high expression of *NRG3* in MMC of some patients is intriguing. One can anticipate *NRG2* to be a myeloma cell growth factor because *NRG1* and *NRG2* are closely related proteins that have the same specificity for ErbB receptors and

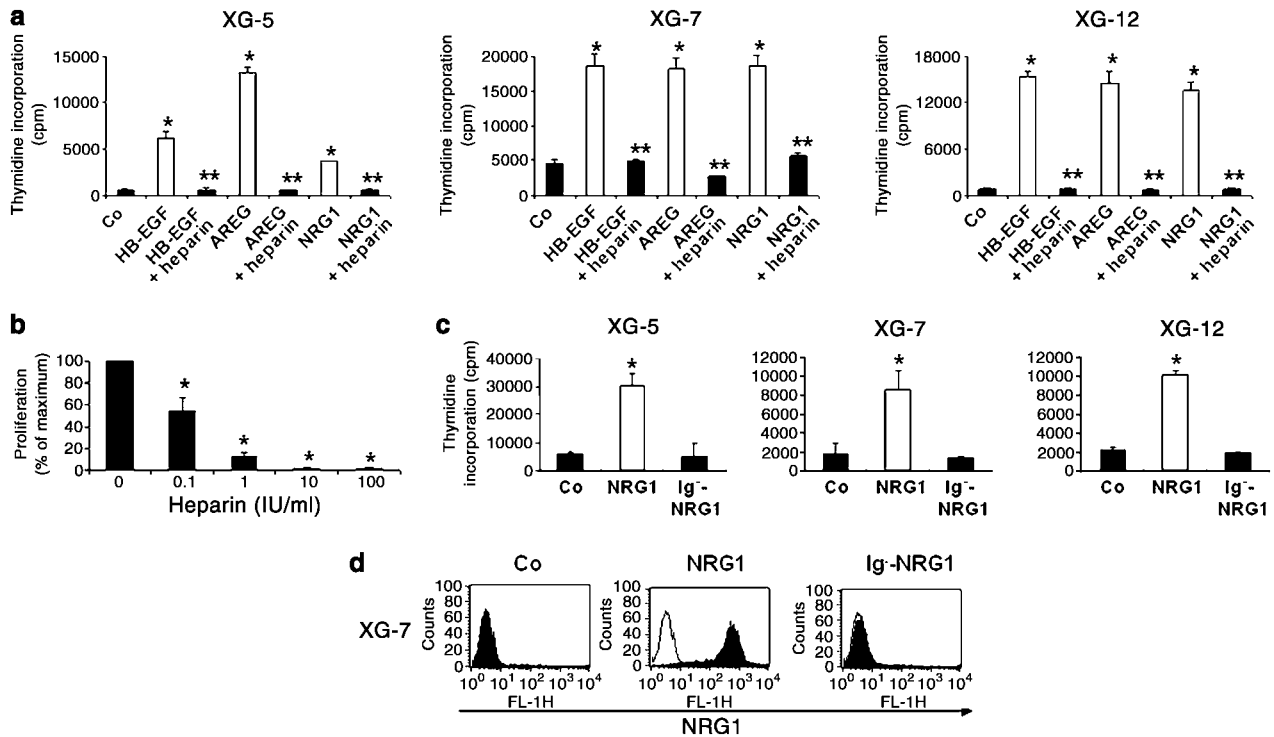


Figure 7 Binding of EGF-family members to HSPG is required for their myeloma cell growth factor activity. **(a)** HMCLs were cultured with 5 pg/ml IL-6, with or without 1 μ g of recombinant HB-EGF, AREG or NRG1. When indicated, growth factors were preincubated for 1 h with 4 IU/ml heparin at 4°C. * indicates that the mean value is statistically significantly different from that obtained without growth factor, using a Student's *t*-test ($P \leq 0.05$). ** indicates that the mean value is statistically significantly different from that obtained without heparin, using a Student's *t*-test ($P \leq 0.05$). **(b)** XG-7 cells were cultured with 5 pg/ml IL-6 and 1 μ g of recombinant HB-EGF, preincubated for 1 h at 4°C with different concentrations of heparin. * indicates that the mean value is statistically significantly different from that obtained without adding heparin, using a Student's *t*-test ($P \leq 0.05$). **(c)** HMCLs cultured with 5 pg/ml IL-6, with or without 1 μ g of recombinant NRG-1 or Ig⁻-NRG1. * indicates that the mean value is statistically significantly different from that obtained without adding NRG1 or Ig⁻-NRG1, using a Student's *t*-test ($P \leq 0.05$). In all experiments, data are means \pm s.d. of the tritiated thymidine incorporation determined on sixuplicate culture wells and are those of one experiment representative of three. **(d)** XG-7 cells were incubated with or without (Co) 1 μ g/ml of recombinant NRG1 or Ig⁻-NRG1, washed, and stained with anti-NRG-1 antibody (black histogram), or isotype control (open histogram), followed by FITC-conjugated secondary antibody, and analysed by FACS. Results are those of one experiment representative of three.

have a HS-binding (Ig-like) domain (Carraway *et al.*, 1997). NRG3 does not have this HS-binding Ig-like domain (Zhang *et al.*, 1997) but we cannot exclude that it can also bind HSPGs. The role of NRG2 and NRG3 will deserve further studies.

The restriction of the MMC growth activity to HS-binding EGF-ligands can be explained by the binding of large amounts of these factors by MMC. The binding was abrogated when MMC were pretreated with heparitinase, that cleaves HS chains, or when growth factor were preincubated with heparin, demonstrating the involvement of HS chains. We demonstrate that in MMC, the binding to a membrane HSPG is absolutely required for the MMC growth activity of EGF-family members. Indeed, heparin abrogated the stimulatory effect of AREG, HB-EGF and NRG1 on MMC proliferation. In addition, a truncated form of NRG1 corresponding to the EGF domain without the HS-binding domain was unable to bind to MMC and to promote myeloma cell growth, contrary to the complete form of NRG1. This NRG1 truncated form was previously reported to be biologically active on MCF-7

cells that expressed ErbB4 (Karey and Sirbasku, 1988). Using real-time RT-PCR, we have previously shown that expression of ErbB receptors is low on MMC. However, their activation is critical for MMC biology as a pan-ErbB inhibitor (which blocks the kinase activity of all three ErbB receptors with transducing activity) induced dramatic apoptosis of patient's myeloma cells cultured *in vitro* together with their environment and without adding exogenous growth factors (Mahtouk *et al.*, 2004). The binding of very high numbers of HS-binding EGF-family molecules to the MMC membrane makes it possible to understand why we found a potent growth factor activity of those ligands although their specific receptors are weakly expressed. HSPG probably increase the effective concentration at the cell membrane and then facilitate ErbB activation. An additional explanation might be that HSPGs induce a conformational change in EGF-ligands which would stabilize them in an active conformation, as it was shown for the chemokine IL-8 (Goger *et al.*, 2002). As the non HS-binding EGF-ligands have no effect on myeloma cells, one can speculate that they are unable to activate their

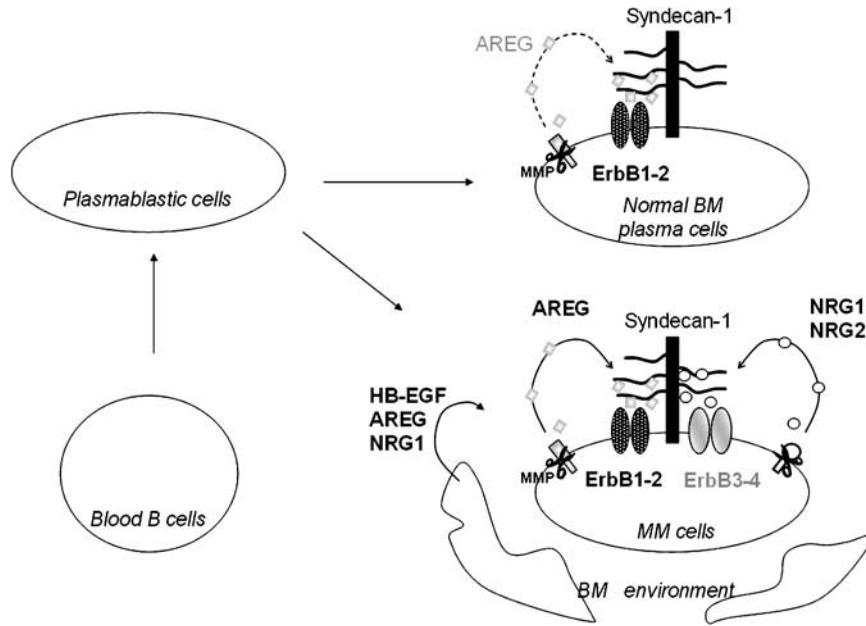


Figure 8 Syndecan-1, ErbB receptors and EGF-ligands are induced during plasma cell differentiation and malignant transformation. Blood B cells and plasmablastic cells neither express syndecan-1, nor ErbB receptors, nor EGF-ligands. Late plasma cell differentiation is associated with the development of a possible functional autocrine loop involving ErbB1 and ErbB2, their specific ligand AREG, and its co-receptor syndecan-1. Malignant transformation is associated with the development of an additional possible autocrine loop involving ErbB3 and ErbB4, their specific ligands NRG1-2, and their co-receptor syndecan-1. NRG1, AREG and HB-EGF are also produced by cells from the myeloma BM environment. Only the four ligands with a HS-binding domain, that is, that are able to promote myeloma cell growth, are represented here. MMP = metalloproteinase.

Table 3 Autocrine loops involving EGF-members and their receptors in HMCLs

HMCLs	<i>ErbB1</i>	<i>ErbB2/ErbB3</i>	<i>ErbB4</i>
XG1		NRG1	HB-EGF/NRG1
XG2		NRG2	
XG3			
XG4	HB-EGF		HB-EGF
XG5			
XG6			
XG7	HB-EGF		HB-EGF
XG10			
XG11			
XG12		NRG1	NRG1
XG13	HB-EGF		
XG14			
XG16			NRG3
XG19			
XG20	HB-EGF	NRG2	HB-EGF/NRG2/NRG3
LP1		NRG2	NRG2
OPM2	HB-EGF		NRG3/HB-EGF
RPMI			
SKMM			
U266			

Gene expression profile of EGF-ligands was determined with Affymetrix U133A+B DNA microarrays in 20 HMCLs. *ErbB* receptor expression was determined by real-time PCR (Mahtouk *et al.*, 2005). Only HS-binding EGF-ligands are indicated in the table. They are also indicated only when the corresponding specific receptor (that is, *ErbB1* for AREG, *ErbB1* and *ErbB4* for HB-EGF, *ErbB2/ErbB3* and *ErbB4* for NRG1 and NRG2 and *ErbB4* for NRG3) is also expressed in the HMCL. *ErbB3* has no transducing activity and is active only as a heterodimer with *ErbB2*.

receptors. However, as previously mentioned (Mahtouk *et al.*, 2004), we could not directly study the phosphorylation of ErbB receptors because their low density on

the MMC surface makes it difficult to immunoprecipitate them.

Several arguments suggest that syndecan-1 is the major membrane HSPG involved in the binding of EGF members: (i) among the 11 known membrane HSPG (Belting, 2003), *syndecan-1* was the main one to be expressed by normal and malignant plasma cells. (ii) The number of HS-binding EGF family members bound on MMC was very large ($1.7\text{--}4.4 \times 10^5$ molecules/cell) and was in the same range than the number of membrane syndecan-1 molecules. (iii) XG-10 myeloma cells, which express virtually no syndecan-1 molecules on the surface (around 100 molecules/cell), could not bind HB-EGF or AREG and were not stimulated by HB-EGF, in spite of *ErbB4* expression (data not shown). It is noteworthy that syndecan-1, ErbB receptors and HS-binding EGF-family ligands are concomitantly expressed during normal plasma cell differentiation at the stage of plasma cells (Figure 8).

The function of syndecan-1 has been largely described in normal and tumor epithelial cells where it plays major roles, partly due to its ability to bind growth factors and chemokines (Bernfield *et al.*, 1999; Sanderson *et al.*, 2004). On MMC, the function of syndecan-1 is not fully elucidated yet. The soluble form of syndecan-1 is detected in the serum of patients with MM (Dhodapkar *et al.*, 1997) and is an indicator of poor prognosis (Klein *et al.*, 1999; Seidel *et al.*, 2000b). Soluble syndecan-1 also accumulates within the tumor BM environment of MM patients (Seidel *et al.*, 2000a; Bayer-Garner *et al.*, 2001). As this soluble form of syndecan-1 is biologically active (Dhodapkar *et al.*, 1997), one can speculate that

MMC are likely 'bathed' in high concentrations of EGF-family ligands (produced by MMC themselves and/or by the BM environment) that are either bound to MMC via membrane syndecan-1 or sequestered within the extracellular matrix via soluble syndecan-1, in the proximity to the MMC. Derksen *et al.*, (2002) reported that syndecan-1 acts as a coreceptor for hepatocyte growth factor (HGF) to promote HGF/met signalling in MM cells. Together with our findings, this suggests that interaction of HS-binding growth factors with syndecan-1 might be a general mechanism to promote MMC growth. Whether our present data can be extended to other myeloma growth factors is under active investigation in the laboratory. Two recent studies demonstrated that APRIL, a growth factor for myeloma cells (Moreaux *et al.*, 2004), can bind HSPG (Hendriks *et al.*, 2005; Ingold *et al.*, 2005). One study reported that IL-6, the major known MMC growth factor (Klein *et al.*, 1989), is a heparin-binding cytokine and that a heparin mimetic inhibits the biological activity of IL-6 and the binding to its receptors (Mummery and Rider, 2000). However, we failed to find a large binding of IL-6 molecules to MMC, which can be detected with anti-IL-6 MoAbs and FACS analysis (unpublished data).

The failing of MMC to bind HB-growth factors in the presence of heparin, and the resulting reduction of EGF-ligand-induced proliferation of MMC suggest that heparin – or its non-anti-coagulant derivatives (Kragh and Loechel, 2005) – could be of interest for MM treatment. Of note, the HB-EGF-induced proliferation of myeloma cells was inhibited by 46% with 0.1 IU/ml of heparin, which is in the range of therapeutic concentrations. One recent study has reported that a short-term treatment with heparin could improve the survival of patients with metastatic epithelial cancers (Klerk *et al.*, 2005). One possible mechanism might be that heparin competes with HS for the binding of growth factors preventing their stimulatory effect on tumor cells.

Taken together with our previous data showing that a pan-ErbB inhibitor induces strong apoptosis of primary MMC, the present study emphasizes that EGF-signalling should be considered as a potential therapeutic target in MM. ErbB-specific inhibitors, heparin, or a combination of those molecules could be of major therapeutic benefit for the treatment of MM patients.

Materials and methods

Cell samples

Multiple myeloma cells were purified from 65 consecutive patients with MM at diagnosis (median age, 59 years) after informed consent was given. According to Durie–Salmon classification, 12 patients were in stage IA, 12 in stage IIA, 38 in stage IIIA, and three in stage IIIB. Eleven patients had IgA κ MM, 7 IgA λ MM, 26 IgG κ MM, 9 IgG λ MM, 7 Bence–Jones κ MM, 3 Bence–Jones λ MM and 2 non-secreting MM. Bone marrow plasma cells were obtained from healthy donors after informed consent was given. Plasma cells were purified with anti-CD138 MACS microbeads (Miltenyi-Biotec, Paris,

France). Plasmablastic cells were generated from purified CD19⁺ peripheral blood B cells *in vitro* as previously described (Tarte *et al.*, 2000). BM mononuclear cells from seven patients were obtained by removing MMC with CD138 Miltenyi microbeads (<1% plasma cells). Human IL-6-dependent myeloma cell lines (HMCLs) were obtained in our laboratory (Zhang *et al.*, 1994; Rebouissou *et al.*, 1998). They were routinely maintained in RPMI1640, 10% fetal calf serum, and 2 ng/ml of IL-6.

Reagents

Recombinant IL-6 was purchased from Abcys SA (Paris, France) and EGF-family growth factors were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies used were PE-conjugated anti-CD138 (Beckman-Coulter, Marseille, France) and anti-EGF-family growth factors all from R&D Systems.

Microarray hybridization

RNA was extracted with the RNeasy Kit (Quiagen, Valencia, CA, USA). Biotinylated complementary RNA (cRNA) was amplified with a double *in-vitro* transcription, and hybridized to the human U133 A and B GeneChip microarrays according to manufacturer's instructions (Affymetrix). Fluorescence intensities were quantified and analysed using the GECOS software (Affymetrix).

Real-time reverse transcriptase-polymerase chain reaction

For real-time RT–PCR, we used the assay-on-demand primers and the TaqMan Universal Master Mix from Applied Biosystems (Courtaboeuf, France) according to the manufacturer's instructions. Real-time RT–PCR was performed using the ABI prism 7000 Sequence Detection system. Data were analysed as previously described (Mahtouk *et al.*, 2005) and were normalized to GAPDH for each sample. MCF-7 cells (for NRG3 and NRG4) or A431 cells (for all other genes) were used as control cell lines and were assigned the arbitrary value of 100.

Myeloma cell proliferation assay

Cells were IL-6 starved for 3 h and cultured for 6 days in 96-well flat-bottomed microtiter plates at 10⁴ cells/well in 200 μ l of RPMI 1640 culture medium and 5% FCS, with a low concentration of IL-6 (5 pg/ml). Growth factors (1 μ g) were added at the beginning of the culture in six culture wells per group. At the end of the culture, cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech, Orsay, France) for 12 h, harvested and counted as reported previously (De Vos *et al.*, 2001).

Detection of apoptotic cells and cell counts

Myeloma cells were cultured for 3 days in 24-well flat-bottomed microtiter plates at 10⁴ cells/well in 1 ml of RPMI 1640 medium with 5% FCS and various growth factors. Cells were counted at day 3 and 6 of the culture. At day 6, they were washed once in PBS and suspended in Annexin-V FITC solution (Boehringer, Mannheim, Germany). Fluorescence was analysed with a FACScan flow cytometer.

Detection of growth factor binding by FACS

Cells were incubated with 1 μ g/ml of HB-EGF, AREG, NRG1 or Ig⁻-NRG1 for 1 h at 4°C and washed twice in PBS before incubation with the corresponding antibodies. The fluorescence was determined using a FACSCalibur flow-activated

cytometer (Becton Dickinson). To cleave the HS chains, myeloma cells were pretreated with 10 mU/ml heparitinase (EC 4.2.2.8, Sigma, St Louis, MO, USA) for 1 h at 37°C. When indicated, added recombinant EGF-ligands were preincubated with 4 IU/ml of heparin (Heparin Choay[®], 25000 UI/5 ml) for 1 h at 4°C.

Quantitative analysis of immunofluorescence staining

The quantification of syndecan-1 or AREG molecules bound to the membrane of MMC was done using the DAKO QIFIKIT (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions.

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Statistical analysis

Gene Expression Profiles were analysed with our bioinformatics platform (RAGE, remote analysis of microarray gene expression, <http://rage.montp.inserm.fr>) designed by T. Reme (INSERM U475, Montpellier, France). Statistical comparisons were made with the non-parametric Mann–Whitney test or the Student's *t*-test.

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Malgré les progrès récents dans le traitement du MM, cette pathologie reste à l'heure actuelle incurable. De nouveaux espoirs pour le traitement des cancers reposent aujourd'hui sur de nouveaux médicaments qui ciblent une protéine jouant un rôle important dans la biologie des cellules tumorales. Dans le MM, cette approche a été développée dès le début des années 90 avec l'utilisation d'anticorps anti-IL-6. Cependant, bien que les anticorps anti-IL-6 aient un effet anti-tumoral chez les malades, un problème majeur est la quantité trop importante d'IL-6 produite chez les patients et qui est difficilement neutralisable par des anticorps. De nouvelles molécules visant à bloquer des facteurs de croissance (IGF1, VEGF, FGFR3...) importants dans la biologie du MM sont actuellement testés (301). Aujourd'hui, le Velcade (inhibiteur du protéasome) apparaît comme un nouveau traitement du MM très prometteur. Le Velcade inhibe la voie NF κ B et stimule la voie proapoptotique liée au stress du réticulum endoplasmique (302). Bien que tous les mécanismes d'action du Velcade ne soient pas encore définis, il illustre parfaitement le fait que le développement de nouveaux traitements reposera sur une meilleure connaissance de la physiopathologie du MM. L'hétérogénéité de la maladie et la multitude de facteurs de croissance impliqués dans le MM rendent difficile la compréhension du rôle de chacun et nous font poser des questions : tous les facteurs de croissances sont ils nécessaires à la croissance des cellules tumorales ? Comment ces facteurs de croissance interagissent et fonctionnent ensemble ? Quelle place occupe l'IL-6 ? Quelle est la place de syndecan-1 ? Toutes ces questions sont importantes afin de mieux comprendre la biologie du MM et de pouvoir proposer aux patients des traitements ciblés efficaces.

III-3. Discussion générale et perspectives

Cette étude illustre l'intérêt des puces à ADN pour l'identification de nouvelles cibles thérapeutiques. Le point de départ de mon travail de thèse a été l'observation, faite à l'aide des puces à ADN, que les plasmocytes tumoraux et normaux surexpriment les récepteurs BCMA et TACI par rapport aux cellules B normales. En l'espace de trois ans, nous avons pu comprendre et décrire de manière générale l'implication des membres de la famille BAFF/APRIL dans le MM et un essai clinique de phase I/II utilisant un inhibiteur de BAFF/APRIL dans la MM a démarré au CHU de Montpellier en décembre 2004. En parallèle, les données des puces à ADN ont permis au laboratoire d'identifier et de décrire l'implication des membres de la famille EGF dans le MM. La mise en place d'un essai clinique de phase I/II utilisant un inhibiteur de la famille EGF est aujourd'hui envisagé dans le MM. Plus récemment, nous avons identifié grâce aux microarrays un nouveau facteur pronostic du MM : le CD200 qui s'avère représenter une nouvelle cible thérapeutique intéressante dans cette pathologie. Bien que l'apport des puces à ADN soit incontestable pour identifier de nouveaux facteurs importants dans la biologie du MM, une analyse biologique poussée est nécessaire pour affiner le concept issu des données du transcriptome. En effet, la découverte du lien entre APRIL/TACI et le protéoglycane syndecan-1 laisse présager des difficultés pour inhiber efficacement cette voie *in vivo*. Dans la moelle osseuse, syndecan-1 soluble est présent à de très fortes concentrations. Syndecan-1 soluble peut contribuer à conserver des taux élevés d'APRIL dans l'environnement tumoral. L'utilisation d'un inhibiteur de type TACI-Ig ou BCMA-Ig visant à séquestrer BAFF et APRIL dans la moelle osseuse de malades pourrait

s'avérer insuffisante. De plus, tout comme pour HGF, la demi-vie d'APRIL, une fois lié à syndecan-1, pourrait être considérablement augmentée.

Désormais, un enjeu important réside dans la manière optimale d'intégrer ces nouvelles molécules parmi les traitements existants et d'identifier des méthodes permettant de sélectionner les patients susceptibles de répondre le mieux au traitement envisagé. C'est notamment ce que nous avons cherché à faire en comparant les profils d'expression génique des cellules de MM de patients exprimant fortement TACI avec ceux de patients exprimant faiblement TACI. L'utilisation de ces profils d'expression génique pourra servir à identifier le groupe de patients pouvant bénéficier d'un traitement basé sur des inhibiteurs de la voie BAFF/APRIL. Des études transcriptomiques identifiant des signatures de gènes prédisant l'évolution et/ou la réponse aux traitements ont été publiés dans certains cancers tels que les sarcomes (303), le cancer du sein (304), le cancer du colon (305), les LAL pédiatriques (306), le cancer de l'ovaire (307), les lymphomes diffus à grandes cellules (308, 309), les lymphomes du manteau (310, 311) et les LAM (312, 313).

L'approche la plus prometteuse sera probablement de coupler plusieurs traitements spécifiques, chacun ciblant un mécanisme biologique bien particulier de la cellule tumorale. **C'est le principe du traitement à la carte, différent pour chaque malade, qui vise les cibles thérapeutiques présentes.** C'est dans cette optique que nous avons initié au CHU de Montpellier le transcriptome systématique pour tout nouveau patient atteint de MM au diagnostic. Ceci s'accompagne d'un rendu aux cliniciens par l'intermédiaire d'une fiche avec les données d'expression de gènes codant pour des protéines liées à un mauvais pronostic, impliquées dans la résistance aux agents de chimiothérapie et constituant des cibles thérapeutiques potentielles.

En conclusion, ce travail de thèse démontre l'importance des membres de la famille BAFF/APRIL dans le MM ainsi que le rôle majeur des puces à ADN pour la compréhension de la biologie de cette pathologie et l'identification de nouvelles cibles thérapeutiques. Il met également en évidence le rôle central de syndecan-1 dans la physiopathologie du MM. Ce travail ouvre de nouvelles perspectives thérapeutiques visant à cibler les membres de la famille BAFF/APRIL, syndecan-1 et CD200 dans le traitement du MM.

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ROLE DES MEMBRES DE LA FAMILLE BAFF/APRIL DANS LE MYELOME MULTIPLE : IMPLICATIONS PHYSIOPATHOLOGIQUES ET INTERET THERAPEUTIQUE

Le myélome multiple (MM) est une néoplasie B caractérisé par l'accumulation d'un clone plasmocytaire dans la moelle osseuse. Cette pathologie demeure incurable d'où la nécessité d'identifier de nouvelles cibles thérapeutiques. C'est notamment dans cette optique que nous avons initié, au sein du laboratoire, un travail de comparaison des profils d'expression génique des plasmocytes tumoraux purifiés de malades avec ceux de plasmocytes normaux et de lymphocytes B, ce qui permettra l'identification de nouvelles voies importantes pour la biologie du MM et donc de nouvelles cibles thérapeutiques potentielles.

Par cette approche, nous avons mis en évidence un rôle essentiel des membres de la famille BAFF/APRIL et de leurs récepteurs (BCMA, BAFF-R et TACI) dans la biologie du MM. Les cellules de MM expriment les récepteurs alors que les ligands sont principalement produits par les cellules de l'environnement médullaire. L'utilisation d'un inhibiteur spécifique de BAFF/APRIL a permis de montrer que ces facteurs de croissance sont importants pour la survie et la prolifération des cellules tumorales. TACI apparaît être le récepteur principal pour médier l'effet de BAFF et APRIL dans le MM. Une forte expression de TACI par les cellules de MM est associée à une signature génique de plasmocytes matures alors que les plasmocytes tumoraux présentant une faibles expression de TACI ont une signature génique de plasmablastes proliférants. Nous avons montré que syndecan-1, un protéoglycane à chaînes héparane sulfaté joue un rôle essentiel dans la biologie du MM en permettant l'accumulation de fortes concentrations de facteurs de croissance à la surface des cellules. Nous avons identifié que syndecan-1 joue un rôle de corécepteur pour APRIL et TACI supportant ainsi la croissance des cellules de MM.

Ces travaux offrent de nouvelles perspectives thérapeutiques pour le MM et ont débouché sur un essai clinique de phase I/II, au CHU de Montpellier, utilisant un inhibiteur de la voie BAFF/APRIL dans le MM.

Mots clés : myélome multiple, facteurs de croissance, famille BAFF/APRIL, inhibiteur de BAFF/APRIL, syndecan-1.