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SURVIVAL AND PROLIFERATION FACTORS OF NORMAL AND MALIGNANT PLASMA CELLS

Bernard Klein[1], Karin Tarte[1], Michel Jourdan[1], Karene Mathouk[1], Jerome Moreaux[1], Eric Jourdan[2], Eric Legouffe[3], John De Vos[1], Jean François Rossi[3]

1. INSERM U475 and Unit for Cellular and Gene therapy, CHU Montpellier, 34285 Montpellier. France

2. Service de Médecine Interne B, CHU de Nîmes, 30900, Nîmes, France.

3. Service d'Hématologie et Oncologie Médicale, CHU Montpellier, Hôpital Lapeyronie, 34295 Montpellier, France

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Corresponding author.

Pr Bernard Klein
INSERM U475
99Rue Puech Villa
34197 Montpellier, France
Email: klein@montp.inserm.fr

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Abstract

Since the first identifications of interleukin(IL)-6 as a myeloma cell growth factor by Dr Kawano's and Dr Klein's groups 14 years ago, numerous studies have emphasized its major role in the emergence of malignant plasma cells in vivo and in the generation of normal plasma cells.

Four transcription factors control B cell differentiation into plasma cells. The B cell transcription factor pax-5 is mainly responsible for a B cell phenotype and bcl-6 represses the plasma cell transcription factor blimp-1 and plasma cell differentiation. *Bcl-6* expression is triggered by CD40 and IL-4 activation. A lack of CD40 and IL-4 activation yields a down regulation of *bcl-6* expression and IL-6 stimulation an upregulation of blimp-1, mainly through STAT3 activation. Blimp-1 will further downregulate *bcl-6* and *pax-5* expression and makes it possible plasma cell differentiation. IL-6 as well as IL-10 upregulate XBP-1. XBP-1 is another transcription factor involved in plasma cell differentiation whose gene expression is shut down by pax-5.

These plasma cell transcription factors blimp-1 and XBP-1 are upregulated and the B cell transcription factors bcl-6 and pax-5 downregulated in malignant cells compared to B cells. Apart for this recent identification of these four transcription factors, the factors involved in normal plasma cell generation are mostly unknown.

Regarding malignant plasma cells, three categories of growth factors have been identified. 1) the IL-6 family cytokines, IL-10 and IFNα that activate the JAK/STAT and MAPK pathways. 2) growth factors activating the PI-3 kinase/AKT and MAPkinase pathways, unlike the JAK/STAT pathway (insulin like growth factor 1, hepatocyte growth factor and members of the epidermal growth factor family able to bind syndecan-1 proteoglycan). 3) BAFF or APRIL that activate the NF-kappaB and PI-3 kinase/AKT pathways. BAFF and APRIL bind to BAFF receptor and TACI and are major B cell survival factors. Recent data indicate that

these various growth factors may cooperate together to provide optimum signalling, eventually because ther are colocalized together and with cytoplasmic transduction elements in caveolin-linked membrane caveolae.

The identification of these myeloma cell growth factors and of the associated transduction pathways should provide novel therapeutic targets in multiple myeloma.

Since 15 years, numerous studies have been devoted to the study of myeloma growth factors. These myeloma growth factors may be specific to the myeloma clone or involved in the generation of normal plasma cells. In a first part, we will briefly review the recent knowledge of the biology of normal plasma cells and then discuss the major growth factors involved in multiple myeloma (MM).

1. Biology of normal plasma cells

Mature plasma cells are mostly located in the bone marrow where they represent 0.25% bone marrow mononuclear cells and in the lamina propria of mucosae. Due to their rarity, the process of generation of normal plasma cells and their biology are poorly known. Plasma cells are generated in the lymph node where B cells with a high affinity antigen receptor (Ig) are selected by the antigen through mutations of the Ig variable genes. Selected B cells are then induced to become either a memory B cell or a plasmablastic cell. The plasmablastic cells are supposed to migrate rapidly to the bone marrow where they can find additional survival and differentiation factors making it possible their long-term survival and differentiation into mature plasma cells. A hallmark of mature plasma cells is their large Ig secretion, a high expression of the syndecan-1 proteoglycan that is not expressed on B cells and a lack of most B cell markers except CD19. These PC also largely express CD38.

The intercellular communication signals that are critical to induce B cell differentiation into plasmablastic cell are poorly known. Plasmablastic cells can be greatly expanded in vivo in some patients with acute inflammation. These plasmablastic cells are highly proliferating and short living. They comprise syndecan-1^- immature plasmablastic cells that can yield syndecan-1^+ plasmablastic cells. [1] We recently developed an in-vitro model of generation of polyclonal plasmablastic cells (PPC) starting from healthy donor's or MM patient's peripheral blood B cells. [2] This model consists of culturing memory B cells with a CD40 ligand

transfectant, interleukin(IL)-4, IL-2, IL-10 and IL-12 for 4 days before induction of plasma cell differentiation is induced by removal of CD40 stimulation and change in cytokine combination: removal of IL-4 and addition of IL-2, IL-6 and IL-10 and IL-12. These plasmablastic cells are highly proliferating but apoptose at days 7-8 of culture. This model should be critical to better understand the mechanisms controlling the survival of plasmablastic cells and mature plasma cells in the bone marrow [3]. In particular, we recently compared gene expression profiles of PPC and bone marrow plasma cells and identified several autocrine and paracrine loops, associated with an induction of anti-apoptotic proteins, that can be involved in the prolonged survival of mature plasma cells [4].

As illustrated up in Figure 1, memory B cells express Bcl-6 and Pax-5 whereas these transcription factors are donwregulated in plasmablastic cells. On the contrary, plasmablastic cells expressed the plasma cell transcription factors Blimp-1 and XBP-1. Actually, according

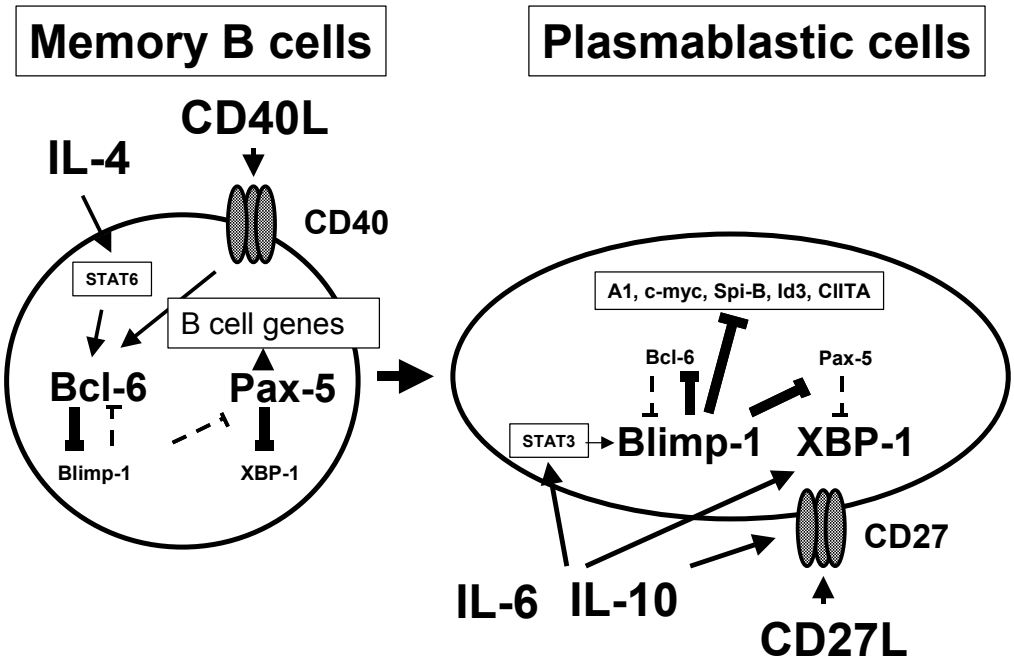

Figure 1: Transcription factors involved in plasma cell differentiation

to the data of the literature, [3] one can hypothesize that, in germinal center B cells, IL-4 upregulates bcl-6 transcription through STAT6 phosphorylation and CD40 stimulation blocks Bcl-6 degradation. Bcl-6 in turn blocks *Blimp-1* gene expression.

Removal of IL-4 and CD40 signals make it possible a downregulation of bcl-6 and an expression of blimp-1 that is triggered by IL-6 and IL-10 activating the JAK/STAT pathway, mainly STAT3. Blimp-1 is a transcriptional repressor that downregulates *bcl-6* and *Pax5* as well as numerous other B cell genes. Pax-5 is critical for B cell maintenance and its overexpression may block plasma cell phenotype in plasma cell lines. Pax-5 directly represses *XBP-1* gene that encodes for a second major plasma cell transcription factor whose gene targets are poorly unidentified. In our model of PPC generation, we found that activated B cells coexpress CD70 and CD27 suggesting that an activation of CD27 together with IL-10 takes part in the process of plasmablastic cell generation. [2] Indeed, CD27 is expressed on memory B cells and highly on plasma cells [5] and triggering CD27 with CD70, the CD27 ligand, together with interleukin (IL)-10 induces plasma cell differentiation in vitro. [6] IL-6 also plays a major role, in part by inducing STAT3 phosphorylation that will trigger *Blimp-1* expression and probably through induction of *XBP-1* transcription[7]. Recently, XBP-1 was described as an inducer of IL-6 production [8] suggesting the existence of an amplification loop between IL-6 and XBP-1.. Jego et al. using plasmablastic cells from patients with reactive plasmacytosis showed a major role of IL-6 in plasma cell differentiation. [1] In this model, the differentiation of syndecan-1$^-$ plasmablastic cells into syndecan-1$^+$ early plasma cells was blocked with antibodies to IL-6. This property of IL-6 is not surprising since IL-6 gene was initially cloned in 1988 as a B cell differentiation factor. [9] In addition, transgenic mice expressing an IL-6 gene driven by an Eμ promoter develop massive polyclonal plasmacytosis [10]whereas IL-6 knock out mice have a defect in the production of high affinity antibodies. [11] [12] As pointed above, the polyclonal plasmablastic cells generated in our in vitro model rapidly apoptose in vitro, on days 7-8 after starting the cultures of B cells, 3-4 days after removal of CD40 stimulation, despite the addition of various cytokines: IL-6, sIL-6R, IL-10, IL-2, IL-12.

This apoptosis is associated with a rapid downregulation of several genes coding for anti-apoptotic proteins, the A1 protein of the bcl-2 family member and the c-IAP2 inhibitor of caspase activity. Conversely, we found an upregulation of the Bik, caspase 3 and caspase 10 genes, coding for pro-apoptotic proteins. The down regulation of A1 is likely a direct consequence of Blimp-1 expression that blocks *A1* transcription.

Thus in conclusion, only two intercellular communication pathways have been described for normal plasma cells: IL-6 and activation of CD27 and IL-10. It is not presently known whether the factors known to induce the growth of malignant plasma cells - IGF-1, EGF family, HGF, BAFF/APRIL - are also involved in the biology of normal plasma cells. Nor are known the transduction pathways that are activated in normal plasma cells resulting in their cell survival and proliferation. We can expect that at least a part of the growth factors recently identified for malignant plasma cells are also involved in normal plasma cell biology.

2. Myeloma cell survival and proliferation factors

Numerous studies have been devoted to the identification of myeloma cell growth factors and to the signalling pathways leading to survival and/or proliferation of myeloma cells. A first category of factors activates the JAK/STAT and MAP kinase pathways (mainly IL-6). Another category involves the PI-3 kinase/AKT as well as MAPkinase and NF-kappa B pathways.

2.1. Factors activating the JAK/STAT and MAP kinase pathways: IL-6, cytokines of IL-6 family, interferon alpha

IL-6 binds to a specific receptor (IL-6R) and the complex IL-6/IL-6R binds and induces the homodimerization of the gp130 IL-6 transducer. [13] A remarkable feature of IL-6R is that its soluble form (sIL-6R) is an agonist molecule. It binds IL-6 with the same affinity as

membrane IL-6R and the complex IL-6/sIL-6R binds and activates gp130. [13] The evidences of a major role of IL-6 in the survival and proliferation of malignant plasma cells accumulated since the initial reports by others and us 15 years ago. [14,15] These evidences are the following:

1) Antibodies to IL-6 block the myeloma cell proliferation and reduce by 50% the number of myeloma cells in culture of patients' bone marrow cells *in vitro* [14-16]
2) Injection of anti-IL-6 monoclonal antibody inhibited myeloma cell proliferation in patients with terminal disease [17,18] if the antibody was injected at a sufficient concentration to block the large IL-6 production in vivo. [19]
3) Serum levels of IL-6 and soluble IL-6R are increased in patients with MM in association with a poor prognosis. [20,21]
4) The bone marrow environment of patients with MM, mainly by monocytes, myeloid cells and stromal cells, overproduces IL-6. [15,22] This production of IL-6 by the tumor environment is mostly mediated by IL-1 that is produced by monocytes and myeloma cells. [22,23] IL-1 induces PGE2 synthesis that further triggers IL-6 production. [23] Thus inhibitors of IL-1 as the IL-1 receptor antagonists or of PGE2 synthesis might be interesting to block IL-6 production in patients with MM. A similar mechanism was shown in the model of murine plasmacytoma in BALB/C mice. The generation of plasmacytomas was blocked by chronic administration of indomethacin that inhibited PGE2 synthesis and the large IL-6 production by the inflammatory environment. [24] Myeloma cells can also directly trigger IL-6 production by direct contact with the bone marrow stromal cells by unidentified mechanisms. [25,26]
5) Cell lines whose survival is dependent on addition of exogenous IL-6 can be obtained from patients with extramedullary proliferation. [27]
6) Mice transgenic with an IL-6 gene driven by the Eμ promoter develop massive polyclonal plasmacytosis. [28] When crossed with murine BALB/c mice that

spontaneously develop plasmacytomas, these crossed mice develop malignant plasma cell prolferation. [29] In addition, knock out of IL-6 gene abrogated the generation of malignant plasmacytomas in BALB/C mice primed with mineral oil. [30]

Other cytokines of the IL-6 family are also myeloma cell growth factors due to the expression of specific receptors: OSM, CNTF, IL-11, LIF. [31] But these factors are likely not involved in the emergence of the disease in vivo as they are weakly produced by the tumor or its environment. [32] In our hands, interferon-alpha (IFNα) is also a myeloma cell survival factor that is independent of IL-6. [33,34] IFNα activated the JAK/STAT and MAP kinase pathways as IL-6, in particular STAT3 phosphorylation. [34] Other groups found that IFNα could block myeloma cell proliferation. This discrepancy might be explained by the ability of IFNα to induce P19 inhibitor in some cell lines yielding to apoptosis. [35] Finally, we found that IL-10, a potent plasma cell differentiation factor, is also a myeloma cell growth factor. [36] IL-10 works through induction of autocrine loops of IL-6 family cytokines. [37]

The myeloma cell survival activity of these cytokines is partly mediated by the phosphorylation of STAT3 by JAK kinases activated by the gp130 IL-6 transducer or IFN receptor. Blockade of JAK/STAT pathway by AG490 inhibits STAT3 phosphorylation and induces myeloma cell apoptosis. [38] STAT3 binding elements are found in the promoters of several anti-apoptotic proteins: Mcl-1, Bcl-2, Bcl-xL. Among ten anti-apoptotic and pro-apoptotic proteins, we found that only Mcl-1 was regulated by IL-6 or IFNα. [39] Other groups suggested that bcl-xL was the main anti-apoptotic protein controlled by IL-6 in myeloma cells [40,41] but a recent study emphasizes that only a blockade of Mcl-1, unlike bcl-2 or bcl-xL could inhibit myeloma cell survival. [42] In addition, we found that induction of the constitutive production of Mcl-1 by retroviral vector is sufficient to promote myeloma cell proliferation independently of IL-6 [43]. IL-6 was reported to activate AKT kinase in myeloma cells that is

able to trigger various signalling pathways. [44] AKT activation can be mediated by STAT3 phosphorylation and ras pathway that can trigger PI-3 kinase activation. [45] In our experience, we found a weak AKT phosphorylation in only some IL-6-dependent cell lines (results not shown). Actually, the IL-6-induced AKT phosphorylation in myeloma cells is weak and transient as compared to that induced by IGF-1. [46] PI-3 kinase mediated AKT phosphorylation appears critical in promoting proliferation of myeloma cell lines since PI-3 kinase inhibitors abrogate it unlike MAP kinase inhibitors. [47,48]

These transduction pathways activated by IL-6 and other myeloma cell growth factors are schematized in the figure 2.

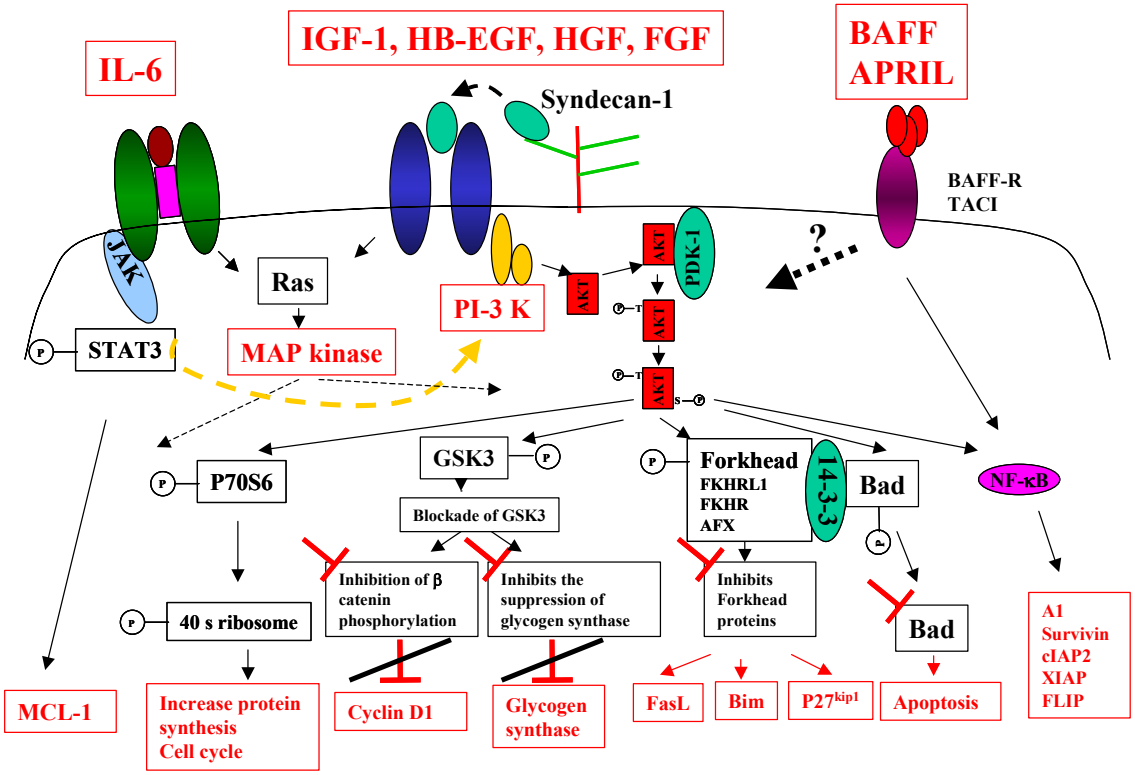

Figure 2: Growth factors and transduction pathways involved in myeloma cell survival and proliferation

2.2. Factors activating the PI-3 kinase/AKT pathway: Insulin like growth factor 1, heparin binding growth factors

2.2.1. Insulin like growth factor 1 (IGF-1). IGF-1 is a survival and proliferation factor for most myeloma cell lines. [49-51] Its effect is independent of an activation of the JAK/STAT

pathway. [51,52] IGF-1 induced the PI-3 kinase pathway and in particular the phosphorylation of AKT protein. [44,52] IGF-1 also induces MAP kinase phosphorylation. [51,52] The myeloma growth factor activity of IGF-1 is blocked by an inhibitor of PI-3 kinase pathway unlike a MAP kinase inhibitor. [47,48] One mechanism of action of AKT is the phosphorylation of the pro-apoptotic protein Bad that induces its sequestration by the 14-3-3 protein and prevents its migration to mitochondrial membrane. [52,53] Other proteins are phosphorylated by the PI-3 kinase/AKT pathway in myeloma cells: the P70S6-kinase, forkhead proteins and the glycogen synthase kinase-3 beta (GSK3β). [47,48,53] Phosphorylation of these proteins contributes to blockade of apoptosis and activation of cell cycle in various models. In particular, IGF-1 induces cyclin D1 and Skp2 expression and downregulation of P27kip1 in myeloma cells. [48] In addition, it was shown in one myeloma cell line that the PI-3K/AKT pathway may activate the NF-kappa B pathway and expression of several targets of NF-kappa B involved in cell survival: A1/Bfl1, cIAP2, XIAP, survivin, FLIP. [46]

Transfection of myeloma cells with an activated AKT enhances tumor growth and protects from DEX-induced apoptosis and expression of a AKT dominant negative results in inhibition of IL-6 induced proliferation of myeloma cells. [54] The importance of the PI-3 kinase/AKT pathway for the survival and proliferation of myeloma cells is emphasized by deletion/mutation of PTEN gene in some myeloma cells. [55] PTEN is a phosphatase inhibiting the PI-3 kinase/AKT pathway and its deletion results in a high activation of PI-3K/AKT pathway.

IGF-1 plays likely a major role in myeloma in vivo. Indeed, IGF-1 serum levels are predictive of a poor survival in patients with MM although they are not increased. [56] IGF-1 is mostly produced by the liver but also by osteoblasts in the bone matrix where myeloma cells survive and proliferate in vivo. The biology of IGF-1 is complex since four IGF binding proteins, mostly IGF-BP3, circulate at high concentrations and neutralize IGF-1. [57] Cells may also

express IGF-binding protein that contribute to the biological activity of IGF-1 and disrupts the circulating IGF/IGF-BP complexes. Using Atlas macroarrays, we found that myeloma cells variably express IGF-BP3 or IGF-BP4 genes. [58]

2.2.2. Heparin binding factors.

A hallmark of plasma cell differentiation is the expression of the proteoglycan syndecan-1. [59,60] This heparan-sulfate protein has many biological activities and in particular, is able to bind heparin-binding growth factors and to present them to their specific receptors. [61] Thus, it is not surprising that several recently-reported myeloma cell growth factors are heparin-binding growth factors.

2.2.2.1. Heparin-binding epidermal growth factor like growth factor (HB-EGF). Using Atlas macroarrays, we found that myeloma cell lines overexpressed HB-EGF gene compared to EBV-transformed B cell lines or normal plasmablastic cells and that inhibitors of HB-EGF can block the IL-6-dependent survival of these myeloma cell lines. [58] Actually, we found that HB-EGF cooperates with IL-6 to trigger an optimal survival of myeloma cells, likely through an interaction between the transducer chains, gp130 and EGF receptors. [62] HB-EGF can activate two of the four members of the EGF receptor family, ErbB1 and ErbB4, which are variably express by myeloma cells. HB-EGF triggers the PI-3K/AKT pathway in myeloma cells, unlike STAT3 phosphorylation (unpublished data). Several coreceptors can enhance the binding of HB-EGF and increase its biological activity: syndecan-1, the tetraspanin CD9 and the integrin $\alpha 3\beta 1$. [63] Using Affymetrix microarrays and FACS analysis, we found that myeloma cells compared to B cells or plasmablastic cells overexpress these coreceptors. [2] In addition, since there are 11 members of the EGF family able to activate the ErbB receptors, [64] it is likely that other EGF members may be involved in myeloma cell biology. Several inhibitors of EGF activity (humanized monoclonal antibodies, inhibitors of ErbB kinase activity) are actually investigated clinically in patients with epithelial cancers. [65] Our recent

data indicate that ErbB inhibitors can potentiate dexamethasone-induced apoptosis of myeloma cell lines and of primary myeloma cells of most patients and suggest that they might improve treatment of patients with MM.

2.2.2.2. Hepatocyte growth factor (HGF). A recent study has shown that HGF is also a growth factor for myeloma cell lines. [66] HGF activity is blocked by removal of heparan sulfate chains of syndecan-1 with heparitinase. This result indicates that syndecan-1 is critical to capture heparin-binding HGF and to present it to its receptor, cMet. Whether HGF cooperates with IL-6 to trigger myeloma cell survival was not investigated. Noteworthy, the XG-1 cell line used in this study was initially obtained in our laboratory and produces a low amount of autocrine IL-6 [33] that is sufficient to induce the HB-EGF activity[62]. HGF is likely involved in the biology of myeloma. Indeed, serum level of HGF is increased and is a prognostic factor in patients with MM. [67] As HGF increases bone resorption, it may also be involved in the abnormal osteoclast resorption in patients with MM. [68]

2.2.2.3. Fibroblast growth factor (FGF). A role of FGF in myeloma is suggested by the finding of a t(4;14) translocation affecting the FGF receptor type 3 (FGR3) in 15% of patients with MM. [69] Whether FGR3 translocations have prognostic value is controversial. [69,70] In addition, mutations of FGR3 making it possible a ligand independent FGR3 activation are found in some myeloma cell lines. [71] These mutations are involved in thanatophoric dysplasia. Although serum levels of FGF2 are increased in myeloma, [72] no direct evidence of a role of FGF or FGFR3 expression on the survival or proliferation of human myeloma cells has been published yet. FGFs likely play an important role in the myeloma biology because they bind syndecan-1 as HB-EGF or HGF and since activation of FGR3 may induces the PI-3K/AKT pathway [73] that is critical for myeloma cell survival and proliferation. Indirect evidences using the murine B9 hybridoma or 3T3 cells suggest that a constitutive FGR3 expression may increase resistance to dexamethasone or tumorogenicity. [74,75]

2.3. Factors activating NF-kappa B: BAFF family.

BAFF and APRIL belong to the TNF family and activate at least three receptors of the TNF receptor family: BAFF-R, BCMA and TACI. BAFF proteins are critical for the survival of normal B cells, tumor B cells of chronic lymphoid leukaemia and may be involved in Systematic Lupus Erythematosus [76]. Activation of BAFF receptor family results in triggering the NF-kappa B pathway and likely other unidentified pathways. [77]Using DNA microarray, others and we found an over expression of two BAFF receptors, BCMA and TACI. [2,78] This observation prompts us to look for a role of the BAFF family in the survival/proliferation of myeloma cells. We found that BAFF or APRIL is a potent survival and proliferation factor of myeloma cells, depending on expression of BAFF-R or TACI on myeloma cells. In addition, BAFF can protect myeloma cells from dexamethasone-induced apoptosis. [79] These data suggest that BAFF inhibitor could be useful in the treatment of patients with MM in association with dexamethasone.

2.4. Cross-activation of growth factor receptors and potential clinical applications

The contribution of these various myeloma growth factors to myeloma disease can be simplified by considering the transduction pathways that are critical to promote myeloma survival and cell cycle. As indicated above, at least four transduction cascades are activated by these various factors, the JAK/STAT pathway induced by IL-6 cytokines and IFNα, the PI-3 kinase/AKT pathway strongly activated by IGF-1 and heparin binding factors and weakly induced by IL-6, the NF-kappa B pathway activated by IGF-1 and BAFF proteins and the MAP kinase pathway induced by all factors. We recently pointed out cooperation between IL-6 and HB-EGF to trigger myeloma cell survival and proliferation. [62] The effect of HB-EGF is dependent on weak gp130 activation by IL-6. This cooperation likely reflects a cross talk of the transduction elements and activation of various anti-apoptotic proteins. [62]

This was recently shown for gp130 IL-6 transducer and IFN receptor or IGF receptor. Indeed, Jelinek's group showed that IFN-α could activate the phosphorylation of endogenous gp130. This cross-activation is not reciprocal as IL-6 cannot cross-phosphorylate endogenous IFN receptor. [80] The same group also showed that IFNα induced a cross-phosphorylation of endogenous ErbB3 receptor in a myeloma cell line. [81] This ability of IFNα to induce these cross-phosphorylations of other transducer chains unlike IL-6 or IGF-1, is linked to its ability to trigger a large and long-lasting activation of JAK1 and Tyk2 kinases compared to IL-6. [80] More recently, it was shown that the gp130 and IGF-1R are both colocalized in caveolin-associated membrane caveolae in human myeloma cells together with PI-3 kinase and src kinase and that abrogation of caveolae by cholesterol inhibitors blocks IL-6 or IGF-1 induced transduction, in particular PI-3K/AKT pathway. [82] Of major interest, the caveolin 1 gene is overexpressed on malignant plasma cells compared to normal B cells or plasma cells. Taken together these data suggest that gp130 IL-6 transducers, IGF-1 receptors and EGF receptors and eventually coreceptors such as syndecan-1 and CD9 are colocalized within caveolin-associated caveolae. In particular, myeloma cells overexpress the CD9 gene [2] coding for a tetraspanin involved in the formation of membrane multimolecular complexes. [83]

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