Toll-like receptors in human multiple myeloma: studies on the cellular interaction with bone marrow microenvironment components

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Toll-like receptors in human multiple myeloma: studies on the cellular interaction with bone marrow microenvironment components

Toll-like receptoren in human multiple myeloma: onderzoek aan de cellulaire interactie met beenmergomgevingsfactoren
(met een samenvatting in het Nederlands)

Proefschrift

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To my wife Tala who means everything to me…….

Table of contents

1

General Introduction

Multiple myeloma

History

Multiple myeloma (MM), a malignant plasma cell disorder accounting for about 10% of hematologic neoplasms, has been probably present for thousands of years, but the first documented case was a woman named Sarah Newbury described by Solly in 1844. In that era, Henry Bence Jones described a protein in the urine of a patient (Alexander McBean) as" hyrdrated deutoxide of albumen". The patient was suffering from "mollities osseum" (softening of the bone). Interestingly, only after about 115 years, Lipari and Korngold reported that two classes of "Bence Jones" proteins were excreted in the urine of myeloma patients, which were given the names kappa and lambda chains. Detection of this protein in urine has continued to be used as a screening diagnostic test in MM. Furthermore, some parameters of the disease including morphology of malignant plasma cells, serum protein M-spike, paraproteinemia, and monoclonal or polyclonal nature, have been identified in the course of a long time. The first drug used for these patients was Urethane in 1947 followed by other drugs such as Melphalan, corticosteroids, Cyclophosphamide, in single or combined formulations through many clinical trials, each with variable effects on patient survival. Moreover, bone marrow transplantation in combination with chemotherapy has a long history in MM, but often ending in relapses in many patients. Only in recent years have two drugs proved to be significantly effective in MM, Bortezomib (Velcade, 26S proteasome inhibitor) and Lenalidomide (Revlimid, an immunomodulatory agent, remarkably more potent analog of Thalidomide). These drugs are still considered to be associated with a high level of response in MM patients. Nonetheless, in spite of all the clinical approaches and effective therapeutical protocols, some categories of patients still persist resistance to treatment, thus facing the therapeutic and clinical fields with a big challenge (adopted from [1]).

Pathogenesis

MM in most cases arises from a non-malignant condition termed monoclonal gammopathy of undetermined clinical significance (MGUS) which is present in 1% of the adult population above the age of 25 or in 10% of above 50 and progresses to overt MM with a rate of 0.5-3.0% per year [2]. Basically, in some patients another asymptomatic condition termed smoldering multiple myeloma (SMM) precedes active MM, however, in some studies almost all the patients diagnosed with MM had already developed MGUS [2,3]. MM is a malignancy of terminally differentiated B cells (plasma cells) which synthesize a complete and/or partial (light chain) monoclonal immunoglobulin protein [4]. Myeloma cells can induce, in the context of extracellular matrix components (e.g. bone marrow stromal cells and fibronectin), critical alterations in the bone marrow microenvironment (BMME), which in turn provide MM cells with survival, anti-apoptosis, and drug resistance signals [2]. Clinical

manifestations of MM vary due to a ***heterogeneous biology***, covering a complete spectrum from indolent disease to highly aggressive myeloma with extramedular features [2,4]. Neoplastic plasma cells synthesize abnormal amounts of immunoglobulin or immunoglobulin fragments as free light chains (FLCs) culminating in hypogammaglobuinemia, vulnerability to infections, and impairment of renal function. Symptoms develop because of tumor mass effects, cytokines produced by MM cells or indirectly by bone marrow stromal cells (BMSCs) and bone cells in response to tumor cells adhesion.

Origin of the malignant plasma cell clone

Increasing evidence suggests that MM and its precursor MGUS are post-germinal center B cell tumors (illustration at figure 1). Furthermore, presence of somatic hypermutations in immunoglobulin variable region genes in plasma cells of MGUS and MM patients is a strong indicator that the malignant transformation has occurred in a more mature B cell which has already traversed the germinal center, implying that these B cells must have memory B cell phenotype as has been indeed demonstrated by several studies [2,4-6]. In line with this, findings of studies involving serial transplantation of MM cells into recipient mice or using xenograft models indicate potential colonogenicity of MM cells, however, only MM cells lacking CD138 but expressing B cell typical markers (CD45, CD22 and CD19) display this capability [6-8], and interestingly these B cells express colonotypic markers identical to MM bone marrow plasma cells [8,9].

Role of (cyto-) genetic aberrations

Despite some evidence supporting co-clustering of MM and MGUS in a few families, the role of genetic background and environment in pathogenesis has not been clearly defined [10]. Like other post-germinal center B cell tumors, in MM and MGUS chromosomal translocations involving immunoglobulin heavy chain (IgH) locus (14q32) or one of the two immunoglobulin light chain (IgL) loci (κ, 2p12, or λ, 22q11) are common [11-14]. These translocations may occur by errors in one of the B cell-specific DNA modification mechanisms: VDJ recombination, IgH switch recombination, or somatic hypermutation.

It has been suggested that above translocations might represent the initiating oncogenic events in MM as normal B cells traverse the germinal centers, however, there are not yet adequate support for the role of these recurrent translocations as essential for tumor survival or as therapeutic targets. Furthermore, several secondary chromosomal and genetic aberrations have been suggested to be involved in MM progression, including inactivation of *TP53* and secondary translocations of *c-MYC*.

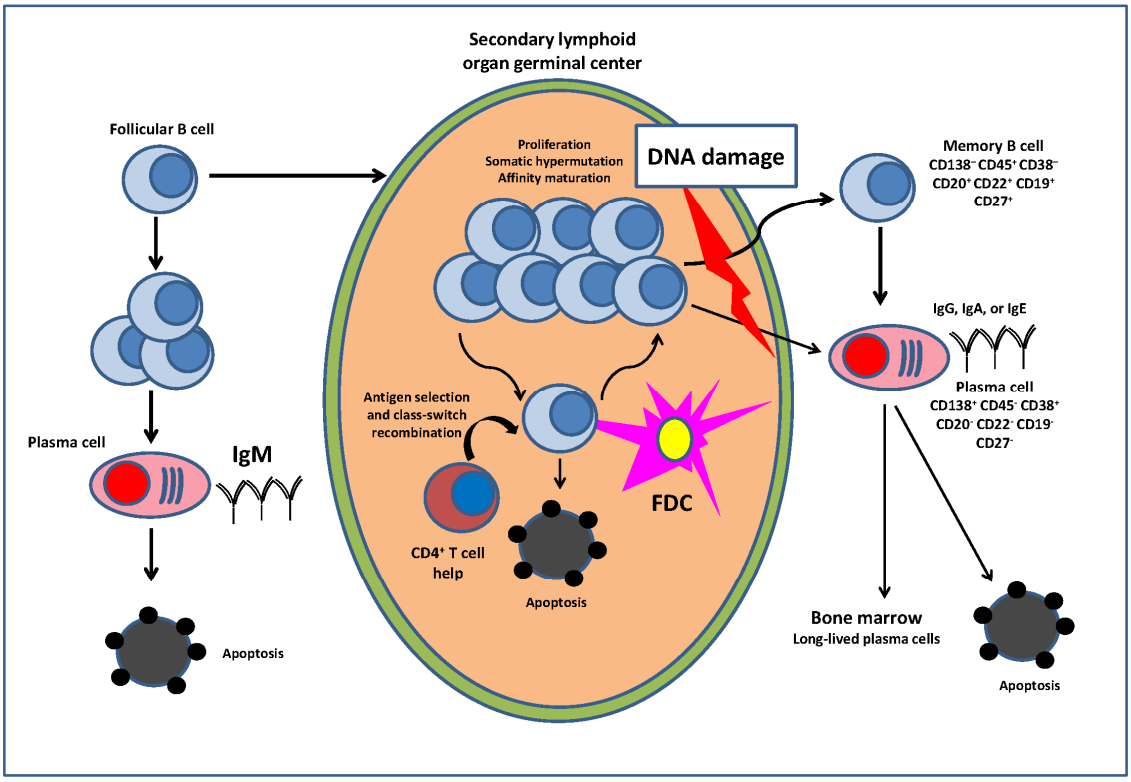

Figure 1. The origin of multiple myeloma (MM) cells. After their initial encounter with antigen, naıve follicular B cells differentiate into short-lived plasma cells, undergoing apoptosis in situ. Some activated follicular B cells form a germinal center (GC), where B cells are primed by BCR (B cell receptor) recognition of antigens presented by FDCs (follicular dendritic cells). Also, $CD4^+$ T cells interact with B cells to help them differentiate into plasma cells, and undergo affinity maturation, somatic hypermutation, and immunoglobulin heavy chain (IgH) switch recombination. In the GC during this process, abnormal DNA recombination events (due to DNA damage?) translocate IgH genes to other chromosomes and generate a malignant phenotype. These translocations usually place IgH gene near oncogenes/cell-cycle proteins on other chromosomes. Secondary changes including duplication/loss of chromosomes and /or mutations in cell growth/tumor suppressor genes will follow. Post-GC plasma cells progress through a memory B cell stage in the primary response or develop directly from GC B cells. Plasma cells that arise from a GC reaction become long lived if they find survival niches, which are located mainly in the bone marrow. The immunoglobulin gene sequences in MM plasma cells are somatically hypermutated and remain constant throughout the clinical course, suggesting that the disease arises from a post-GC B cell. Recent studies indicate that MM cancer stem cells lack CD138 and express the memory B cell markers CD19 and CD27.

Role of NFκB pathway activation

NFκB is the most important factor controlling the expression of various genes involved in immune response, growth and proliferation, survival and apoptosis. However, it plays a critical role in directing inflammation through orchestrating an induction in a plethora of pro-inflammatory cytokines. NFκB is activated through two pathways, canonical (classic) and non-canonical (alternative). The first pathway which is mostly triggered by engagement of **Toll-like receptors (TLRs)** is crucial for initiation and controlling the innate immune response and its integration into adaptive immune response. The alternative pathway is involved in the development of lymphoid system, and is basically triggered by members from TNF superfamily including BAFF, CD40L, LTαβ and RANKL. This is indeed not unexpected if any aberration in structure or function of NFκB could lead to a variety of human diseases specially auto-immunity and cancer. Constitutive activation of this transcription factor has also been observed in several hematologic malignancies such as some lymphoid and myeloid leukemias [15,16] and MM [17-19]. Recent nucleotide array-based studies have uncovered gene mutations in NFκB pathway in 40% of human myeloma cell lines (HMCLs) and 17% of MM primary tumor cells, which are associated with high NFκB activity [17]. On the other hand, constitutive activation of NFκB was long thought to be due to ligand-dependent interaction of MM cells with bone marrow milieu. However, recent thorough investigations contribute this to an activated non-canonical pathway due to mutations in *NFKB1, NFKB2, NIK, CD40, LTBR,* and *TACI,* which are all positive regulators of NFκB pathway [18,19]. It has also been suggested that above mutations make MM cells independent of BMME signals required for survival, while there are many MM cases with a high baseline NFκB activity without any genetic lesions in NFκB pathway indicating that most MM cells will be dependent on extrinsic signals such as cytokines (e.g. BAFF produced by BMSCs) to survive and expand [19].

Role of BMME: adhesion systems, blocks for building up a supportive architecture

In spite of the fact that many chromosomal translocations, genetic mutations in growth factor and tumor suppressor genes have been identified in MM, various studies have only detected trivial differences in above abnormalities between MGUS and MM, suggesting they may only have a slight contribution to MM progression [2,3,20,21]. On the other hand, knowledge of the role of BMME in MM pathogenesis/progression has remarkably increased, particularly in terms of tumor survival, expansion, drug resistance and immune evasion [2]. Indeed, adhesion of MM cells to BMME components including BMSCs and ECM proteins (fibronectin, laminin, collagens, hyaluronic acid, etc.), triggers upregulation of a plethora of cytokines, growth factors and chemokines in autocrine or paracrine manners (Fig 2). The mostly studied factors, IL-6, IGF-1, VEGF, BAFF, FGF, SDF-1α and TNF-α, which are induced both by MM cells and stromal cells, activate various pathways including

Ras/Raf/MEK/MAPK, PI3k/Act, JAK/Stat-3, NFκB and Wnt, contributing to growth, survival and drug resistance of MM cells [20] as well as to osteoclastogenesis [22] and angiogenesis [23]. Indeed, BMME plays critical role in development of bone destruction in MM. Following adhesion of MM cells to BMSCs, latter cells secrete IL-6, IL-1β, and TNF-β which are basically activators of osteoclasts and provoke stromal cells and osteoblast to produce TRANCE (RANKL) which is in turn a differentiation and maturation factor for osteoclast progenitors. Normally, TRANCE secretion is controlled by the delicate balance between osteoprotegerin (OPG) and RANKL, but in MM this balance is deranged leading to increase in TRANCE secretion, and thus increase in osteoclast activity and bone destruction.

The main cellular receptor for FN is α5β1 (also known as VLA5 or CD49e) being expressed on normal plasma cells and can be detected in most MM tumor samples in the initial stages of the disease [24]. However, it has been shown that during MM progression and also in circulating malignant plasma cells, expression of α5β1 declines [24], indicating that α5β1 integrin is mostly involved in regulating MM physiology within BM milieu. On the other hand, α4 subunit can make a complex with β1 to bind FN (CS-1domain) or to VCAM-1 (on BMSCs), or pair with β7 to bind with MAdCAM-1 [25]. Unlike α5β1, α4β1 is expressed on all normal and malignant plasma cells [26], and its overexpression has been observed in drug resistant MM cells [27]. Expression and activity of adhesion molecules in MM can be regulated by extracellular signals (such as cytokines) and BM environment condition, or intrinsically by oncogenes. For example, mutated oncogenes N-*ras* and K-*ras* in MM increased adhesion of MM cells to FN in a cox-2 dependent manner [28]. It has also been shown that *c-maf,* another MM-associated oncogene, upregulates expression of β7 integrin and thus MM cells adhesion to BMME [29].

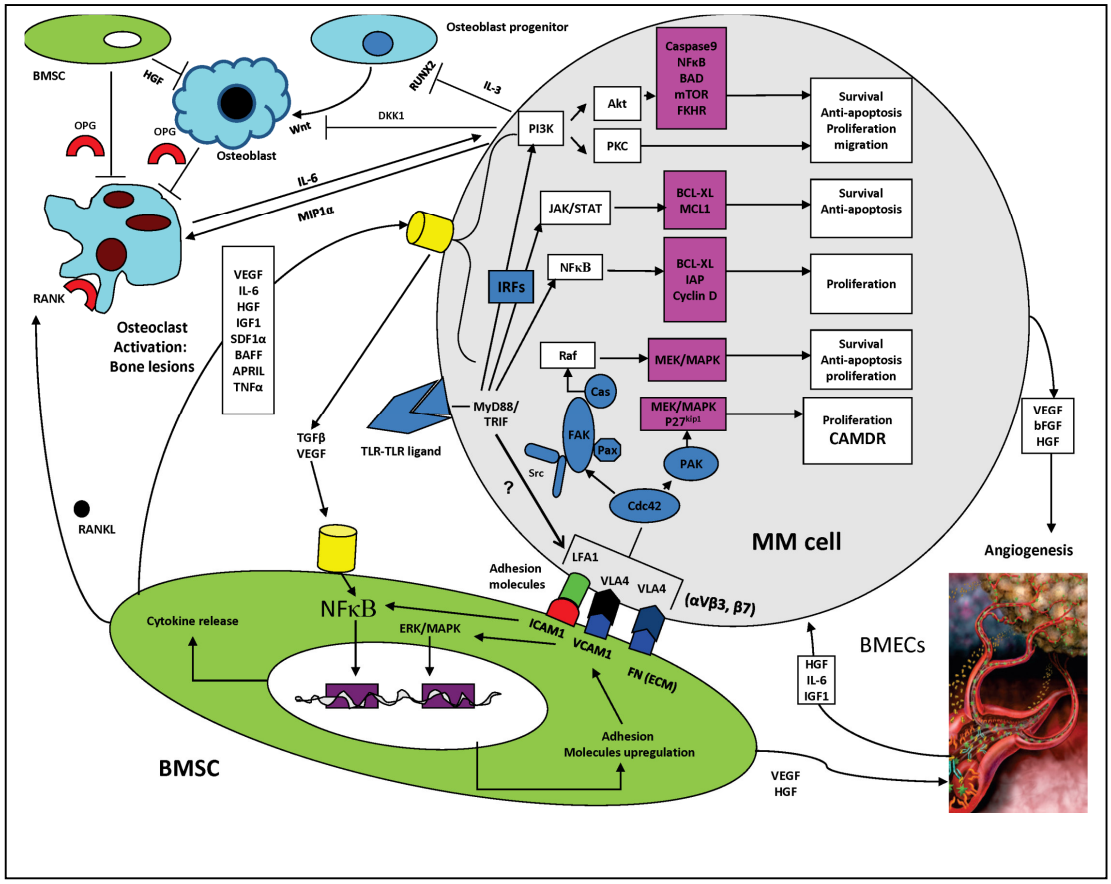

Figure 2. Various functional outcomes of MM cell interaction with bone marrow milieu. Adhesion of MM cell to BMSCs or FN gives rise to three main outcomes induced either by physiologic events following integrin engagement or by secretion of a plethora of cytokines: 1)-MM cell proliferation, survival, invasion, and CAMDR, due to activation of PI3K/Act, MAPK/MEK, JAK/STAT and NFκB. 2)-Osteolytic lesions or bone destruction due to suppressive effects of IL-3 and DKK-1 (Dickkopf-related protein-1) (both secreted by MM cells) on osteoblast progenitors differentiation, and due to activating effects of MIP-1α on osteoclasts. These two effects increase osteoclast activation and derangement in RANKL/OPG balance leading to bone resorption. 3)-Angiogenesis due to mutual effects of VEGF, IL-6, bFGF and HGF secreted by MM cell, BMSC or endothelial cells. TLRs will act through activation and recruitment of MyD88 or TRIFF to leave several effects on MM cell. Apart from some functional responses following TLR activation in MM cell, including increase in proliferation, induction of IL-6 secretion and drug resistance, nothing is known about the effects of TLR triggering on adhesion of MM cell to FN or BMSCs or on modulation of CAMDR.

Furthermore, MM cells express CXCR4 both inside the bone marrow environment and in extramedullary sites [30,31], and its expression is enhanced by cytokines (such as TNF-α, TGFβ and VEGF), dexamethasone and hypoxia ([32]. Notably, the SDF-1α-CXCR4 axis plays a key role in homing, adhesion and motility of MM cells [33]. MM cells physiology in the BM environment is largely governed by interplay between cytokines, adhesion molecules and cellular or extracellular matrix ligands. Homotypic or heterotypic adhesion of MM cells to BM milieu is mediated by a wide array of adhesion molecules including: CD44 (major hyaluronan), VLA4 (α4β1, CD49d), VLA5 (α5β1, CD49e), LFA-1 (CD11a), NCAM (CD56), ICAM-1, syndecan-1 (CD138), α4β7 and αVβ3. Adhesion of MM cells to BMSCs induces NFκB-dependent secretion of IL-6 in latter cells, in response MM cells also produce VEGF, TNF-α, and TGFβ which in turn promote a paracrine secretion of IL-6 in BMSCs. It has been demonstrated that NFκB controls expression of a variety of adhesion molecules, and its activation following adhesion of MM cells to BM milieu further reinforces the latter adhesion resulting in induction of IL-6 production by BMSCs. Additionally, binding of CD40 on MM cells to CD40L in BM milieu, upregulates VLA4 and LFA-1 on MM cells, increases their adhesion to BMSCs and secretion of IL-6 by the latter cells [34].

Cell adhesion-mediated drug resistance (CAMDR), first designated by Damiano and Dalton et al. has a fundamental importance in MM pathogenesis and has now been extended to other cancer types, as part of drug resistance mediated by the tumor environment [27,35-42]. In the FN-α4β1-drug resistance axis induction of several biochemical mediators has been detected. Adhesion of B-CLL cells to FN conferred drug resistance in these cells, which was associated with Bcl-xL upregulation [43]. In one study it was shown that CAMDR in MM cells was associated with upregulation of $p27^{kip1}$ and was indicated to be required for maintaining drug resistant phenotype, possibly due to its involvement in cell cycle regulation [35]. Furthermore, activation of NFκB was observed following adhesion of MM cells to FN, and was postulated to be related to CAMDR [44]. Finally, upregulation of HSP70 has also been observed following MM cells adhesion to FN and suggested to be associated with CAMDR [45]. It should be noted that CAMDR is not associated with reduction in drug-induced DNA damage but mostly with protection from mitochondrial transformation and caspase activation [46]. MM cells adhesion to ECM has also been associated with promotion of invasiveness of malignant plasma cells mediated by the adhesion molecule αVβ3 [47,48].

Role of infections/inflammation: the new concept of Toll-like receptors

Myeloma patients are highly vulnerable to infectious conditions and indeed infections are still the leading cause of mortality in most MM patients [49]. Human herpes virus-type 8 (HHV-8), which is also referred to as Kaposi sarcoma associated herpes virus (KSHV), has been implicated in the pathogenesis of pleural cavity lymphoma [50], Kaposi sarcoma [51] and Castelman disease [52]. This virus has also been detected in the bone marrow of MM patients and is implicated in MM pathogenesis [53]. The main reason for high susceptibility to infections is the failure to launch an efficient immune response due to reported defective dendritic cells (DCs) [54,55], T cells [56,57], B cells [58] and NK cells [59], and a remarkable reduction in normal polyclonal immunoglobulins [2]. Malignant plasma cells in MM appear to exploit various (yet unexplained) mechanisms to cause above defects, several cytokines secreted following adhesion of MM cells to BMSCs or to FN are suggested to be involved (Fig 3).

TLRs are a heterogeneous group of cell surface or cytosolic molecules expressed primarily in innate immune cells such as DCs, monocytes/macrophages and B cells [60]. However, TLRs have been shown not to be confined to immune cells but to be expressed on a variety of cancer cells as well [61-63]. TLRs are mainly involved in detection of conserved moieties termed" **pathogen associated molecular patterns or PAMPs"** on microorganisms and thus triggering innate immune response and bridging it to adaptive immune response [60,64]. Furthermore, it is now known that TLRs are also activated by non-microbial (endogenous) ligands which are released by necrotic or dying cancer cells at the site of the tumor, and are known as "**danger associated molecular patterns or DAMPs"** [62,65-67].

MM cells express a wide range of (mostly functional) TLRs at a level significantly higher than normal plasma cells from normal individuals, as has been demonstrated in a few recent studies [68-71]. These findings indicate that MM cells may have been primed by an inflammatory environment and thus will shed light on the contribution of inflammatory conditions to MM pathogenesis or progression. Various functional responses depending on cellular context and cancer type have been reported for TLRs in *in vitro* and *in vivo* models [72], but research on TLRs in MM is still in its infancy. It has been shown that TLR triggering on MM cells can increase growth and proliferation of some HMCLs, which may be mediated by induction of autocrine IL-6 secretion [70,71], and also reduce dexamethasone or serum deprivation-induced apoptosis [71]. On the other hand, pro-apoptotic effects of TLR signaling on MM cells has also been reported [73]. The heterogeneous pattern of TLR expression or activation-induced responses in MM further highlights the complicated biology of the disease. Of note, while TLR activation in MM cells has mostly been associated with favorable results for these cells, in other-B cell malignancies including B-lymphomas and B-CLL the outcome of this stimulation may antagonize MM cell physiology [69,74-76], and expression pattern of TLRs in MM is shown to differ from that in normal B cells [69].

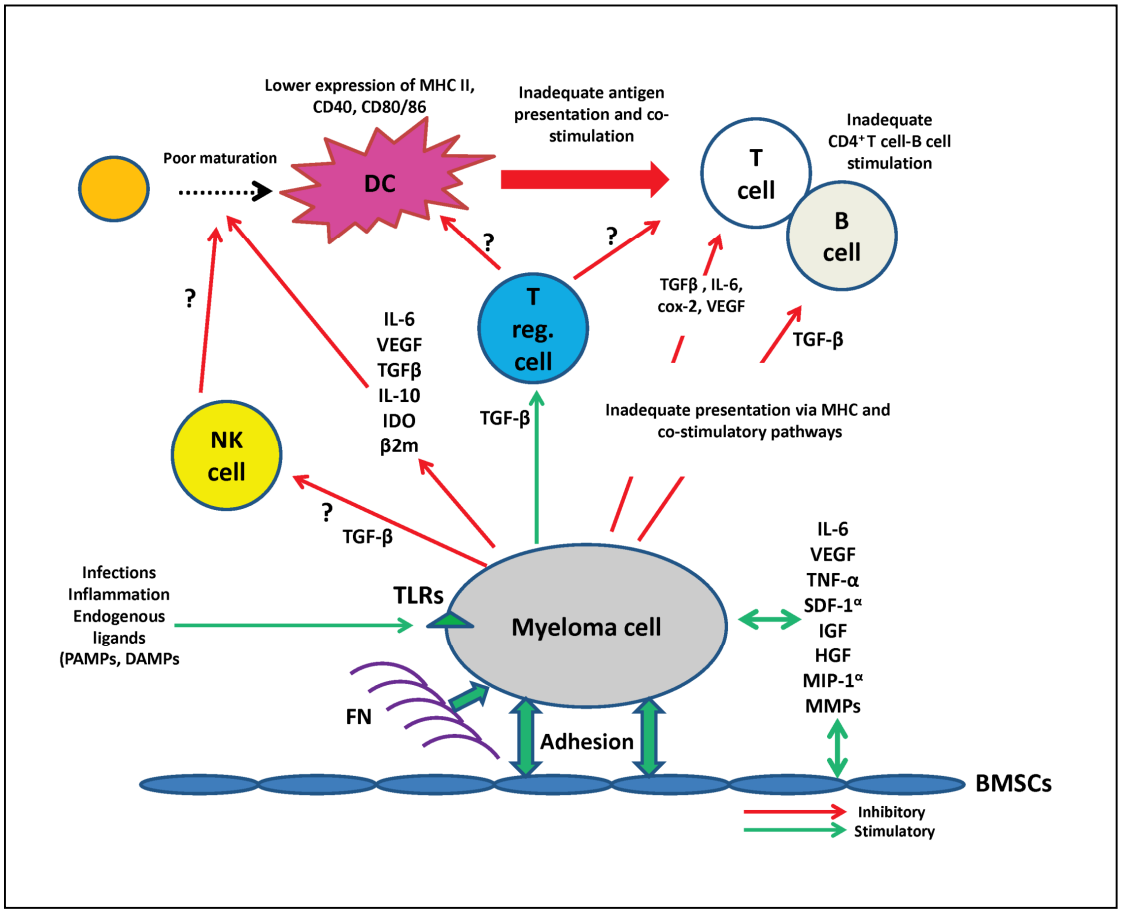

Figure 3. Schematic outline of postulated mechanisms engaged by MM cells within bone marrow milieu to provoke immune response defects. Following adhesion of MM cells to FN or BMSCs a wide array of cytokines or growth factors are induced in both cell types using both autocrine or paracrine systems. It has been suggested that some of these cytokines might have inhibitory effects on immune cells as NK cells, T cells, B cells or DCs. For example, IL-6, TGFβ and VEGF have negative effects on DC maturation, T cells, NK cells and B cells, the outcome of which will be poor maturation of DCs (decline of MHC and co-stimulatory molecules), and thus inadequate antigen presentation to T cells by DCs, insufficient activation of B cells, T cells and inhibiting cell cytotoxicity activity of NK cells. On the other hand, TGFβ is an activating signal for T regulatory lymphocytes which in turn are (questionably) suppressive for DCs and T cells. Activation of TLRs in this context will also be possible, due to inflammatory environment of bone marrow, microbial ligands, and possibly endogenous ligands that might be released after tumor-induced tissue injury or by necrotic malignant cells. TLR triggering will lead to induction of IL-6 in MM cells, as is already shown [70,71]. In spite of the fact that several vague points with respect to immunosuppressive activity of MM cells still persist, deciphering the role of TLR activation in this context will open a new avenue to understanding of MM cells-immune system interaction.

On the other hand, a recent study indicated low functionality of TLRs on myeloid leukemic cells [77]. These findings would lead us to realize that MM cells may exploit specific pathways or systems to deploy TLRs.

Investigating the relationships between infectious and auto-immune diseases and MM has recently become an interesting research focus. A retrospective study on more than 4 million male American and African soldiers yielded an increased incidence of essential monoclonal gammopathy or MM in patients suffering from auto-immune or inflammatory disorders [78]. This indicates that immune-mediated mechanisms may play role in triggering plasma cell dyscrasia.

Chronic inflammation-related mechanisms have been suggested in incidence and promotion of lymphoid malignancies such as B-CLL, mucosa-associated lymphoid tissue (MALT) lymphoma, large B cell lymphoma, Burkitt's lymphoma and Hodgkin's lymphoma [79,80]. It has been indicated that chronic antigenic stimulation along with BAFF (a TNF family member) could be the putative mechanism for inflammation-driven B-CLL promotion, with cytokines (IL-4, VEGF), chemokines (SDF-1α) and interaction with BMSCs supporting its expansion [79,81]. The situation in MM is more or less the same, where it is mostly dependent on extrinsic signals such as IL-6 (an important inflammatory cytokine) and BMME components despite confirmed constitutive NFκB activation [17,18,79]. However, NFκB is now known to be the critical factor linking inflammation to carcinogenesis [82-84], and it is the main transcriptions factor activated following TLR triggering. Hence, deciphering the role of TLRs in MM will enforce our understanding of contribution of inflammation to disease progression or pathogenesis. In line with the fact that inflammation could function as a double-edged sword in tumor context [85,86], TLR activation in tumor context may also leave positive or negative effects, with the former optimizing tumor condition and the latter antagonizing it [62], and indeed MM will not be an exception. All studies performed to date on MM cells, have explored the expression status of TLR genes and proteins and some functional responses following their triggering in *in vitro* assessments. As a matter of fact, more extensive research is required to delineate the potential effects of TLR signaling in the physiology or pathology of MM. We still need to know how TLRs are upregulated in MM bone marrow, what the possible activators of TLRs might be, whether TLR engagement has a real negative or positive effect on immune cells of the bone marrow, whether TLR triggering could have a role in maintaining the established adhesion of MM cells to BMME components, and finally whether TLR triggering might contribute to adhesion-induced or *de novo* drug resistance of MM cells. None of these questions have sufficiently been addressed to date, and indeed finding an answer to these questions may help to a large extent to identify and exploit novel therapeutic targets for MM.

Aims and outline of the thesis

In spite of the fact that TLR signaling has been more extensively investigated in normal B cells and other B lymphoid malignancies, with even exploiting TLR ligands in some clinical trials, TLR topic in MM is quite new. Accordingly, to promote our insight into the patterns of TLR expression and functional responses following their triggering in a variety of B cell cancers and to make a comparison with (as yet not detailed) studies on TLRs in MM, we compiled current findings from the literature into a full-of-content review given in **Chapter 2**. In this chapter, we set forth an overview of TLR signaling, some as yet unanswered questions pertaining TLR triggering effects in MM, speculative contributions of TLR activation to MM angiogenesis and bone lesions, and also the concept of endogenous ligands. The few recent studies addressed TLR activation-induced responses including proliferation and IL-6 production in stroma-free situations, which could not give a clear implication for MM patient bone marrow. Whether TLR activation in MM cells might have any effect on their interaction with BMME components has never been investigated before. In this project we were prompted to explore if TLR triggering on MM cells could modulate: *their adhesion to BMSCs and FN*, *surface expression of several adhesion molecules*, *viability and drug resistance in the context of BMSCs and FN*. Because normal B cells also express multiple TLRs, to gain an idea of how TLR activation might modulate above interaction in normal human B lymphocytes, we performed a study on TLR9 triggering effects of adhesion of B cells to FN, which is given in **Chapter 3**. The pattern of TLR expression in MM cells in recent studies has been limited to mRNA analysis, thus to create a complete picture of TLR expression profile at **mRNA and protein** levels, we characterized TLR expression profile of 10 human myeloma cell lines (HMCLs) and primary myeloma cells, which is presented in **Chapter 4**. We sought to investigate the effect of TLR activation on MM cells adhesion to FN and BMSCs in **Chapter 5 and Chapter 6**. We first carried out an extensive analysis of surface expression pattern of adhesion molecules on MM cells and then explored modulation of adhesion to FN and BMSCs following TLR activation. Furthermore, TLR activation effects on MM cells' viability and drug (Bortezomib, Velcade) resistance in the context of FN and BMSCs were also assessed. Regarding the profound role of **immunoglobulin free light chains (FLCs)** in clinical complications of MM, and since targeting FLCs is a promising step in MM combined therapeutic protocols, we performed a parallel and novel study on the effect of TLR activation on FLC production in TLR-activated and Bortezomib-treated MM cells in the context of FN or BMSCs. These findings on FLCs are described in **Chapter 7**. In light of the fact that anti-inflammatory agents have been frequently included in MM supplementary and combined therapeutic protocols, we also performed a pilot study on the effect of **Omega-3** components (**EPA and DHA**) on viability and drug sensitivity of myeloma cells lines. These two components are of known anti-inflammatory functions and have been applied to various cancer clinical trials but not to date in MM (**Chapter 8**).

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2

The role of Toll-like receptor mediated signaling in the pathogenesis of multiple myeloma

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Abstract

Toll-like receptors are critical structures in sensing the invading pathogens via conserved moieties termed pathogen associated molecular patterns and in directing the innate and adaptive immune responses. Studies have shown that Toll-like receptors are not limited to normal immune cells but are expressed on tumor cells as well, including those of lymphoid neoplasms particularly B-cell malignancies, multiple myeloma and chronic lymphocytic leukemia. Neoplastic plasma cells in multiple myeloma usually show a different pattern of Toll-like receptor expression compared to normal B cells. These receptors on multiple myeloma cells, have been indicated to have a role in their proliferation, differentiation and survival, probably through induction of autocrine IL-6 secretion, and in their immunomodulatory functions. Moreover, it is speculated that these molecules may contribute to osteolytic lesions through activation of osteoclasts, and to angiogenesis through induction of pro-angiogenic factors. Knowledge on Toll-like receptor signaling in the biology of malignant plasma cells or their cellular microenvironment may give new insights into pathogenesis of multiple myeloma and may open new avenues for the therapy of this disease.

1. Introduction

Multiple Myeloma (MM) is an as yet incurable B cell malignancy characterized by proliferation and infiltration in the bone marrow of malignant plasma cells leading to marked hypogammaglobulinemia and failure in normal immune responses [1,2]. Almost all the patients experience a premalignant and often clinically undetectable phase, monoclonal gammopathy of undetermined significance (MGUS), which is seen in more than 3% of the population above 50 years of age and progresses to MM with a rate of 1% per year[1,2]. Immune dysfunction in MM patients makes them vulnerable to a variety of infections including bacterial, fungal and viral [3,4]. Detection by the immune system of various pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs) plays a key role in establishing innate and adaptive immune responses, among PRRs, Toll-like receptors (TLRs) are the most instrumental [5]. Furthermore, several reports indicate that tumor cells use infections/inflammation to divert immune responses for their own benefit; this indicates that tumor cells have to be in a dynamic interaction with their microenvironment to evade the immune system surveillance [6]. TLRs, critical for innate and adaptive immune responses, are not restricted to immune cells, but are also expressed on tumor cells [7]. TLR signaling may promote tumor growth and survival, growth inhibition or apoptosis, and even induce immune evasion [7-10]. These effects have also been reported in MM cells following TLR activation *in vitro* [10-14]. MM cells show a distinct expression pattern for TLRs compared to their normal counterparts, and their triggering acts mostly in favor of the malignant cells apparently through induction of cytokines [11-14]. This implies

that MM cells will benefit from the high incidence of infections in myeloma patients. Moreover, since the expression and effects of TLR activation on osteoclasts/osteoblasts and also on endothelial cells have been established in other situations it is speculated that TLR triggering on these cells in the myeloma bone marrow environment may contribute to bone lesions and angiogenesis [15-17]. In this review we will discuss the role of TLRs in the pathogenesis of MM, taking into consideration the immunologic, biologic and possible therapeutic aspects.

2. TLR signaling and tumorigenesis

2.1. TLR signaling overview

Toll-like receptors sensing a vast variety of pathogens (bacteria, viruses, fungi and parasites) through PAMPs, direct the innate and establish the adaptive immune responses [5,18-21]. Recently 13 TLR analogs have been identified of which TLRs 11, 12 and 13 are not expressed in humans but are functional in mice, and TLR10 is expressed in humans but not in mice [18-20]. TLRs are type I transmembrane proteins expressed mainly on innate immune cells (macrophages, neutrophils and dendritic cells) to act as immunoadjuvants in activating the antigen-presenting cells (APCs), although many other cells express most of these molecules [19]. TLR extracellular domains contain leucine-rich repeats for ligand sensing and binding, and the cytoplasmic region contains TIR (Toll-like/IL-1R) domains involved in recruiting adapter molecules, MyD88, TIRAP/MAL, TRIF and TRAM to receptor complexes for downstream signaling [19,20]. TLRs which sense lipids such as LPS (TLR4), lipoteichoic acid, peptidoglycan and lipoprotein (TLR1,2,6) and proteins such as flagellin (TLR5) are located on the cell membrane, while those that recognize nucleic acids such as ssRNA (TLR7,8), dsRNA (TLR3) and CpG DNA (TLR9) are resided in intra-cellular endosomal vesicles [20]. Broadly speaking, two general pathways are used by TLRs [18,20,21] (see Fig. 1): MyD88-dependent (all TLRs except TLR3) and TRIF-dependent (TLR3 and TLR4) pathways. In MD88-dependent pathways, upon TLR triggering, MyD88 recruits IRAK4 which in turn activates and phoshorylates IRAK1. IRAK4 and IRAK1 then dissociate from MyD88 and interact with the E3 ubiquitin-ligase TRAF6, ubiquitinating itself and NEMO. The latter complex recruits an enzymatic complex involving TAK1 and TABs which in turn activate two distinct pathways, IKK complex (IKKα/β) and MAPK (p38, JNK and ERK) ending in activation of transcription factors NFκB and AP-1, respectively. In TRIF-dependent pathways, TRIF will bind directly to TRAF6 and/or RIP1 to activate TAK1 ending in NFκB activation. Additionally, studies show that TRIF-dependent pathways can also activate another complex involving IKKi/TBK1 which has an essential role in type-I IFN production through activation of transcription factors IRF3 and IRF7 [21]. NFκB activation following TLR triggering controls many genes involved in innate and adaptive immunity, survival and

proliferation of various cells, cellular stress responses, anti-apoptosis and cancer progression [19]. In addition to the above functions, TLR signaling has been reported to induce apoptosis in many cell lineages initiated mostly by recruiting Fas-associated death domain (FADD) to MyD88 or TRIF adaptors and mediated by caspase activation or type-I IFN secretion [22]. Thus it is plausible that TLR activation could function as a double-edged sword in terms of its potential contribution to host immunity and tumorigenesis.

2.2. TLR signaling in tumorigenic conditions – effects on malignant cells

The effect of TLR signaling on neoplastic cells stems from the first observations in the 18th century when microbial infections were reported to have anti-tumoral features [8]. Later on, the same effects were found in Coley's toxin [8]. Today it is generally accepted that these effects had been due to presence of LPS in this toxin leading to TLR4 activation. Additionally, variants and polymorphisms of TLR genes have been associated with human cancers [23-25], indicating the importance of these receptors in body homeostasis. The first indication that TLRs may have a role in tumorigenesis came from administration of LPS concomitant with metastasizing carcinoma cell lines to animal models [8,25]. In these experiments it was observed that LPS both causes migration and enhances invasion of the tumor. TLR activation may impose its effects on the tumor cells themselves or on the microenvironment through induction of tumor growth promoting factors. For example, direct TLR ligation on a TLR-4 expressing tumor cell line led to growth of the tumor independent of exogenous administration of LPS [8,9]. Moreover, LPS treatment of tumor cells *in vitro* led to their growth inhibition after injection into mouse models [26]. This growth inhibition was not due to a direct effect of LPS binding, but was mediated by T cells. On the other hand, in another study it was shown that LPS exposure resulted in induction of tumor growth and drug resistance, which was due to LPS binding and dependent on MyD88 and NFκB [27]. Moreover, systemic administration of LPS in an animal model enhanced the growth of adoptively transferred tumor cells by increasing the circulating TNF level which up-regulated anti-apoptotic factors such as Bcl-XL, cIAP1, and cIAP2 [8]. Taken together, TLR triggering has heterogeneous effects on tumor cells depending on experimental conditions and may lead to tumor growth induction [27] or inhibition [26], apoptosis [28,29], invasion [30], and drug-resistance [27,31].

3. TLR signaling in MM cells

3.1. TLR expression pattern

Human B cells express several TLRs, with TLR7, 9 and 10 being strongly expressed [32-34]. On the other hand, among different B cell subsets the pattern of TLR expression and activation varies, for example circulating naïve B cells show weaker expression and response to some TLR activation than memory B cells [33-35]. Interestingly, normal human plasma cells also show a different pattern of TLR expression compared to normal B cells, as plasma cells express some TLRs which are not expressed by B cells or vice versa [36]. This indicates that TLR expression in human B cells may be developmentally regulated [33,35]. TLR expression is not limited to normal immune cells but it has also been demonstrated on tumor cells, including solid tumors [31,37], leukemias such as acute and chronic lymphocytic leukemias (ALL and CLL) [33,38,39], lymphoma [33,40] and MM [11-14,33]. Myeloma cells, as the malignant counterpart of normal B or plasma cells, display a distinct pattern of TLR expression and response to ligand binding as has been reported in several studies [11,12,14,33,41]. MM plasma cells express a broad range of TLRs including TLR1-9, but with a heterogeneous distribution among cell lines and primary cells from patient bone marrow or other tissues. Moreover, it has been reported that TLR1,7 and 9 are the most significantly expressed and TLR2,8 the least expressed TLRs on MM primary cells [13]. When compared with normal B cells, MM cells loose TLR10 which is expressed significantly by B cells whereas they acquire TLR3,4,8 which are almost not expressed by B cells [11,33]. This pattern of TLR expression in MM cells is apparently similar to that in normal plasma cells from tonsils and cord blood [36]. TLR expression on plasma cells may be tissue-specific. Peripheral blood and tonsilar plasma cells exhibit the same expression with respect to TLR1–TLR9, but TLR9 is more expressed in blood [36]. Bone marrow plasma cells from normal donors express only TLR1 [12] or they only show weak expression of a few TLRs [14]. Given the weak expression on normal plasma cells and the high expression of TLRs on MM cells in the bone marrow, these receptors may be involved in the pathogenesis or progression of the disease. It is tempting to speculate that the microenvironment of MM cells may determine the TLR expression in these cells. Malignant plasma cells are highly dependent on bone marrow stromal cells (BMSCs) and extracellular matrix (ECM) for growth and survival [2]. Adhesion of MM cells to BMSCs has been associated with significant IL-6 [42,43], VEGF (vascular endothelial growth factor) [44] and bFGF (basic fibroblastic growth factor) secretion [45] and NFκB activation [46]. However, the importance of this cellular adhesion for modulation of TLR expression needs further research. Mutations in NFκB pathway components might also contribute to TLR modulation on MM cells, because such mutations have been reported to result in constitutive activation of both classic and alternative pathways of NFκB signaling [47,48]. However, other factors such as viral

infections and endogenous ligands (see Section 5) could also be involved in TLR modulation on MM cells. Furthermore, the source of human myeloma cell line (HMCL) isolation appears to influence TLR expression patterns on these cells. For example, expression of TLR4 and TLR7 on cells isolated from pleural effusion was found higher than on cells isolated from bone marrow in a single patient, while TLR9 was detectable only in the bone marrow [11]. The authors also relate this discrepancy to disease stage and differences in the cellular microenvironment. It is important to note that heterogeneity in TLR expression on patient plasma cells and HMCLs is also apparent between studies. For instance, TLR4 was found expressed on 33% of MM primary cells in one study [11] and on 70% in another [10].

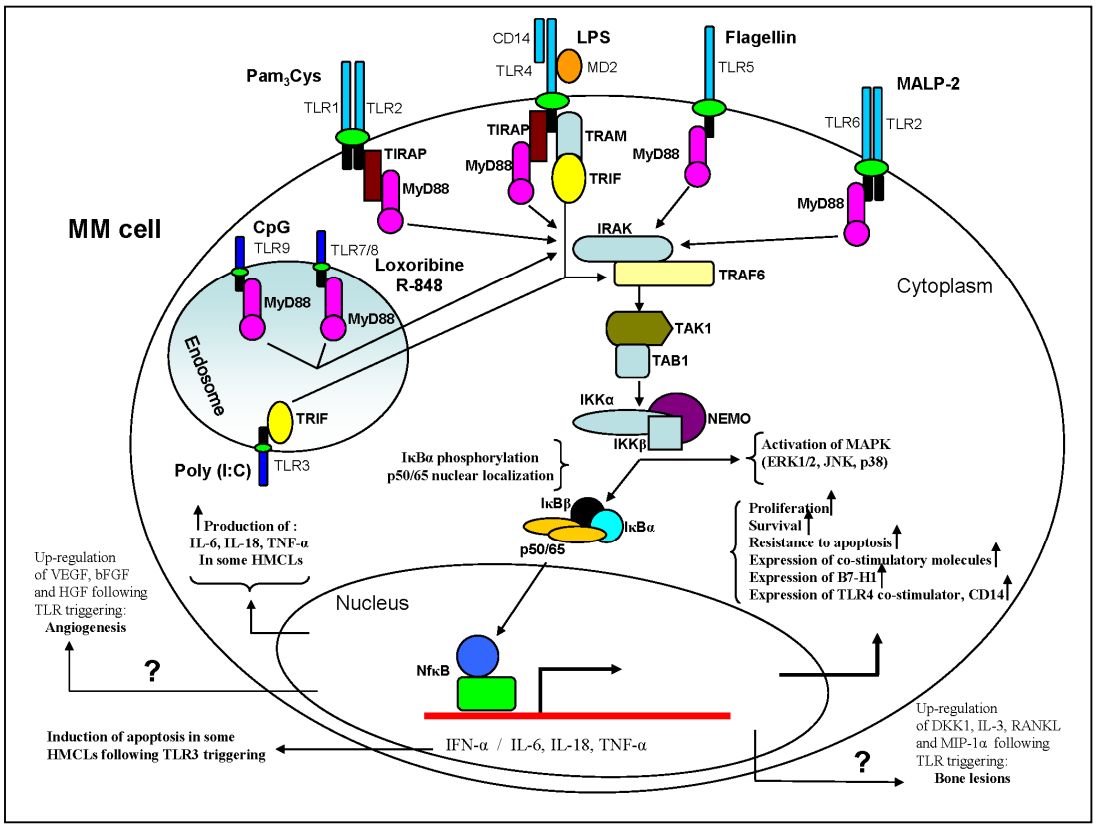

Figure1. Effects of TLR activation on MM cells. Various ligands which have been used in some studies are depicted here. Following triggering of most TLRs on HMCL or MM primary cells with their relevant ligands, heterogeneous effects have been reported. In some studies, increase in proliferation, survival and resistance to drug-induced apoptosis have been the most conspicuous effects, with activation of NFκB and MAPKs being involved. With some HMCLs, apoptotic effects were observed following TLR3 ligation. Up-regulation of co-stimulatory molecules and B7 family members has also been observed following TLR activation mediated by MyD88 / TRAF-6 leading to immune response evasion. In addition, almost all the studies have indicated secretion of pro-inflammatory cytokines by HMCLs and MM primary cells following TLR activation, especially IL-6 as the most important growth factor for these cells. Additionally, expression of most adaptor molecules including MyD88, TRIF and TRAF6 in HMCLs has been reported. Whether TLR activation on MM cells and HMCLs or on cells in myeloma context contributes to angiogenic switch (e.g. through induction of VEGF, bFGF and HGF) or to osteolytic lesions (e.g. through induction of MIP-1α, IL-3, RANKL and DKK-1) remains to be determined. ***Abbreviations*** – IKK: IκB kinase, IRAK: IL-1 receptor-associated kinase, MyD88: Myeloid differentiation 88, NEMO: NFκB essential modulator, TAB: TAK1-binding protein, TAK: transforming growth factor- (TGF-β)-activated kinase, TIRAP / MAL: TIR domain-containing adaptor protein / MyD88-adaptor-like, TRAM: Toll-like receptor associated molecule, TRIF: TIR domain-containing adaptor protein inducing IFN-β

3.2. TLR triggering effects: proliferation and survival

TLR activation, mostly TLR1, 6, 7, 9 and 10, on normal human B cells has been associated with diverse effects including proliferation and survival [32,33,48], release of pro-inflammatory cytokines (IL-6, IL-10, type-I IFN) [32,33,49], and differentiation into antibody secreting cells or plasma cells [35,50]. Normal plasma cells also respond to TLR activation with an increase in intracellular immunoglobulin and immunoglobulin release [36]. Normal human B cells express B lymphocyte maturation protein-1 (BLIMP-1) in response to LPS and CpG treatment to differentiate into plasma cells, while inhibition of NFκB following TLR4 and TLR9 triggering will block the above effects [50-52]. Surprisingly, NFκB binding sites have been detected within the *prdm1* (BLIMP-1 encoding) gene sequence [51,52]. BLIMP-1 is also indicated to be required for maintenance of long-lived myeloma plasma cells inside the bone marrow [53], implicating a probable contribution of TLR activation to this functional response. This BLIMP-1 feature in malignant plasma cells is a hallmark of plasma cell differentiation which has been conserved during oncogenic process [52]. In support of this, MyD88, the main adapter in TLR signaling, plays a central role in plasma cell differentiation inside the bone marrow [54]. Moreover, although IL-6 supports MM cells survival *in vitro*, *IL-6*$^{-}$*/IL-6*$^{-}$ mice had normal plasma cell maintenance *in vivo*, indicating other factors in the myeloma microenvironment, leading to NFκB activation, e.g. following TLR or TNFR triggering, might provide signals for BLIMP-1 up-regulation and plasma cell maintenance [53]. Taken together, it is conceivable that expression and activation of TLRs on human B and plasma cells play a role in development and effector functions. The functional response to TLR activation in MM cells has not been studied as extensively as in B cells. In some studies performed to date, TLR triggering on HMCL and MM primary cells has been associated with heterogeneous effects including increase in proliferation [11-14,33,41,55], survival [11-14,33,55], cytokine and chemokine production [32,41], induction of apoptosis or saving from apoptosis [11-13,33,55], drug-resistance [11] and immune escape [32,33,55] (Table 1, and illustration at Fig. 1). For instance, Pam3Cys and flagellin induced greater proliferation than IL-6 (the most important growth and survival factor for MM cells). MALP-2, LPS and R-848 were as effective as IL-6, whereas poly (I:C) and CpG had negative effects on proliferation in some cell lines [12]. Stimulation with TLR specific ligands also resulted in significant proliferation of MM primary cells with R-848 being the most potent of all, while CpG ODN and poly (I:C) had negative effects on some cells [12]. In another study [11], Loxoribine and CpG protected the cells from apoptosis, Loxoribine had no effect on proliferation but CpG induced significant proliferation of IL-6-dependent cell lines. CpG ODN also induced significant proliferation and production of TNF-α, but not IL-6 or IL-10, in RPMI 8226 cell line (IL-6-independent) [41]. In the above studies, although few TLR ligands had negative effects on proliferation, most of them had positive effects on survival of

the cells. Furthermore, in the study of Zhen et al. (data presented at 51st ASH annual meeting and exposition, December 5–8, 2009), TLR4 signaling resulted in various effects on myeloma primary cells and HMCLs, including increase in proliferation, protection from apoptosis induced by adriamycin, activation of MAPK pathway, and increase in the level of IL-6 and IL-18. Increased serum level of IL-18 is clinically informative in MM, as it has been shown to indicate a poor prognosis [56]. Surprisingly, CpG ODN treatment of B-CLL cells increased apoptosis and reduced their number over time, whereas the same ligand increased the number of normal B cells [40]. Most TLR ligands have been shown to have apoptotic and immunosensitizing effects on B-CLL or B lymphoma cells (Table 2). The reasons why MM cells behave differently in terms of TLR activation compared to malignant B cells in CLL or B lymphomas, are not clear.

3.2.1. Role of IL-6 in TLR-induced MM cell proliferation and survival

The molecular basis of TLR activation of MM cells has not been clearly defined. IL-6 is the only factor reported to date to partly mediate the proliferative and anti-apoptotic effects of TLR signaling apparently through an autocrine mechanism [11-14]. This is supported by the observation that when HMCLs were cultured in the absence of Loxoribine and CpG, no IL-6 was detected in the supernatant. CpG and Loxoribine induced IL-6 production and neutralization of IL-6/IL-6R blocked proliferation. It should be noted, however, that in the IL-6-independent RPMI 8226 cell line, LPS, CpG ODN and MALP-2 induced a significant proliferation [12,41], and some TLR ligands including LPS and Pam3Cys could replace IL-6 in long-term (28 days) culture of IL-6-dependent cell lines [12]. The molecular pathways and mediators governing this TLR triggering contribution to MM cell survival and proliferation have not been clarified in these experiments. Indeed other studies have deciphered the role of IL-6 in proliferation [57], survival [58], resistance against drug- [59] and FASL- [60] induced apoptosis of MM cells, and the intracellular IL-6-containing myeloma cell clones have been associated with persistent and active disease [61]. Nonetheless, MM pathogenesis cannot be explained based on IL-6 alone, because IL-6 transgenic mice do not always develop plasmacytomas [62] and apoptosis of MM cells induced by cytotoxic drugs, tamoxifen or irradiation is not affected by IL-6 [62,63]. However, IL-6 functions as a key factor in MM progress because IL-6 gene knock-out mice fail to develop pristane-induced plasmacytomas [62]. It has been demonstrated that IL-6 modulates growth and survival of MM cells and these two effects are regulated by distinct pathways, with MAPK cascade controlling cell growth and stat-3 pathway having anti-apoptotic effects [64,65]. Interestingly, it is suggested that IL-6 signaling pathway in IL-6-dependent HMCLs is different from that in IL-6-independent ones, with Ras-dependent MAPK cascade activation in the former and STAT-1, STAT-3 or both in the latter [66,67]. Indeed, existence of different signaling pathways for

TLR and IL-6 induced survival and growth of MM cells might be plausible, as it has been shown that TLR4 triggering-induced proliferation of plasmacytoma or hybridoma cell lines is mediated by MAPK pathway activation, while IL-6 functions through STAT3 activation in these cells [68]. Regarding the key role of IL-6 in MM pathogenesis, the main origin or inducer of IL-6 is controversial, although stromal cells of bone marrow microenvironment (paracrine) [42,43,45] and MM cells themselves (autocrine) [61,69] have been indicated to be involved.

3.3. TLR triggering effects: apoptosis

Signaling through TLR3, which uses a MyD88-independent TRIF-dependent pathway, has been reported to have heterogeneous effects on HMCLs leading to increased proliferation and survival in some cells and to cell death and apoptosis in others [13,70]. Present studies suggest that the apoptotic effect is controlled by p38MAPK and mediated by IFN-α, and the proliferative effects are due to NFκB activation. This is not unexpected because the association of NFκB activation with malignant B cell proliferation and survival [47,48,71] and the inhibitory effects of IFN-α on growth and proliferation of normal and malignant haematopoiesis are well established [72,73]. On the other hand, IFN-α can also protect HMCLs against dexamethasone-induced apoptosis [74]. This effect was mediated by AP-1 and STAT activation, thus limiting the use of corticosteroids in MM patients. In fact type-I IFNs may have various effects on malignant cells depending on the cell type, tumor environment, secreted products and degree of cell differentiation [75], and whether this could explain part of disparities in TLR triggering responses between various MM cells is not known. TLR3 triggering also increased cycloheximide-induced apoptosis in other tumor cell lines which was shown to be mediated by IFN-β [29]. However, IFN-β neutralization had little effect on apoptosis, so other molecules downstream to TLR3 triggering may have been involved. Other mediators in TLR3 signaling such as TBK1, RIP-1 and IKKi or transcription factors such as IRF3 and IRF7 could also be important, because TRAF-6 (or RIP-1)/TBK1/IKKi has been accepted as the alternative pathway in TLR3-induced type-1 IFN production leading to apoptosis [22]. Whether this pathway is operative in MM cells or HMCLs remains to be investigated.

TLR activation has also been associated with apoptosis in most B cell malignancies (CLL and B lymphoma), although some ligands such as CpG may induce significant proliferation but it usually ends in apoptosis [40](Table 2). TLR ligation has heterogeneous effects on MM cells which will vary depending on experimental conditions and the types of the cells investigated (primary or cell line). The expression and triggering effects of TLRs on B cell malignancies (ALL, CLL) and MM cells are summarized in Tables 1 and 2.

3.4. Immunomodulatory effects of TLR signaling in myeloma environment

To survive, expand and metastasize, tumor cells are dependent on signals received from their micro-environment and they also have to overcome constant immune surveillance by the host immune cells. This dictates a compulsory dynamic interaction with the immunologic environment of the tumor [7]. Likewise, MM cells have to challenge the immune surveillance in their environment to survive and expand. Several mechanisms have been reported for MM tumor to achieve this, such as impairing the function of dendritic cells [76], NK cells [77], and T regulatory cells [78]. However, these mechanisms are less defined on a molecular basis. Huang et al. showed that stimulation of mouse tumor cell lines with LPS enabled the cells to suppress CTL lysis, impair NK cell function and T cell specific immune response [7,9]. TLR activation, especially TLR7 and 9, in B lymphoma and B-CLL cells has also been associated with remarkable immunomodulatory effects (Table 1). Interestingly, signaling via TLR2, 4 or 9 and IFN-γ in HMCLs or primary cells of MM patients has been associated with an increase in the expression of B7-H1 (PD-L1 or CD274) through a MyD88/TRAF-6-mediated and MEK dependent pathway, thus making MM cells able to inactivate T cells and prevent CTL lysis [10]. B7-H1 is not expressed by normal plasma cells but only by a few MGUS primary cells and is thought to play a role in MGUS progression to MM by immunosuppressive effects [10]. Additionally, being equipped with several TLRs and related adaptors and proteins, MM cells will be able to function like dendritic cells or B cells to initiate an immune response by responding to PAMPs in their microenvironment [13]. Moreover, MM cells can act as APCs because expression of MHC-I/II and costimulatory molecules, CD40, CD80 and CD86, is increased following IFN-γ treatment and they launch a more proliferative T-cell response [79]. Interestingly, when chloroquine was used to inhibit endosomal acidification, T-cell stimulation and antigen presentation were completely abolished. In support of this, CpG ODN treatment of RPMI 8226 cell line was found to up-regulate the surface molecules, CD54, CD80, CD86 and HLA-DR and increase allogeneic T-cell proliferative response in mixed lymphocyte reaction (MLR) [41]. In this sense, MM cells must be recognized by the immune system machinery to induce effective immune responses, but this is apparently hampered by various evasive mechanisms. Immune dysfunction is a hallmark of MM, which is characterized at least by a defective T and dendritic cell function [76,78]. Bone marrow derived plasmacytoid dendritic cells (pDCs) from MM patients show reduced ability to stimulate T-cells and TLR9 activation of these pDCs, restores their ability to induce T-cell specific response and block growth of MM cells *in vitro* [80]. On the other hand, in MM due to influence of malignant clone on number and function of T regulatory cells, hyper-reactive T cells with defective TCRs will emerge which fail to launch an effective immune response [78]. Furthermore, in the study of Prabhala et al. when monocytes from MM patients and normal donors were stimulated with LPS, the MM-derived cells

produced much less TNF-α. This abnormal monocyte response in MM could lead to generation of Th17 cells, which are recently reported to play a key role in many immune related diseases and cancer. In another study by the same authors, it was shown that LPS will overcome the suppression of T-cell proliferation by Treg cells in normal controls but has little effect in MM or MGUS. Moreover, peripheral blood mononuclear cells (PBMCs) from MM patients showed much less T-cell proliferative responses in the presence of ligands for TLR2,3,5,7,9 compared with PBMCs from normal donors [Prabhala et al., data presented at XIth. international myeloma workshop & IVth. international workshop on Waldenström macroglobulinemia, 2007]. Taken together, defective immune response in MM patients may be multi-factorial and TLR activation in myeloma environment may contribute to this abnormality through immunomodulation.

3.5. Cytogenetic relevance of TLR expression in MM

Interestingly, it has been indicated that the expression pattern of TLRs and their relevant adapter molecules can be prognostic in MM [13,33,81]. For example, over-expression of TLR4 in malignant plasma cells has been reported to be associated with activation of proto-oncogenes, *MAFB* and *c-MAF*, which usually indicates a poor prognosis. On the other hand, it is reported that hyperdiploid patients who usually have a more favorable prognosis, mostly overexpress TLR3 and TLR7 but underexpress TLR4 and TLR9 [13]. Whether this association is an influence of the oncogenesis process in MM or other factors is presently not known. Interestingly, the expression level of MyD88 and TLR increases in transition from MGUS to MM [33]. In some MM patients and cell lines a chromosomal translocation (t(6;14)(p25;q32) juxtaposes the immunoglobulin heavy chain locus to *IRF4*/*MUM1* resulting in over-expression of IRF4 [82], which is an adapter molecule in TLR signaling and has been suggested to play a negative regulatory role [20,83].

4. TLR signaling and pathophysiology of MM: angiogenesis and bone lesions

4.1. Angiogenesis

The potential role of TLR signaling in angiogenesis is indicated by the findings that: (a) MALP-2 directly induces angiogenesis and endothelial cells proliferation in the presence of GM-CSF [84]; (b) LPS exposure following injection of lung tumor cells into mice leads to an increased tumor burden in the lung [25]; (c) signaling through TLR3 leads to suppression of angiogenesis [85]; (d) The TBK1/TRIF/IRF3 signaling pathway controls the expression of pro-angiogenic factors, VEGF and bFGF [86].

4.1.1. Does hypoxic environment in myeloma bone marrow contribute to TLR activation

It is reported that unlike other tumors, MM is highly dependent on bone marrow microenvironment which is hypoxic in nature [87,88]. Indeed MM clones benefit from hypoxia [89]. Interestingly, it has been recently indicated that hypoxia can optimize tumor environment for survival and expansion following TLR triggering, mostly through the induction of hypoxia-inducible factor-1alpha (HIF-1α) protein [90]. HIF-1α is a transcription factor which controls the genes encoding glycolytic enzymes, VEGF and genes associated with innate immune function such as TNF-α and NO [91,92]. Moreover, HIF-1α is up-regulated in inflammatory cells (especially macrophages) to make them more adapted for anaerobic metabolism and launching immune responses in a hypoxic environment [92,93]. A possible contribution of TLR signaling to angiogenesis in hypoxic MM environment may be through the induction of inflammatory cytokines by innate immune cells especially macrophages, which in turn leads to optimization of tumor environment. Two factors have been reported to be associated with angiogenesis in myeloma, HIF-1α [87-89] and HSP-90 or GP96 [94,95]. In hypoxia-exposed MM cells and BMSCs of myeloma patients, HIF-1α was up-regulated which in turn increased the production of VEGF and IL-8 (Simon et al., data presented at the 50th ASH meeting and exposition, 2008). HIF-1α has been reported to modulate expression and activation of TLRs [96]. Moreover, release of angiogenic factors such as bFGF and hepatocyte growth factor (HGF) following TLR activation has been well indicated in endothelial and fibroblasts (two important bone marrow components) [17,97]. Additionally, hypoxia can induce heat shock proteins (HSPs) including HSP90 which has been demonstrated to have a positive effect on myeloma cell growth and survival [94,95], possibly by acting as endogenous ligands for TLRs (see Section 5). It has been suggested that HSP90 can bind to CpG DNA to induce TLR9 signaling [98]. In support of this, HSP90 inhibition led to a decrease in TLR4 and 9-induced production of inflammatory cytokines [99]. Taken together, it could be speculated that malignant clones may aggravate hypoxic condition in bone marrow of MM patients and up-regulate HIF-1α in MM cells and BMSCs affecting expression and activation of TLRs.

4.2. Bone lesions: possible contribution of TLR activation on MM cells

It has been well described that bone lesions in MM can be induced following interaction of myeloma cells with BMSCs, osteoblasts/osteoclasts, and also with ECM leading to the production of osteoclast-activating factors such as IL-1β, IL-6, TNF-β and particularly receptor activator of NF-kB ligand (RANKL) [2,100]. TLR9 activation on RPMI 8226 cells induced significant TNF-α production playing a key role in MM osteoclastogenesis [41,100]. However, the most critical element of the induction of bone lesions in MM is the RANK/RANKL/osteoprotegerin (OPG) interaction [2], with RANK being expressed on

osteoclasts and RANKL on osteoblasts or T-cells [2,15,16,100,101]. OPG is secreted by BMSCs and osteoblasts, and competes as a decoy receptor with RANK for RANKL. Whether TLR triggering in the myeloma environment could influence interaction of MM cells with BMSCs and osteoblasts/osteoclasts remains to be determined. It would be of interest to investigate if an interaction of TLR-activated MM cells with ECM or myeloma-derived BMSCs and bone cells results in osteoclast activating cytokines. In experiments with mouse osteoclasts, TLR4 signaling combined with hyaluronic acid signaling inhibited RANKL-induced osteoclast differentiation by interfering with M-CSF-dependent pathways involved in RANK expression [102]. In support of these findings, c-Fms (M-CSF receptor) has been reported to be critical in osteoclastogenesis [103].

4.2.1. Could TLRs be critical in linking inflammation/infection to osteolytic lesions?

TLR triggering on MM cells following infection, inflammation or endogenous ligands release (see Section 5) may enhance secretion of osteoclast-activating cytokines. Activation of TLRs on osteoblasts/osteoclasts should also be addressed. Osteoclasts share a common progenitor with dendritic cells and monocytes/macrophages, thus it is likely that bone cells express functional TLRs [16]. In support of this, a recent study demonstrated that human osteoclasts expressed TLR4 and could function as APCs like dendritic cells to present antigens to T-cells [15]. This function was enhanced by LPS and IFN-γ treatment. Furthermore, T cells also express RANKL which interacts with RANK on osteoclasts modulating their activity [16,100,101]. Indeed, LPS binding acts as a potent stimulator of bone loss or osteolytic lesions [104,105], and CpG ODN binding on osteoblasts contributes to osteoclastogenesis by increasing RANKL expression [106]. However, it has also been indicated that TLR activation on bone cells may function as negative regulator of osteoclastogenesis [16,107]. The reason for this discrepancy is not clear, although infections involving the bone environment are associated with increase in bone resorption due to osteoclast activation [16]. Taken together, it is speculated that the combined effects of TLR ligation on MM cells, which may be associated with induction of osteoclast-activation factors, and TLR activation on bone and immune cells (especially T-cells) would orchestrate a scenario which could aggravate the bone resorption and loss in MM patients. This concept suggests the importance of close monitoring of MM patients in cases of infections.

5. How is TLR signaling activated in the context of myeloma tumors?

5.1. TLRs and the concept of endogenous ligands

It has been reported that microbial ligands are not the only activators of TLRs, because TLR activation may occur in "sterile inflammation", e.g. inflammation induced by cellular necrosis in trauma or malignancy [8,108,109]. Thus the concept of endogenous ligands, so-called "danger associated molecular patterns (DAMPs)" or "alarmins", for TLRs has emerged as the "danger signaling model" [110]. Some of the identified alarmins are: high mobility group box 1 (HMGB1), HSPs (HSPs 60, 70, 90 or GP96) and ECM proteins (fibronectin, fibrinogen, hyaluronan and heparans) [8,108,109]. In MM environment (primarily bone marrow) where matrix destruction is ensuing following MMP activation [111,112], ECM degradation products must emerge which may trigger TLRs [109,113,114], e.g. on malignant plasma cells and other cells in their microenvironment. Moreover, nucleic acids released from necrotic tissues, e.g. in a malignant environment, may provide an amplification loop in MM [115]. Soluble syndecan-1 (CD138) a heparan sulphate shed by MM cells has also been suggested to trigger TLRs on MM cells or those of the microenvironment [13]. Indeed TLR activation has been associated with above endogenous ligands, although their direct role is still controversial [108,109]. HMGB1 interacts with several TLRs and induces osteoclastogenic and angiogenic factors in surrounding cells [116], but it is not known whether it binds directly to these receptors [117]. No study has shown HMGB1 effects in MM, although they have been delineated in some leukemic conditions and solid tumors [33]. HSPs, which can also be associated with TLR activation and angiogenesis [94,95], have been indicated to play a role in malignant plasma cell biology and MM pathogenesis [118]. Thus, release of TLR endogenous ligands in MM environment can lead to activation of TLRs and could potentially contribute to the optimization of tumor growth conditions.

5.2. Inflammation/infection, TLRs and myeloma progression

The description of expression and activation of several TLRs on MM cells has opened a new insight into the interaction with their inflammatory environment in bone marrow [13,115]. Several reports indicate a history of previous infection or inflammation in MM patients [119,120]. Furthermore, human herpes virus-8 (HHV-8, a DNA virus also known as Kaposi sarcoma associated virus) infection has been documented in most MM cases, and its genome has been detected in non-malignant dendritic cells and serum of MM patients [121]. HHV-8 has been implicated in MM pathogenesis possibly through induction of viral IL-6 (vIL-6) in the bone marrow, although this is still controversial [122]. Interestingly, HHV-8 has been shown to activate TLRs [123], but whether it may activate TLRs in dendritic cells of MM bone marrow to influence their interaction with MM cells remains to be determined. Another study showed that cytomegalovirus (CMV) infection of HMCLs could protect these cells

from apoptosis through increase in IL-6 production [124]. Immune dysfunction and infections in MM [3,4,76,78] will provide a vicious cycle involving TLR activation-induced MM cell growth and proliferation, immunosuppression and disease progression [115]. In general, if the causes of chronic inflammation in tumor environment are bacterial and viral factors which have not been cleared efficiently, danger molecules such as LPS, dsRNA and dsDNA (CPG) are also able to engage the TLRs on tumor cells or macrophages and neutrophils leading to tumor progression [18,125]. Thus, pathogen-induced TLR activation may function as a link between pathogens, inflammation and cancer [8,126]. In support of this, NFκB activation has been observed in some inflammation-associated cancers [8,127], regarding NFκB as the main transcription factor downstream of TLR signaling. Constitutive NFκB activation has been reported in some blood malignancies including leukemias [71], and MM [47,48,70,128]. It has been indicated that NFκB activation in MM cells up-regulates the expression of some critical elements including IL-6, VEGF, bFGF and adhesion molecules [126]. Moreover, significant role of NFκB activation in MM pathogenesis has been deciphered at gene level [47,48].

6. TLR ligands for immunotherapy in MM: is that really applicable?

While clinical application of TLR ligands has been well established in a variety of malignancies including B-CLL and B lymphomas [129,130], it has not been clinically tested in myeloma patients. Although TLR triggering in B-CLL and B cell lymphomas has mostly led to apoptosis and growth arrest *in vitro*, it is too early to predict its applicability for therapy of MM because of the limited number of scientific studies available. The main reason for this obstacle could be the overt heterogeneity in TLR expression and activation among HMCLs or patient primary cells [11-14,41,70], and particularly the potential of myeloma cells in production of IL-6 following TLR activation. IL-6 is the major growth and survival factor for these cells and has been interestingly referred to as *the pro-tumorigenic effect of TLR activation* in MM [13]. Thus, based on the present studies use of TLR ligands for targeting MM cells does not seem to be promising. On the other hand, use of CpG as an adjuvant with heat shock protein GP96 to enhance immune responses against myeloma tumors in xenograft mouse models showed encouraging results [131]. However, the effect was also dependent upon T cells and IFN-γ and in high tumor burden this may compromise a beneficial outcome. In another study CpG was used to stimulate pDCs and this led to restoring of pDC immune function (induction of T-cell proliferation) and blocking of MM cells growth [80]. The authors suggest this as a therapeutic strategy to target pDC-MM interaction. Alternatively, down-regulation of TLRs on MM cells may be putative targeting approach?. For instance, blocking TEAF6 (downstream to TLR signaling) has anti-proliferative effects on myeloma cells [132,133].

Taken together, although *in vitro* studies have deciphered anti-apoptotic and proliferative effects of TLR ligands on MM cells, well-established *in vivo* animal models of myeloma and further *in vivo* studies are needed to provide these findings with more support. Moreover, TLR ligands therapy in MM will tend to create a "double-edged" sword as long as more detailed research does not resolve the molecular basis of the difference in TLR activation between MM cells and other malignant B cells, especially B-CLL and B-lymphoma. Finally, a new question arises as to whether natural negative regulatory mechanisms, for example single immunoglobulin IL-1R-related molecule (SIGIRR), are active in malignant plasma cell. This signaling pathway being referred to as "a break" in TLR activation [134], has been reported to have a pivotal role in controlling the cell response to TLR activation [115,135]. In SIGIRR-deficiency, there will be an enhanced inflammatory response indicated by hyperresponsiveness to TLR triggering [135], however no study has as yet investigated this in MM. (For ease of comparison, the effects of different TLR ligands on various MM cells and other B cell neoplasms are given in tables 1 and 2, including also findings from refs: [39,136-144]

7. Concluding remarks and future prospects

MM patients are vulnerable to a variety of infections because of a derangement in immune responses and decrease in normal immunoglobulins due to the effect of malignant clones. TLR triggering by PAMPs or DAMPs in infections or inflammation is normally exploited by immune cells such as dendritic cells to challenge the pathogens or tumor cells in co-operation with T-cells. However, this may not be effective in MM patients because of the evasive mechanisms used by the malignant plasma cells and since TLR activation has been shown to support malignant cell survival and proliferation, at least in *in vitro* studies. At this point, infections and inflammation will always be in favor of the malignant cells. On the other hand, since TLRs are expressed on a variety of cells, including those of the myeloma cell environment, it is expected that stimuli such as danger signals (infection or tumor-induced) could also lead to TLR activation on these cells contributing to complications such as angiogenesis and bone lesions. TLR ligands have recently achieved a good position as immunoadjuvants in cancer immunotherapy, but using these ligands in the therapy of human myeloma has not gained support yet. Indeed more research on the molecular pathways and mechanisms involved in the response of myeloma cells to TLR ligands as well as *in vivo* studies are required to gain further insight into the immunotherapeutic prospects of TLR agonists/antagonists?

Table1. Various effects of TLR triggering on MM cells and cell lines

Cell type	TLR activated- TLR ligand used	Cellular response	Refs.
MM cells, HMCLs	TLR2 - PGN TLR4 - LPS TLR9 - CpG ODN	- Up-regulation of B7-H1 molecule →→ ***immune response evasion***	[10]
MM cells , HMCLs	TLR7- Loxoribine TLR9- CpG ODN	- Increase in proliferation - Saving the cells from serum deprivation and dex. induced apoptosis - Autocrine secretion of IL-6 →→ ***growth and survival of malignant plasma cells***	[11]
MM cells, HMCLs	TLR1/2 - Pam3Cys TLR3 - Poly I:C TLR4 - LPS TLR5 - Flagellin TLR7/8 - R 848 TLR9 - CpG ODN	- Increase in proliferation of most cells - Decrease in proliferation of some cells - Replacing IL-6 in long-term culture - IL-6 autocrine secretion →→ ***growth and survival of malignant plasma cells***	[12]
HMCL	TLR9 – CpG ODN	- Increase in proliferation through NFκB activation - Up-regulation of surface molecules, CD54, CD80, CD86 and HLA-DR - Increase in allogeneic T-cell proliferation in MLR - Increase in TNF- α production →→ ***proliferation of malignant plasma cells*** →→***increasing the immunogenicity of malignant plasma cells***	[41]

Table 1. *continued…..*

Plasmacytoma cell line	TLR4 - LPS	- Increase in proliferation through p38MAPK activation →→ ***growth and proliferation of malignant plasma cells***	[68]
HMCLs	TLR3 - Poly I:C	- Increase in proliferation of some cells through activation of NFκB - Apoptosis in some cells through p38MAPK and IFN-α secretion →→***variable effects on malignant plasma cells***	[70]
HMCLs	TLR4 - LPS	- Increase in proliferation through NFκB activation - Up-regulation of CD14 - Saving from adriamycin-induced apoptosis - Increasing the number of cells in S phase - Increase in IL-6 and IL-18 secretion - Increase in phosphorylation of ERK1/2, p38 and JNK - Decrease in allogeneic T cell proliferation in MLR →→ ***growth and survival of malignant plasma cells*** →→ ***immune response escape***	Zhan Cai, et al. Data presented at the 51st ASH annual meeting and exposition

Table2. The effects of TLR triggering on B cell malignancies (CLL, ALL) and B lymphomas

Malignant B cells from B-CLL patients	TLR7 - R 837 TLR9 - CpG	Increase in the expression of co-stimulatory molecules →→ ***increasing the immunogenicity of the tumor cells and enhancing T cell response***	[38,137]
Malignant B-ALL and PB-ALL cell lines	TLR1/2 - Pam3Cys TLR2 - PGN TLR3 - P:IC TLR4 - LPS TLR5-flagellin TLR7 - Loxoribine TLR9 - CpG	- Increase in the expression of co-stimulatory molecule CD40, especially with PGN and Pam3 (both TLR2 ligands), but none of cell lines showed changes in CD80 or CD86 expression - Only TLR2 ligand induced a strong T cell response (IL-5 and IFN-γ production) to BCP-ALL cell lines. →→ ***increasing the immunogenicity of the tumor cells and enhancing T cell response***	[39]
Malignant B cells from B-CLL and different B lymphomas	TLR9 - CpG ODN	- Activation of all types of malignant B cells, but with variations between different cells - Increase in proliferation - Increase in co-stimulatory molecules (CD40, CD80, CD86, CD54) and MHC-I/II →→ ***increasing the immunogenicity of the tumor cells and enhancing T cell response***	[40]
Malignant B cells from B-CLL patients	TLR7 - S28690	- Increase in the expression of co-stimulatory molecules - Activation of PKC - Increase in the proliferation of T cells and in the ability of CLL B cells in antigen presenting →→ ***making the tumor cells more sensitive to immunotherapies and cytotoxic killing***	[136]

Table 2. *continued…..*

Malignant B cells from B-CLL patients	TLR9 – Immuno stimulatory ODN with or without CpG motifs and with PD or PS backbones	- Increase in apoptosis of B-CLL cells which is associated with specific chromosomal deletions (13q) - Apoptosis is caspase-dependent and mediated by death receptors (Fas and TRAIL) →→ ***blocking tumor cell growth***	[138]
Malignant B cells from B ymphoma	TLR9 - CPG ODN	- Increase in the expression of co-stimulatory molecules (CD40-CD80-CD86-CD54), MHC-I/II - No effect on reactive B cells →→ ***making the tumor cells more sensitive to immunotherapies***	[139]
Malignant B cells from B-CLL patients	TLR9 - CpG ODN + Nutlin-3 (inhibitor of mdm2 / p53 interaction	- Increase in the expression of co-stimulatory molecules (above) - Enhancing the cytotoxic effects of Nutlin-3 through up-regulation of CD95 and TRAIL-2 receptors →→ ***making the tumor cells more immunogenic and more sensitive to cytotoxic drugs***	[140]
Malignant B cells from B-CLL patients	TLR7 - S28690	- Decrease in TNF-α production, TLR7 mRNA and IRAK-1 level in **re-stimulation** with TLR7 ligand: a state of " **tolerance**" in malignant B cells -This tolerization was characterized by block in NFκB and stress-activated PKC activation →→ ***making the tumor cells more sensitive to cytotoxic drugs***	[141]

Table 2. *continued……..*

Malignant B-ALL and PB-ALL cell lines	TLR9 - CpG ODN	No significant increase in co-stimulatory molecules (CD80, CD86) expression, but significant increase in CD40 expression, on both cell groups. Correlation between CD40 and TLR9 expression levels →→ ***making the malignant pre-B cells more responsive to T cells and so launching a more potent immune response***	[142]
Malignant B cells from B-CLL patients	TLR7 - S28690	-Increase in the expression of co-stimulatory molecules(CD80, CD86, CD83) on CLL cells with IL-2 - Increase in the expression of IL-2R(CD25), →→ ***making the cells more immunogenic***	[143]
Malignant B cells from B-CLL patients	TLR9 – CpG ODN	-Up-regulation of CD25 (IL-2R) -Synergistic effect with IL-2 on the proliferation of CLL B cells →→ ***making these cells more sensitive to T cell immune response***	[144]

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3

Toll-like receptor-9 triggering modulates expression of alpha-4 integrin on human B lymphocytes and their adhesion to extracellular matrix proteins

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Abstract

Objective. The interaction of human B lymphocytes as recirculating cells with their microenvironment components including fibronectin is an instrumental process which directs their further responses in an inflammatory milieu or during their development in secondary lymphoid organs. Factors derived from extracellular environment including those of pathogens, termed pathogen associated molecular patterns, may have effects on this interaction, yet no study has addressed to date these effects. In this study, we explored the effect of TLR9 triggering on the interaction of normal B cells with fibronectin and collagen.

Materials and methods. The synthetic analog of TLR9 ligand, CpG-C, was used for stimulating the cells. The expression pattern of VLA-4 integrin was studied by FACS and western blotting experiments, and cell adhesion was analyzed by fluorometric adhesion assay.

Results. CpG at 0.5μM upregulated fibronectin receptor (VLA-4) expression and cell adhesion, and Increasing the CpG concentration did not have further effect . Blocking experiments with TLR9 signaling inhibitor, TTAGGG, anti-α4 antibody, and IκBα phosphorylation inhibitor, Bay 11-7082, confirmed that the CpG-induced induction level was TLR9, VLA4 and NFκB-mediated, respectively.

Conclusion. This study indicates that TLR9 triggering on B cells influences their interaction with extracellular matrix, which will be critical in modulating activation of these cells in conditions such as infections, and gives a basic insight into the contribution of innate immunity elements in B cell functional responses.

Introduction

Cell adhesion is involved in many physiologic and pathologic processes including embryogenesis [1], hemato-lymphopoiesis [2-7], migration of immune cells to sites of infection /inflammation [7-9], and invasion / metastasis of tumor cells [10,11]. For example, binding of $\alpha_4\beta_1$ (VLA-4) integrin on multiple myeloma B cells to VCAM-1 on bone marrow stromal cells or to fibronectin plays a critical role in pathogenesis of disease [12-14]. The VLA-4 and VCAM-1 interaction also contributes largely to homing and detainment of recirculating B cells in the microenvironment of secondary lymphoid organs [15-17], or to homing of post-germinal center memory B cells to the bone marrow or mucosal tissues to differentiate into plasma cells [18].

The role of several cytokines and mediators has been well deciphered in B cell interaction with the microenvironment [19,20], but the effect of other environmental factors including pathogen associated molecular patterns (PAMPs) on this interaction has not been well characterized. PAMPs are conserved moieties in a variety of microorganisms exploited by immune cells to establish and integrate immune responses [21]. Most PAMPs are *sensed* by the Toll-like receptor (TLR) family of pattern recognition receptors expressed on innate immune cells [21].

Bacterial CpG DNA is recognized by the endosomal pathogen recognition receptor, TLR9, which has been demonstrated to be highly expressed on human B cells [22], and is noticeably functional [22-24]. During a bacterial infection, it is possible that B cells dynamics in the circulation, secondary lymphoid tissues and bone marrow microenvironment is affected by bacterial products which may prime them for interaction with the microenvironment. Moreover, it has been indicated that microbial products (bacterial DNA) can mobilize hematopoietic progenitor cells from the bone marrow [25], or deplete bone marrow B lymphocytes [7]. On the other hand, there is no information whether bacterial products such as hypomethylated CpG motifs in DNA affect B cell interaction with stromal cells or ECM proteins. In this study, we explore the effect of TLR triggering with CpG ODN on the interaction of human B cells with fibronectin and collagen, two important ubiquitous extracellular components.

Materials and methods

Reagents& antibodies

TLR9 ligand (CPG ODN type C) with the sequence5'- tcgtcgttttcggcgc:gcgccg -3', TLR9 signaling inhibitor TTAGGG with the sequence 5'-tttagggttagggttagggttaggg-3', both containing phosphorothioate backbones, were from Invivogen. Calcein-AM, human B cell isolation (negative selection) kit, and collagen/fibronectin were from AnaSpec, Miltenyi Biotech, and Sigma-Aldrich, respectively. FITC-conjugated anti-human CD19, PE-conjugated anti-human CD49d ($\alpha_4$integrin, clone 9F10) and appropriate isotype controls were all from eBioscience. Antibodies used in western blotting were polyclonal rabbit anti-human α4 integrin (Cell Signaling Technology, 4600S), monoclonal mouse anti-human beta-actin (Santacruz Biotechnology), polyclonal HRP-conjugated rabbit immunoglobulins (DAKO) and goat anti-mouse IgG1 (Santacruz Biotechnology). Inhibitor of IκBα phosphorylation, Bay 11-7082, was purchased from Sigma-Aldrich.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from normal human buffy coat (Sanquin Blood Bank, Amsterdam, the Netherlands) by Lymphoprep^{TM}(Axis-Shield, Oslo, Norway) density gradient centrifugation. B cells were isolated using a B cell isolation kit (negative selection kit Miltenyi Biotech). PBMCs were incubated with a cocktail of biotinylated antibodies (anti-CD2, CD14, CD16, CD36, CD43, CD235a) followed by incubation with anti-biotin micro beads as described in the manufacturer's instructions. B cell purity checked by CD19 staining was 95.14% ± 3.76 (2SD). The cells were suspended in RPMI-1640 containing 2mM L-glutamine supplemented with 10% FBS, 100u/ml penicillin, and 100μg/ml streptomycin and kept at 37^0C. To eliminate a possible stimulatory effect of the isolation procedure, the cells were left for at least one hour at 37^0C to rest before further treatment. One million B cells were incubated with CpG ODN (0.25, 0.5, 1.0, 1.5, 2.0 and 5.0 μM) for 24hrs, and TTAGGG (TLR9 inhibitor) was used at 5.0μM with CpG ODN. A condition without CpG (unstimulated cells) was also included as the main control. After incubation, the viability of cells in each condition was more than 90% as tested by trypan blue vital staining.

Flow cytometry

One hundred thousand B cells were incubated with human IgG immunoglobulin (20μg/ml) in cold PBS+0.5% BSA for 5 min at room temperature to block FcγRs, and then stained with PE-conjugated anti-human CD49d (VLA-4) or anti-human CD19 or their relevant isotype controls for 30 min on ice. The expression of α4 integrin was run in a FACS Calibur^{TM}(BD

Biosciences, CA, USA) by acquiring 10000 events of the gated live population of B cells and data were analyzed using the CellQuest™ software (BD Biosciences, CA, USA).

Adhesion assay

B cells (~5×10^5/ml) were suspended in RPMI and labeled for 30 min at room temperature with 0.5μM calcein-AM, with a gentle shaking at 15 min. The cells were then washed with cold RPMI and re-suspended in RT RPMI+2% FBS.

For adhesion to ECM, 96-micro well plates (Costar) were coated overnight with 20μg/ml fibronectin (from human foreskin fibroblasts, Sigma-Aldrich) dissolved in HBSS or with 20μg/ml type I collagen (from human lung, Sigma-Aldrich) dissolved in 0.01% acetic acid, at 4^0C. The coated wells were then incubated with 10mg/ml heat-denatured BSA in PBS for 30 min. at 37^0C, and washed twice with RT PBS. For each condition, 1×10^5 B cells were seeded on pre-coated micro wells and incubated for one hr at 37^0C. After one hour incubation, the total, adhered and spontaneous fluorescence were determined in a plate reader. Fluorescence reading of cells adhered to BSA-coated wells was considered as background. In inhibition experiments, B cells were pre-incubated with anti-human α_4 blocking antibody (clone P1H4, Millipore) and then added to wells. The following formula was used:

[(Adhered B cells fluorescence-background fluorescence (BSA))/(Total fluorescence - Spontaneous fluorescence)] ×100.

Western blotting

For each sample, two million B cells (in RPMI 1640medium) were incubated under the following conditions: 1.CpG ODN (0.5μM); 2. CpG (0.5μM) + inhibitor of IκBα phosphorylation Bay 11-7082 (at 0.25, 0.5, 1.0, 2.5 and 5.0μM); 3. CpG ODN (0.5μM) +TTAGGG (5.0μM); 4. unstimulated. The cells were incubated for 24 hrs at 37 ^{o}C. Cell lysates were prepared in RIPA buffer containing protease and phosphatase inhibitors (Complete Mini, Roche). After centrifugation for 10 min at 10,000g (4^0C), the supernatants were removed and their protein concentration was determined by BCA kit (Pierce). Twenty micrograms of protein was electrophoresed on an 8% SDS PAGE gel and electroblotted onto PVDF membrane (Bio-Rad). Membrane was blocked for one hr at RT with 3% non-fat dry milk and then incubated with primary antibodies (1/1000) against α4 integrin or beta-actin followed by relevant secondary antibodies (1/5000). Finally the immunoreactive bands were detected by ECL or Super-Signal West Femto Substrate kits (both from Pierce). All the results were normalized with beta-actin using quantitative densitometry (Biorad) and reported as relative band densities.

Statistics

All obtained data were analyzed with unpaired *t*-test using Graph Pad software.

Results

Human B cells express VLA-4 and CpG ODN up-regulates VLA-4 expression in a dose-dependent manner

Baseline expression of α_4 on freshly isolated human B cells was analyzed by staining with PE-conjugated anti-CD49d (VLA-4) (Fig. 1). Subsequent incubation of B cells with CpG ODN led to a dose-dependent up-regulation of VLA-4 expression and a maximum response was seen with 0.5 µM CpG ODN (Fig 2A,B).

To investigate whether the CpG ODN-induced upregulation of VLA-4 was TLR9-mediated, a TLR9 signaling inhibitor TTAGGG (5.0µM) was coincubated with 0.5µM CpG ODN (10:1 molar ratio). As shown in figure 3, the induction of VLA-4 expression by CpG ODN was completely blocked by the TLR9 inhibitor.

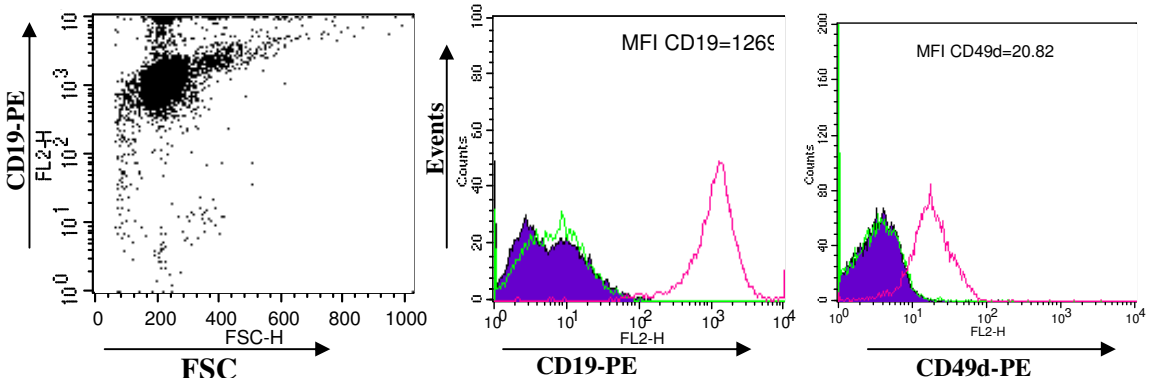

Figure 1. Expression on VLA-4 on unstimulated B cells. Purity of B cell preparation was measured by CD19 expression (left and middle panel). Right panel, expression of VLA-4 (CD49d) on freshly isolated human B cells (pink histogram). Filled histogram: unstained cell, green: isotype control (mouse IgG1, κ)

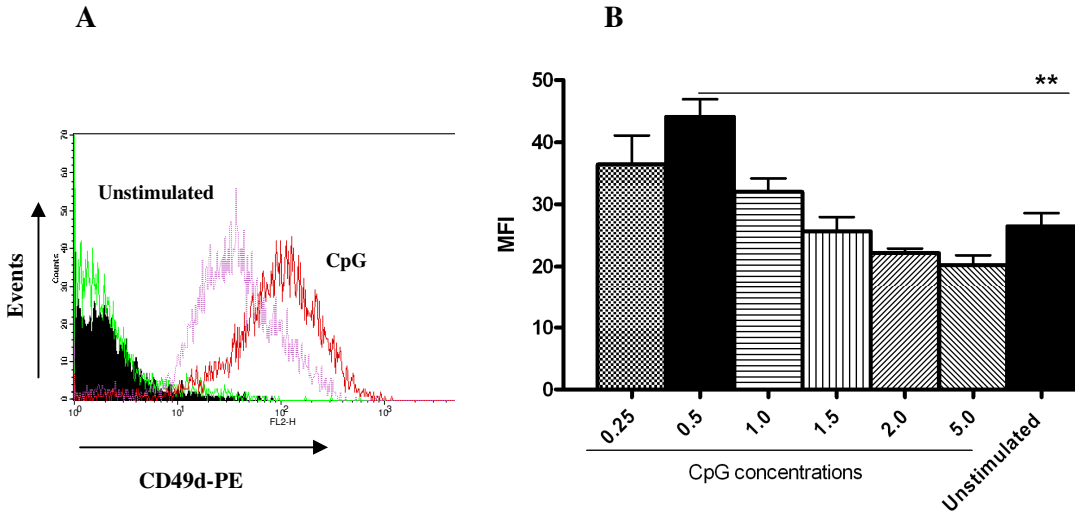

Figure 2. (**A**) The effect of CpG ODN (0.75 µM) on the expression of VLA-4 on human B lymphocytes. Specific isotype control (green line) and negative control (solid histogram) are also indicated. (**B**) Dose-dependent effect of CpG ODN (µM) on expression of VLA-4 on human B lymphocytes. Data in the graph are the mean fluorescence intensity (MFI) ± SEM from the analyses in four independent experiments; $^{**}p< 0.01$. PE=phycoerythrin.

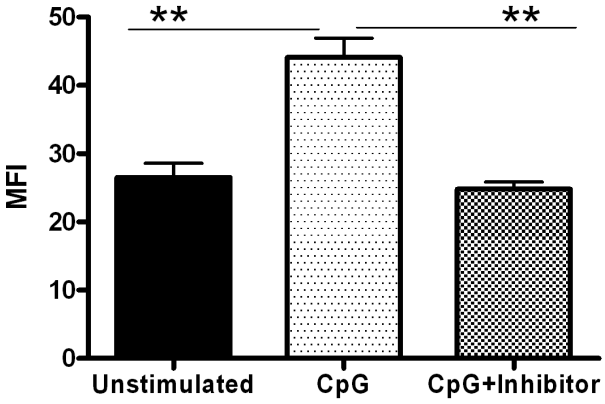

Figure 3. Inhibition of TLR9 with TTAGGG (5.0 µM) completely blocks CpG-induced upregulation of VLA-4 expression on B lymphocytes. Data are expressed as mean ± SEM; $^{**}p< 0.01$. MFI= Mean Fluorescence Intensity.

CpG ODN stimulates adhesion of human B cells to fibronectin

In the next experiments, we determined if the increased VLA-4 expression induced by CpG ODN was accompanied with an increased adhesion by the B cells. Cells were preincubated with different concentrations of CpG ODN for 24 hrs and subsequently the adhesion to fibronectin and collagen was measured. CpG ODN treatment stimulated adhesion to fibronectin with a concentration of 0.5μM having a significant effect on the adhesion (Fig 4A). The adhesion to collagen appeared increased at 0.5 μM CpG, but did not reach statistical significance (Fig 4B).

To investigate whether this induction of adhesion was TLR9-mediated, the cells were incubated with a combination of 0.5μM CpGODN+5.0μM TTAGGG. The TLR9 inhibitor completely blocked the CpG-induced increase in adhesion (Fig. 5A). To investigate whether the induction was mediated via VLA-4, a blocking antibody against the α4 subunit of VLA-4 was used. This blocking antibody greatly reduced the CpG-induced cell adhesion. At 20μg/ml, the antibody also blocked a fraction of the baseline adhesion (Fig 5B).

CpG ODN-induced upregulation of VLA-4 is NFκB-mediated

In the next experiments, we investigated if NFκB is involved in the CpG-induced upregulation of VLA-4. The NFκB inhibitor Bay 11-7082(0.25, 0.5, 1.0μM) inhibited the induced expression of VLA-4 in a dose-dependent manner (Figure 6 A,B). Concentrations 2.5μM and higher of Bay 11-7082 appeared toxicto the cells.

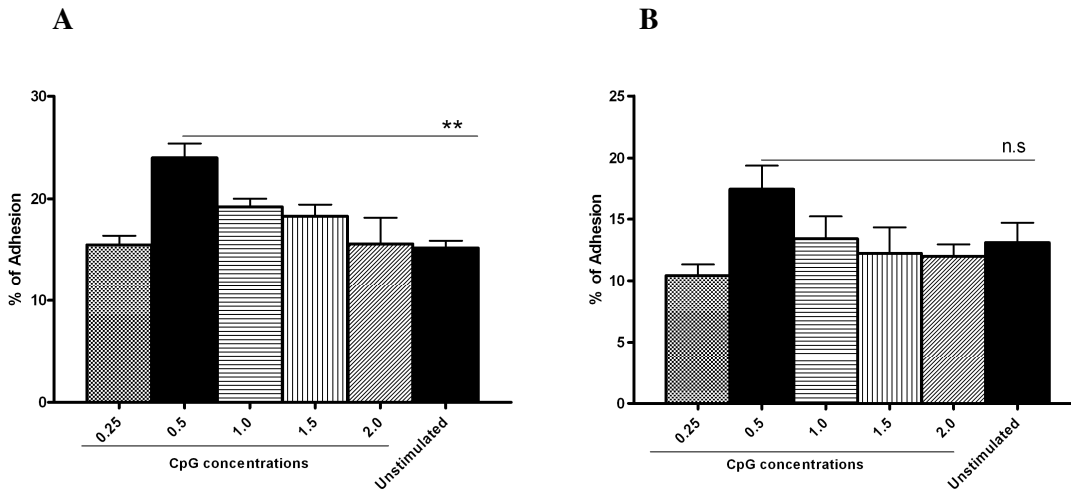

Figure 4. Effect of different concentrations of CpG on adhesion of human B cells to **(A)** fibronectin and **(B)** collagen. Figures represent mean ± standard error of mean of four independent experiments.
***p< 0.01.*

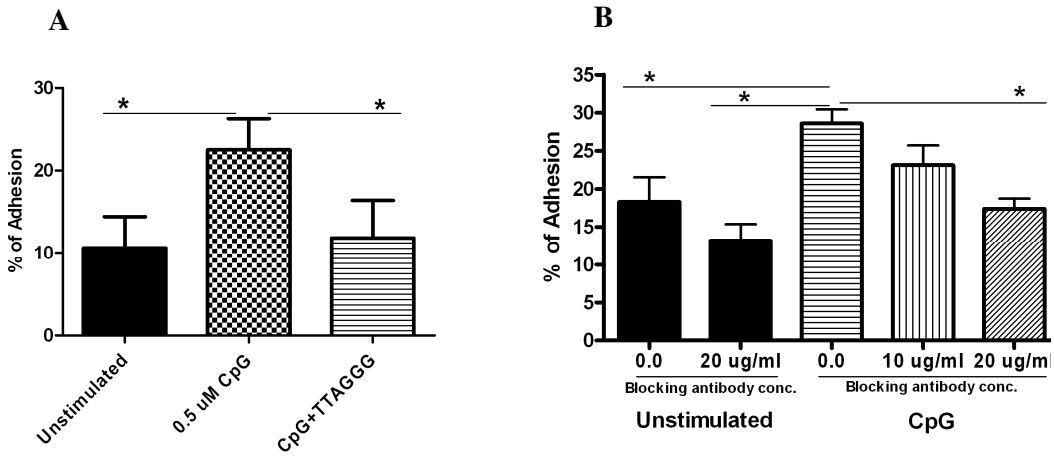

Figure 5. CpG-induced adhesion of human B cells is dependent on VLA-4 and TLR9. **(A)** TLR9 signaling inhibitor, TTAGGG (5.0 µM), blocked CpG (0.5 µM)-induced adhesion of B cells to fibronectin. **(B)** Effect of anti-human α4 blocking antibody, clone P1H4, on the adhesion. Data represent mean 6 standard error of mean; **p< 0.05.*

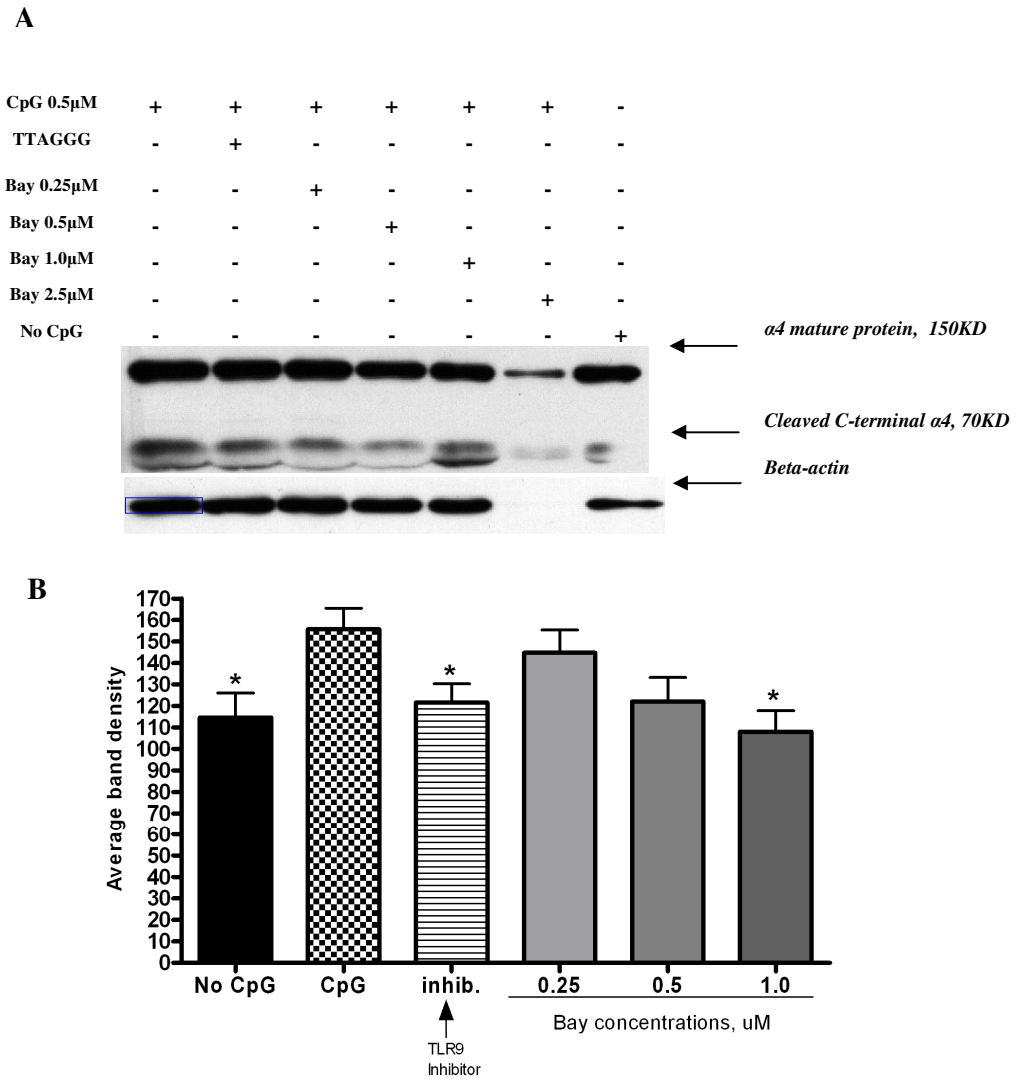

Figure 6. The effect of CpG on VLA-4 (α4) protein expression in blotting experiments. **(A)** CpG stimulation was associated with an upregulation of the protein compared to the unstimulated (no CpG) condition. This induction is almost not observed with CpG+TTAGGG. The NF-kB activation inhibitor, Bay 11-7082, caused a significant reduction, but at 2.5 µM it had toxic effects on the cells, as indicated by the loading control. Three concentrations of Bay, 0.25, 0.5, and 1.0 µM did not have any cytotoxic effects, yet they led to gradual depression in VLA-4 production, with the 1.0 µM concentration showing the most significant effect. The concentration of TTAGGG is 5.0 µM. (B) Statistical analysis of average blot band densities of seven experiments extracted from a densitometer, after normalization with β-actin bands in each experiment. All the comparisons have been made with the stimulated (CpG) condition. TTAGGG (TLR9 signaling inhibitor) could not completely block the CpG-induced level of α4 protein, although it shows a significant difference with the stimulated condition. The gradual decreasing effect of different Bay concentrations is strikingly clear with the 1.0 µM being the optimal concentration because it has completely blocked the CpG-induced level of the protein.

Discussion

In case of infections, released microbial products, including bacterial CpG DNA, may prime the circulating B cells to interact with ECM and endothelial cells to enter tissues such as lymph nodes, mucosa or even bone marrow. In the present study, B lymphocytes isolated from the fresh PBMCs of healthy donors were stimulated with CpG ODN and then exposed to two ubiquitous ECM proteins, fibronectin and collagen. Our results show that CpG DNA modulates both adhesion to fibronectin and expression of α4 integrin on human B cells. CpG at 0.5μM was optimal and further increase of CpG did not induce increased expression nor adhesion of B lymphocytes, which is in correspondence with reported effects of CpG on immunoglobulin production [23]. As expected, B lymphocytes did not adhere significantly to collagen, which corresponds with the reported weak expression of VLA-1 and -2 on B lymphocytes [26].

This inductive effect was shown to be induced by TLR9 and for the major part VLA-4-mediated. B lymphocytes represent the majority (>99.5%) of the TLR9-expressing cells in the circulation [23], which makes it unlikely that contaminating cells may account for the CpG-induced effects. The anti-α4 blocking antibody, inhibited not only the TLR9 triggering-induced adhesion but also a fraction of the baseline adhesion. This indicates that other α4-containing integrins e.g. α4β7 may also be involved, and indeed this has been shown by others to play role in B cell-fibronectin adhesion [27,28]. The CpG-induced increased adhesion of B cells to fibronectin may also be related to a change in the avidity of α4 integrin for its ligand, but this needs to be studied in future experiments.

By using a specific inhibitor of NFκB activation (Bay 11-7082), the inductive effect was determined to be NFκB-mediated. It is well established that signaling to NFκB occurs following all TLR triggering [29]. NFκB activation was found linked to VLA-4 upregulation. Although others have shown that NFκB activation could account for constitutive and cytokine-induced expression of VCAM-1 and ICAM-1 on B lymphocytes [30], or mediates CpG-induced adhesion of neutrophils to endothelial cells (HUVECs) [31], this is the first study to show that VLA-4 up-regulation on B lymphocytes following TLR triggering is NFκB-controlled. It is important to mention that the transcription factor NFκB may not be the converging core point downstream TLR triggering; other transcription factors might also be activated, including AP-1 and IRFs which were not addressed here.

In correspondence to our findings, other studies indicated that CpG also upregulates activation markers (CD54, CD80 and CD86) on human B cells [31], and increases proliferation [32,33], survival [34], and cytokine / chemokine release [22-24,35] in human or mouse B cells. Our study shows that TLR9 triggering on human B cells will modulate their interaction with the ubiquitous ECM protein fibronectin through VLA4. VLA4-mediated

adhesion to fibronectin plays prominent role in survival of B cells [36]and in integration of their immune response by modulating B cell receptor [20]. This adds further insight into the basic biologic aspects of innate immunity contribution to the modulation of activation of these cells in an inflammatory context or during lymphoid development.

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4

Characterization of the Toll-like receptor expression profile in human multiple myeloma cells

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Abstract

Expression and function of Toll-like receptors (TLRs) in multiple myeloma (MM) has recently become the focus of several studies. Knowledge of expression and biology of these receptors in MM will provide us with a new insight into the role of an inflammatory environment in disease progression or pathogenesis of MM. However, to date a quite heterogeneous expression pattern of TLRs in MM particularly at gene level has been described while information on the TLR expression at the protein level is largely unavailable. In this study, we investigated the TLR expression in human myeloma cell lines (HMCLs) Fravel, L363, UM6, UM9, OPM1, OPM2, U266, RPMI 8226, XG1, and NCI H929 and primary cells from MM patients at both mRNA and protein level (western blot and flow cytometry). We found that all cell lines and primary cells expressed TLR1, TLR3, TLR4, TLR7, TLR8, and TLR9 mRNA and protein. TLR2 and TLR5 were expressed by the majority of HMCLs at mRNA but were not detectable at protein level, while primary samples showed a low level of TLR2, TLR3 and TLR5 protein expression. Our results indicate that MM cells express a broad range of TLRs with a degree of disparity between gene and protein expression pattern. The clear expression of TLRs in MM cells indicates a propensity for responding to tumor-induced inflammatory signals, which seem inevitable in the MM bone marrow environment.

Introduction

Multiple myeloma (MM) is a lymphoid neoplasm characterized by infiltration in the bone marrow of malignant plasma cells [1]. The presence of monoclonal immunoglobulins and defective innate or adaptive immune responses render MM patients vulnerable to infectious or inflammatory conditions, and in most cases these complications hamper the therapeutic approaches [2-4]. Furthermore, a history of infectious and chronic inflammatory diseases has been reported in certain MM patients [5]. Thus, contribution of inflammatory or infectious conditions to MM pathogenesis or progression seems plausible; however, the underlying molecular mechanisms have not been clearly deciphered. Indeed the link between inflammation and malignant conditions has long been pursued by many researchers [6-9]. In recent years, Toll-like receptors (TLRs), which are instrumental in integrating the innate and adaptive immune responses, have been addressed as the potential linking elements. These receptors have been detected in many cancer cells with various functional responses following their triggering. In MM, TLRs have been reported to be expressed heterogeneously on freshly isolated myeloma cells and MM cell lines, and their expression is significantly higher than on normal plasma cells [10-15]. However, most of the analyses have been limited to mRNA level showing inconsistencies in TLR patterns expressed by MM cells and the cellular responses following their triggering. Consequently, information on the functional protein expression patterns of these molecules is limited. Here, we present a comprehensive study on the expression profile of TLRs on established and commonly used human myeloma cell lines (HMCLs) and MM primary cells. We show strong expression of TLRs in primary MM cells as well as in all MM cell lines, which indicates a propensity for responding to tumor-induced inflammatory signals, which seem inevitable in the MM bone marrow environment.

Materials and Methods

Reagents and antibodies

All the antibodies used in this study for TLR detection were from IMGENEX (San Diego, CA, USA): TLR1 (IMG-5012), TLR2 (IMG-416A), TLR3 (IMG-315A), TLR4 (IMG-5031A), TLR5 (IMG-663A), TLR7 (IMG-581A), TLR8 (IMG-321A) and TLR9 (IMG-305E). The following secondary antibodies and isotype controls were used in the FACS experiments: F(ab')anti-rabbit IgG-FITC, anti-mouse IgG-FITC, mouse IgG2b, κ, all from eBioscience, mouse IgG2a, κ from Biolegend (San Diego, CA, USA) and rabbit normal immunoglobulin from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-beta actin and the following secondary antibodies used for blotting experiments were from Santa Cruz Biotechnology: horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG. HRP-conjugated goat anti-rabbit immunoglobulin was from DAKO (DK-2600 Glostrup, Denmark). Monoclonal anti-human CD138-APC was from Biolegend. All PCR and cDNA synthesis reagents including Platinum® *Taq* DNA polymerase were from Invitrogen.

Cells and cell culture

Human multiple myeloma cell lines, Fravel, L363, OPM-1, OPM-2, U266, RPMI-8226, XG1 and NCI-H929 were obtained from American Type Culture Collection (Manassas, VA, USA). UM-6 and UM-9 had been established by the Department of Clinical Chemistry & Hematology, University Medical Center Utrecht, Utrecht, the Netherlands [16,17]. All the cell lines were maintained in RPMI-1640 culture medium containing 2-mM L-glutamine supplemented with 5 or 10% fetal bovine serum and intermittently with antibiotics, in a 37^0C incubator with 5% CO_2. XG1 and UM6 cell lines were cultured with 1ng/mL and 5ng/mL of recombinant human IL-6 (from eBioscience, San Diego, CA, USA), respectively. NCI-H929 cell line was cultured in the presence of 1mM sodium pyruvate and 50μM 2-mercaptoethanol. The cell cultures were within five to ten passages after thawing for the expression experiments.

Bone marrow mononuclear cell (BMNC) samples from 3 MM patients were thawed from frozen stocks. BMNC samples were surplus material from bone marrow isolated for diagnostic procedures. All patients approved use of surplus material for scientific purposes by informed consent. Use of surplus material has been discussed with and approved by the review board of the University Medical Center Utrecht. The samples were first suspended in fresh RPMI medium and kept in an incubator for a few hours. Cellular debris were removed by using Ficoll hypaque centrifugation, and rest of the samples were cultured overnight in RPMI medium supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin in a 37^0C incubator with 5% CO_2.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from the cells by RNeasy Minikit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. For cDNA first strand synthesis, 2.5µg of total RNA was reverse transcribed using SuperScript^{III} reverse transcriptase. The primer sequences used for TLR 1, 2, 4, 5, 7, 8 were described before [12].

Other primer sequences obtained from Isogen (De Meern, the Netherlands) were as follows:
TLR3-forward: 5'-TGCCGTCTATTTGCCACACACT-3',
TLR3-reverse: 5'-CAGGTGGCTGCAGTCAGCAA-3',
TLR9-forward: 5'-TCATGACTGTGCCTGCGCTG-3',
TLR9-reverse: 5'-AGGCGCCAGTTTGACGATGC-3', beta-actin primers had also been already described [18]. To perform the reactions in a total volume of 25µl, we used the following conditions: primary denaturation at 95^0C for 3 minutes, 35 cycles of 30 sec at 94^0C, 40 sec at 58^0C, 40 sec at 72^0C, and 10 minutes at 72^0C.

Quantitative real time polymerase chain reaction

Quantitative PCR was performed for the expression of TLR3 mRNA in Fravel, L363 and NCI- H929. Five hundred ng total RNA from indicated cell lines was transcribed using a first strand synthesis kit (Qiagen). Two microliters from each sample was applied to SYBR green real time PCR using primers which were as described previously [19]. The beta actin primer was described above. Standard curves for targets and beta actin genes were created with two-fold dilutions of a measured concentration of pooled cDNA. The amount of amplicon for TLR3 gene in samples was then determined using log of concentration and slope of the curve and normalized to that of actin. We used a two-step PCR protocol with the following temperature profile: 5 min 95^0C, 40 cycles of (15 sec 95^0C, 30 sec 58 or 65^0C), including a melting curve at the end.

Western blotting

Five to ten million cells from each cell line were harvested, washed in cold PBS and suspended in lysis buffer (150mM NaCl, 1% IGEPAL (Sigma), 50mM Tris, pH 8.0) followed by addition of protease inhibitor cocktail (Complete Mini pills, Roche). The lysates were then left on ice for 30 minutes, spun at 10,000×g for 10 minutes and supernatants were isolated. The protein concentration of lysates was determined with a BCA kit (Pierce) and 30µg total protein from each lysate was electrophoresed on an 8 or 12% SDS-PAGE gel and subsequently electro blotted onto a PVDF membrane. The membranes were incubated with primary antibodies (1-3µg) overnight at 4^0C followed by incubation with HRP-conjugated secondary antibodies (1:2000-1:5000). The signals were detected by using ECL PLUS or

ECL Prime (Amersham). Membranes were reprobed for a maximal of 3 times with different TLR antibodies and anti-beta-actin, after stripping the membranes with Restore Western Blot Stripping buffer (Pierce).

Flow cytometry

In FACS experiments, indirect (intra)cellular staining was performed using TLR-specific antibodies. Briefly, 10^5 cells from each cell line or BMNCs were harvested and washed in FACS buffer, PBS+0.5% BSA+0.01% sodium azide, pelleted and suspended in permeabilization/fixation buffer (from eBioscience), and left for 20 minutes at room temperature. The cells were then washed in permeabilization buffer, pelleted and incubated with 1-2µg of indicated primary antibody at room temperature (or 4^0C) for one hour. FITC-conjugated secondary antibodies, anti-rabbit IgG and anti-mouse IgG, were added at the next step. Finally, the samples were washed, suspended in FACS buffer and then analyzed using a FACSCanto^{TM}II flow cytometer (BD Biosciences) for TLR detection, and captured data of gated live populations were analyzed using CellQuest software. Human peripheral blood mononuclear cells (PBMCs) isolated from buffy coat (Sanquin, the Netherlands) were used as positive control in some experiments. Patient BMNCs were washed and stained with APC-conjugated anti-human CD138 in FACS buffer followed by staining with TLR-specific antibodies containing as described above. FACS analysis for TLR expression was performed on live gated CD138-positive cells.

Results

Analysis of TLR mRNA expression in HMCLs

Expression of mRNA for TLR1, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 was found in all myeloma cell, but levels of mRNA expression differed amongst different cell lines (Fig 1). TLR2 mRNA was not detected in Fravel, and L363. Expression of TLR3 mRNA was also analyzed using qPCR in 3 cell lines: Fravel, L363 and NCI-H929. The TLR3/actin expression of Fravel, L363, and NCI-H929 was 0.083, 0.043 and 0.086, respectively. Absolute quantities of TLR3 amplicon were 4.84±1.82 ng, 1.35±0.66ng and 4.81±1.8ng, respectively.

Analysis of TLR protein expression in HMCLs using Western blot

In all cell lines, expression of TLR1, TLR3, TLR4, TLR7, TLR8, and TLR9 was detected using immunoblotting (Figs 2,3). On the other hand, TLR2 and TLR5 proteins were not found in any of cell lines. The absence of signal was not due to a lack of reactivity of the antibodies used, because strong expression of these TLRs was found in human intestine tissue lysate (Fig 4). Furthermore, using densitometry the ratio of TLR protein densities to those of

beta actin was determined and showed that relative expression levels of TLRs varied amongst different cell lines (Fig 5). For example, in TLR4 group, a prominent difference in relative protein level is obvious between RPMI 8226, Fravel and U266 cell lines. In the TLR8 group, L363 displays the highest level of the protein, and in TLR1, TLR3, TLR7, TLR9 groups the highest level of the relevant proteins is seen in the NCI-H929 cell line.

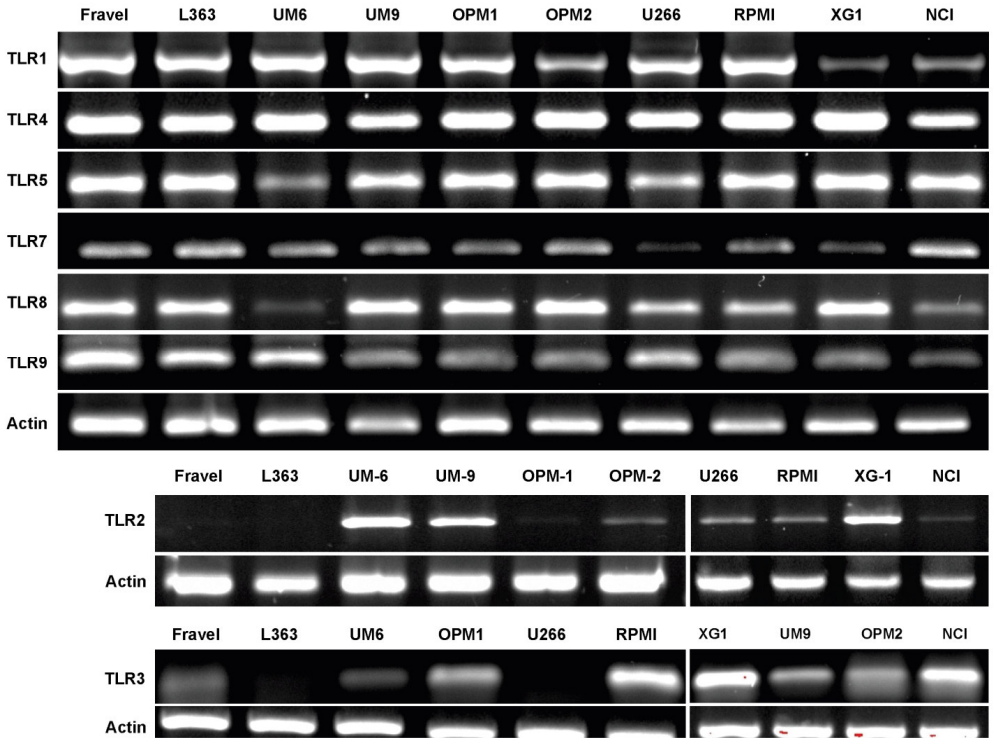

Figure 1. Expression of TLR mRNA in HMCLs. **A)** All the cell lines expressed mRNA for TLR1, TLR4, TLR5, TLR7, TLR8, TLR9. **B)** TLR2 mRNA was detected in all cell lines except L363 and Fravel. TLR3 showed varied expression levels in HMCLs

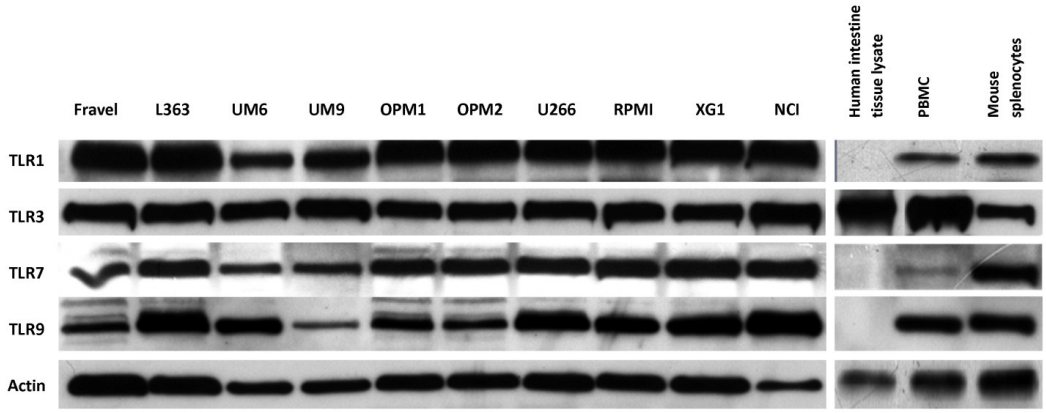

Figure 2. Expression analysis of TLR protein in HMCLs by Western blotting. All cell lines displayed a strong expression of TLR1, TLR3, TLR4, TLR7, TLR8, TLR9 proteins. Cell lysates were electrophoresed and blotted to PVDF membrane, which was probed with TLR-specific antibodies. To confirm the immunoreactivity of the antibodies, different positive controls were included. Beta-actin was used as loading control and for normalizing the expression levels between cells (see Figure 5). Data are representative for analysis of ≥2 independent experiments.

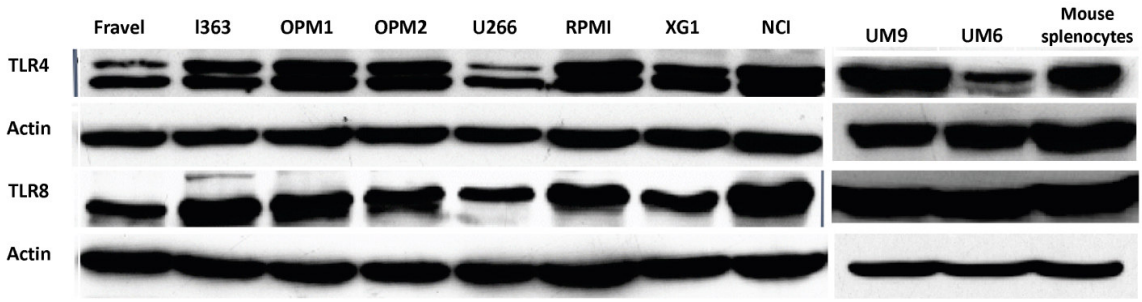

Figure 3. Expression analysis of TLR4 and TLR8 protein in HMCLs by Western blotting. Lysates of mouse splenocytes was used as positive control. Beta-actin was used as loading control and for normalizing the expression levels between cells (see Figure 5). Data are representative for analysis of ≥2 independent experiments

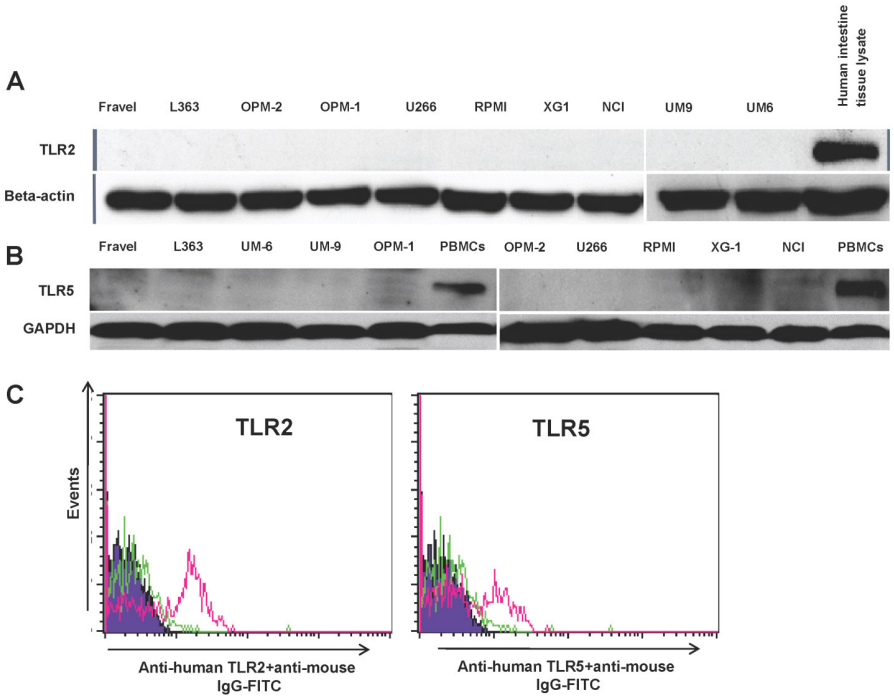

Figure 4. HCML did not express TLR2 and TLR5 at protein level as shown in western blotting (panel A and B) and FACS (panel C). The immunoreactivity of the anti-TLR2 and -5 antibodies in western blotting and FACS analysis was confirmed with human intestinal lysate (panel A), and human PMBCs (panel B,C) as positive controls. Data are representative for analysis of ≥2 independent experiments.

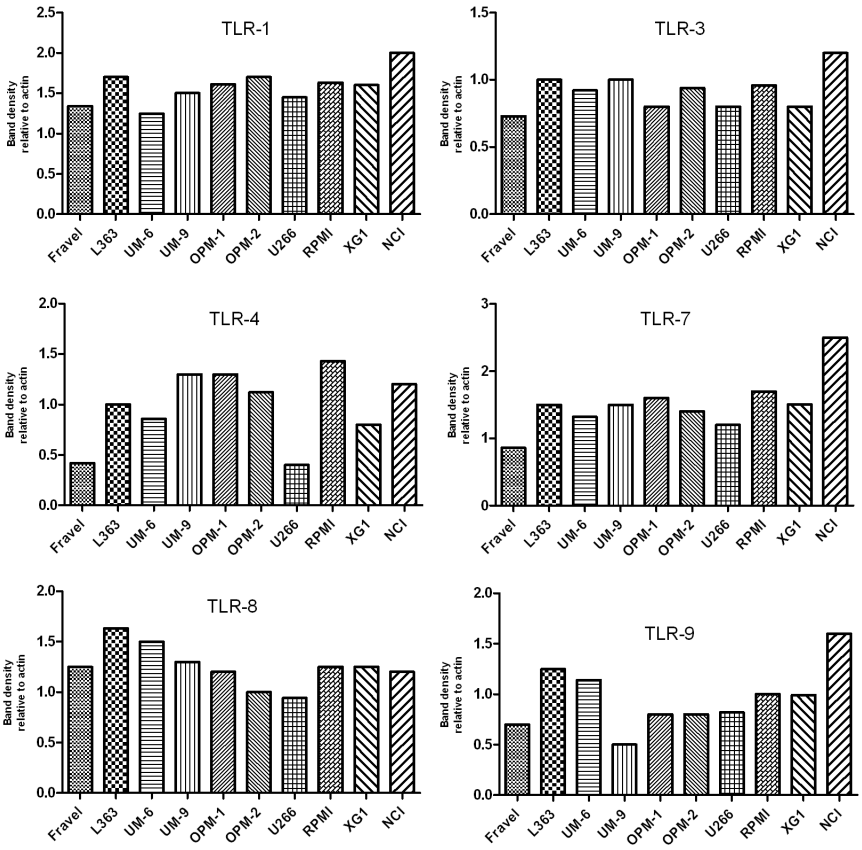

Figure 5. Expression of TLR1, TLR3, TLR4, TLR7, TLR8, and TLR9 (calculated as densitometric ratio of TLR to actin) for HMCLs. Density of TLR expression was divided by the corresponding density of the actin signal.

Analysis of TLR expression in HMCLs using flow cytometry

In the next experiments, the expression of TLR protein was analyzed by flow cytometry. TLR1, TLR7, TLR8, TLR9 were strongly expressed by HMCLs (Figs 6 and 7). In agreement with the Western blotting analysis, TLR2 and TLR5 were found not expressed by any of HMCLs in FACS analysis. In control experiments, we confirmed that antibodies used were able to detect TLR2 and TLR5 expression in human peripheral blood mononuclear cells (Fig 4). Interestingly, expression of TLR-3 and TLR-4 in some cell lines (Fravel and NCI-H929) was very low, while the bands of these proteins in immunoblotting analysis were prominent, probably suggesting posttranslational modifications.

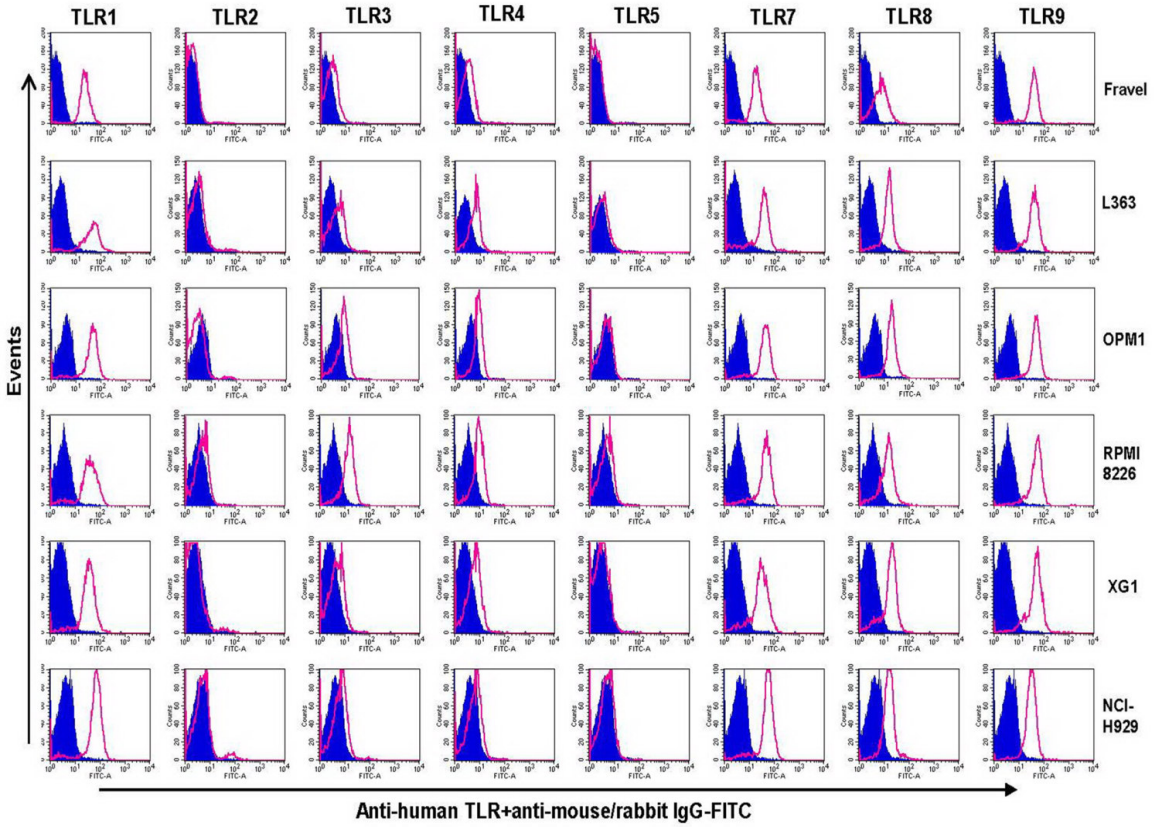

Figure 6. Expression of TLRs in Fravel, L363, OPM1, RPMI8226, XG1, and NCI-H929 as determined by flow cytometry. HCMLs were stained using an intracellular staining protocol with TLR-specific antibodies followed by relevant secondary fluorescent-conjugated antibodies. TLR2 and TLR5 proteins were not detectable in any of cell lines. Filled histograms (purple) represent the isotype controls and the open histograms (red) indicate specific antibodies. Data are representative for analysis of ≥2 independent experiments

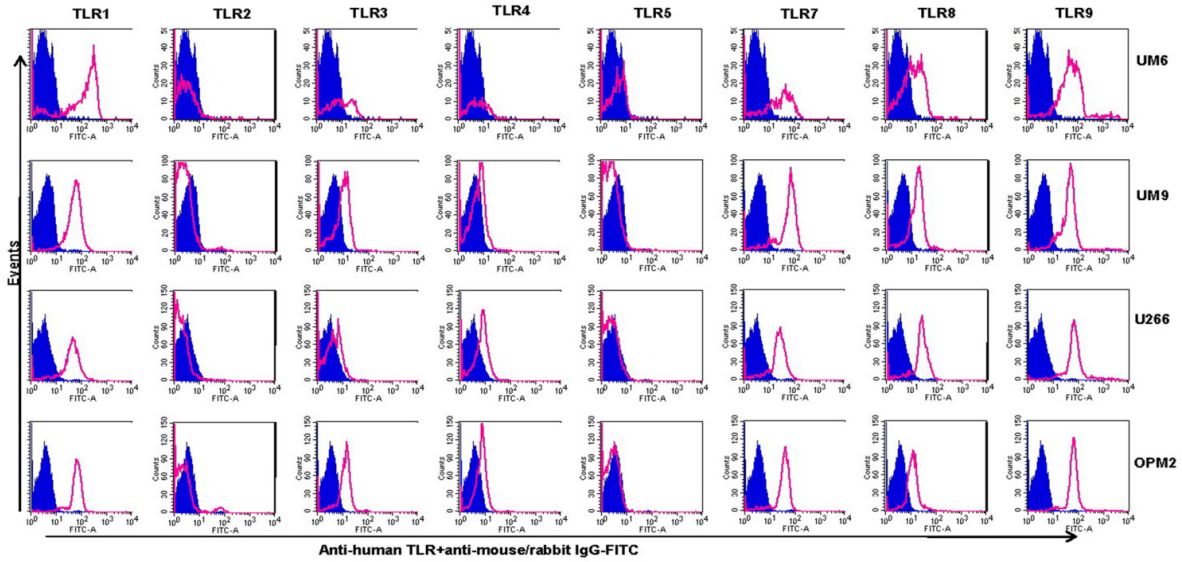

Figure 7. Expression of TLRs in UM6, UM9, U266, and OPM2 as determined by flow cytometry. HMCLs were stained using an intracellular staining protocol with TLR-specific antibodies followed by relevant secondary fluorescent-conjugated antibodies. TLR2 and TLR5 were not expressed by any of cell lines, confirmed also by using positive controls (see Fig 4). Filled histograms (purple) represent the isotype controls and the open histograms (red) indicate TLR-specific antibodies. Data are representative for analysis of ≥2 independent experiments

For primary MM cells, in BMNCs, live CD138-positive cells were gated and the percentage of cells binding to TLR-specific antibodies was calculated (Fig 8). $CD138^{+}$-BMNCs displayed a strong expression of TLR1, TLR7, TLR8 and TLR9. Some variation in the expression of TLR8 and TLR9 was found in samples of different patients. The percentage of TLR9 positive cells in MM1, MM2 and MM3 was 47.2, 97.8, and 53, respectively, while 16.7%, 52.7% and 20.8% of cells were positive for TLR8 in these patients.

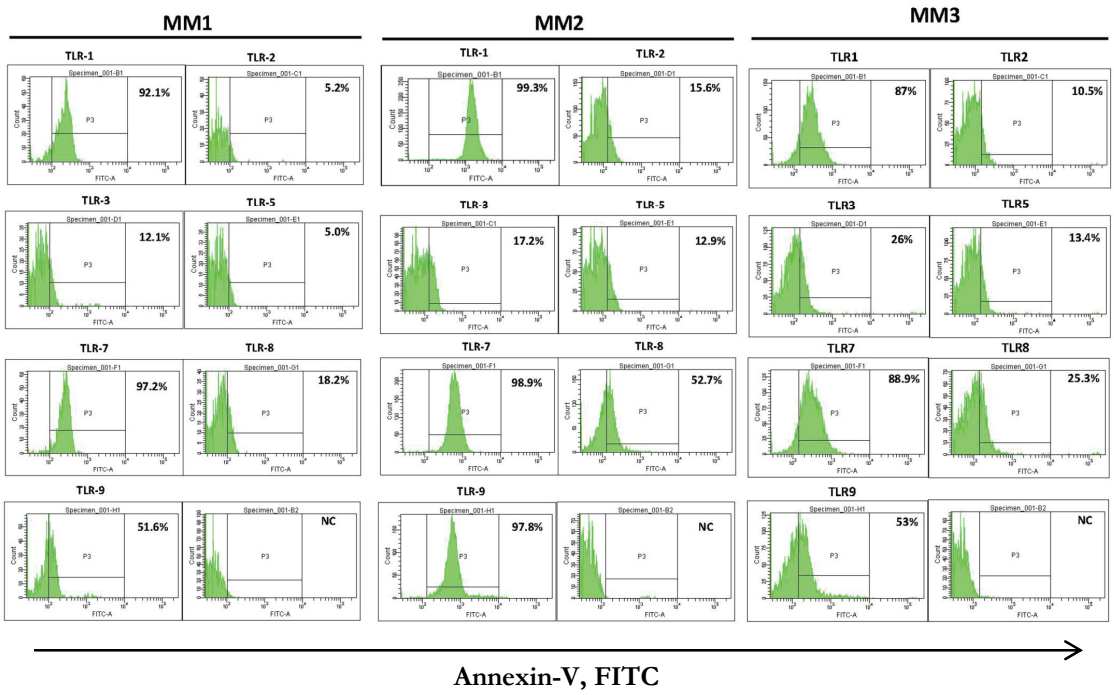

Figure 8. TLR protein expression pattern in primary BMNCs from 3 MM patients analyzed by flow cytometry. CD138-positive cells were gated from the total cell population. Staining with specific antibodies for TLR1, TLR2, TLR3, TLR5, TLR7, TLR8, and TLR9 was compared with isotype-matched controls (NC).

Discussion

The expression of TLRs on cells of B-lymphoid malignancies, MM and CLL, has been documented in different recent studies [10,12-15,20]. These studies, however, show inconsistencies in TLR patterns expressed by MM cells and the cellular responses following their triggering. This is the first study in which TLR expression in different HMCLs and primary MM cells has been evaluated at mRNA (RT-PCR) and protein (Western blot and flow cytometric analysis) level (Table 1, page 77). All analyzed HMCLs express mRNA for TLR1, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9. Although RT-PCR analysis is not a quantitative method, analysis of TLR3 expression in some HMCLs with realtime PCR provided a comparable expression profile. The pattern of TLR1, TLR2, TLR7, and TLR9 mRNA expression for NCI-H929, XG1, RPMI 8226, and L363 is in agreement with that described by Jego *et al* [12]. However, our results show differences with those obtained by Bohnhorst *et al* for OPM2, RPMI 8226, NCI-H929 and U266 cell lines [14]. While the pattern of TLR1, TLR2, TLR5, TLR9 mRNA expression is the same as in our cell lines, no mRNA for TLR3, TLR7, TLR8 in OPM-2, and TLR4, TLR7 in U266 cells was found in their study [14].

Analysis of TLR expression at protein level showed that TLR1, TLR3, TLR4, TLR7, TLR8, and TLR9, were expressed in most HCMLs. TLR analysis using western blotting closely correlated with the expression pattern found by flow cytometric analysis. Comparison of TLR expression at transcriptional and translation level showed discrepancies between presence of TLR mRNA and protein. For example, some cell lines expressed very low levels of TLR3 mRNA (e.g. Fravel, L363, and U266), while TLR3 protein was clearly expressed. On the other hand, presence of mRNA did not predict the expression of functional protein for some TLRs. Most notably was the marked presence of TLR5 mRNA in all HMCLs, while no expression of TLR5 at protein level was detected. This discordant relation between mRNA and protein expression may be caused by a low stability of the specific mRNA and translation and post-translational modifications of the specific protein. Similarly, Arvaniti *et al*. found that some B-CLL cells did not express TLR6 protein in spite of a high mRNA level, and also most samples displayed a high expression of proteins for TLR2 and TLR8 in spite of a low mRNA [21].

Expression of TLR1, TLR7, TLR8, and TLR9 in primary cells from MM patients was comparable with the profile in HMCLs, although some variation between patients was found in the extent of TLR8 and TLR9 expression. Primary MM cells showed a low level of TLR2, TLR3 and TLR5 expression as compared to HMCLs, while in all 10 HMCLs a strong signal for TLR-3 but no expression of TLR2 and TLR5 was found. Such heterogeneity in MM TLR expression and the observed differences between HMCLs and MM primary cells has also been described in recent studies. The pattern of TLR gene expression in MM cells is

strikingly different from normal bone marrow plasma cells [14] or normal B cells [10]. For instance, TLR2, TLR3, TLR4, TLR5, TLR8 genes are not expressed in normal B cells [20], but expressed by most HMCLs as shown in our study and others [12,14], or in MM primary cells [22]. This difference may be attributed to the malignant transformation of B cells during MM oncogenic alterations. Others have found similar changes in TLR expression when normal peripheral blood plasma cells are compared to normal B cells [19]. This may also suggest that the origin of the tissue may have determined the TLR expression pattern. Of note, mRNA for TLR3, TLR4, and TLR8 was not detected in B cells of B-CLL patients [23], implying that MM cells may differently regulate the expression of specific TLRs.

Taken together, our expression analyses indicate that HMCLs display a broad range of TLRs at gene and protein levels. This study also shows that analysis of mRNA alone may not provide a correct prediction of functional TLR protein expression in HMCLs [24]. Indeed, strong expression of TLRs in HMCLs and primary tumor cells indicates a propensity for responding to tumor-induced inflammatory signals which seem inevitable in MM bone marrow environment. TLR triggering on HMCL and MM primary cells has been associated with heterogeneous effects including increase in proliferation, survival, cytokine and chemokine production, induction of apoptosis or protection from apoptosis, drug-resistance and immune escape [11]. Our study suggests that effects on tumor cells through stimulation of TLRs by endogenous ligands such as soluble syndecan-1, matrix metalloproteinase products, heat-shock proteins, HMGB-1, which are released due to cell necrosis, should also be considered in MM [25].

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Table 1. Expression of toll-like receptors in myeloma cell lines and primary bone marrow mononuclear cells from MM patients

	TLR-1			TLR-2			TLR-3			TLR-4			TLR-5			TLR-7			TLR-8			TLR-9		
cell line	PCR	FACS	WB	PCR	FACS	WB	PCR	FACS	WB	PCR	FACS	WB	PCR	FACS	WB	PCR	FACS	WB	PCR	FACS	WB	PCR	FACS	WB
Fravel	+	+	+	-	-	-	w+	w+	+	+	w+	+	+	-	-	+	+	+	+	+	+	+	+	+
L363	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
UM-6	+	+	+	+	-	-	w+	+	+	+	w+	+	+	-	-	+	+	+	+	+	+	+	+	+
UM-9	+	+	+	+	-	-	+	+	+	+	w+	+	+	-	-	+	+	+	+	+	+	+	+	+
OPM-1	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
OPM-2	+	+	+	+	-	-	w+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
U266	+	+	+	+	-	-	-	w+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
RPMI	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
XG-1	+	+	+	+	-	-	+	w+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
NCI-H929	+	+	+	w+	-	-	+	w+	+	+	w+	+	+	-	-	+	+	+	+	+	+	+	+	+
primary cells																								
BMNC	nd	+	nd	nd	w+	nd	nd	w+	nd	nd	nd	nd	nd	w+	nd	nd	+	nd	nd	+	nd	nd	+	nd

+: positive; w+: weakly positive; -: negative; nd: not determined

BMNC: CD138-positive cells gated from bone marrow mononuclear cells derived from MM patients (3 patients)

5

Stimulation of Toll-like receptor-1/2 combined with Velcade increases cytotoxicity to human multiple myeloma cells

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Abstract

An increasing body of evidence supports the important role of adhesion to bone marrow microenvironment components for survival and drug resistance of multiple myeloma cells. Previous studies suggested that stimulation of Toll-like receptors by endogenous ligands released during inflammation and tissue damage may be pro-tumorigenic, but no studies have been performed in relation to modulation of cell adhesion and drug cytotoxicity. Here, we investigated the effect of TLR1/2 activation on adhesion of human myeloma cells to fibronectin and their sensitivity to the proteasome inhibitor Velcade (bortezomib). We found that TLR1/2 activation either increased or decreased adhesion of human myeloma cells to fibronectin. On the other hand, TLR1/2 stimulation always increased cellular cytotoxicity. The increase in cytotoxicity by TLR1/2 stimulation was found partly mediated by p53, NFkB and MAPK signaling pathways. These findings indicate that TLR activation of MM cells could bypass protective effects of cell adhesion and suggest that TLR signaling may also have anti-tumorigenic potential.

Introduction

Signal transduction following integrin binding increases survival, invasion and resistance to apoptosis in many cancer cells [1-5]. In multiple myeloma (MM), this interaction is primarily seen in the bone marrow microenvironment where malignant plasma cells adhere to fibronectin (FN) or stromal cells contributing to induction of cytokines involved in angiogenesis and osteoclastogenesis [6,7], and tumor cells survival, invasion and drug resistance [5,7-10]. Although the detailed molecular mechanism(s) underlying this resistance has yet to be delineated, most recent studies have documented VLA-4 (CD49d/CD29, α4β1) and to a lesser extent VLA-5 (CD49d/CD29, α5β1) integrin molecules on MM cells to be involved in their adhesion to FN and hence induce the so called "cell adhesion mediated drug resistance (CAMDR)"[11-17]. Notably, increase in drug resistance of MM cells correlates with their increased adhesion to FN and over-expression of VLA-4.[11,12] Moreover, cytokines secreted by bone marrow stromal cells (BMSCs) could enhance adhesion of MM cells to FN, contributing to MM cell survival and drug resistance [18]. Thus, modulating MM cell adhesion to stroma or FN may have beneficial therapeutic effects in combination with anti-neoplastic drugs.

In recent years a special focus has been made on the role of Toll-like receptor (TLR)-mediated signaling effects in MM cells and human myeloma cell lines (HMCLs) biology. TLRs are germ line encoded and conserved receptors detecting pathogen-associated molecular patterns (PAMPs) to elicit innate immune responses and shape the adaptive immunity[19]. Following triggering by their relevant exogenous or endogenous ligands, TLRs provoke secretion of a variety of pro-inflammatory cytokines which if takes place in a tumor environment might optimize or derange tumor activity, a feature designated as "double edged sword" of innate immunity[20]. The function of TLRs may be of particular interest linking inflammation to MM pathogenesis[21]. A few recent studies have reported higher levels of TLR expression on MM cells compared to normal plasma cells and induction of survival, proliferation, drug resistance and immune escape upon TLR triggering in stroma-free conditions[22-27]. However, current knowledge suggests that TLR activation of B-cell neoplasia can result in both positive and negative outcomes [28]. In this study, we investigated the effect of TLR1/2 triggering on survival and drug resistance of adhered MM cells. We show that Pam3CSK4, a well-known TLR1/2 agonist [19,29], has differential effects on adhesion of HMCLs to FN, but TLR1/2 stimulation combined with Velcade increases the cell death of FN-adhered MM cells.

Materials & methods

Cell lines and cell culture

Human myeloma cell lines (HMCLs), Fravel, L363, UM-6, OPM-1, OPM-2, U266, RPMI-8226 and NCI-H929 were obtained from American Type Culture Collection (Manassas, VA, USA). UM-6 had been established by the Department of Clinical Chemistry & Hematology, Utrecht University Medical Center, the Netherlands. The cell lines were maintained in RPMI medium containing 2mM L-glutamine, supplemented with 5% fetal bovine serum (FBS) and intermittently with 100U/ml penicillin and 100µg/ml streptomycin at a humidified 37^0C incubator providing 5% CO_2. To UM6 cell line was added 1ng/mL of recombinant human IL-6 (from eBioscience, San Diego, CA, USA). NCI-H929 cell line was also treated with 1mM sodium pyruvate and 50µM 2-mercaptoethanol. Cells were cultured for maximal 15 passages after thawing.

Reagents & antibodies

TLR1/2 ligand (Pam3CSK4) was obtained from Invivogen and dissolved in sterile water according to manufacturer's instructions to make a 1 mg/ml stock. Velcade (Bortezomib) was from LC laboratories (Woburn, MA, USA) and dissolved in DMSO to prepare 100mM stocks. Pathway inhibitors Bay 11-7082 (inhibitor NFkB), Doramapimod (inhibitor MAPK) and NVP-BEZ235 (inhibitor PI3K/mTOR), were obtained from Invivogen and LC Laboratories, respectively. They were dissolved in DMSO (Bay 11-7082 and Doramapimod) and dimethylformamide (NVP-BEZ235) to make 100mM stocks.

AMC (7-Amino-4-methylcoumarin) powder and caspase-3 substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) were obtained from Sigma and Bachem, respectively, and also dissolved in DMSO. The final concentration of solvents in all experimental conditions never exceeded 0.1%. The antibodies used in western blotting experiments were as follows: rabbit monoclonal, anti-human cleaved caspase-3 (clone D3E9, Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-human pro-caspase-3 (clone E83-103, Epitomics, Burlingame, CA, USA), rabbit polyclonal anti-human p53, p73, BCL-2, Bax (GeneTex, Irvine, CA, USA), HRP-conjugated goat anti-rabbit immunoglobulins from DAKO or anti-rabbit IgG and IgG1 from Santa Cruz Biotechnology. Mouse anti-human beta-actin Ab was also from Santa Cruz Biotechnology.

Fluorometric adhesion assay

Adhesion to FN was measured as described previously.[30] In detail, cells were treated with or without Pam3CSK4 (1-5 µg/ml) for 24 hours, washed and added to FN-coated 96 well-plates for adhesion assay as follows. Ninety-six-well plates (Costar) were coated with 10µg/ml FN (human plasma-derived, Sigma) in HBSS and left for one hour at 37^0C. To block

non-specific binding, heat-denatured BSA (10mg/ml) in PBS was added after removing FN and plates were left for 30 minutes at 37^0C or room temperature. Immediately before use, plates were washed once with HBSS buffer. Then, one million cells out of each condition (treated or untreated) were harvested, washed twice in PBS buffer and suspended in 1ml of room temperature RPMI medium without any additive. Cell suspensions were labeled with Calcein-AM (1-2µM) for 30 minutes at room temperature with gentle mixing after 15 min. To stop labeling, samples were washed/spun twice with ice cold PBS at 4^0C. One milliliter of RPMI plus 2% FBS was added to all samples and 10^5 cells were seeded on FN-coated 96-well plates which were incubated at 37^0C for one hour. At the end of incubation time, total and spontaneous fluorescence were measured with a plate reader (Mithras LB 940; Berthold Technologies; Germany). For measuring adhered fluorescence, non-adhered cells were removed with two gentle washes using warm RPMI, 100µL RPMI was added to each well and the plate was read as above. For background readings, fluorescence of the wells containing cells adhered only to BSA was considered. The following formula was used to calculate percentage of adhesion: (Fluorescence reading of adhered cells- background reading) × 100 / (Total fluorescence reading-spontaneous reading).

Cell viability: growth inhibition assay (drug cytotoxicity)

Drug cytotoxicity studies were modified after a previously described protocol for *acute drug exposure*.[12] In detail, one day before drug exposure, 96-well plates (Costar) were coated overnight (4^0C) with 20µg/ml FN in HBSS buffer and blocked with 10mg/ml sterile heat denatured BSA in PBS at 37^0C for 30 minutes. HMCLs were first stimulated with 2.5µg/ml (OPM-2) or 5µg/ml (L363 and U266) Pam3CSK4 for 24 hours. Cells were washed with PBS twice and 5×10^4cells from each cell line were treated with different concentrations of Velcade in RPMI+5%FBS in separate 96-well round bottom plates for one hour at a 37^0C incubator, with gentle shaking after 30 minutes. Cells were then washed with RPMI, resuspended in RPMI+FBS and transferred to the 96-well flat bottom plates pre-coated as above. The plates were further incubated for 48 hours. At the last 4 hours of incubation, 25µl from XTT reagent with phenazinemethosulfate (PMS) was added to each well and after 4 hours the absorbance (at 450nm) of each well was measured using a plate reader. The percent survival of the cells was calculated by using non-linear regression. In each plate run, wells for solvent control (medium+cells+DMSO), blank (medium+DMSO), and growth control (medium+cells) were also included. The readings of the blank wells were subtracted from those of all test samples.

Cell viability: annexin-V apoptosis assay

Myeloma cells were incubated in presence or absence of Pam3CSK4 for 24 hours, washed and transferred to FN-coated 12-well plates for 1-2 hours. Unattached cells were removed, fresh medium was added and after 8-12 hours incubation, Velcade (5nM) was added and the incubation was extended for another 48 hours (chronic exposure). In parallel, cells were seeded in uncoated wells and treated similarly.

For FACS analysis, cells were removed with cold 5mM EDTA in PBS, washed once with normal FACS buffer (cold PBS containing 1% BSA and 0.01% sodium azide) and once with binding buffer (eBioscience). The cell pellets were then suspended in 200µl binding buffer containing 5µl FITC-conjugated annexin-V and incubated for 10 minutes at room temperature. After washing with binding buffer, 5µl propidium iodide in 200µl buffer was added to each well and samples were analyzed using a Becton Dickinson FACSCantoII flow cytometer. The percent specific apoptosis was calculated using the following formula (adopted from ref.[31]): (Test-control)×100/(100-control). Test refers to treatment with Pam3CSK4, Velcade or Pam3CSK4+Velcade, and control is the cells without any stimulation (baseline).

In separate experiments, HMCLs were first treated with subtoxic concentrations of inhibitors for 1-2 hours. Then without washing, Pam3CSK4 was added, the incubation was extended to 24 hours and procedure was continued as above. Subtoxic concentrations of inhibitors were determined separately after 24-hour incubation and performing Calcein-AM labeling assay, as previously described.[32]

Caspase-3 enzymatic activity

Cleaved caspase-3 enzymatic activity was measured as described previously.[33] The assay is based on the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety following hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by the activated enzyme. Briefly, cells were lysed in a buffer consisting of 10mM HEPES (pH 7.5), 1% IGEPAL, 10% sucrose, 50mM NaCl, 40mM β-glycerophosphate, 2mM MgCl2, 5mM EDTA and supplemented with a cocktail of protease inhibitors (Complete Mini, Roche) and left on ice for 30 minutes. After spinning the samples at 10000×g for 15 minutes at 4^0C, supernatants were collected and incubated with 1µM of caspase-3 substrate Ac-DEVD-AMC in each well of a 96-well plate. The plate was placed in a fluorescent plate reader with a built-in 37^0C incubator (Fluoroskan *A*scent FL, Labsystems) for 1 hour. During this time, substrate was cleaved (AMC release) by active caspase-3 and the fluorescent signals were recorded (excitation 340nm, emission 460nm). The activity of caspase-3 was determined as nMAMC/min/ml of cell lysate. A calibration curve was also created using free AMC.

Immunoblotting

Myeloma cells (treated in the same way as for FACS analysis) were lysed in RIPA buffer (150mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0) containing a cocktail of protease inhibitors (Complete Mini, Roche). After determining the protein concentration with a BCA kit (Pierce), 20-30µg total protein was fractionated using 12% SDS gel electrophoresis. Proteins were transferred to a PVDF membrane and probed with indicated primary antibodies (1:1000-1:2000) followed by specific secondary antibodies (1:2000-1:4000). The signals were finally developed with ECL (Amersham).

Gene expression profiling of the p53 signaling pathway

RT^2*Profiler*^{TM} PCR Array kit (PAHS-027, SABiosciences) was used to analyze the expression pattern of an array of 84 genes involved in tumor suppressor protein p53 signaling pathway, including 5 different housekeeping genes (*B2M, HPRT1, RPL13A, GAPDH, and ACTB*). Briefly, OPM-2 cells were stimulated with 2.5µg/ml of Pam3CSK4 for 8 hours; cells were washed and exposed to FN-coated wells of a 6-well plate. After 90 minutes the unattached cells were removed and fresh medium was added to the wells and incubation was extended to 24 hours. For the last 8 hours, 10nM of Velcade was added and finally total RNA was isolated with RNeasy Mini kit (Invitrogen) for cDNA synthesis which was then applied to the array plate according to the manufacturer's instructions. The same protocol was also applied to cells without Pam3 stimulation and considered as control group in the assay. For data analysis, threshold cycle values (Ct values) were obtained and fold regulations (up or down) of the test group were calculated over the control group using $2^{-\Delta\Delta Ct}$ algorithm.

Real time polymerase chain reaction

To confirm differential gene expression detected in the profiling array expression of changed genes in p53 pathway was analyzed by real time PCR. OPM-2 cells were treated as indicated above and total RNA was isolated using RNeasy Mini kit (Invitrogen). About 500ng RNA was reverse transcribed using iScript first strand cDNA synthesis kit (Biorad) and amplified using the following primers (Isogen, the Netherlands): EGR-1, GML, TP63, CDKN1A, IFNB1, FASLG and GAPDH, the primer sequences are given in supplementary table 1. We used a two-step protocol in real time PCR with the following thermal profiles for the primers. For EGR-1, CDKN1A, and IFNB1 primers: 95^0C, 3' for initial denaturation, 40 cycles of (95^0C, 10"; 65^0C, 30"), for P63, FASLG, and GML primers, 40 cycles of (95^0C, 10"; 60^0C, 30"). A melting curve to validate the amplification was applied to each run and only one peak was observed in all assays.

Statistics

ANOVA (one way) and unpaired *t*-test were performed using GraphPad software (version 5.0). P values less than 0.05 were considered as significant.

Table 1. Sequence of primers designed for performing supplementary real time PCR

Gene	**Primer sequence (5'to 3')**
EGR1-forward	AGCCAAACCACTCGACTGCC
EGR1-reverse	TTGCCACTGTTGGGTGCAGG
GML-forward	TCCAGGCTCCTGCGTGAAGT
GML-reverse	ACTGGCTGCCACCAATGGGA
TP63-forward	TTCACCCTCCGCCAGACCAT
TP63-reverse	TGCGGCGAGCATCCATGTCA
CDKN1A-forward	AAACGGCGGCAGACCAGCAT
CDKN1A-reverse	TGGGCGGATTAGGGCTTCCT
IFNB1-forward	AGTGTCAGAAGCTCCTGTGGCAA
IFNB1-reverse	ATGCGGCGTCCTCCTTCTGGA
FASLG-forward	AGGATTGGGCCTGGGGATGT
FASLG-reverse	TGCTGTGTGCATCTGGCTGG
GAPDH-forward	TTCTTTTGCGTCGCCAGCCG
GAPDH-reverse	TGACCAGGCGCCCAATACGA

Results

TLR-1 triggering in HMCLs has differential modulatory effects on their adhesion to FN

Increase in drug resistance of MM cells correlates with their increased adhesion to FN [10,12,13,18]. We first tested the effect of Pam3CSK4 on HMCLs adhesion to FN. TLR1/2 stimulation with Pam3CSK4 resulted in a decrease of adhesion to FN by Fravel, OPM-1, OPM-2, RPMI-8226 and NCI-H929 cell lines in a dose dependent manner (Fig 1). The concentration for optimal inhibition of adhesion varied between 1 and 5 µg/ml for these cell lines. In contrast, 3 HMCLs L363, UM-6 and U266 showed an increased adhesion in response to Pam3CSK4. In next experiments OPM-2, L363, and U266 were used as representative cell lines of both groups to further investigate the functional consequences of TLR1/2 stimulation.

Pam3CSK4 sensitizes HMCLs to Velcade in the context of FN

To investigate the effect of TLR-1/2 activation on CAMDR, Pam3CSK4-stimulated HMCLs were incubated in uncoated vs FN-coated plates and then exposed to different concentrations of Velcade. In line with previous studies[12, 13] the IC50 of Velcade was higher for cells adhered to FN compared to that for non-adhered cells, suggesting the induction of a cell adhesion mediated drug resistance (Fig 2). Although TLR-1/2 activation by Pam3CSK4 induced some toxicity, combination of Pam3CSK4+Velcade increased the cell death in all cell lines, as illustrated by a lower IC50 for Velcade compared to control-treated conditions (Fig 2).

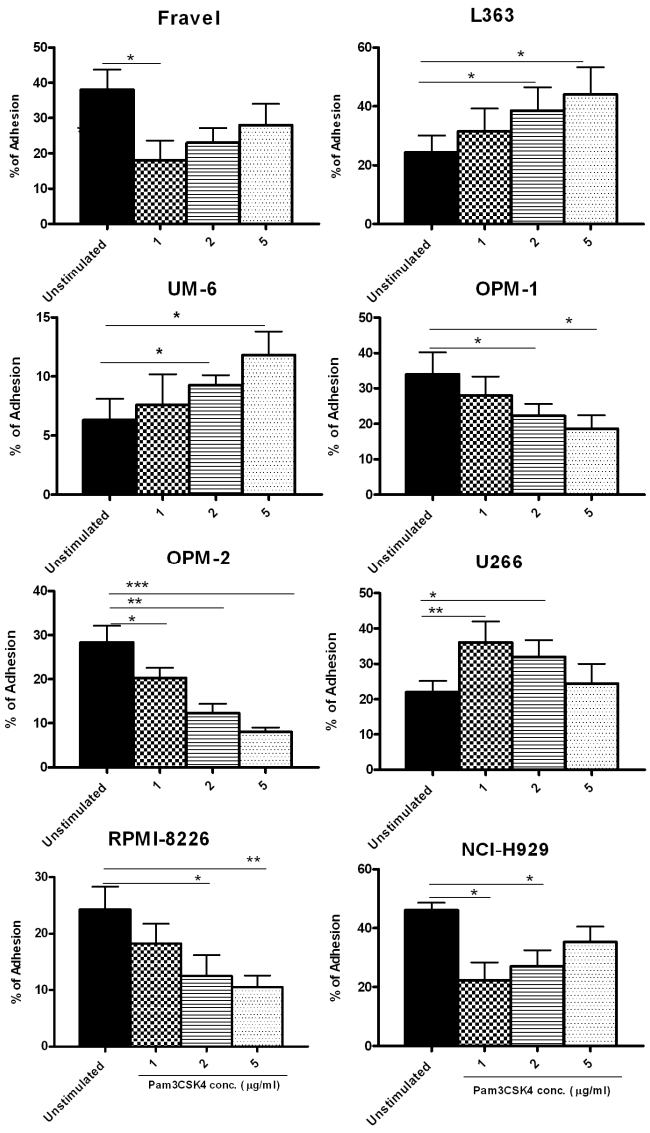

Figure 1. The effect of Pam3CSK4 on adhesion of HMCLs to FN. Calcein-AM labeled cells were seeded on FN-coated 96-well plates which were incubated at 37^0C for one hour. At the end of incubation time, total and spontaneous fluorescence were measured with a plate reader. Data represent the results (mean ± SEM) of at least 3 separate experiments, * *P<0.05, ** P<0.01, *** P<0.001*

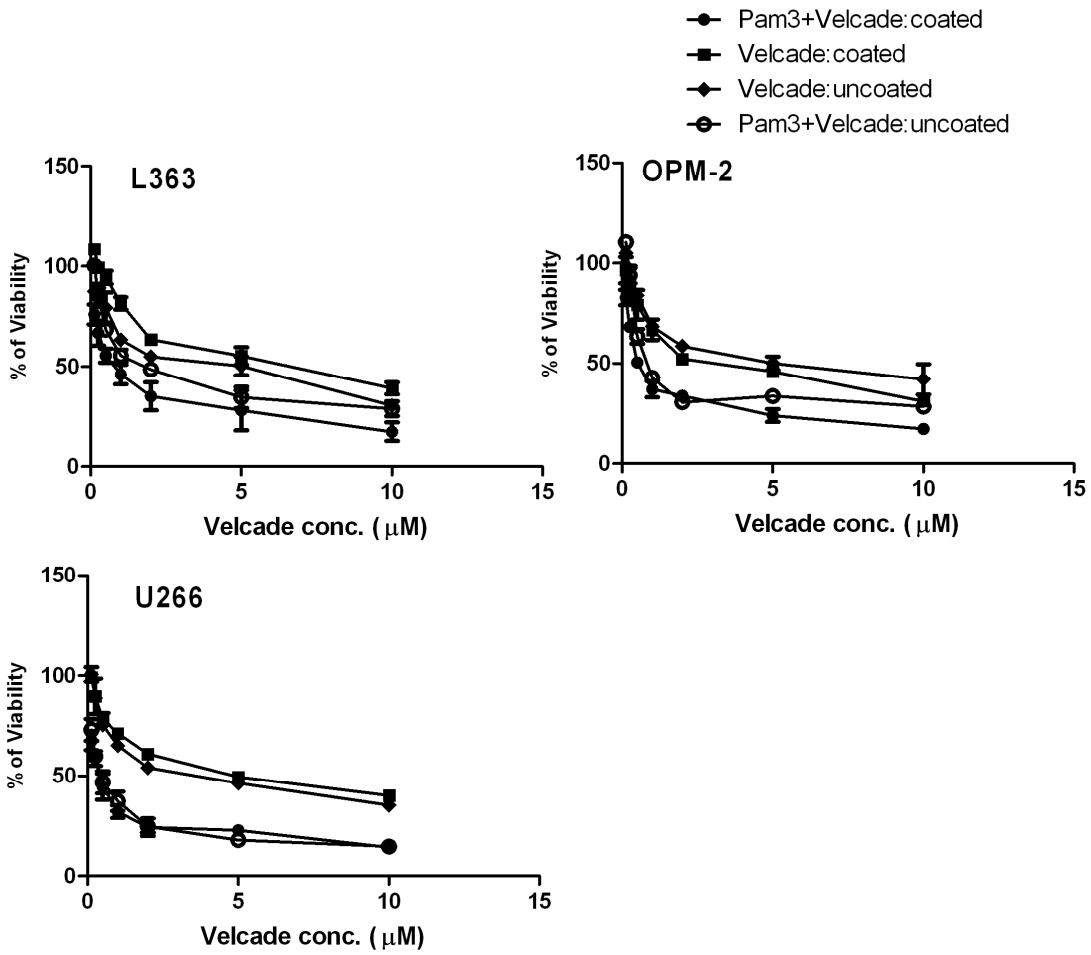

Figure 2. Pam3CSK4 sensitizes HMCLs to cytotoxic effects of Velcade. HMCLs were treated with Pam3CSK4 or solvent (control) for 24 hours, exposed to different concentrations of Velcade for one hour, washed and seeded in uncoated or FN-coated 96-well plates. Cells were incubated for 72 hours and at the last 4 hours, XTT containing PMS was added. IC50 for Velcade were determined from the concentration-cell death curves. **L363**: IC50 (control, non-adhered)=6.8 μM, IC50 (control, adhered)=13.3μM; IC50 (+Pam3, non-adhered=1.0 μM), IC50 (+Pam3, adhered)=3.5μM; **OPM-2**: IC50 (control, non-adhered)=2.75 μM, IC50 (control, adhered)=6.55 μM; IC50 (+Pam3, non-adhered=0.06 μM), IC50 (+Pam3, adhered)=0.288 μM; **U266**: IC50 (control, non-adhered)=2.35 μM, IC50 (control, adhered)=5.49 μM, IC50 (+Pam3, non-adhered=1.45μM), IC50 (+Pam3, adhered)=0.88 μM. Data represent calculated mean±SEM of two separate experiments with duplicate measurements in each.

Pam3CSK4 increases the level of apoptosis following exposure to Velcade and reverses FN-induced CAMDR

Next we investigated whether enhancing the cytotoxic effect of Velcade following Pam3CSK4 stimulation was due to the induction of apoptosis. HMCLs were stimulated with Pam3CSK4 and exposed to Velcade for 24 hours as described in materials and methods. Pam3CSK4 alone caused a substantial level of apoptosis in all HMCLs, but combination of TLR1/2 stimulation with Velcade further increased apoptosis. These findings indicated that TLR-1/2 activation in adhered HMCLs increased their apoptotic response to Velcade treatment.

Apoptosis triggered by Pam3CSK4 is at least partly mediated by caspase-3 activation

We next asked whether the increased apoptotic response induced by Pam3CSK4 was accompanied by activation of the caspase cascade. Intracellular levels of cleaved and pro-enzyme forms of caspase-3 were measured using immunoblotting (Fig 4). Pam3CSK4 alone weakly induced procaspase-3 cleavage in OPM-2 and U266 cell lines but not in L363. A prominent upregulation of cleaved caspase-3 protein was observed in Velcade-treated or Pam3CSK4+Velcade HMCLs, which was accompanied by a complete disappearance of procaspase-3 protein. Next, to understand if Pam3CSK4 alone or its combination with Velcade influenced the enzymatic activity of caspase-3, we assayed its activity using a specific peptide substrate. The enzymatic activity was not significantly increased by Pam3CSK4 alone, while it was most prominently increased in Pam3CSK4+Velcade treated cells (Fig 5).

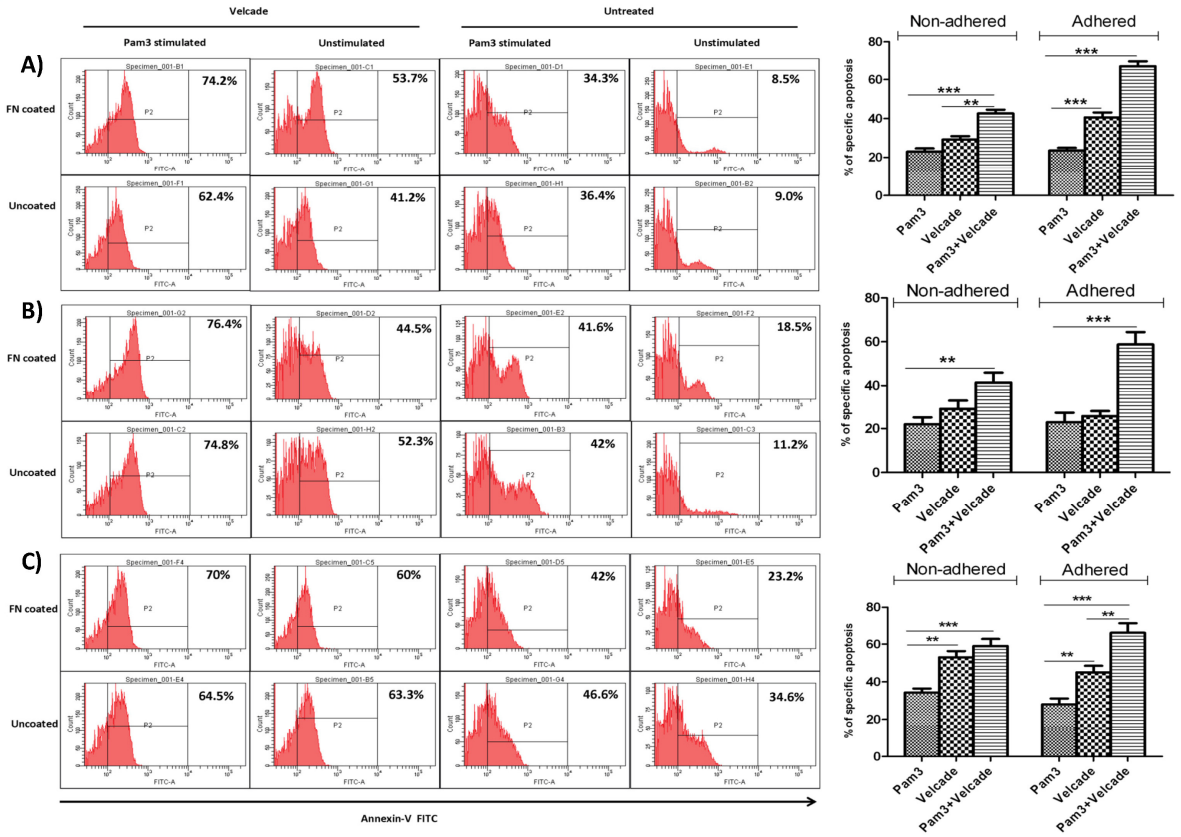

Figure 3. Pam3 enhances apoptotic response of L363 (A), OPM-2 (B), and U266 (C) to Velcade in the presence or absence of FN. Left panel shows the FACS analysis of annexin-FITC staining of L363, OPM-2 and U266. HMCLs were stimulated with Pam3 for 24 hours, washed, adhered to FN for 8-12 hours, and exposed to 5nM Velcade for 24 hours. Right panel: percent specific apoptosis was determined with annexin-V/PI staining as described in materials and methods. Data represent calculated mean±SEM of at least three separate experiments. ** P<0.05, ** P<0.01, *** P<0.001*

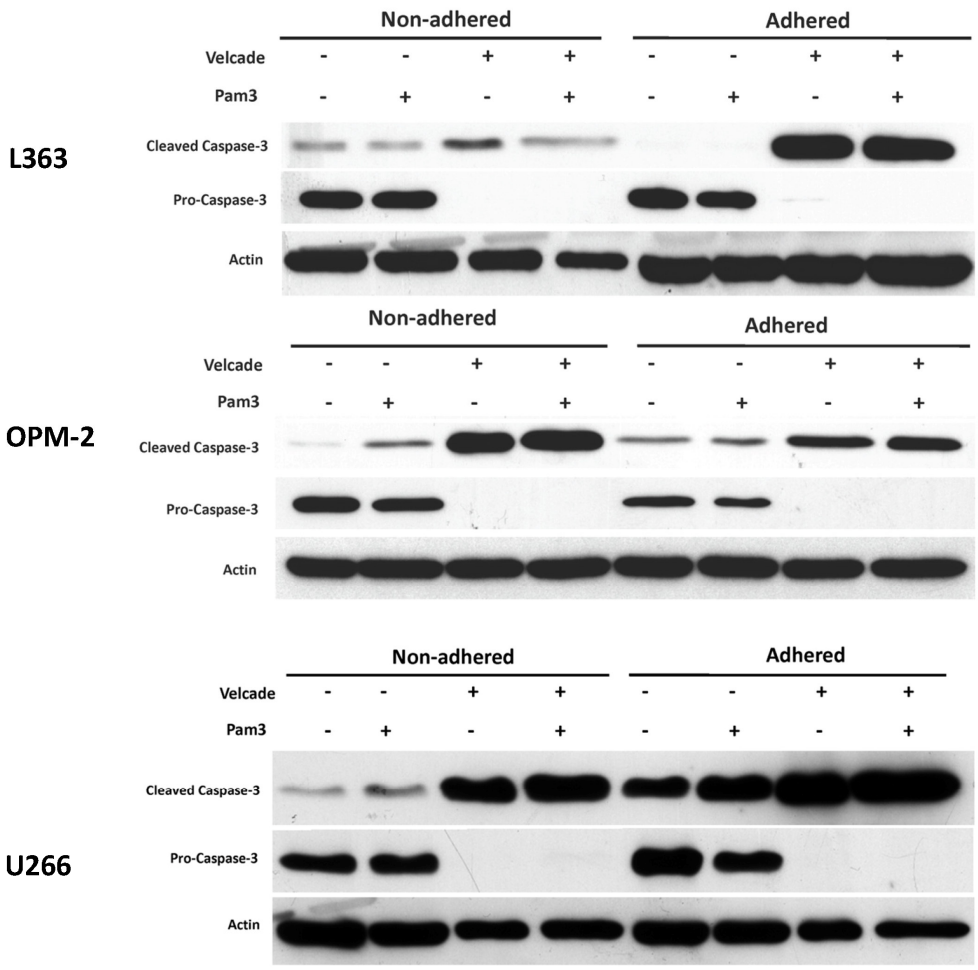

Figure 4. The effect of Pam3 and Pam3+Velcade on cleavage of pro-Caspase-3 protein in the presence or absence of FN in HMCLs. After treatment with Pam3 with or without Velcade, non-adhered and FN-adhered HMCLs were analyzed for cleaved and pro-enzyme forms of Caspase-3 using Western blotting as described in Materials and Methods. Beta-actin was determined as housekeeping protein.

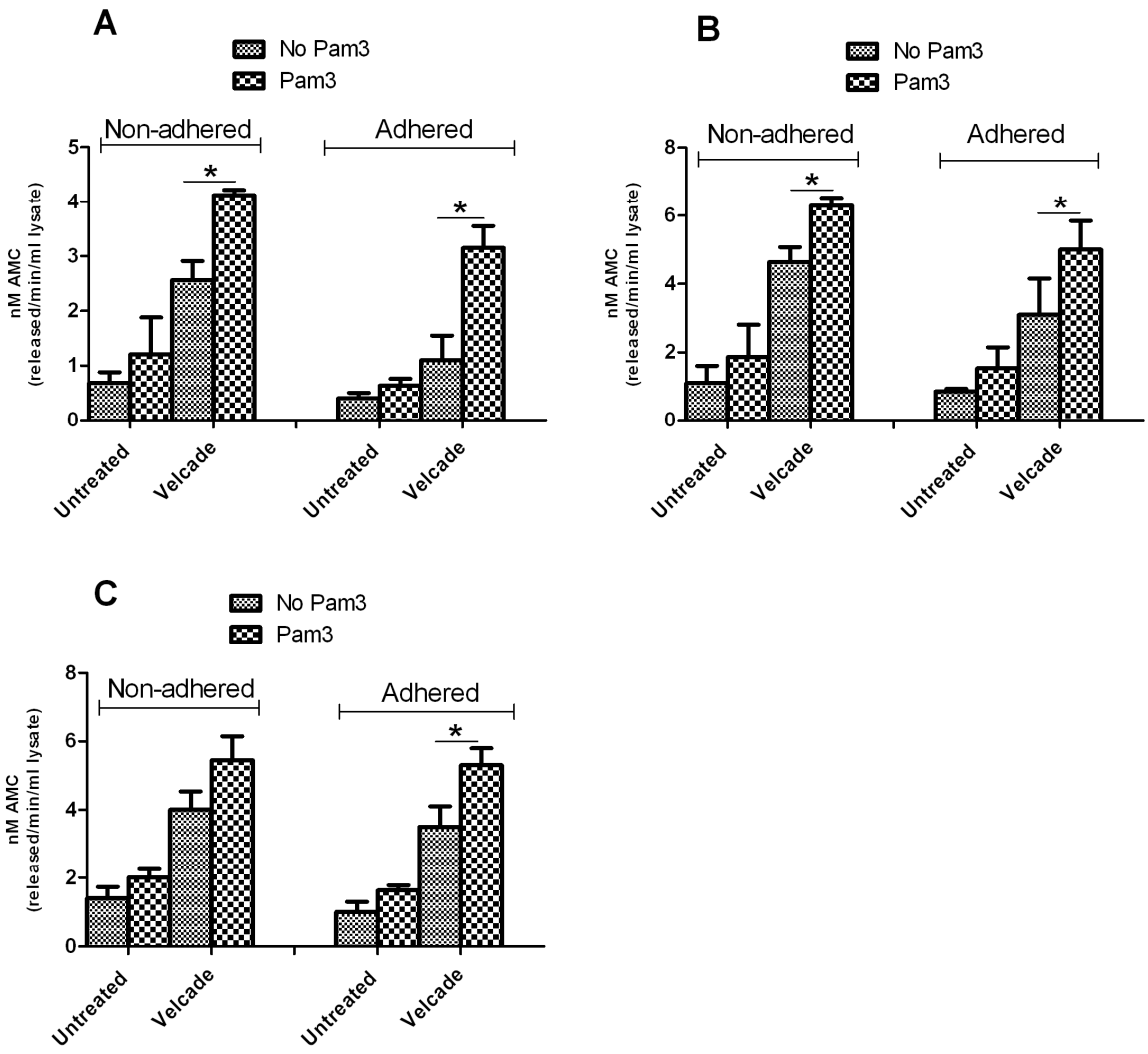

Figure 5. The effect of Pam3 and Pam3+Velcade on Caspase-3 enzymatic activity in the presence or absence of FN in L363 (A), OPM-2 (B) and U266 (C) cell lines. Caspase 3 activity was determined by the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety following hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by the activated enzyme. Data represent calculated mean±SEM of two separate experiments. * *P<0.05.*

p53 pathway may be indirectly involved in apoptosis-enhancing effect of Pam3CSK4 in FN-adhered HMCLs

It has recently been demonstrated that the transcription factor NFκB and tumor suppressor protein p53 cross talk to control apoptotic or survival signals transduced through NFκB[34,35]. Since many TLRs activate NFκB pathway [36], we explored if p53 signaling pathway was involved in controlling the increased apoptosis by Pam3CSK4 in OPM-2 cells. For this purpose, we first determined using a gene array the expression of 84 genes involved in the p53 signaling pathway after treatment of Velcade of control- and Pam3CSK4-stimulated OPM-2 cells. Fourteen genes, which were involved in apoptosis, cell cycle and proliferation, displayed 1.5 to 37 folds up-regulation in Pam3CSK4+Velcade-treated cells compared to Velcade-treated *only* (supplementary table 2). Real time PCR analysis of six of these genes (*IFNB1, EGR1, GML, FASLG, TP63, and CDKN1A*) confirmed the upregulation for *EGR1* (4.0 folds) and *CDKN1A* (3.88 folds). Other genes showed partial upregulation (Supplementary figure 1).

Interestingly, some other genes related to p53 function also displayed at least 1.5 fold upregulation. These genes included *GML* (glycosylphosphatidylinositol-anchored molecule-like protein [37-39]), *RPRM* (REPRIMO, TP53 dependent G2 arrest mediator candidate [40]), and *KAT2B* (lysine acetyltransferase 2B orP300/CBP-associated factor (PCAF)[41,42]).

Three genes *MYC* (cell cycle/proliferation), SESN1 (cell cycle) and *TNF* (apoptosis) displayed 1.55, 1.66 and 1.50 folds downregulation, respectively. *TP53* and some of its related or target genes such as *BCL2, BIRC5, MDM2,* and *BAX* were unchanged, whereas *TP73,* a p53 family member, showed a 27-fold up-regulation.

Analysis at protein level indicated that TLR1/2 stimulation with Pam3CSK4 downregulated p53 and p73 proteins in L363 and OPM-2 cell lines, whereas in U266 it had no effect on p53 and p73 protein levels. Velcade and more significantly Pam3CSK4+Velcade decreased the level of p53 protein in FN-adhered and non-adhered cells. This is in contrast to the recent finding that Velcade would upregulate p53 protein in MM cells in stroma-free conditions [31]. Interestingly, in all cell lines Velcade alone or its combination with Pam3CSK4 increased the level of p73 protein. Next, we analyzed BCL-2 and Bax proteins whose balance play a critical role in controlling apoptosis, with upregulation of Bax and downregulation of BCL-2 signifying an apoptotic response [43,44]. As illustrated in figure 6, in all HMCLs stimulation with Pam3CSK4 did not change BCL-2 and Bax proteins compared to the baseline. Velcade and more significantly Pam3CSK4+Velcade downregulated both proteins. Taken all together, these findings imply that TLR-1/2 triggering could amplify the Velcade-induced expression of genes which mostly inhibit cell cycle (cell proliferation) or control apoptosis, in part through the p53 signaling pathway.

Table 2. Fold upregulation of p53 related genes in p53 pathway gene profiling in OPM-2 cell line stimulated with Pam3 and exposed to Velcade in FN context, compared to cells without Pam3

Gene name	Gene category	Fold-regulation
SESN1	Cell cycle	1.66 (down)
MYC	Cell cycle/proliferation	1.55 (down)
TNF	Apoptosis	1.50 (down)
RPRM	Cell cycle	1,5 (up)
KAT2B	Cell cycle	1,6 (up)
CDKN1A (P21)	Cell cycle	1,6 (up)
CCNG2	Cell cycle	1,74 (up)
TNFRSF10D	Apoptosis	2,5 (up)
TP63	Apoptosis/cell cycle	3 (up)
IL-6	Cell growth /proliferation/differentiation	3 (up)
FASLG	Apoptosis	3,6 (up)
GML	Cell cycle	4,2 (up)
EGR1	Transcription factors & regulators	5,3 (up)
IFNB1	Cell growth /proliferation/differentiation	6 (up)
MYOD1	Transcription factors & regulators	7,3 (up)
TP73	Apoptosis/ Cell cycle	27 (up)
ESR1	Apoptosis/ Cell growth /proliferation/differentiation	37 (up)

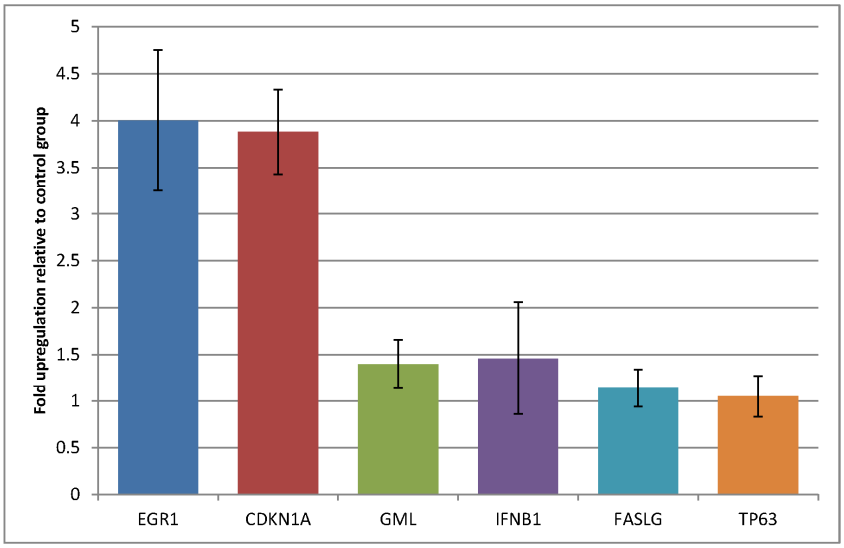

Figure 6. Fold change in expression of p53-related genes gene profiling array. OPM-2 cells were stimulated with Pam3CSK4 and exposed to Velcade, their cDNA was applied to real time PCR and fold change of gene expression was compared to OPM-2 cells not stimulated with Pam3CSK4. Two genes showed an average fold upregulation of 4 (EGR1) and 3.88 (CDKN1A). The graph illustrates data analysis from three separate experiments.

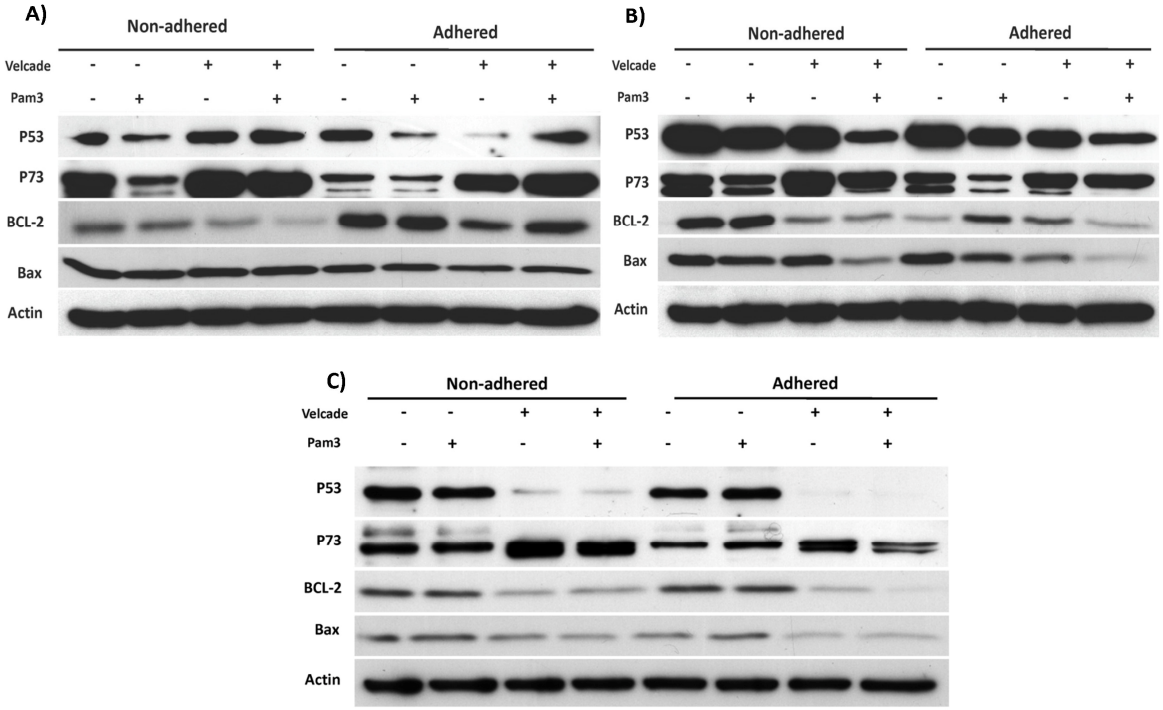

Figure 7. Western blot analysis of bcl-2, bax, p53 and p73 proteins in HMCLs L363 (A), OPM-2 (B) and U266 (C) after Pam3CSK4 and Velcade treatment. HMCLs were stimulated for 24 hours with Pam3CSK4, washed, adhered to FN and finally exposed to low dose Velcade, as explained in materials and methods.

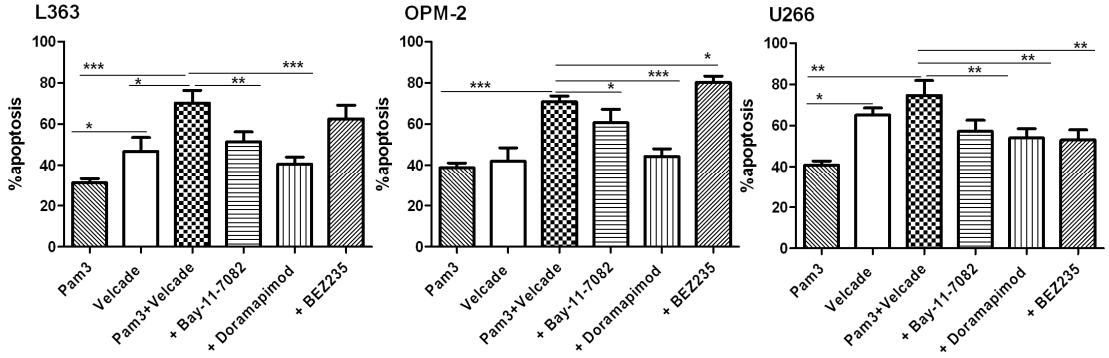

Figure 8. *Involvement of MAPK and NFkB pathway in apoptosis promoting effect of Pam3CSK4 in HMCLs.* L363, OPM-2 and U266 were treated with inhibitors of NFκB (Bay 11-7082), MAPK (Doramapimod), and PI3K/Act (BEZ235) before Pam3CSK4 treatment and drug exposure. Apoptosis was determined by FACS analysis of annexin-V FITC staining as described in materials and methods. Data represent calculated mean±SEM of at least three separate experiments.

Apoptosis promoting effect of Pam3CSK4 in FN-adhered HMCLs is inhibited by MAPK and NFkB inhibitors

Activation of PI3K/Akt, MAPK and NFκB pathways occurs downstream to integrin engagement and adhesion-induced drug resistance could partly be explained by activation of prosurvival signals through Ras/MEK/MAPK pathway [7]. We tested if interplay of these pathways could explain the apoptosis inducing effect of Pam3CSK4+Velcade in FN-adhered cells. HMCLs were first pretreated with pathway inhibitors before TLR-1/2 stimulation and Velcade exposure as outlined in materials and methods. Blocking of PI3K (NVP-BEZ235) pathway inhibited apoptosis in U266, but had no effect in L363 or even increased apoptosis in OPM-2 (Fig.7). Most consistent inhibitory response in all HMCLs was found with the MAPK inhibitor doramapimod, which decreased the level of apoptosis induced by Pam3+Velcade to that induced by Velcade only. Inhibition of the NF-kB pathway with Bay-11-7082 also reduced the Pam3CSK4-induced increase in apoptosis in all HMCLs.

Discussion

Induction of apoptotic responses by TLR activation has only been sparsely examined in lymphoid cancers. A recent study indicated that TLR3 activation of MM cells imposed an apoptotic response, which was found to be IFN-α mediated [24]. Culture of MM cells with TLR 7 and 9 ligands induces IL-6 secretion which endows them with resistance against dexamethasone or serum deprivation-induced apoptosis [25]. Bohnhorst *et* al. found that stimulation of OH-2 and ANBL-6 myeloma cell lines with Pam3CSK4 induced cellular proliferation, but in this study no effects on the sensitivity to cytotoxic drugs were investigated [26]. Our study is the first to show that cell death of MM cells can be increased by TLR triggering. We demonstrate that TLR1/2 stimulation of HMCLs increase the cytotoxicity of Velcade. Also, the partial drug resistance observed in cells adhered to FN is completely reversed by Pam3CSK4 both in drug cytotoxicity and apoptosis assays. Furthermore, increased drug sensitivity and apoptosis induced by TLR1/2 stimulation is not explained by decreased adhesion of HMCLs. For instance, activation of PI3K/Akt, MAPK and NFκB pathways occurs downstream to integrin engagement and adhesion-induced drug resistance could partly be explained by activation of pro-survival signals through Ras/MEK/MAPK pathway [7]. Our study shows that inhibition of NFkB and MAPK pathway greatly inhibits the TLR-1/2-induced increased drug sensitivity in HMCLs. Induction of apoptosis by Pam3CSK4 has already been confirmed in human monocytes [45,46], but involvement of the caspase activation was not clear in these studies. Here we demonstrate that stimulation of TLR1/2 by Pam3CSK4 potentiates apoptosis induced by Velcade, which may be regulated at least partly via an increase in caspase-3 activity. Immunoblotting experiments demonstrated that cleavage of pro-caspase-3 into activated caspase-3 is already

maximal in Velcade-treated cells and stimulation of TLR1/2 with Pam3CSK4 did increase the presence of activated caspase-3 (Fig 4). However, TLR1/2 stimulation significantly enhanced the proteolytic activity of caspase-3 in Velcade-treated cells (Fig 5). Caspase-3 activity can be regulated by changes in redox status [29], S-nitrosylation [47], or phosphorylation [45]. Interestingly, phosphorylation by protein kinase Cδ (PKCδ) was specific to caspase-3 and phosphorylation was required to induce apoptosis in monocytes [45]. TLR2 triggering has been shown to activate PKCδ in other cells,[48] but whether this pathway is involved in the enhancement of caspase-3 activity via PamCSK4 in MM cells is currently under investigation.

Our study may also imply that other pathways in cell survival and apoptosis could be regulated via TLR1/2 stimulation. Recently it was shown there could be crosstalk between NFκB and p53 pathways to control cell cycle and apoptosis in cancer cells [34,35]. To further understand the mechanism underlying enhancement of Velcade-induced apoptosis by Pam3CSK4, the expression of genes of p53 signaling pathway was compared in Velcade-treated and Pam3CSK4+Velcade-treated OPM-2 cells. p53 protein can up-regulate the expression of downstream genes including *CDKN1A, BAX,* and *FAS/APO-1,* which are implicated in cell growth inhibition and apoptotic cell death [49]. Although no effect on *BAX* gene expression was found, *CDKN1A* showed a high up-regulation (3.88 fold) implying that p53 might display at least part of its function through up-regulation of CDKN1A/p21, which has been shown to mediate p53 growth inhibitory effects [50]. Furthermore, we found another gene up-regulated, *EGR1*, which exerts its apoptotic function mediated by p53 protein [49]. Interestingly, a recent study demonstrated that *EGR1* was activated downstream to *JUN* oncogene in MM cells and promoted apoptosis through interaction with *JUN* in these cells; furthermore, overexpression of *EGR1* was associated with an increased susceptibility to Velcade and a favorable prognosis in MM patients[51].

TP53 and its related genes, *BCL-2, BIRC5 (survivin)* and *MDM2* did not change in the expression analysis, while its two family members, *TP63* and *TP73* were up-regulated. We analyzed p53, Bax, BCL-2 and p73 proteins in Western blotting to evaluate changes in expression at a post-transcriptional level. We found that TLR1/2 stimulation downregulated protein expression of p53 and p73 in L363 and OPM-2 cell lines but not in U266 indicating a heterogeneity in the response of different myeloma cells to Pam3CSK4. Combination of TLR1/2 stimulation with Velcade further decreased the expression of Bax and BCL-2 protein in all HMCls as compared to Velcade only. To what extent these changes in both pro-apoptotic and anti-apoptotic molecules contribute to the TLR1/2-induced enhanced cytotoxic response remains to be elucidated. TLR1/2-induced signaling via MAPK and NFκB may indeed integrate with apoptosis pathways in MM cells (Fig 7).

Taken together, our study indicates that stimulation of TLR1/2 results in enhanced cell death when combined with Velcade conceivably by enhancing the caspase-3 activity in myeloma cells. Further research into the molecular mechanisms linking TLR activation to drug-induced apoptotic pathways in MM is needed to evaluate if TLR1/2 stimulation by Pam3CSK4 could be useful in the therapy of MM.

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6

Toll-like receptor (TLR)-1/2 triggering on multiple myeloma cells modulates their adhesion to bone marrow stromal cells and enhances bortezomib-induced apoptosis in this context

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Abstract

In multiple myeloma (MM), the malignant plasma cells usually localize to the bone marrow where adhesion to stromal cells and various environmental signals make myeloma cells drug resistant. Hence, modulation of this interaction is expected to influence drug sensitivity of myeloma cells. Some drugs including bortezomib have proven to decrease above interaction; however, specific categories of patients tend to develop drug resistance. Toll-like receptor (TLR) ligands have displayed heterogeneous effects on B-cell malignancies and also on MM cells in a few recent studies, but their effects on adhesion and drug sensitivity of myeloma cells in the context of bone marrow stromal cells (BMSCs) have never been investigated. In the present study, we explored the modulatory effects of TLR1 ligand (Pam3CSK4) on adhesion of human myeloma cells to BMSCs. We found that TLR1 triggering on myeloma cells differentially modulated their adhesion to BMSCs and also their surface expression of integrin molecules. Furthermore, this treatment increased cytotoxic and apoptotic effects of bortezomib on myeloma cells in above context in an adhesion-independent manner. Our findings uncover a novel role of TLR activation in MM cells in the context of bone marrow microenvironment, which bypasses the protective shield of BMSCs and imply a therapeutical application.

Introduction

Adhesion of multiple myeloma (MM) cells to bone marrow stromal cells (BMSCs), mediated mostly by the integrin family of adhesion molecules, renders the tumor cells resistant against drugs and apoptotic stimuli, and contributes to other complications of the disease including osteolytic lesions and angiogenesis [1-3]. Several cytokines derived from both bone marrow stromal cells and MM cells have been indicated to maintain this interaction [4-6]. Toll-like receptors (TLRs) are a family of pathogen recognition receptors expressed mainly by the innate immune cells but also by a variety of human cancer cells including those of B cell malignancies especially MM [7-13]. TLR activation by microbial or endogenous ligands has been implicated in linking inflammation to cancer, with the transcription factor NFκB activation as the main establishing event [14-18]. However, activation of NFκB in human myeloma cell lines (HMCLs) and primary MM cells has been explained partly by detection of some mutations in NFκB-controlled / related genes (mostly in alternative pathway) [19,20], and are probably independent of TLR signaling which is normally through the canonical pathway [21,22].

Possible contribution of TLRs to inflammation-related malignancy is indicated mostly by induction of pro-inflammatory cytokines in tumor environment [23], upregulation of cell adhesion molecules on cancer cells and their adhesion or migration following TLR triggering [24-27]. Recent studies in cells of B lymphoid malignancies including MM also demonstrated that TLR triggering would result in both positive and negative outcomes, including induction of growth and proliferation, drug resistance, immune evasion and cell death. Nonetheless, the modulatory effect of TLR activation in MM cells on their adhesion to bone marrow microenvironment components including BMSCs has not been explored to date. Hence, regarding the fact that TLRs of MM cells may be activated in the inflammatory environment of bone marrow, possibly by microbial / endogenous ligands, we hypothesized that TLR triggering on MM cells might modulate their adhesion to BMSCs and subsequently modulate MM cells survival and drug resistance. Through an *in vitro* model system, we found that TLR-1/2 triggering on MM cells by Pam3CSK4 modulated their interaction with BMSCs involving adhesion molecules of β1 integrin family. Furthermore, Pam3CSK4 treatment of HMCLs increased their apoptotic response to bortezomib in the context of BMSCs.

Materials & methods

Reagents and antibodies

TLR-1/2 specific ligand, Pam3CSK4, was obtained from Invivogen (San Diego, CA, USA). Rat anti-human beta 7 integrin (clone FIB504, for both FACS and blocking), mouse anti-human αVβ3 integrin (CD51/CD61, clone 23C6, for both FACS and blocking), mouse anti-human VCAM-1 (CD106)-PE (clone STA), mouse anti-human CD49e (α5 integrin, clone P1D6)-PE, mouse anti-human CD49d (α4 integrin, clone 9F10)-PE, anti-mouse IgG-FITC, and mouse IgG2b, κ isotype control were all from eBioscience. APC-conjugated anti-human CD138 (clone DL-1) was obtained from Biolegend (San Diego, USA). PerCp-conjugated anti-human CD19, anti-human CD3 and anti-human CD14 antibodies were provided by Hematology department of Utrecht University Medical Center. Monoclonal rabbit anti-human MyD88 (clone D80F5) and anti-human cleaved caspase-3 (clone D3E9) were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-human CD49d (clone HP2/1, for blocking) was from ABD Serotec (MorphoSys, Oxford, U.K). Alexa Fluor 488 rabbit anti-rat IgG (H+L) was purchased from Invitrogen. Anti-beta actin and horseradish peroxidase-conjugated goat anti-rabbit IgG were also from Santa Cruz Biotechnology, CA, USA. Bortezomib (Velcade) was obtained from LC Laboratories (Woburn, MA, USA) and dissolved in DMSO to make 100mM stock. DMSO concentrations in all drug exposure tests never exceeded 0.05%. Phenazine methosulfate (PMS) and XTT reagents were also from Sigma.

Cell lines and cell culture

The HMCLs, Fravel, L363, OPM1, OPM2, U266, and NCI-H929, were obtained from American Type Culture Collection (Manassas, VA, USA). UM-6 and UM-9 were established by the Department of Clinical Chemistry & Hematology, University Medical Center Utrecht, Utrecht, the Netherlands [28,29]. XG1 and UM6 are IL-6 dependent and others are IL-6 independent. All the cell lines were maintained in RPMI-1640 culture medium containing 2-mM L-glutamine supplemented with 5 or 10% fetal bovine serum and intermittently with antibiotics, in a 37^0C incubator with 5% CO_2. To UM6 cell line was added 5ng/mL of recombinant human IL-6 (from eBioscience, San Diego, CA, USA). NCI-H929 cell line was also treated with 1mM sodium pyruvate and 50μM 2-mercaptoethanol. Normal human bone marrow stromal cell line, HS-5, was obtained from American Type Culture Collection. This cell line was maintained in DMEM medium supplemented with 10% FBS and intermittently with antibiotics. For isolating primary stromal cells, frozen vials of patient bone marrow samples were thawed, suspended in fresh

warm DMEM medium and applied to Ficoll Hypaque density gradient centrifugation to possibly remove cellular debris and dead cells. The remaining fractions were suspended in DMEM medium and kept in a T-75 culture flask for a few hours in a 37^0C incubator. Then the floating cells were gently aspirated and DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100µg/ml streptomycin was added. The cultures were maintained by refreshing the medium twice per week, this provided a confluent growth of bone marrow stromal cells in 3-4 weeks. To make one passage by using trypsin-EDTA, we seeded the cells in 12-well plates for survival experiments. Human peripheral blood mononuclear cells (PBMCs) were isolated from Buffy coats of normal blood donors (Sanquin, Netherlands) with Ficoll-Hypaque density gradient centrifugation and suspended in RPMI medium containing 2mM L-glutamine and supplemented with 10% FBS, 100µg/ml streptomycin and 100U/ml penicillin.

Cell stimulation

To stimulate HMCLs, Pam3CSK4 was used in 1.0, 2.0 and 5.0µg/ml concentrations. Before any treatment, cells were washed once with PBS, suspended in warm RPMI medium supplemented with 5% FBS. Incubation time in a 37^0C incubator with 5% CO_2 was 24 hours.

Flow cytometry

In FACS experiments, direct or indirect staining was performed. Briefly, 10^5 cells from indicated conditions were washed and suspended in FACS staining buffer (PBS+0.5% BSA+0.01% sodium azide). Cells were incubated with primary antibodies (β7 and αVβ3) followed by relevant fluorescent conjugated secondary antibodies. Direct staining method was also used for anti-VCAM-1 (CD106), anti-CD49d (α4) and anti-CD49e (α5) with fluorochrome-conjugated antibodies. Finally, the samples were washed, suspended in FACS buffer and analyzed with a FACS Canto™ II flow cytometer (BD Biosciences). Gated live cell populations were analyzed using Cell Quest or FACS Diva software. For PBMCs, live populations of B cells, T cells and monocytes were gated with specific fluorescent-conjugated antibodies and captured data were analyzed.

Fluorometric adhesion assay

Two to three days before adhesion experiments, 3×10^4 cells of the bone marrow stromal cell line, HS-5 were seeded on 96-well plates. Immediately before adhesion, the plates were washed twice with warm PBS. For adhesion analysis, 10^6 HMCLs (treated or untreated) were harvested, washed twice in PBS buffer and suspended in 1ml of room temperature RPMI medium without

any additive. Cell suspensions were labeled with Calcein-AM (1µM) for 30 minutes at room temperature with gentle mixing after 15 min. To stop labeling, samples were treated with ice cold RPMI and spun twice at 4^0C. One milliliter of RPMI plus 2% FBS at room temperature was added to all samples and 10^5 cells were seeded on stromal cell coated 96-well plates and incubated at 37^0C for 2 hours. At the end of incubation time, total and background fluorescence were measured with a plate reader (Mithras LB 940, Berthold Technologies, Germany). For measuring fluorescence of adhered cells, non-adhered cells were removed with three gentle washes with warm RPMI, 100µL RPMI was added to each well and the fluorescence was measured. In some experiments to determine the adhesion molecules involved, anti-β7 (5µg/ml), anti-αVβ3 and anti-α4 (10µg/ml) antibodies were added to appropriate number of cells for 15 min at 4^0C, the cells were then washed once with cold RPMI to remove free antibody molecules and then added to coated plates. For background readings, fluorescence of the wells containing cells adhered only to BSA was considered. The following formula was used to calculate percentage of adhesion: (Fluorescence reading of adhered cells-background reading) × 100 / (total fluorescence reading-spontaneous reading)

Cell survival: drug cytotoxicity assay

Drug sensitivity measurements were performed using modification of an *acute exposure* approach as described previously [30]. HMCLs were first stimulated with 2µg/ml (OPM-1, OPM-2, and NCI-H929) or 5µg/ml (L363 and U266) Pam3CSK4 for 24 hours. Cells were washed twice with PBS and 5×10^4 cells were treated with indicated concentrations of bortezomib in RPMI+5%FBS in separate 96-well round bottom plates for one hour at a 37^0C incubator, with gentle shaking after 30 minutes. Cells were then washed with warm RPMI, resuspended in drug-free RPMI+FBS and transferred completely to the 96-well flat bottom plates pre-coated with HS-5. These plates were further incubated for 2-3 days. At the last 4 hours of incubation, 25µl from XTT reagent which already contained PMS was added and incubation continued. Finally, the absorbance of each well was measured using a plate reader. The percent survival of cells was calculated by using non-linear regression. In each plate run, wells for solvent control (medium+cells+DMSO, for assay validity and also as 100% viability), blank (medium+DMSO), and growth control (medium+cells, for quality control) were also included. The readings of the blank wells were subtracted from those of all test samples.

Cell survival: annexin-V/PI and cleaved caspase-3 apoptosis assay

Five hundred thousand cells from each HMCL were incubated in the presence or absence of Pam3CSK4 for 24 hours, washed and treated with 5μM bortezomib in RPMI+FBS for one hour (*acute exposure).* Conditions without drug treatment were also included. Cells were then washed, resuspended in drug-free RPMI containing FBS and added to 12-well plates pre-coated with 1×10^5 cells from HS-5 cell line or patient BMSCs for 2 hours. Then unattached cells were removed and fresh medium containing protein was added and plates were incubated for 24-48 hours. In parallel, cells were also put in uncoated wells and treated as mentioned. Finally, cells were removed with cold 5mM EDTA in PBS, washed and suspended in FACS buffer (cold PBS containing 1% BSA and 0.01% sodium azide). Samples were first stained with anti-CD138-APC for 45 minutes on ice, washed once with above FACS buffer and once with binding buffer (eBioscience). The cell pellets were then suspended in 200μl binding buffer containing 5μl FITC-conjugated annexin-V and incubated for 10 minutes at room temperature. After washing with binding buffer, 5μl propidium iodide in 200μl of this buffer was added to each well and samples were applied to FACS analysis in a BD FACSCanto™ II machine. Percentage of apoptotic cells was calculated by selecting the gate of CD138 positive cells. For cleaved caspase-3 analysis, cells were first stained with andi-CD138 as above, fixed with a permeabilization/fixation buffer (eBioscience) for 30 minutes on ice, and then stained with anti-human cleaved caspase-3 for one hour at 4^0C. At the next step, FITC-conjugated secondary antibody (anti-rabbit IgG) was added for 30 minutes on ice. After washing and pelleting, samples were applied to FACS analysis as above. The following formula was applied to determine percent specific apoptosis (adopted from ref.[31]): (test-control)×100/(100-control). Test refers to treatment with Pam3CSK4, bortezomib or Pam3CSK4+bortezomib, and control is the cells without any stimulation (baseline).

MyD88 siRNA transfection

To knockdown MyD88 gene efficiently with the least off-target effects, we used a set of three siRNA duplexes produced through Stealth RNAi™ technology by Invitrogen. The oligonucleotide sequences were as follows:

Duplex: AGAUGGACUUUGAGUACUUGGAGA, UCUCCAAGUACUCAAAGUCCAUCUC,

Duplex2: GAAGCCUUUACAGGUGGCUGUA, UACAGCGGCCACCUGUAAAGGCUUC,

Duplex3: CCGGAUGGUGGUGGUUGUCUCUGAU, AUCAGAGACAACCACCACCAUCCGG.

A Stealth™ negative control duplex (scrambled siRNA) was also included in the analyses. Lipofectamine® RNAiMAX transfection reagent and a fluorescent oligonucleotide were used for

transfecting the cells and optimizing the transfection efficiency, respectively. One day before transfection the cells were cultured in RPMI medium with 5% FBS but no antibiotics. On transfection day, 1µl Lipofectamine® RNAiMAX reagent was mixed with 50µl OptiMEM medium (Invitrogen) and after 5 minutes the total volume was added to 50µl of OptiMEM medium containing almost 50pmol from each siRNA duplex (to make a final siRNA concentration of about 100nM after adding to cell suspensions). The mixture was left at room temperature for 20 minutes to let the siRNA-lipid complexes form, and then added drop wise to the cell suspensions (2×10^5 cells in 400µl of culture medium plus protein) in each well of a 24-well plate. Following 24 hours incubation at 34^0C in a humidified incubator (5% CO_2), the medium was exchanged with fresh medium and the second transfection treatment was performed as above, then the cells were incubated for another 24 hours. At the end of this incubation time, gene knock-down was evaluated at protein level with Western blotting by using an antibody against human MyD88 protein. To carry out further downstream analysis, medium was refreshed and the cells were stimulated with 5µg/ml Pam3CSK4 for 24 hours and finally applied to FACS for protein expression analysis.

Western blotting

Immunoblotting experiments were performed to confirm knockdown of MyD88 protein. Briefly, cell pellets from siRNA-treated or untreated conditions were washed in cold PBS and suspended in lysis buffer (150mM NaCl, 1% IGEPAL (Sigma), 50mM Tris, pH 8.0, 2mM EDTA and 10% glycerol) followed by addition of protease inhibitor cocktail (Complete Mini, Roche). The lysates were then left on ice for 30 minutes and spun at 10,000×g for 10 minutes. The protein concentration of supernatants was determined with a BCA kit (Pierce) and almost 10µg total protein was loaded on a 12% SDS-PAGE gel and electroblotted onto a PVDF membrane. The membrane was incubated with primary antibody (anti-MyD88, 1/1000) overnight at 4^0C followed by addition of HRP-conjugated secondary antibody (1:2000). The signals were then detected by using ECL PLUS or ECL Prime (Amersham).

Statistical analysis

We used unpaired t-test or ANOVA (one way) in GraphPad prism 5 software for statistical analysis, and the values with a $p<0.05$ were considered as significant.

Results

TLR1 triggering modulates expression of integrin molecules on T cells, B cells and Monocytes

To gain an idea of the mode of integrin expression changes after TLR activation, normal human PBMCs were stimulated with Pam3CSK4 (1,2,5 μg/ml) for 24 hours and expression of α4 and β7 integrins was analyzed on live $CD3^+$, $CD19^+$ and $CD14^+$ populations based on MFI (Fig 1). TLR1 ligand increased expression of these molecules dose-dependently on all three cell types.

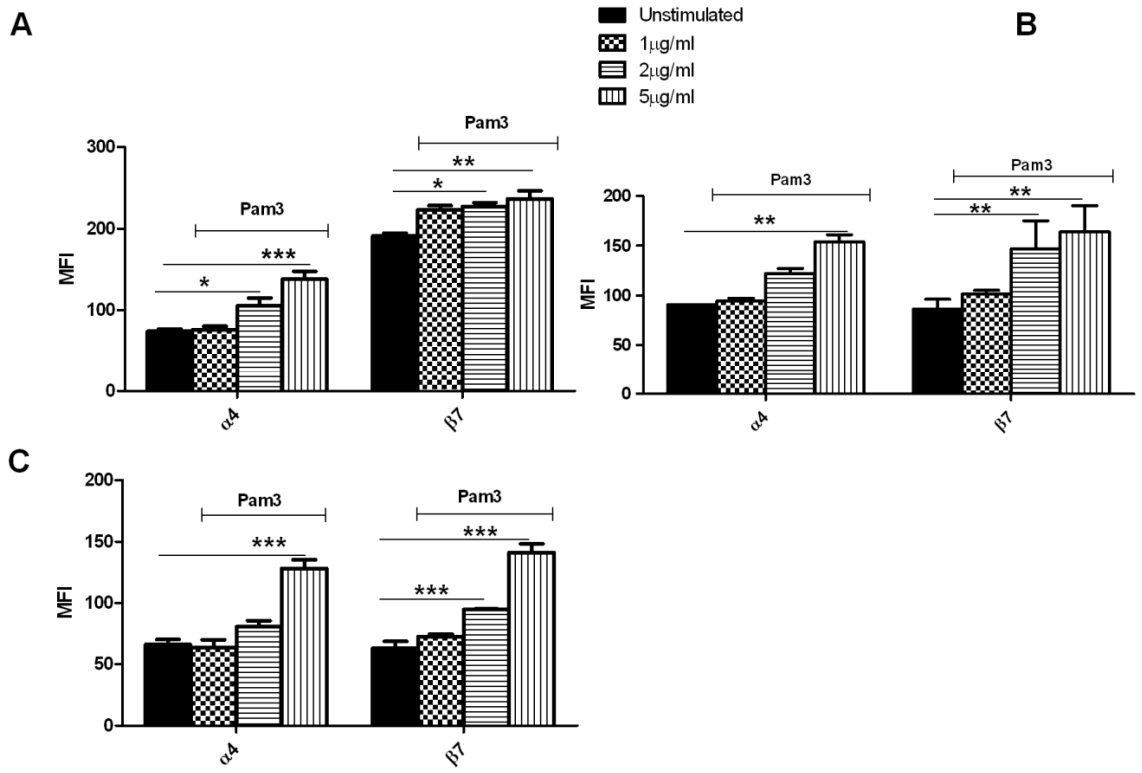


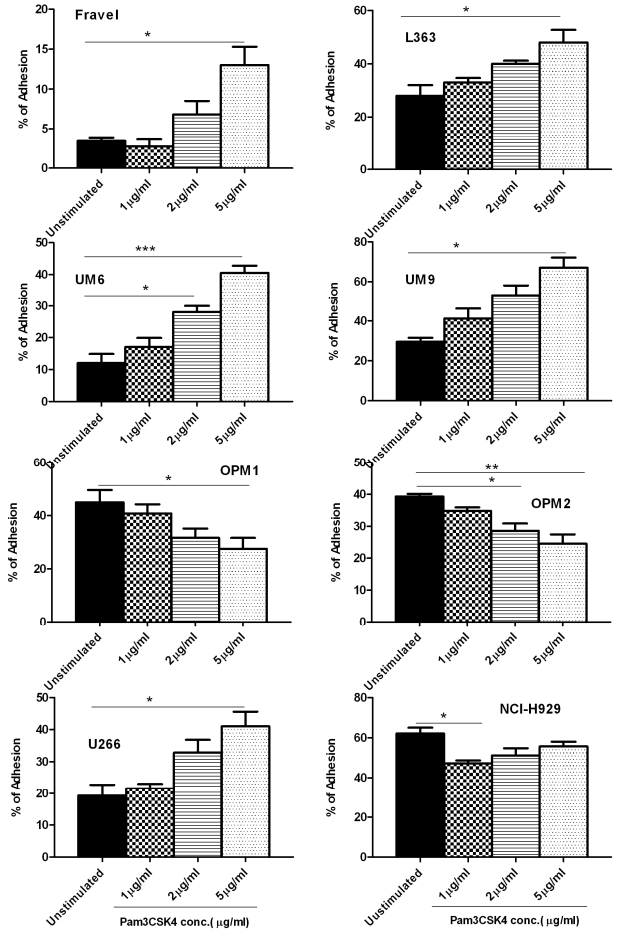
Figure 1. The effect of TLR1 activation in T cells, B cells and monocytes on surface expression of integrins. Normal human PBMCs were stimulated for 24 hours with Pam3CSK4 and expression level of α4 and β7 integrins (based on MFI) was analyzed with gating CD3+, CD19+ and CD14+ populations out of a live population. Pam3CSK4 upregulated dose-dependently the expression of above integrins on all three cell types.

TLR-1 triggering in HMCLs has different modulatory effects on their adhesion to BMSCs

We first investigated the effect of TLR1/2 ligand Pam3CSK4 on the interaction of MM cells with BMSCs, regarding the critical importance of this interaction in MM biology and pathogenesis. TLR1/2 activation modulated adhesion of HMCLs to BMSCs, yet with a heterogeneous pattern (Fig 2). Fravel, L363, UM-6, UM-9 and U266 showed a dose-dependent increase in adhesion. Interestingly, Fravel and UM-6 showed quite low baseline adhesions (3.4% and 12%, respectively) which were highly increased with 5µg/ml Pam3CSK4 (13% and 40%, respectively). OPM-1 and OPM-2 showed a dose-dependent decrease in adhesion. NCI-H929 showed maximal reduction of adhesion already at 1µg/ml Pam3CSK4.

TLR-1/2 triggering in HMCLs modulates surface expression of different adhesion molecules

We next determined the effect of Pam3CSK4 on surface expression of integrin molecules β7, αVβ3, CD49d (α4) and CD49e (α5) using FACS analysis. Incubation with Pam3CSK4 resulted in down-regulation of β7 integrin on Fravel, OPM-1, OPM-2 and NCI-H929 cell lines in a dose-dependent manner (Fig 3), with a pattern closely matching their adhesion behavior (see Fig 2). No change in β7 expression (or only minimal increase with the 5µg/ml concentration) was observed in other cell lines. All the cell lines displayed a dose-dependent increase in the expression of α4 and αVβ3 integrins, except RPMI-8226 which showed only small non-significant changes (Fig 4 and 5). The α5 integrin was not detected on any of the cell lines except RPMI-8226 in which α5 expression was not affected by Pam3CSK4 treatment (data not shown).



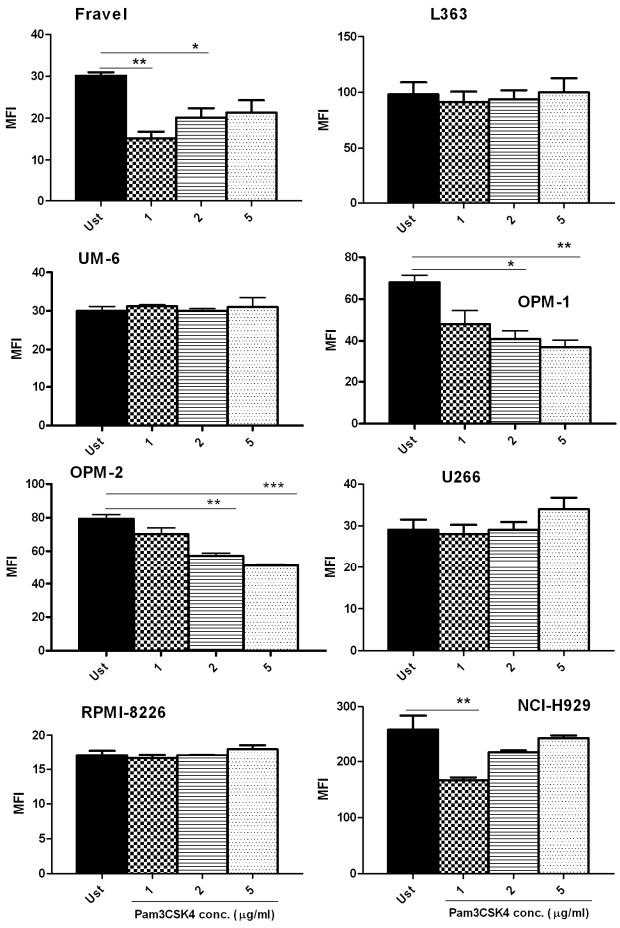


Figure 2. The effect of Pam3CSK4 on adhesion of HMCLs to BMSCs. Pam3CSK4 decreased adhesion of OPM-1, OPM-2, and NCI-H929 cell lines to HS-5 in a dose-dependent manner. Baseline adhesion of Fravel is quite low and is increased dose-dependently following Pam3 treatment. Likewise, adhesion of L363, UM-6, UM-9 and U266 cell lines increased dose-dependently. The results are the statistical analyses of data in at least 3 separate experiments expressed as mean ± SEM, * *P<0.05, ** P<0.01, *** P<0.001*

Figure 3. TLR-1 activation effects on expression of β7 integrin on HMCLs based on MFI. In a dose-dependent manner, Pam3 down-regulated β7 expression on Fravel, OPM-1, OPM-2, and NCI-H929 with a pattern closely matching their adhesion to FN and / or HS-5. No change in β7 expression was observed in any other cell line. The results are the statistical analyses of data in 3 separate experiments expressed as mean ± SEM, * *P<0.05, ** P<0.01, *** P<0.001.*
(Ust: Unstimulated)

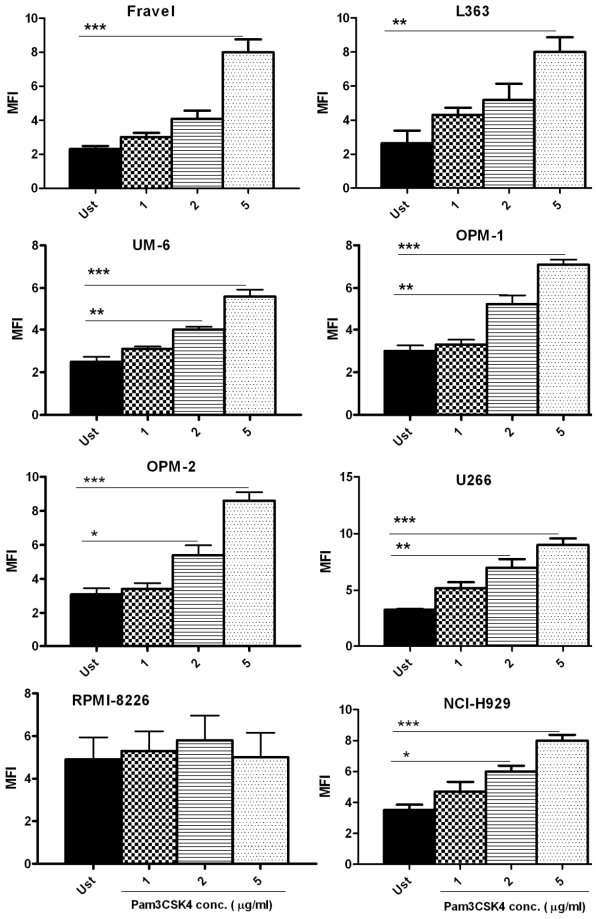


Figure 4. TLR-1 activation effects on expression of αVβ3 integrin on HMCLs based on MFI. In a dose-dependent manner, Pam3 up-regulated αVβ3 expression on all HMCLs except RPMI-8226. The results are the statistical analyses of data in three separate experiments expressed as mean ± SEM, * *P<0.05, ** P<0.01, *** P<0.001.*
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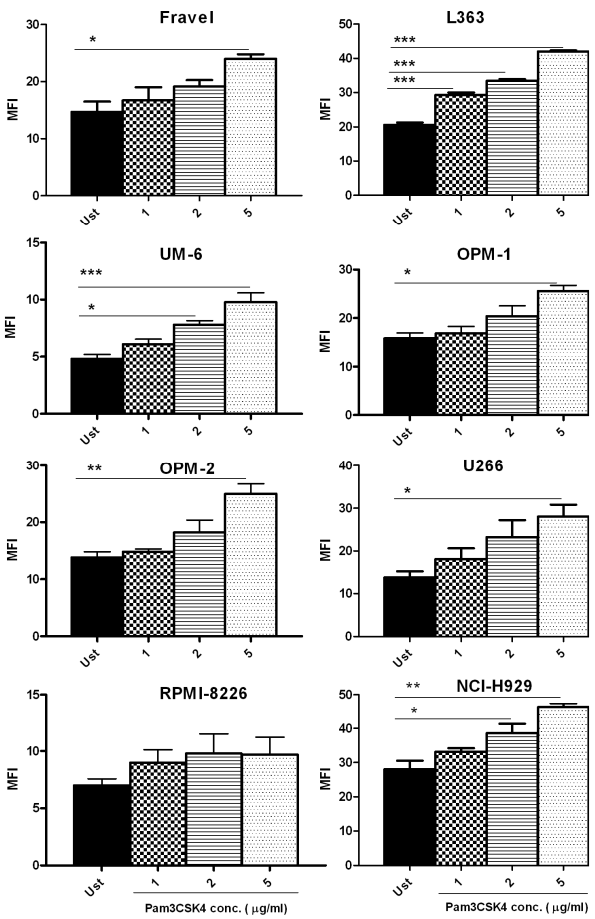


Figure 5. TLR-1 activation effects on expression of α4 integrin on HMCLs based on MFI.
In a dose-dependent manner, Pam3 up-regulated α4 expression on all HMCLs except RPMI-8226. The results are the statistical analyses of data in 3 separate experiments expressed as mean ± SEM, * *P<0.05, ** P<0.01, *** P<0.001.*
(Ust: Unstimulated)

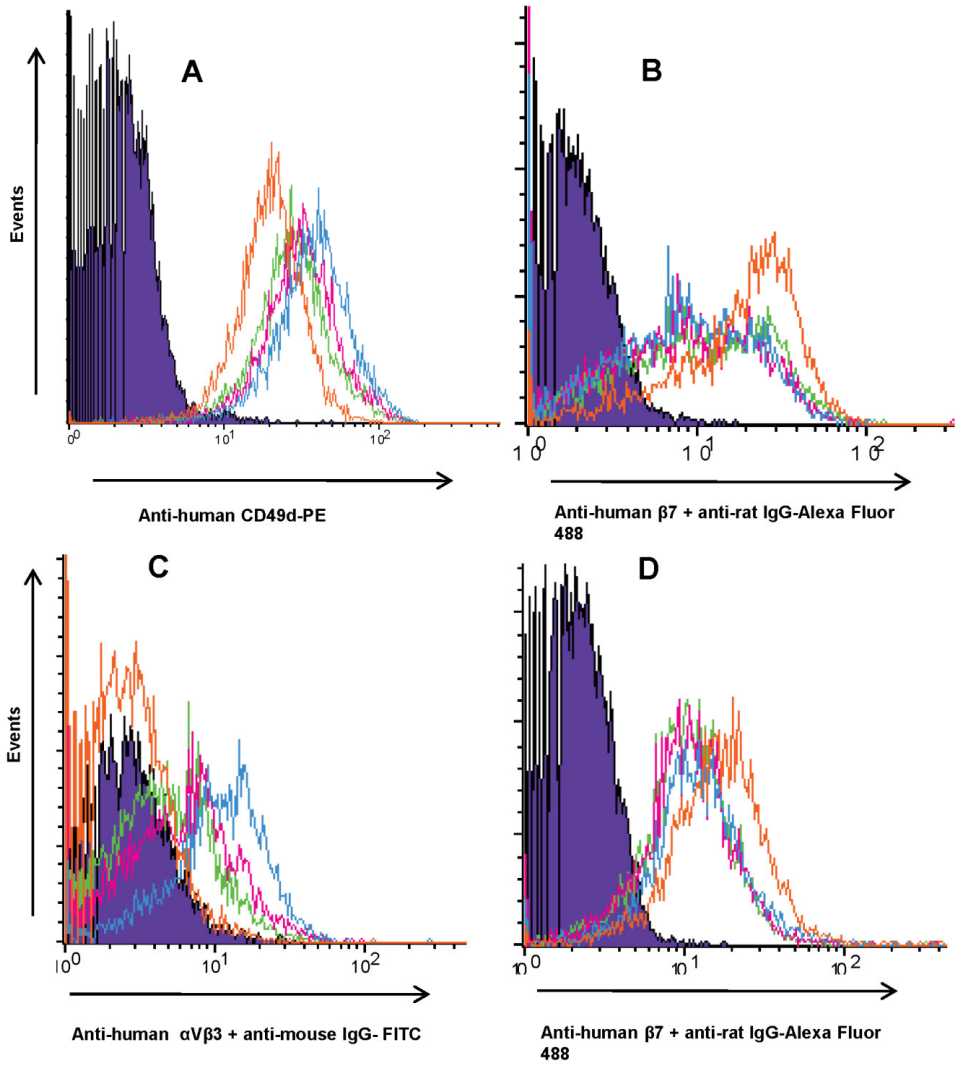


Figure 6. Examples of TLR-1 activation effects on the expression of integrins on L363 (**A**, α4), OPM-1 (**B**, β7), U266 (**C**, αVβ3) and Fravel (**D**, β7). Histogram color representations are as follows: Filled histogram (isotype control), orange (unstimulated), green (1µg/ml Pam3), red (2µg/ml pam3), blue (5µg/ml

Modulation of HMCLs adhesion to BMSCs following TLR-1 activation possibly involves different integrin molecules

Based on the above findings, it seemed conceivable that β7 integrin could mediate down-regulatory and αVβ3 and α4 integrins up-regulatory effects of Pam3CSK4 on adhesion of HMCLs to BMSCs. Next, we used anti-β7 antibody to block baseline adhesion to BMSCs in OPM-1, OPM-2, and NCI-H929. The anti- β7 antibody decreased baseline adhesion as much as 17%, 20%, and 25%, respectively (Fig 7), indicating that β7 was involved in the adhesion of these HMCLs.

To investigate the involvement of αVβ3 and α4 integrins in adhesion, anti-αVβ3 and anti-α4 antibodies were used to inhibit adhesion of Fravel, L363, UM-6 and U266 cell lines (Fig 8). The experiments indicated that Pam3CSK4-induced upregulation of adhesion was mediated by α4 and/or αVβ3 integrins. Adhesion of Fravel, L363 and U266 cells was shown to be mainly α4-mediated, as anti-α4 fully blocked the up-regulated level plus a large part of baseline adhesion. Blockade of αVβ3 integrin did not reduce Pam3CSK4-induced adhesion, significantly. This suggests that α4 integrin subunit is one of the main adhesion molecules engaged by Fravel, L363 and U266 cells in adhesion to BMSCs. Adhesion of UM-6 was regulated by both α4 and αVβ3 integrins. These data confirm the contribution of β7, α4, αVβ3 integrins to adhesion of HCMLs to stromal cells and suggest that there may be a heterogeneous response to TLR1/2 stimulation on their functional expression in HCMLs.

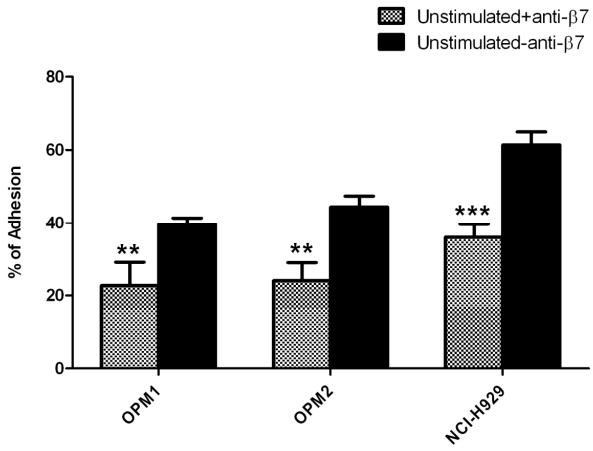


Figure 7. Blocking experiments with anti-β7 antibody for adhesion to HS-5. At a 5μg/ml concentration, anti-β7 significantly decreased baseline FN adhesion of OPM-1, OPM-2, and NCI-H929. These findings are more supported by FACS where β7 integrin was significantly down-regulated by Pam3 treatment (Fig 3). The results are the statistical analyses of data in 3 separate experiments expressed as mean ± SEM, * *P<0.05, ** P<0.01, *** P<0.001*

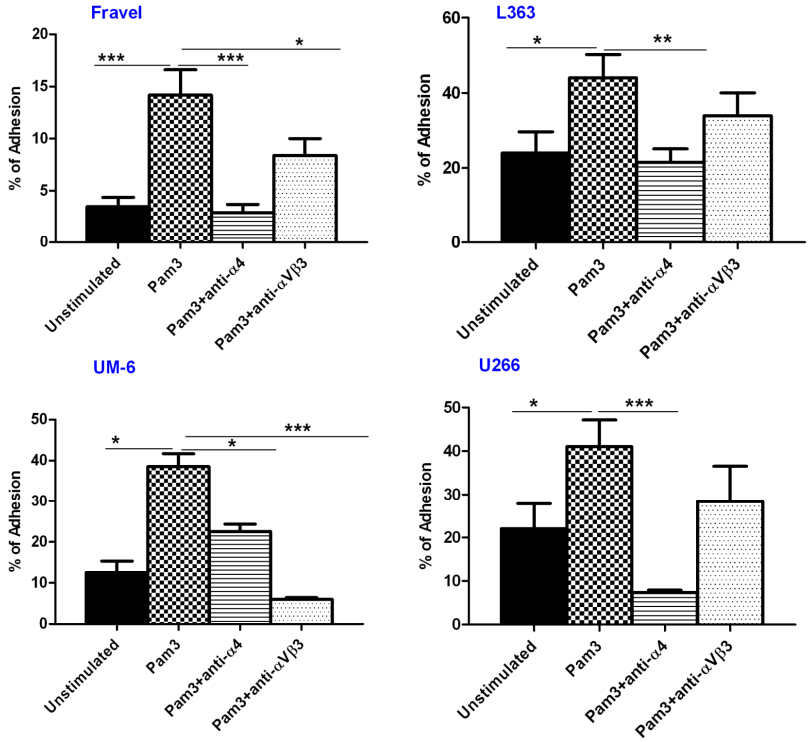


Figure 8. Blocking experiments with anti-α4 and anti-αVβ3 antibodies for adhesion to HS-5. At a 10μg/ml concentration, anti-α4 fully blocked the up-regulated level of adhesion to HS-5 in Fravel, L363 and U266, also blocked a large part of baseline adhesion only in U266 and a small part of up-regulated adhesion in UM-6. In Fravel, anti-αVβ3 (10μg/ml) also blocked part of the up-regulated adhesion but it fully inhibited both up-regulated and a part of baseline adhesions in UM-6 indicating that αVβ3 was the main integrin involved in UM-6 adhesion to HS-5 following TLR-1 activation. These cell lines also displayed up-regulation in αVβ3 and α4 integrins in FACS (Figs 4,5). The results are the statistical analyses of data in 3 separate experiments expressed as mean ± SEM, * *P<0.05, ** P<0.01, *** P<0.001*

The adapter protein MyD88 mediates TLR1-activation induced modulation of adhesion molecules on HMCLs

To ascertain whether alterations in integrin expression pattern are indeed due to TLR-1 activation, and regarding the fact that MyD88 is the main adapter protein downstream to most TLR activation, L363, OPM-1 and OPM-2 cell lines were transfected with siRNA to downregulate expression of MyD88 (Fig 9A and 9B). As depicted in figure 9B, Western blotting confirms that siRNA transfection reduced the level of MyD88 protein. This effect was not apparent in the negative control (scrambled siRNA). The enhancing effect of Pam3CSK4 on α4 and αVβ3 expression and its reducing effect on β7 expression were greatly attenuated following MyD88 knockdown. These findings indicate that modulatory effects of TLR1 activation on HMCLs with respect to expression of above integrin molecules are indeed mediated by MyD88.

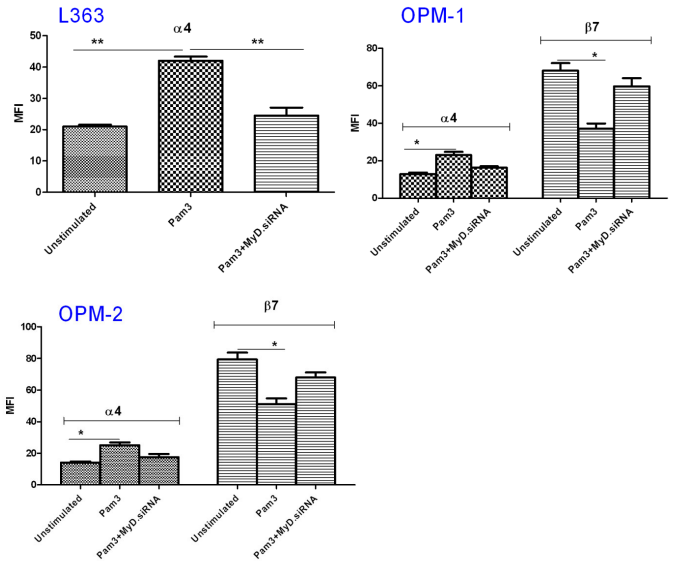
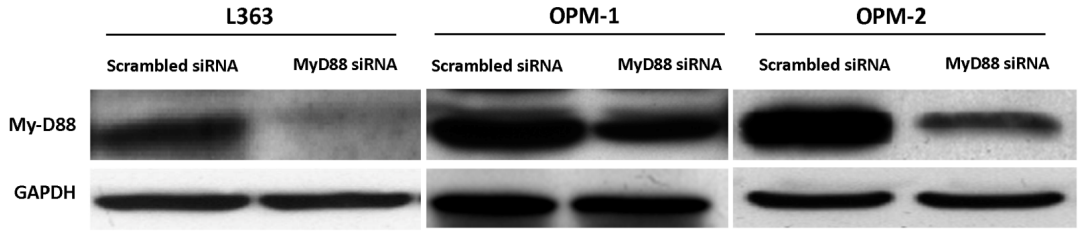


Figure 9. The effect of MyD88 protein knocking-down using siRNA transfection on expression of integrin molecules following Pam3 treatment.
A)-MyD88 silencing attenuated up-regulation of α4 integrin in L363, OPM-1 and OPM-2 and down-regulation of β7 integrin in OPM-1 and OPM-2 cell lines following Pam3 treatment.
B)-MyD88 silencing reduced its protein level, this effect was not observed with scrambled siRNA (negative control).



Surface integrin modulation following TLR-1 activation is mostly mediated by NFkB activation

To test NFκB pathway involvement, we treated Fravel, L363, OPM-1, OPM-2 and NCI-H929 cell lines with Bay 11-7082 (2,5,10µM) before stimulation with 1µg/ml (Fravel and NCI-H929) or 5µg/ml (Fravel and L363) Pam3CSK4 for 24 hours. Some unstimulated conditions were also treated with a specific concentration of Bay 11-7082. As shown in figure 10, blocking NFkB pathway in Fravel, OPM-1, OPM-2 and NCI-H929 cell lines decreased both baseline expression and Pam3CSK4-induced downregulation of β7, indicating that NFκB pathway is probably not entirely involved in β7 downregulation. Furthermore, in Fravel and L363 cell lines, the Pam3CSK4-induced α4 upregulation was completely antagonized by NFkB blocking but only with the highest concentration of Bay (10µM)(Fig 11). Taken together, these findings provide us with an implication that NFkB pathway in HMCLs could mediate TLR1 activation-induced upregulation of α4 integrin but probably not downregulation of β7 integrin.

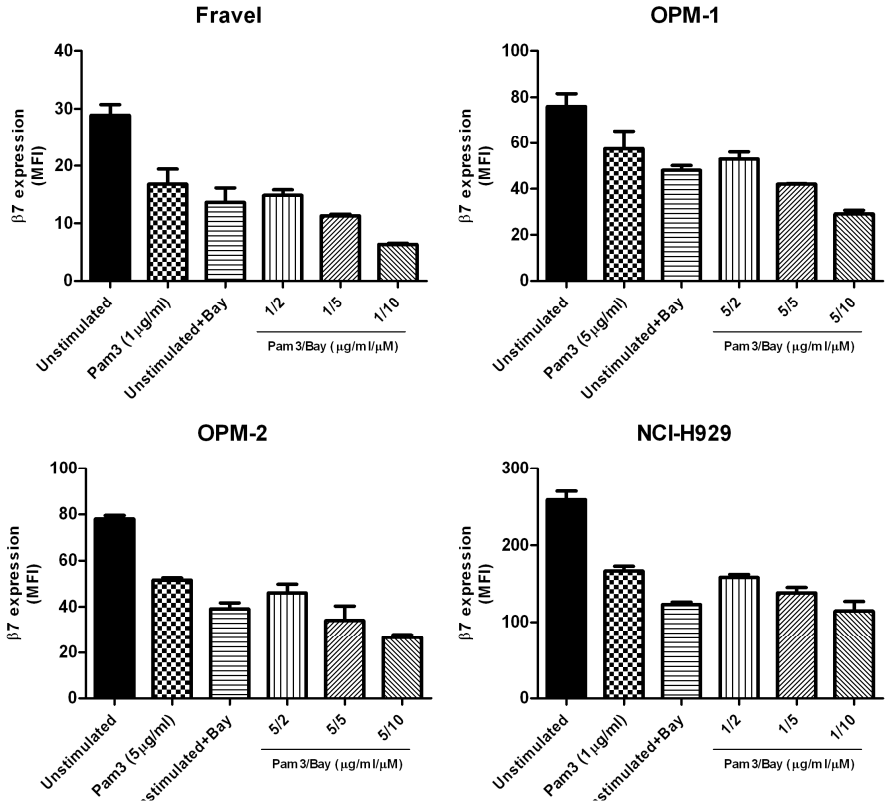


Figure 10. The role of NFκB pathway inhibition in Pam3-induced downregulation of β7 integrin. HMCLs were pre-treated with Bay 11-7082 and then stimulated with specified concentrations of Pam3. Blocking NFκB pathway could not antagonize Pam3-induced β7 downregulation but further reduced it and also the baseline expression of β7. This implies that other mechanism than NFκB might control Pam3-induced β7 downregulation.

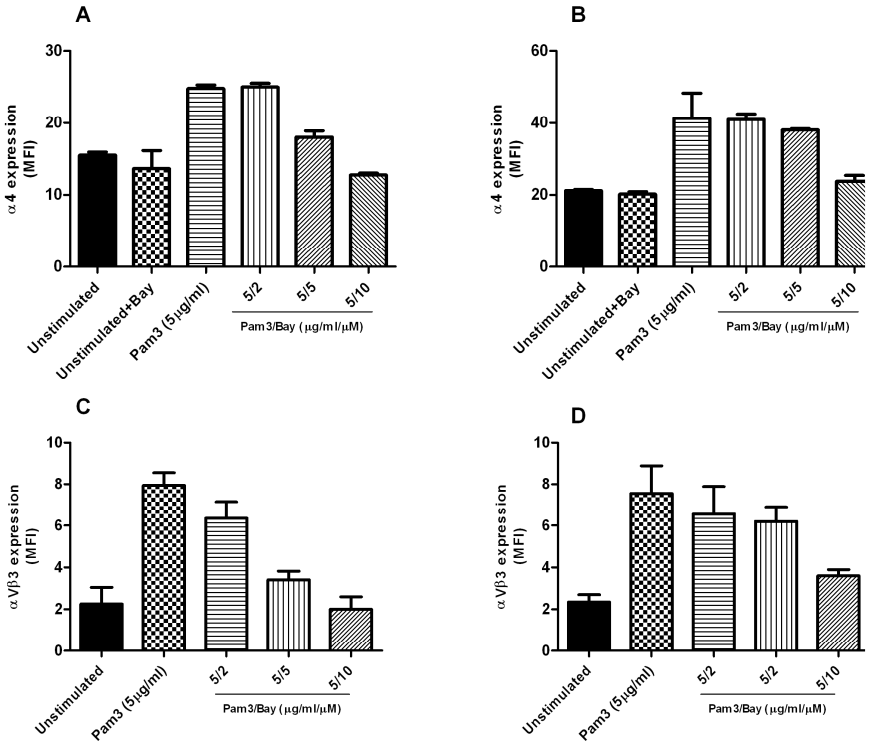


Figure 11. The role of NFκB pathway inhibition in Pam3-induced upregulation of α4 and αVβ3 integrins. HMCLs were pre-treated with Bay 11-7082 and then stimulated with the specified concentration of Pam3.
Blocking NFκB pathway could antagonize Pam3-induced upregulation of α4 (A) and αVβ3 (C) integrins in Fravel. But in L363 (B,D) this inhibition did not completely blocked Pam3 effect even at high concentration of Bay 11-7082, indicating either a probably resistant phenotype of NFκB or another additional mechanism in the latter cell line.

TLR1 activation in HMCLs enhances cytotoxic effects of bortezomib in the context of bone marrow stromal cells

Drug resistance of HMCLs can be greatly influenced by adhesion. In the next experiments, we investigated if the changed adhesion due to TLR1/2 stimulation would influence the drug sensitivity of HMCLs. L363 and U266 which showed upregulated adhesion and OPM-2 with Pam3CSK4-induced downregulated adhesion were selected for further investigation. As expected, IC50 of the HMCLs for bortezomib was higher when they adhered to stromal cells (Fig 12). However, Pam3CSK4 treatment increased drug sensitivity of all HMCLs to bortezomib in the presence or absence of stromal cells suggesting that the Pam3CSK4-induced increases in bortezomib cytotoxicity were adhesion-independent.

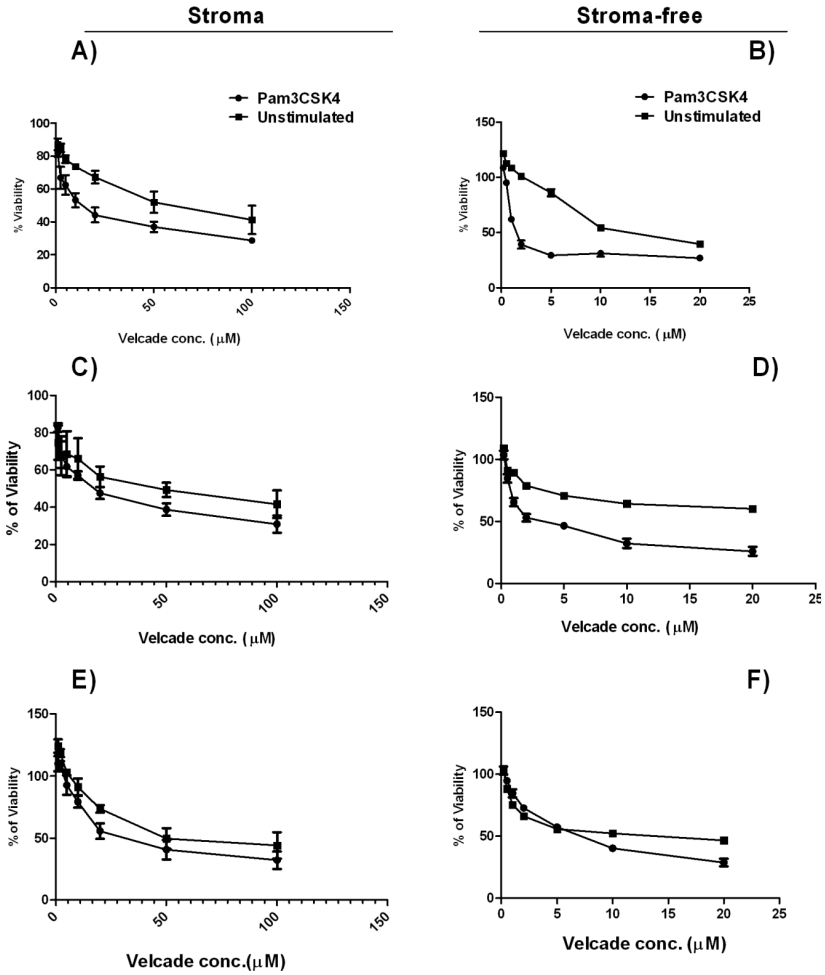


Figure 12. Pam3CSK4 increased sensitivity of HMCLs to Velcade in the context of BMSCs. Myeloma cells were stimulated with Pam3CSK4 for 24 hours, and exposed to increasing drug concentrations in an acute manner. Graphs represent OPM-2 (A,B), L363 (C,D) and U266 (E,F) cells lines. Pam3CSK4 stimulated cells displayed a higher sensitivity to Velcade in the presence or absence of stroma, however, the level of sensitivity was lower for HMCLs in BMSCs context than cells in the stroma-free condition. This will be more obvious by comparing IC50s (μM of the drug) for all conditions:
OPM-2 stroma: Pam3 (7.5), no Pam3 (38.5), stroma-free: Pam3 (1.85), no Pam3 (15.6)
L363, stroma: Pam3 (12.81), no Pam3 (41.12), stroma-free: Pam3 (4.96), no Pam3 (14.89)
U266 stroma: Pam3 (13.46), no Pam3 (14.85), stroma-free: Pam3 (5.6), no Pam3 (11.2)

TLR1 activation in HMCLs enhances the apoptotic response to bortezomib in the context of bone marrow stromal cells

At the next steps, we tried to explore if increased cytotoxicity to bortezomib in HMCLs after treatment with Pam3CSK4 was due to an increase in apoptosis. HMCLs were first stimulated with Pam3CSK4 for 24 hours, washed and exposed to acute bortezomib treatment and seeded onto patient BMSCs or HS-5 cells, as described in materials and methods. In gated $CD138^+$ cells, the percentage of annexin-V positive cells was determined to calculate specific apoptosis. As depicted in figure 13, Pam3CSK4 treatment increased the level of bortezomib-induced apoptosis in all cell lines in the presence or absence of HS-5 cells, which further confirmed the adhesion-independent effect of TLR1/2 stimulation on HMCLs viability. Of note, Pam3CSK4 itself also left a partial apoptotic effect which was more pronounced in U266 cell line. Interestingly, varying levels of CAMDR were detected in the context of patient primary BMSCs for all cell lines, which was reversed by the Pam3CSK4+bortezomib treatment (Fig 14). These findings demonstrate that Pam3CSK4 increases the apoptotic effect of bortezomib in the context of BMSCs.

Apoptosis enhancing effects of Pam3CSK4 on HMCLs in BMSCs context may be mediated by caspase-3 activation

Only limited details are known on the effect of TLR activation on apoptotic signaling pathways. We further investigated if Pam3CSK4 could mediate increased apoptosis via caspase-3 activation. HMCLs were first stimulated and drug treated as detailed in materials and methods. Using FACS analysis the percent apoptotic cells was determined after gating CD138-positive cells (Fig 15). As expected bortezomib increased cleaved caspase-3 in all conditions for all cell lines indicating it activated caspase-3 pathway [31]. Treatment with Pam3CSK4 alone augmented the cleaved caspase-3 level to different extents in the 3 HMCLs and combination of Pam3CSK4 with bortezomib increased the level of cleaved caspase-3. Above findings suggest that Pam3CSK4 may contribute to apoptosis through the activation of caspase-3.

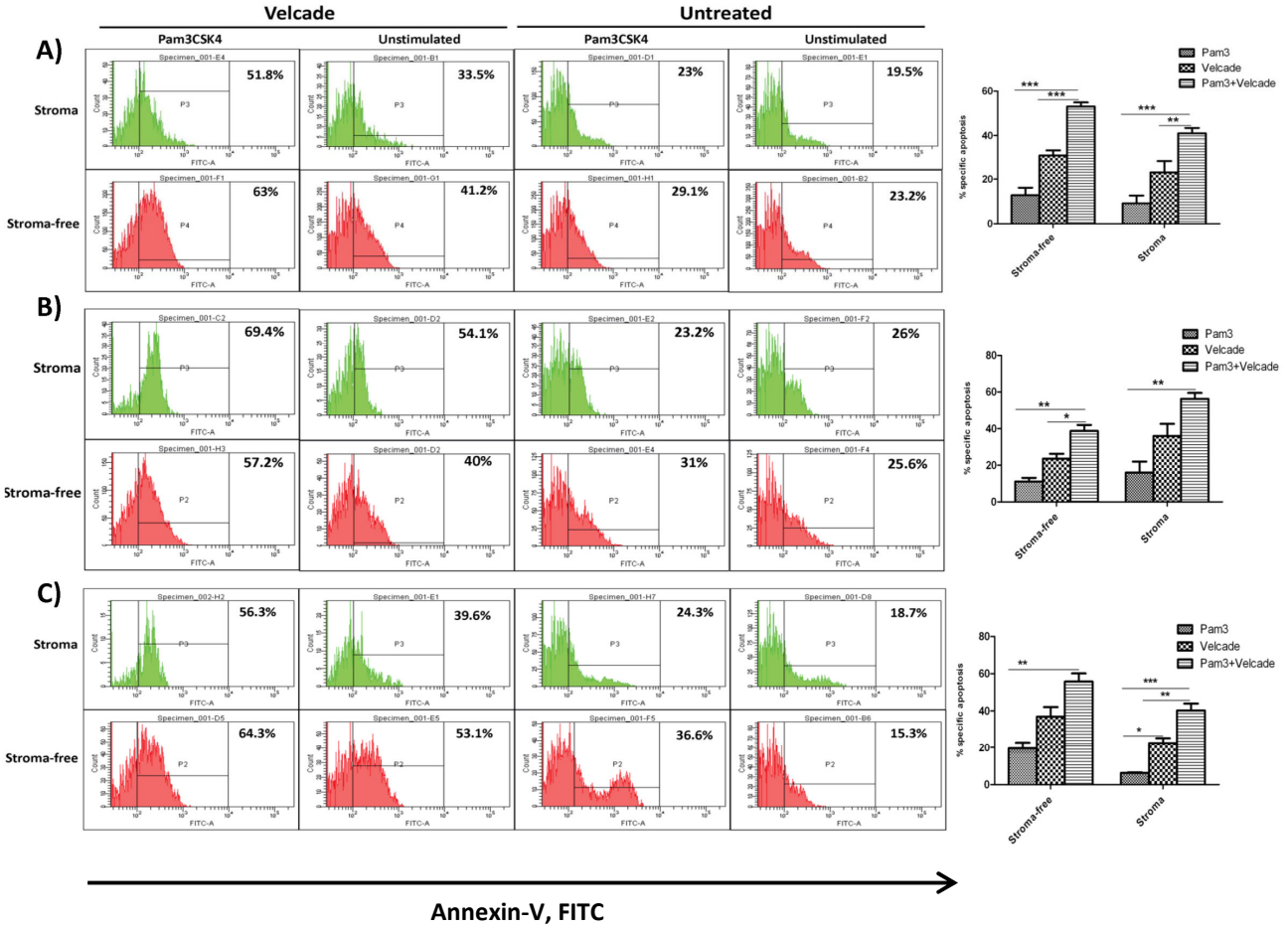


Figure 13. The apoptosis enhancing effect of Pam3CSK4 on L363 (A), OPM-2 (B) and U266 (C) in the context of HS-5. HMCLs were stimulated for 24 hours with Pam3CSK4, adhered to HS-5 cells and then exposed to drug treatment. Cells were then applied to annexin-V/PI FACS analysis by gating on $CD138^{+}$cells as explained in materials and methods. Left panels are one representative out of three separate experiments for each cell line and the right panels are statistical analyses of all experiments. Pam3CSK4 alone had an apoptotic effect only in U266 which was lower in stroma than in stroma-free condition possibly due to stromal resistance, however, it increased the apoptotic response of all cell lines to bortezomib in the presence or absence of HS-5. A level of CAMDR was observed for L363 and U266 cells lines (higher bortezomib-induced apoptosis in stroma-free than stroma conditions) but not for OPM-2 in which apoptosis was higher in stroma condition. This drug resistance was completely removed by Pam3CSK4+bortezomib treatment.

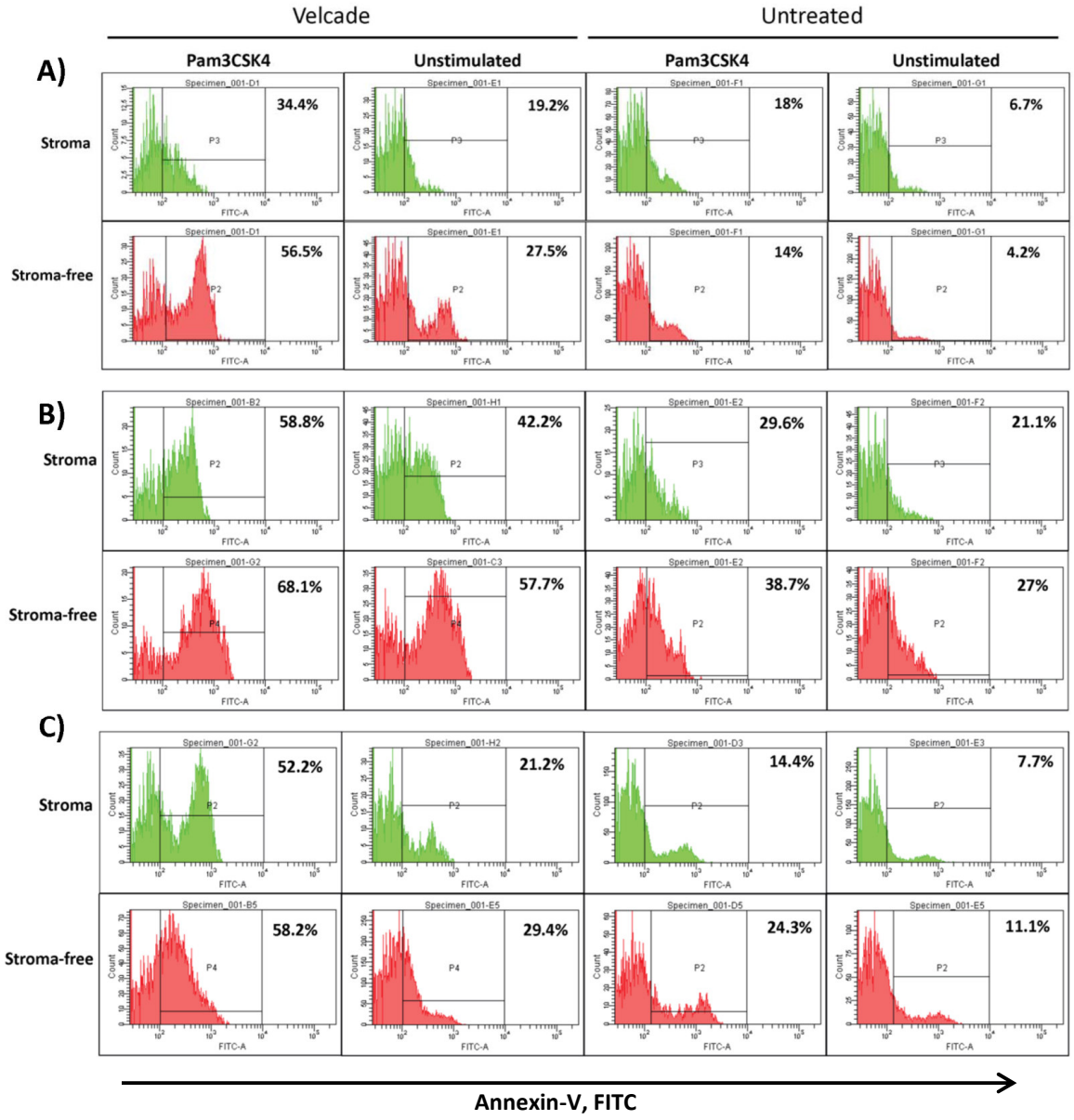


Figure 14. The apoptosis enhancing effect of Pam3CSK4 on L363 (A), OPM-2 (B) and U266 (C) in the context of myeloma patient BMSCs. HMCLs were stimulated for 24 hours with Pam3CSK4, adhered to patient bone marrow isolated stromal cells and then exposed to drug treatment. Cells were then applied to annexin-V/PI FACS analysis by gating on $CD138^{+}$cells as explained in materials and methods. Pam3CSK4 alone had an apoptotic effect which was a little lower in stroma than in stroma-free condition possibly due to stromal resistance, however, it increased the apoptotic response of all cell lines to bortezomib in the presence or absence of BMSCs. Different levels of CAMDR were detected for all lines (higher bortezomib-induced apoptosis in stroma-free than stroma conditions). This drug resistance was completely removed by Pam3CSK4+bortezomib treatment.

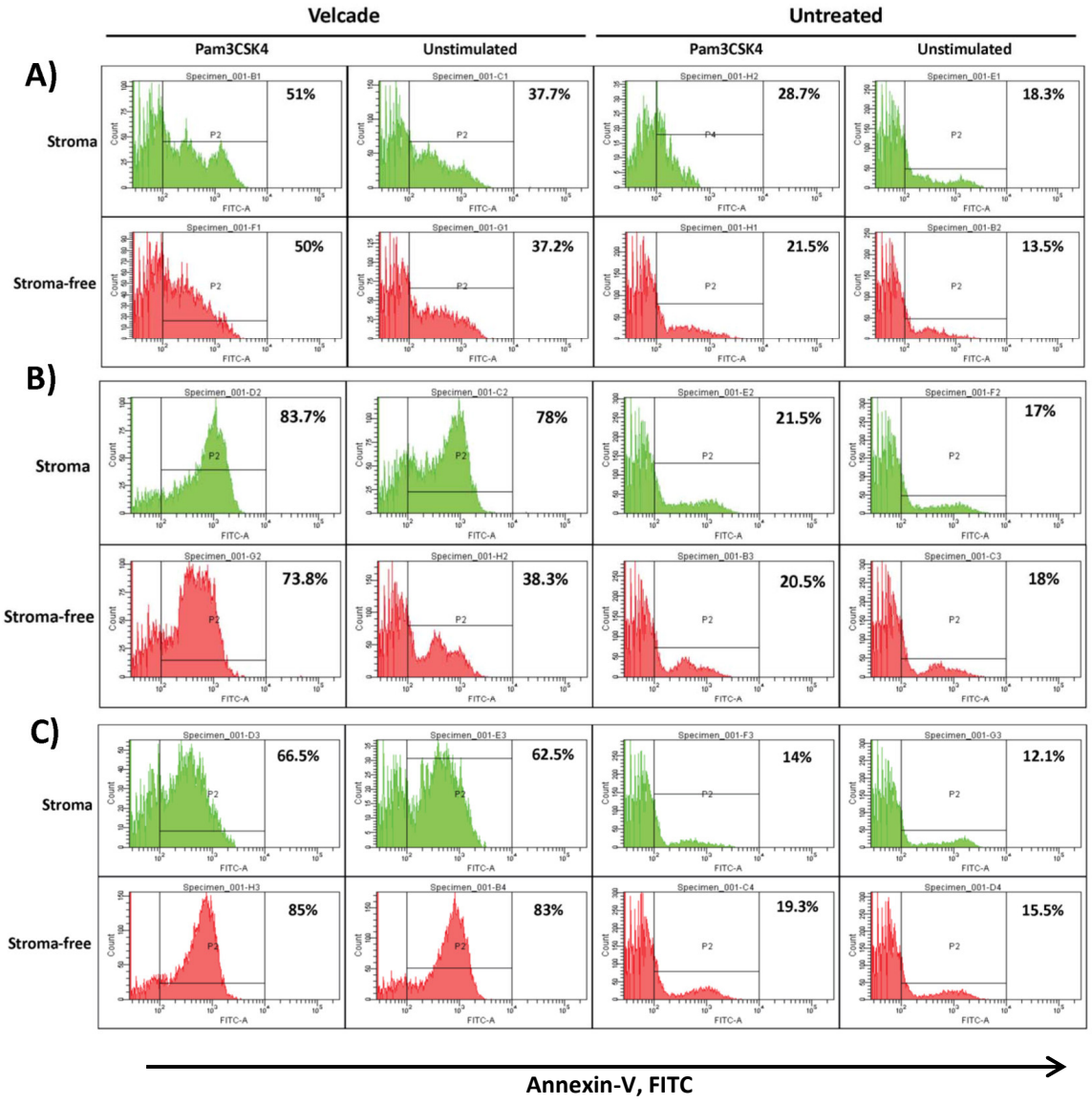


Figure 15. Pam3CSK4 may impose its apoptosis enhancing effects in bortezomib-treated HMCLs partly through caspas-3 activation. HMCLs were stimulated for 24 hours with Pam3CSK4, adhered to patient bone marrow isolated stromal cells and then exposed to drug treatment. Cells were then applied to FACS analysis by intracellular staining for cleaved caspase-3 FACS analysis and gating on $CD138^{+}$cells as explained in materials and methods. Pam3CSK4 alone weakly induced cleaved caspase-3 expression (compared to baseline), however, its combination with bortezomib induced a higher level of cleaved caspase-3 protein in L363 and OPM-2 cell lines. This figure is a representative analysis of one out of two MM primary BMSC samples.

Discussion

In the present study we found that TLR1/2 triggering results in a heterogeneous functional response of HMCLs in terms of integrin surface expression and adhesion to BMSCs. OPM-1, OPM-2 and NCI-H929 myeloma cell lines showed a decrease in adhesion to BMSCs after TLR-1/2 activation, which was accompanied with a down-regulation in surface expression of β7 integrin. Furthermore, blocking experiments confirmed a significant contribution of β7 integrin in their adhesion, although this may not exclude the involvement of other integrins such as α4 and αVβ3 in their basal adhesion. Additionally, the OPM-1, OPM-2 and NCI-H929 displayed significant increases in surface expression of α4 and αVβ3 following TLR-1 activation, but this upregulation appeared to have no functional effects in the adhesion to BMSCs. The integrin α4β7 has also been involved in adhesion of MM peripheral B cells to FN and BMSC [32]. Of note, the anti-β7 antibody used for blocking experiments is well known to detect only β7 epitopes regardless of its heterodimer partners [33] and thus affirms the involvement of this integrin in adhesion to BMSCs. Fravel, L363, UM-6, UM-9 and U266 cell lines showed an increase in adhesion to BMSCs upon TLR1/2 activation. This up-regulated adhesion was demonstrated to be α4 and/or αVβ3 mediated and these two integrins are also involved in part of the baseline adhesion. It is well established that these two integrins can mediate binding of MM cells to FN and BMSCs [34,35], and αVβ3 has been shown to be involved in invasiveness of MM cells [35]. Moreover, overexpression of αVβ3 in cervical cancer has been associated with poor prognosis [36]. On the other hand, in normal human PBMCs, TLR1 activation increased surface expression of α4 and β7 integrins in T cells, B cells and monocytes, which rather support a common mechanism by TLR1 activation for α4 modulation but a specific mechanism (possibly unique to MM cells) for β7 modulation. This suggests that TLR signaling could contribute to MM pathogenesis or progression. Taken together, these findings imply that TLR1/2 triggering can differentially modulate α4-, αVβ3- and β7 surface expression on MM cells and α4-, αVβ3- and β7-mediated adhesion to stromal cells.

The transcription factor NFκB is known to control several adhesion molecules in various cell types; however, whether TLR signaling also engages NFκB to modulate expression of adhesion molecules has not been clearly defined. In this study, we blocked NFκB pathway using an inhibitor of IκBα phosphorylation (Bay 11-7082) prior to Pam3 treatment and then surface expression of α4, β7 and αVβ3 integrins on HMCLs was analyzed. We found that Bay could not antagonize downregulation of β7 by Pam3, but it further enhanced it. On the contrary, Bay (although at higher concentration) completely antagonized upregulation of α4 and αVβ3 integrins by Pam3. These findings suggest specific mechanisms in MM cells controlling

modulation of above integrins following TLR1 activation; in fact highlighting further the heterogeneous biology of latter cells.

Adhesion of MM cells to BMSCs is well known to render myeloma cells resistant against cytotoxic and apoptotic signals [37-41]. Furthermore, adhesion-induced drug resistance is suggested to be associated with increased adhesion to fibronectin and with up-regulation of α4, thus cells with a higher expression of this integrin molecule display a drug-resistant phenotype [30]. In this study it is shown that Pam3CSK4 treatment upregulates expression of α4 in all cell lines. However, some cell lines showed increased adhesion to BMSCs, while others showed decreased adhesion. The effect of TLR1/2 activation in HMCLs on viability and drug sensitivity was further shown in three cell lines L363, OPM-2 and U266. Interestingly, Pam3CSK4 increased sensitivity (lower IC50) to bortezomib in the presence or absence of BMSCs, which was accompanied with increased apoptosis. Pam3CSK4 alone stimulated a low apoptotic effect in all HMCLs in the presence or absence of HS-5 or primary BMSCs, while combination with bortezomib induced a higher level of apoptosis. It should be noted that CAMDR was completely eliminated by combined treatment of Pam3CSK4+bortezomib. These findings suggest that the effect of TLR1/2 stimulation of MM cells does not control their drug resistance or sensitivity following adhesion to BMSCs. Thus, upregulation of adhesion to BMSCs which was shown to be mostly α4 integrin-mediated did not reduce drug-induced cell death. Likewise, downregulation of adhesion to BMSCs which was shown to be β7 integrin-mediated did not increase drug-induced cell death. A recent study demonstrated that knockdown the β7 integrin gene in MM cells decreased their adhesion to FN and BMSCs and reversed CAMDR [33]. Additionally, blocking α4 integrin with specific antibodies increased their drug sensitivity in MM cells [30,42], and bortezomib reversed CAMDR in MM cells through downregulation of α4 integrin [43]. These studies support involvement of α4 and β7 integrins in controlling drug sensitivity of MM cells. However, in this study, Pam3CSK4 apparently bypassed this involvement and increased drug sensitivity of HMLCs irrespective of their adhesion pattern.

Bortezomib stimulates caspase-3 activation in MM cells, triggering apoptosis [31]. The TLR1/2 ligand (Pam3CSK4) has been shown to induce apoptosis in monocytes [44], but whether it activates caspase-3 in MM has not been demonstrated. Here we show that Pam3CSK4 increases the level of activated caspase-3 in HMCLs in the presence or absence of BMSCs. Combining bortezomib with Pam3CSK4 increased the level of cleaved caspase-3 in L363 and OPM-2 but not in U266. Of note, the level of cleaved caspase-3 induced by bortezomib alone paralled CAMDR in L363 and OPM-2 cell lines (stroma compared to stroma-free conditions) but not in U266. These results suggest: a)- Pam3CSK4 may engage other mechanisms than caspase-3 to

enhance bortezomib-induced apoptosis in HMCLs, b)- inhibition of caspase signaling may only partly explain CAMDR at least in some HMCLs.

Finally, this study is the first to delineate the modulatory effects of TLR1 triggering on adhesion of HMCLs to BMSCs and identify the integrin molecules involved in this interaction. It shows that following TLR1/2 activation on HMCLs, expression of β7 integrin is downregulated. Pam3CSK4 increases drug sensitivity of HMCLs in the context of BMSCs in an adhesion-independent manner. On this basis, our findings recommend TLR1/2 as a potential target in MM, although further research is essential.

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7

Combined effect of Toll-like receptor-1 activation and bortezomib on immunoglobulin free light chain production of human myeloma cell lines in the context of bone marrow stromal cells or fibronectin

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Abstract

Investigation on the modulatory effects of TLR activation on immunoglobulin free light chain (FLC) production by human myeloma cells in the context of bone marrow stromal cells (BMSCs) or fibronectin (FN) has as yet not been performed. Here, the effect of TLR1/2 ligand (Pam3CSK4) on FLC production of human myeloma cell lines, L363, OPM-2, U266 and NCI-H929, in the presence or absence of above context has been investigated. Further, we probed for the combined effect of Pam3CSK4+bortezomib in above context on FLC production. We found that Pam3CSK4 decreased FLC production in the presence or absence of BMSCs or FN and this effect was enhanced in combination with bortezomib. Our findings imply that activation of TLR1/2 downregulates FLC production in MM cells in the context of bone marrow microenvironment components and suggest that Pam3CSK4 might be considered in combined therapeutic protocols in MM.

Introduction

Confinement of multiple myeloma (MM) cells to bone marrow environment establishes their close interaction with bone marrow stromal cells (BMSCs) or extra cellular matrix proteins such as fibronectin (FN), collagen, laminin, and tenascin [1,2]. This interaction will play a fundamental role in maintaining the malignant clone in terms of survival and drug resistance, mediated mostly through physical contact [2-4] or secretion of a plethora of cytokines and humoral factors by BMSCs or MM cells, including IL-6, VGEF, IGF-1, HGF, IL-3 and RANK [5]. Indeed the role of these cytokines in MM pathogenesis has been deciphered, including contribution to angiogenesis and osteolytic bone lesions [5].

On top of above products, the malignant clone also produces immunoglobulin free light chain (FLC) which is considered as having critical performance in MM complications. Indeed negative modulation of FLC production would have a positive impact on MM patient, which should be expected following chemotherapeutic or combined therapeutic protocols in MM. However, the regulation of FLC production has not been convincingly scrutinized in cellular experimental systems. Interestingly, recent studies have shown that Toll-like receptor (TLR) activation in MM primary cells and human myeloma cell lines (HMCLs) increases production of IL-6, yet it is not known whether triggering TLRs could also affect Immunoglobulin free light chain (FLC) production by MM cells.

In almost 20% of MM patients referred to as light chain type myeloma, only one monoclonal immunoglobulin light chain is detectable, however, in most types of MM excess light chain proteinuria might occur. When re-absorption of FLCs exceeds the maximum potential of renal tubular system, they form in situ protein complexes resulting in nephropathy [6]. Thus it is of interest to find new therapeutic protocols to target mechanisms mediating the production

of FLCs by the malignant clone. In this thesis, we already showed that treatment of HMCLs with Pam3CSK4 (TLR1/2 ligand) sensitized the cells to cytotoxic effect of bortezomib in the context of BMSCs or FN (unpublished data). In the present research, we sought to explore the effect of TLR activation on FLC production by TLR1/2 ligand-(Pam3CSK4) stimulated HMCLs following exposure to bortezomib, with HMCLs adhered (or not) to FN or BMSCs. We show that Pam3CSK4 decreased production of FLC in HMCLs adhered (or not) to FN or BMSCs. Indeed this may add interesting and helpful aspects to a new combined therapeutic protocol in MM.

Materials & methods

Cell lines and cell culture

The myeloma cell lines, Fravel, L363, OPM-1, OPM-2, U266, RPMI-8226, XG1 and NCI-H929 were obtained from American Type Culture Collection (Manassas, VA, USA). UM-6 and UM-9 had been established by the Department of Clinical Chemistry & Hematology, University Medical Center Utrecht, Utrecht, the Netherlands [7,8]. UM-6 growth was promoted by adding 5ng/ml recombinant human IL-6 (eBioscience) as this cell line is IL-6-dependent and others are IL-6 independent. Cultures were maintained in RPMI medium supplemented with 5-10 % FBS, 2mM glycine, and intermittently with antibiotics. HS-5, a normal human bone marrow stromal cell line, was obtained from American Type Culture Collection and maintained in DMEM medium supplemented with 10% FBS, and intermittently with antibiotics.

Reagents

Pam3CSK4 (TLR-1/2 ligand) was obtained from Invivogen. Bortezomib was from LC Laboratories (Woburn, MA, USA) which was dissolved in DMSO for preparing a 100mM stock. The final DNSO concentration never exceeded 0.01% in all experimental conditions. Human plasma-derived fibronectin was from Sigma.

Cell stimulation

HMCLs were harvested from cultures of low passage numbers (maximum 15 after thawing). Cells were incubated with 2.5µg/ml Pam3CSK4 for 24 hours. After this incubation time, cells were washed and resuspended in fresh RPMI medium and seeded onto fibronectin- or BMSCs-coated wells to proceed with drug exposure experiments as explained below.

Co-culture of MM cells with HS-5

To assess FLC production by TLR1/2-activated HMCLs in the context of bone marrow stromal cells, 10^5 cells from HS-5 cell line were seeded in 12-well plates for 2 days to achieve 50-70% confluency. Five hundred thousand cells from HMCLs pre-activated for 24 hours with Pam3CSK4 were washed, suspended in fresh medium and exposed to stromal cell-coated wells for 2 hours. Unattached cells were removed and fresh medium containing protein was added and plates were further left for 24 hours. At the end of incubation, supernatants were collected and assayed for FLC measurement. In separate experiments, to assess the combined effect of bortezomib+TLR ligand on FLC production in HS-5 context, HMCLs were incubated with 1µM of bortezomib in medium+protein for one hour (*acute exposure*) before seeding. Then cells were washed and resuspended in warm drug-free medium, added to stromal layer and incubation was further extended to 24 hours.

Culture of HMCLs on FN-coated plates

In other experiments, to analyze the effect of TLR ligand alone or in combination with bortezomib on FLC production by HMCLs, cells were incubated in fibronectin-coated wells. Briefly, 12-well plates were coated with 20µg/ml fibronectin overnight at 4^0C. Plates were blocked with sterile heat-denatured BSA (10mg/ml in PBS) for one hour at room temperature and washed. Pre-TLR1 activated (for 24 hours) HMCLs were washed, suspended and seeded on coated plates for one hour. Unattached cells were removed and fresh medium containing protein was added and the plates were further incubated for 24 hours. In separate experiments, to assess the combined effect of bortezomib+TLR ligand on FLC production in FN context, bortezomib (5nM) in medium+protein was added after removing the unattached cells and incubation extended to 24 hours.

ELISA

Kappa or lambda FLCs were assayed as previously described [9]. Briefly, 96 well plates were coated overnight at 4^0C with goat anti-mouse IgG antibody in bicarbonate buffer. Subsequently, plates were blocked for 1 hour (RT) and incubated with mouse-anti human kappa or lambda Ig-FLC mAbs (obtained from Dr. A. Solomon, Tennessee). After incubation with different dilutions of samples and standards (The Binding Site), plates were incubated with HRP-labelled goat $F(ab')_2$-anti human kappa or lambda Ig light chain Abs (AHI1804 and AHI1904, respectively, Biosource, USA). Finally the reactions were developed using TMB and measured through an ELISA plate reader.

Statistics

As needed, unpaired *t*-test in GraphPad software was applied to data analysis and $p<0.05$ was considered as significant.

Results

Immunoglobulin free light chain secretion by HMCLs is reduced following TLR1/2 triggering

HMCLs were classified into two groups based on the isotype of free light chain produced. L363, OPM-1, OPM-2, U266, RPMI-8226 and UM-9 were lambda free light chain (LLC) producers, while NCI-H929 and UM-6 were kappa free light chain (KLC) producers. L363, OPM-2, U266 and NCI-H929 cell lines were selected for the analysis of FLC production following Pam3CSK4 treatment. First, the cells were stimulated with Pam3CSK4 (2.5µg/ml) for 24 hours and FLC concentration was measured in culture supernatants. Pam3CSK4 decreased FLC production in all four cell lines; however this response was more prominent in L363 and OPM-2 cell lines (**Fig 1**).

FLC production reducing effect of Pam3CSK4 is enhanced in combination with bortezomib in the context of bone marrow microenvironment

At the next step, regarding the fact that adhesion to FN or BMSCs induces secretion of various cytokines in MM cells and renders them drug resistant [4,5], we investigated if FLC production also was affected following Pam3CSK4 treatment in presence or absence of bortezomib. Pam3CSK4 inhibited FLC secretion by all cell lines; however, in U266 and OPM-2 cell lines this reduction was higher in BMSC-coated than in uncoated conditions, while the opposite was the case in L363 and NCI-H929 cell lines. Stimulation with Pam3CSK4 equaled the inhibitory effect of bortezomib on FLC production, while combination of Pam3CSK4 and bortezomib further reduced the FLC production in U266, OPM-2 and NCI-H929. Furthermore, the same pattern was also observed in the context of fibronectin for the same cell lines (**Figs 2,3**). Taken together, all our findings indicate that signaling through TLR1/2 inhibits FLC production in the presence or absence of bone marrow microenvironment.

Table 1. Categorization of HMCLs based on their free light chain isotype production

Cell line	Light chain isotype
Fravel	Kappa (low)
L363	Lambda
UM-6	Kappa
UM-9	Lambda
OPM-1	Lambda
OPM-2	Lambda
U266	Lambda
RPMI-8226	Lambda
XG1	Kappa
NCI-H929	Kappa

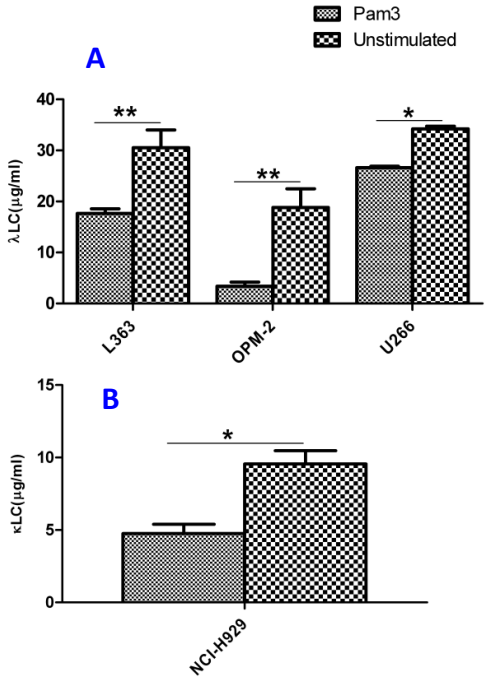

Figure 1. Change in FLC production in HMCLs following activation of TLR1. Pam3CSK4 decreased level of FLCs in HMCLs significantly after a 24-hour stimulation of cells with 2.5µg/ml. Data are the mean±SEM from analysis of three separate experiments, *$p<0.05$, **$p<0.01$.

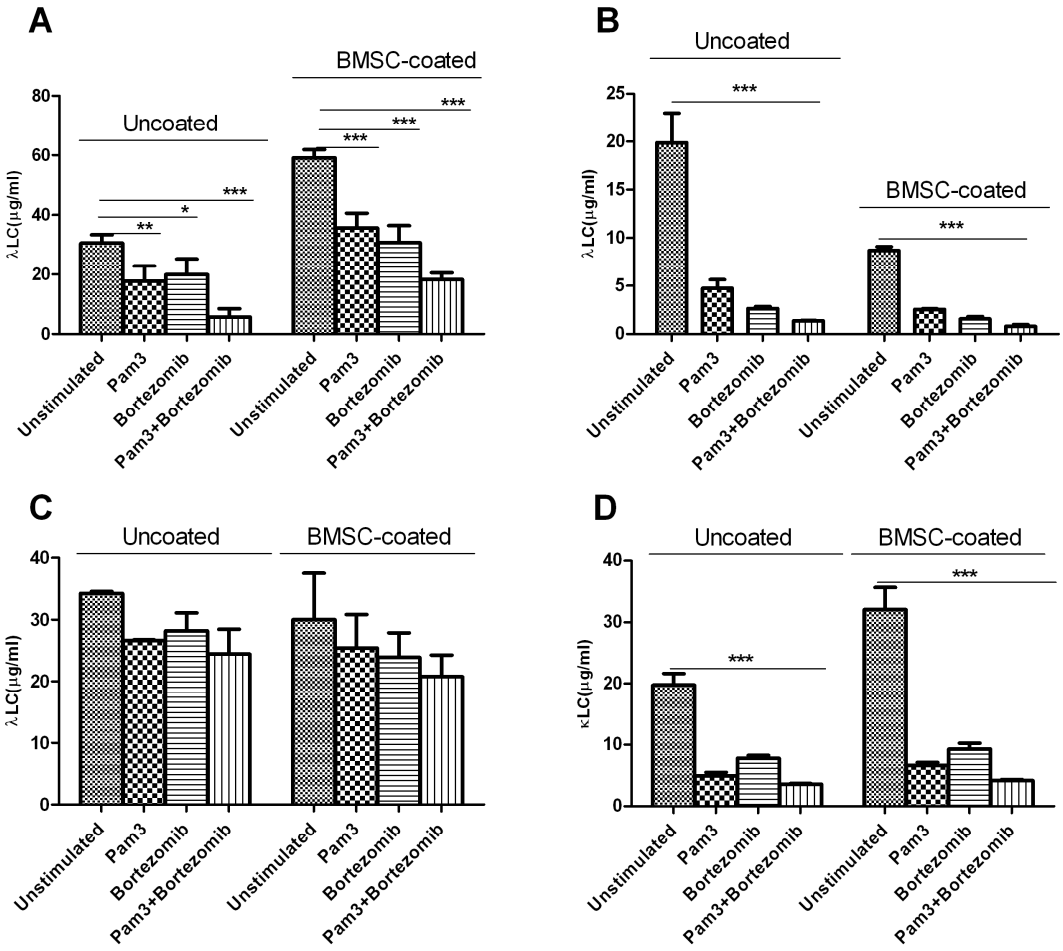

Figure 2. Enhancing the inhibitory effect of Pam3CSK4 on FLC production in HMCLs (λ in L363 (**A**), OPM-2 (**B**). U266 (**C**), and κ in NCI-H929 (**D**)) after exposure to bortezomib in the context of bone marrow stromal cells. HMCLs were stimulated (or not) with 2.5μg/ml Pam3CSK4 for 24 hours, washed, adhered (or not) to BMSCs and exposed (or not) to 1μM of bortezomib for one hour (more explanation in the text). Pam3CSK4 decreased dramatically FLC secretion in all cell lines, with even more decrease in combination with bortezomib. In OPM-2 and U266 cell lines, the reduction in FLC level was higher in the presence of stroma while in NCI-H929 and L363 cell lines this happened in the stroma-free condition. However, in U266 changes in FLC level in all conditions were non-significant compared to unstimulated condition. Data are the mean±SEM from analysis of three separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

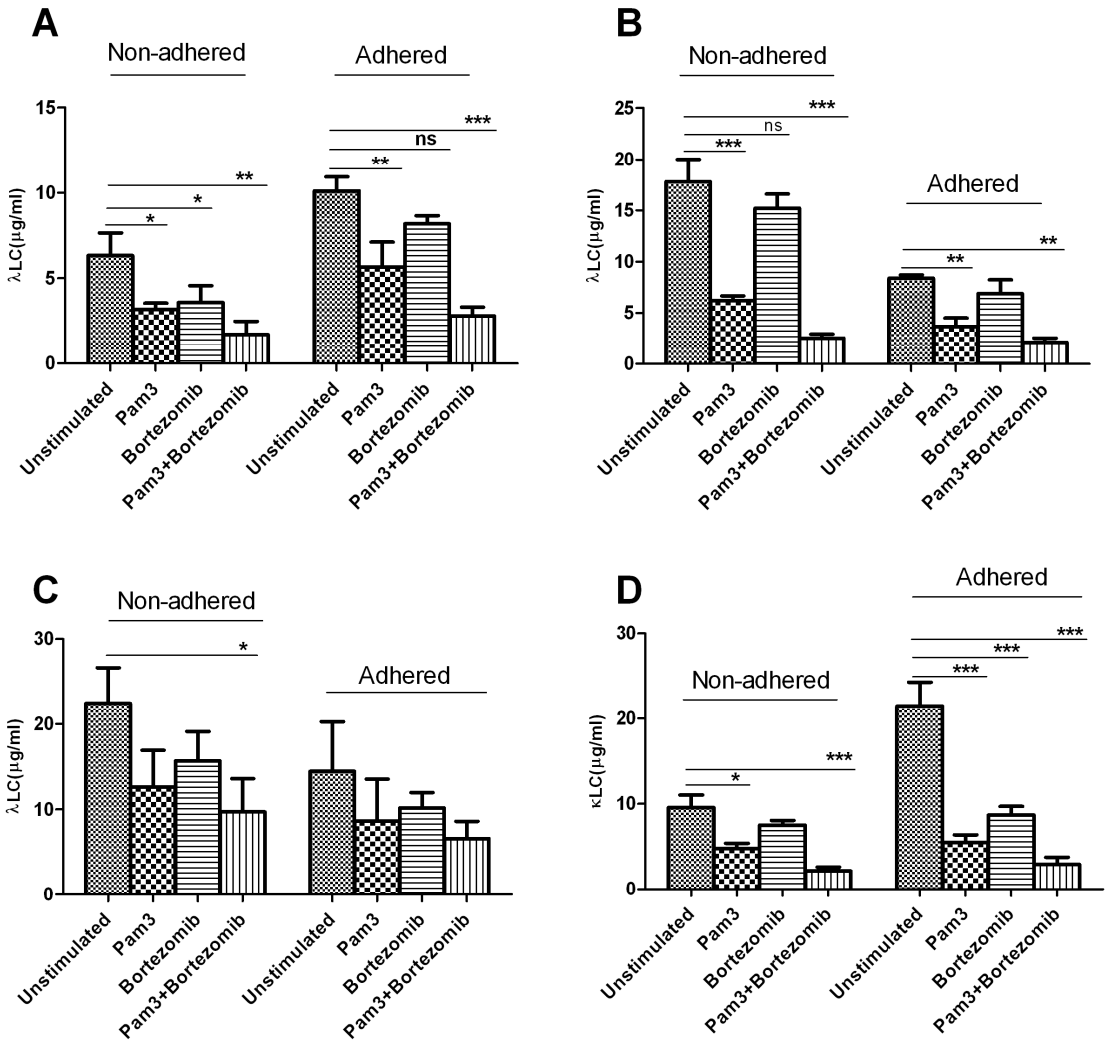

Figure 3. Enhancing the inhibitory effect of Pam3CSK4 on FLC production in HMCLs (λ in L363 (**A**), OPM-2 (**B**), U266 (**C**), and κ in NCI-H929 (**D**)) after exposure to bortezomib in the context of fibronectin. HMCLs were stimulated (or not) with 2.5µg/ml Pam3CSK4 for 24 hours, washed, adhered (or not) to fibronectin and exposed (or not) to 5nM bortezomib for 24 hours as explained in materials and methods. Pam3CSK4 decreased dramatically FLC secretion in all cell lines, with even greater reducing effects in combination with bortezomib. In OPM-2 and U266 cell lines, the reduction in FLC level was higher in the context of fibronectin (adhered) while in NCI-H929 and L363 cell lines this happened in uncoated (non-adhered) condition. Data are the mean±SEM from analysis of three separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Discussion

This study is the first to indicate that TLR1/2 activation could modulate FLC production in myeloma cells. We found that Pam3CSK4 treatment of HMCLs L363, OPM-2, U266 and NCI-H929 in the context of BMSCs or FN greatly reduced their secretion of FLC. It remains to be investigated if stimulation of other TLRs may have similar effects on the FLC production (work in progress). Pam3CSK4 in combination with bortezomib was even superior in inhibiting the FLC production than either compound alone. bortezomib is a potent proteasome inhibitor with outstanding therapeutic response in most MM patients, although recent studies have mechanistically uncovered resistance to this compound [10-12].

Numerous studies have demonstrated the protective role of BMSCs and FN on MM cells leading to cell adhesion mediated drug resistance (CAMDR) [1,2,5,13-16]. However, no study to date has explored whether cell adhesion also influences the FLC production in MM cells. In this study adhered HMCLs showed heterogeneous response with respect to FLC production. Adhered L363 and NCI-H929 cells produced increased amounts of FLC, while adhered OPM-2 or U266 had a decreased production of FLC. Also no relation between up- or downregulation with the isotype FLC produced was observed. OPM-2 and U266 cell lines showed a more profound inhibitory effect of Pam3CSK4 and/or Pam3CSK4+Bortezomib on FLC secretion in the context of BMSCs or FN than in uncoated conditions. On the other hand, in L363 and NCI-H929 cells the effect was more striking in the absence of BMSCs and FN, implying a possible resistance to this treatment. How TLR stimulation and adhesion of MM cells to BMSCs or FN modulate FLC secretion per se, needs further investigation.

In conclusion, it is well known that FLCs cause serious clinical and pathological complications in MM patients. This study suggests that stimulation of TLR1/2 by Pam3CSK4 may be further explored to reduce the burden of FLC production in the pathology of MM.

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8

Omega-3 components, EPA and DHA, induce apoptosis in multiple myeloma cells and enhance bortezomib-induced apoptosis

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Work in progress, part of this chapter has been presented in the following congress:
Toll-like receptor-1 ligand and omega-3components (EPA and DHA) enhance the anti-myeloma activity of Bortezomib in the context of bone marrow stromal cells. **Lymphoma & Myeloma 2012: An International Congress on Hematologic Malignancies.** October 25-27, 2012, the Waldorf=Astoria, New York, New York. **P-31**

Abstract

Including agents with anti-inflammatory potentials in supplementary therapeutic protocols for multiple myeloma has long been followed. These agents may function through hampering production of pro-inflammatory cytokines, inhibiting NFκB the main inflammatory response pathway, or interfering with oxidative transformations. Two main omega-3 components, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are now known to display strong anti-inflammatory effects and have been applied to a variety of experimental settings and clinical trials in cancers. However, EPA and DHA have as yet not been included in multiple myeloma therapeutical protcols and myeloma cells experimental studies. Here we studied the effects of EPA and DHA on viability and drug (bortezomib) sensitivity of human myeloma cell lines. We found that these agents not only are apoptotic for myeloma cells but increase the cytotoxic and apoptotic effects of bortezomib as well, and also they can inhibit NFκB activity. These preliminary findings in myeloma cells are the first to suggest application of EPA and DHA in myeloma treatment regimens.

Introduction

The link between inflammation and tumorigenesis has been well documented in various cancers [1-3]; however, it has not been fully elucidated in multiple myeloma (MM) pathogenesis/progression. In fact, a history of inflammatory diseases has been reported in some MM patients [4]. A mounting body of evidence suggests that the transcription factor NFκB functions as the most important trigger linking inflammation to tumorigenesis [5-8]. Indeed NFκB is the master controller of most inflammatory response, controlling induction of various inflammatory cytokines and also regulates expression of several genes involved in anti-apoptotic and survival pathways [1]. Inflammatory responses can also promote carcinogenesis through oxidative damage to DNA or induction of tissue repair response [3]. Notably, the NFκB pathway plays a prominent role in MM pathogenesis, as indicated by detection through gene profiling technology of several gene mutations in NFκB pathway [9-11]. It has been suggested that these mutations might maintain constitutive activation of NFκB, probably rendering MM cells independent on bone marrow microenvironment survival signals. Furthermore, constitutive activation of NFκB transcription factor in myeloid and lymphoid leukemias highlights its potential contribution to pathogenesis of most hematologic malignancies [12]. Interestingly, part of cytotoxic effects of bortezomib, which is a potent proteasome inhibitor, is exerted through its inhibition of NFκB pathway [13]. However, several studies indicate NFκB resistance to bortezomibin MM primary cells or human myeloma cell lines (HMCLs) [14,15]. Hence, anti-inflammatory agents acting either through reduction of inflammatory cytokines or targeting NFκB pathway could be considered as an efficient combined treatment together with anti-myeloma drugs especially bortezomib. Here, we assess the effect of omega-3 components, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with established anti-inflammatory functions, on MM cells. These two interesting candidates have been reported to display anti-tumor effects in a variety of clinical and experimental settings [16-20]. However, the effects of these agents in MM have not yet been investigated in clinical and experimental settings. In the present study, we show that EPA and DHA have apoptotic effects on HMCLs and also potentiate the killing effects of bortezomib. Furthermore, we demonstrate that these agents can inhibit NFκB activity.

Materials & methods

Reagents and antibodies

Omega-3 main components, EPA and DHA, were provided by Danone research center oncology platform (Wageningen, Netherlands) as 100mM stock solutions in ethanol. The maximum concentration of ethanol in all experiments was kept at 0.05%, however, to exclude any possible effect of the solvent, a controls with only ethanol was also run with each experiment. FITC-conjugated annexin-V and propidium iodide were from eBioscience. Monoclonal rabbit anti-human caspase-3 (cleaved) and HRP-conjugated anti-rabbit immunoglobulins were obtained from Cell Signaling Technology, Epitomics, and DAKO, respectively. Bortezomib was from LC Laboratories (Woburn, USA) and dissolved in DMSO to make a 100mM stock, with DMSO final concentration kept below 0.01%. XTT and phenazine methosulfate (PMS) were also obtained from Sigma.

Cell lines and cell culture

The HMCLs, L363, OPM-1, OPM-2, U266, and NCI-H929, were obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in RPMI medium containing 2mM L-glutamine, supplemented with 5% FBS and intermittently with 100U/ml penicillin and 100µg/ml streptomycin at a humidified 37^0C incubator providing 5% CO_2. NCI-H929 cell line was treated also with 1mM sodium pyruvate and 50µM 2-mercaptoethanol.

Drug cytotoxicity assay

HMCLs were first stimulated with 50µM EPA or DHA in RPMI medium supplemented with 5% FBS for 72 hours. The cells were then washed and treated with different concentrations of bortezomib for one hour in 96-well plates, washed and put back in the fresh drug-free medium and further incubated for 48 hours. At the last 4 hours, 50µL of XTT reagent premixed with PMS was added to each well and after 4 hours the absorbance of each well was determined with a plate reader. Some wells were also specified for blank (medium+ethanol), solvent control (cells+medium+ethanol), and growth control (cells + medium). Percent survival (% of viability) was calculated using non-linear regression.

Annexin-V apoptosis assay

HMCLs were first stimulated (or not) with 5-100µM of EPA or DHA in RPMI medium with 5% FBS for 72 hours. Cells were then washed, pelleted and suspended in ice-cold FACS buffer

(PBS+1%BSA+0.01% sodium azide) containing 5µL FITC-annexin-V and left at room temperature in the dark for 10 minutes. After washing and suspending in FACS buffer, 5µL propidium iodide was added and samples were applied to FACS analysis in a BD FACSCanto™ II machine. Data were analyzed using FACS Diva software. In separate experiments, cells were first stimulated with 30µM of EPA or DHA for 72 hours, washed and exposed to 5nM of bortezomib for 48 hours. After this time, samples were washed and applied to apoptosis FACS analysis as mentioned above.

Preparation of nuclear fractions

HMCLs from different experiments were washed in cold PBS, pelleted and suspended in buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% IGEPAL (Sigma), pH 7.9) containing a cocktail of protease inhibitors (Complete Mini, Roche), and left on ice for 10 minutes. After spinning at 4^0C, 3000 rpm, supernatants were collected and pellets were lysed in buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 30% glycerol (v/v), pH 7.9) for 30 minutes on ice. Finally, supernatants were collected after spinning the lysates at 20000×g, 4^0C, for 15 minutes. Protein concentration was determined using BCA kit (Pierce). A fixed amount of protein was subjected to SDS-PAGE/blotting as mentioned below.

Western blotting

HMCLs were harvested from EPA/DHA-stimulated (or not) conditions, washed in cold PBS and pelleted. Cell pellets were then lysed in cold RIPA buffer (150mM NaCl, 1% IGEPAL, 0.5% sodium deoxy cholate, 0.1% SDS, 50mM Tris, pH 8.0) containing a cocktail of protease inhibitors (Complete Min, Roche) and left on ice for 30 minutes. After spinning at 10000×g, 4^0C for 15 minutes, supernatants were removed and protein concentration was measured with a BCA kit (Pierce). Thirty microgram of total protein was applied to a 12% SDS gel which was then electroblotted onto a PVDF membrane. After incubation in blocking buffer, membranes were probed with rabbit anti-human NFκB-p65 or anti-human beta actin followed by specific secondary antibodies. Finally, the signals were detected using normal ECL or ECL prime (Amersham).

Results

Apoptotic effects of EPA and DHA on myeloma cells are dose-dependent

We treated HMCLs with 5-100µM EPA or DHA for 72 hours and assessed the percent of apoptotic cells as mentioned in materials and methods. As depicted in figure 1, EPA and DHA induced a dose dependent apoptosis with highly significant effects at 50µM and higher. These effects are specific to EPA and DHA, as the ethanol (solvent, ETOH) conditions show a negligible effect. As examples, histograms representing levels of apoptosis at 100µM concentration of EPA and DHA are shown in figure 2A-D. It seemed that the overall effect of EPA was somewhat higher than that of DHA, and this difference was much more pronounced in U266 cell line.

EPA and DHA sensitize HMCLs to cytotoxic effects of bortezomib

At the next step we sought to explore if EPA and DHA also increase the cytotoxic effects of bortezomib. HMCLs pre-stimulated with EPA and DHA (25µM) displayed a significantly lower viability following exposure to increasing doses of bortezomib (0.1-5.0µM) (Fig 3). However, considering IC50 of the drug in EPA/DHA-treated or untreated conditions, the level of sensitivity differed between cells. L363 and OPM-2 cell lines were much more sensitive than U266, however, the IC50 for above conditions were lower in OPM-2 than in L363 implying an overall higher level of sensitivity in OPM-2 than other cells. On the other hand, U266 did not show a significant change in drug sensitivity for the three conditions indicating that EPA/DHA concentration was possibly not sufficient (25µM) for this cell line to elicit the efficient level of sensitivity and the observed decrease in viability was apparently due to the drug alone.

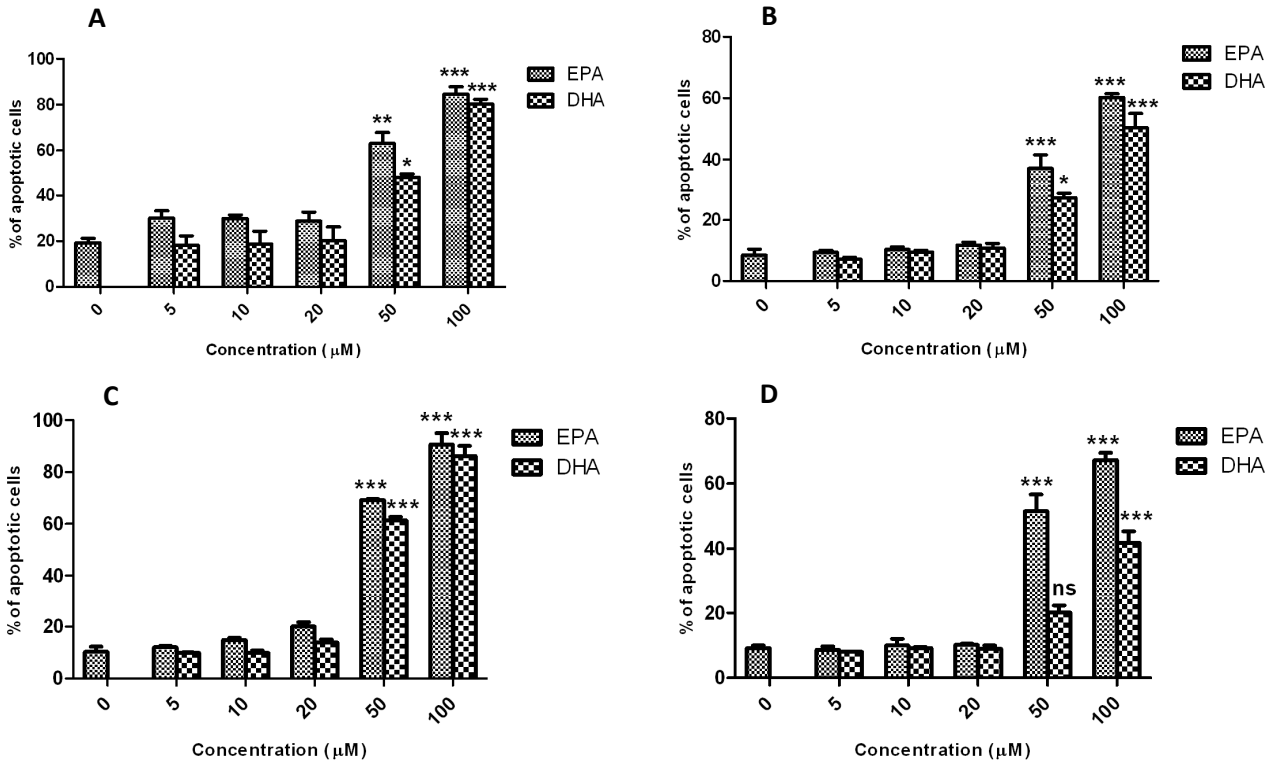

Figure 1. The apoptotic effect of different concentrations of EPA and DHA on L363 (**A**), OPM-1 (**B**), OPM-2 (**C**), and U266 (**D**). These omega-3 components appear to leave their apoptotic effects on HMCLs in a dose-dependent manner; however, the latter effects are highly significant at or above 50μM compared to the untreated (zero concentration) conditions. Data are mean±SEM of two separate experiments. * *p< 0.05,* ** *p<0.01,* *** *p<0.001.*

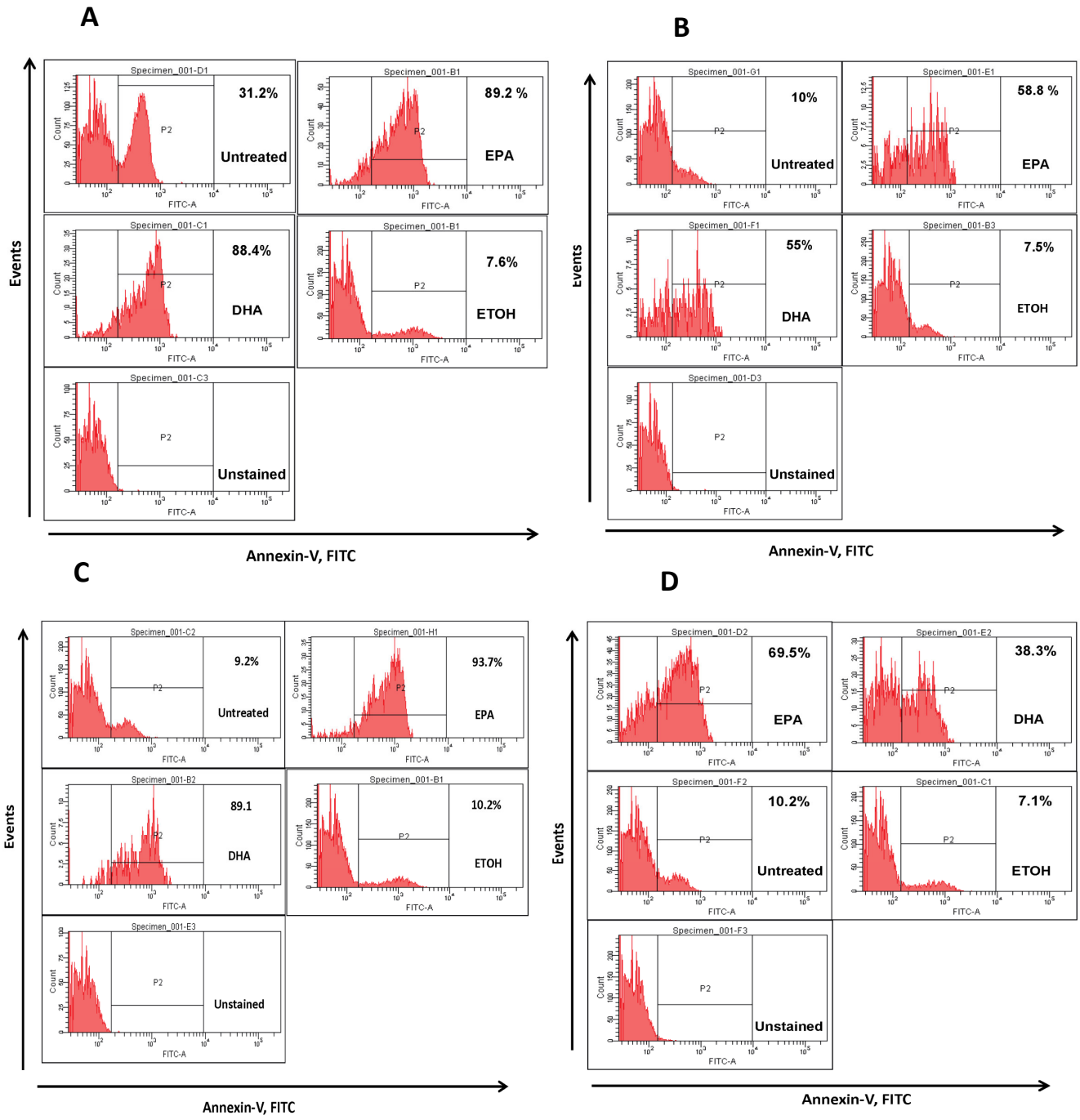

Figure 2. Examples of increase in apoptotic response of L363 (A), OPM-1 (B), OPM-2 (C), and U266 (D) to 100µM of EPA and DHA. The incubation time for all cell lines was 72 hours. The solvent (ethanol, ETOH) condition confirms that the apoptotic responses are specifically induced by EPA and DHA.

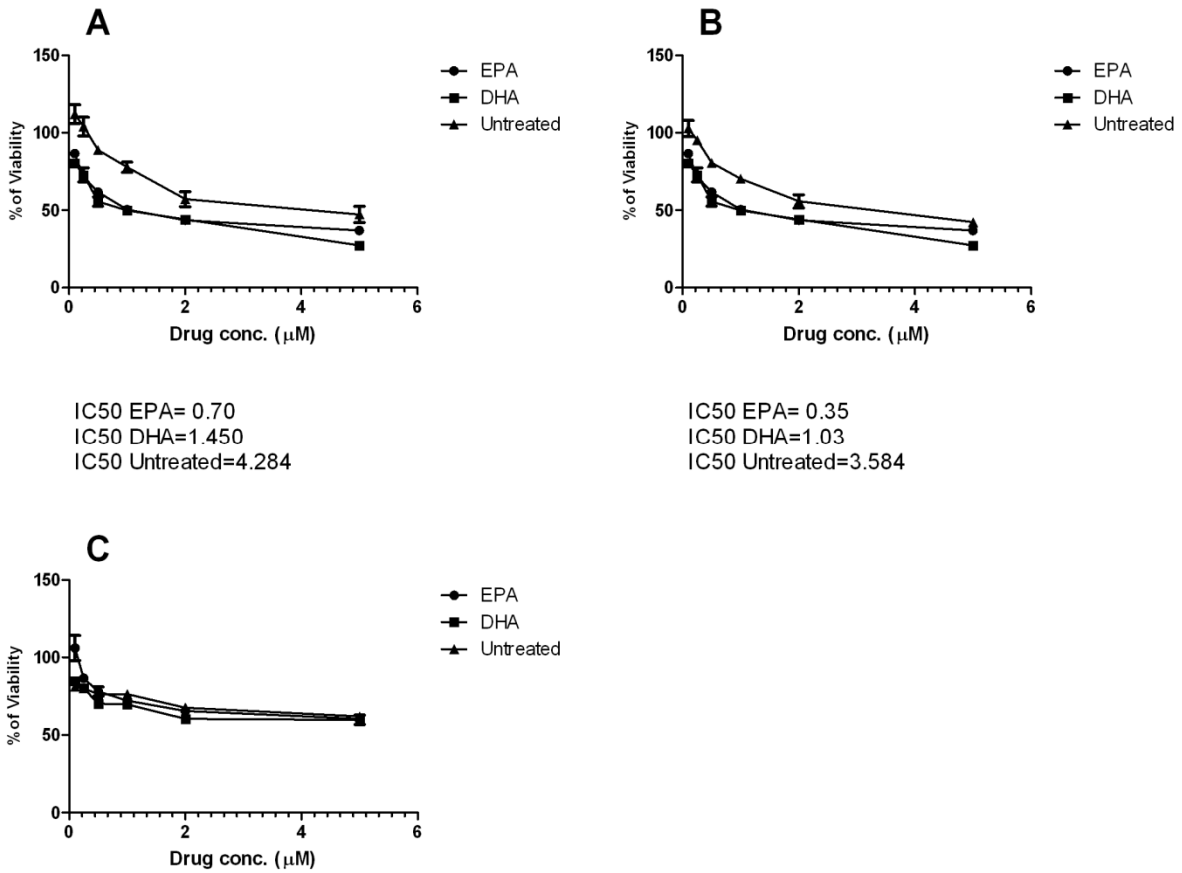

Figure 3. The effect of EPA and DHA on bortezomib sensitivity in L363 (**A**), OPM-2 (**B**), and U266 (**C**). Cells were pretreated with 25μM EPA or DHA for 72 hours and after washing were exposed to several dilutions of bortezomib (0.1-5.0μM) for one hour (*acute exposure*). The incubation was extended to 48 hours in a drug-free medium afterwards. L363 and OPM-2 cell lines displayed a high sensitivity to bortezomib following treatment with EPA and DHA as indicated by higher IC50s in EPA/DHA-treated than in untreated cells. However, in U266 cells no effect of EPA/DHA on drug cytotoxicity was observed. Graphs were obtained from data analysis of two independent experiments.

EPA and DHA increase the apoptotic effect of bortezomib in HMCLs

Having shown that EPA and DHA increase the apoptotic response of HMCLs and sensitize these cells to cytotoxic effects of bortezomib, we next tried to find out whether they also increase the apoptotic effects of bortezomib in HMCLs. HMCLs were first stimulated with 25μM EPA or DHA for 72 hours, washed and exposed to a low dose of bortezomib (5nM) for 48 hours. As shown in figure 4, EPA and DHA enhanced the apoptotic effect of bortezomib significantly in L363 and OMP-2 cell lines, but not in U266 in which the pattern conformed to that in cytotoxicity assay. The latter finding possibly indicates that the concentration of EPA and DHA was not enough to promote the apoptotic effect of bortezomib in U266, while at higher concentration, they induce apoptosis per se (Fig 2). Moreover, in OPM-2 the level of apoptosis enhancement due to both EPA and DHA was higher than that in L363, the same pattern was also observed in drug cytotoxicity assay. These findings further support the apoptosis-promoting effects of EPA and DHA in combination with bortezomib.

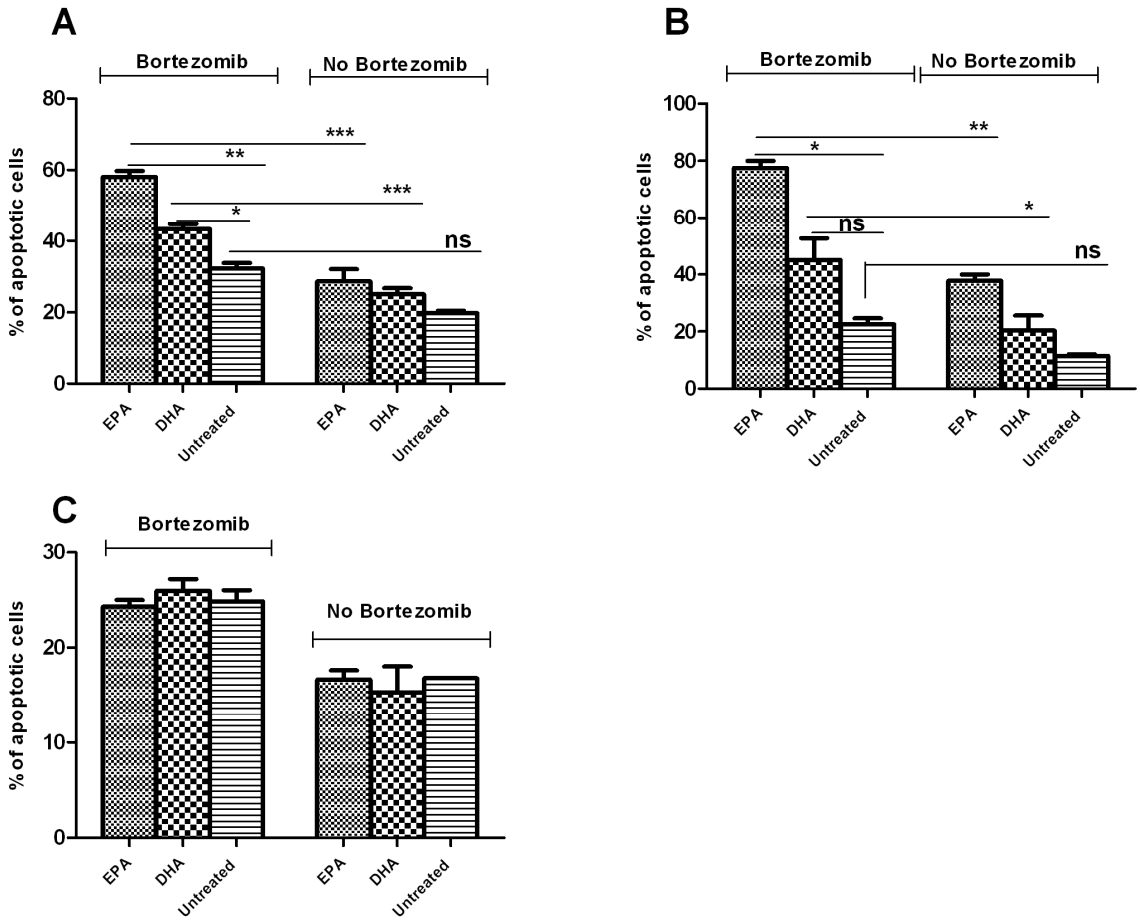

Figure 4. Combined effect of EPA/DHA and bortezomib on apoptotic response of L363 (**A**), OPM-2 (**B**) and U266 (**C**) cell lines. HMCLs were stimulated for 72 hours with 25µM EPA or DHA, washed and exposed to bortezomib (5nM) for 48 hours and analyzed for annexin-V apoptosis in FACS. EPA had a more profound sensitizing effect than DHA in L363 and OPM2; however, OPM-2 displayed a higher level of sensitivity than L363. In U266, although there is difference in apoptosis level between cells exposed to bortezomib and cells not, EPA and DHA could not sensitize the cells to bortezomib, probably due to a low concentration of EPA/DHA. Data are mean±SEM of two independent experiments. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$.

EPA and DHA may impose their effects through inhibition of NFκB pathway

In line of the fact that omega-3 components exert their effects mostly through inhibiting the induction of pro-inflammatory cytokines controlled mainly by NFκB pathway, or by directly hampering the latter pathway [21-25], we were prompted to investigate the effect of EPA and DHA on NFκB pathway in HMCLs at steady state, considering that constitutive activation of NFκB pathway has been reported in most HMCLs [9-12]. We stimulated HMCLs for 72 hours with 50μM EPA or DHA, and expression of NFκB-p65 protein was analyzed in nuclear fractions with Western blotting (Fig 5). It turned out that EPA or DHA down-regulated NFκB-p65 protein in OPM-2 and L363 cell lines, suggesting they probably inhibited nuclear translocation of NFκB subunits and thus NFκB deactivation. However, this effect was more pronounced in OPM2 cells. On the other hand, no change was observed at NFκB-p65 protein level in U266 cell line.

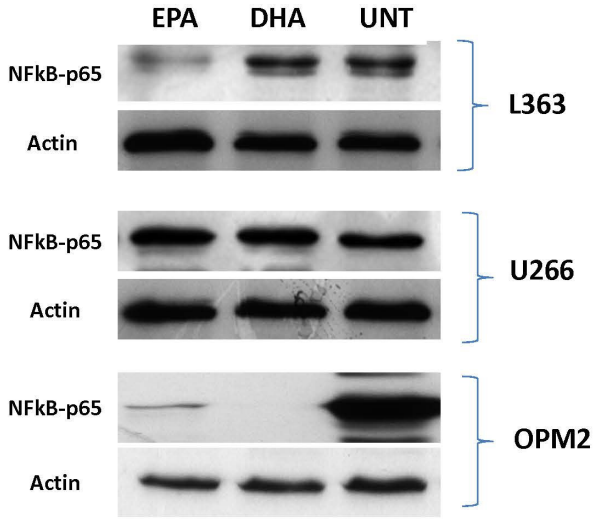

Figure 5. The effect of EPA and DHA on the level of NFκB-p65 protein in nuclear fractions. EPA and DHA resulted in a huge decrease in NFκB-p65 protein in OPM-2, but only EPA had such an effect in L363 cell line. They could not change this protein in U266, implying a probable resistant situation in NFκB pathway in latter cell line.

Discussion

In the present study, the effect of two omega-3 components, EPA and DHA, on survival and drug (bortezomib) sensitivity of human myeloma cell lines, L363, OPM-1, OPM-2 and U266 was investigated. We found that DHA and EPA triggered apoptosis in all cell lines at 50μM or higher concentrations while EPA showed a somewhat stronger effect than DHA. The most striking effect of EPA was observed in OPM-2 cell line with respect to apoptosis induction, drug cytotoxicity and enhancement of drug-induced apoptosis. U266 cells displayed a different pattern with lower drug sensitivity and apoptosis. This is the first study to describe the pro-apoptotic effects of EPA and DHA in myeloma cells and are corroborated by findings in other cancer types [16,20,26,27].

There is only one clinical trial still engaged in recruiting volunteers, which is aimed at preventing MM progression using omega-3 as a supplementary component (http://clinicaltrials.gov/show/NCT00899353), yet knowledge on biologic effects of omega-3 components on MM cells in experimental settings is lacking.

In our study, combination of bortezomib and low doses of EPA/DHA increases the anti-myeloma activity of the drug with respect to apoptosis and drug sensitivity. Interestingly, we also found that NFκB activation is inhibited by EPA (L363) or both EPA and DHA (OPM-2), as indicated by a remarkable reduction in the level of NFκB-p65 protein. On the other hand, bortezomib which is still considered as the most effective drug in MM has been reported to be associated with drug resistance in MM cells mostly due to NFκB resistance [14,15,28,29]. These findings are not in line with earlier studies which had positioned NFκB inhibition as the main mechanism underlying therapeutic effects of bortezomib [30]. However, recent research indicates that anti-myeloma activity of bortezomib cannot be fully attributed to inhibition of NFκB canonical pathway [9]. At present, it remains to be determined if increase in drug sensitivity and apoptosis in HMCLs following EPA/DHA treatment is due to NFκB inhibition and further research on pathways controlling this effect is ongoing. Interestingly, stimulation of L363, OPM-2 and U266 with 50μM EPA or DHA for 72 hours induced apoptosis in all three cell lines, but only a decrease in NFκB-p65 protein in L363 and OPM-2 and not in U266 cells was observed, tempting us to speculate that this resistant NFκB phenotype in U266 might explain its low drug sensitivity or apoptosis in EPA/DHA-treated conditions. Thus EPA/DHA may induce apoptotic cell death at least in some HMCLs via NFκB inhibition. Taken all together, our study suggests that including omega-3 components in combined therapeutic protocols in MM will be beneficial to the patients; however, our findings require further support through investigation on MM primary cells.

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9

General Discussion

TLRs and MM cell physiology

☞***(Tables 1,2 at the end of this book contain all the characteristics and functional responses of human myeloma cells used in this thesis)***

A limited number of studies have recently reported that MM) primary cells and human myeloma cell lines (HMCLs) express a wide range of TLRs at mRNA level, with their expression and activation-induced functional responses displaying heterogeneity [1-6]. Our TLR expression profiling (**Chapter 4**) also showed that HMCLs and MM primary cells do express multiple TLRs (TLR1-9) both at mRNA and protein levels, except TLT2 and TLR5 whose proteins were not detected in spite of mRNA expression. Activation of TLRs in MM cells has been associated with some functional responses including induction of cytokine release, increase /decrease in growth and proliferation, induction of apoptosis, protection against drug or serum deprivation-induced apoptosis, and provoking immune evasion surface markers.

As discussed in **Chapter 2**, more or less similar to MM, TLR functional expression has also been documented for other B cell malignancies including B cell-lymphomas [3,7,8], B-acute lymphoblastic leukemia[3,9,10], and B-chronic lymphocytic leukemia [11-17]. However, in some aspects, functional responses in MM may be unique, as for example, TLR7 and 9 triggering in MM cells has been mostly associated with induction of cell proliferation [1], while in B-chronic lymphocytic leukemia it mostly induced cell death [8,13]. Moreover, the pattern of TLR expression in MM appears to differ from that in normal human B cells, with MM cells expressing TLR1-9 and loosing TLR10, while in normal B cells TLR3,4,8 are not expressed but TLR10 is strongly expressed [3,18]. Notably, plasma cells isolated from bone marrow of normal donors did not express the majority of TLRs [2]; on the other hand, another study detected various TLRs on normal plasma cells isolated from tonsils and cord blood [19]. Although it is still not known whether TLR expression pattern in MM is a sequel to malignant transformation, above findings support that TLR expression in MM is influenced by developmental stage of B cells and possibly by tissue of MM cells origin. In all, our findings along with others' on TLR expression in MM cells will provide primary evidence supporting the role of an inflammatory environment such as bone marrow in MM pathogenesis/progression through engaging these molecules. This is indeed not surprising to place TLR activation as the relay between inflammation and MM pathogenesis, as this link is advocated in other malignant situations through a large body of evidence [20,21]. The latter link is even more supported by the fact that NFκB, the main transcription factor controlling TLR signaling effects, has been found activated in various inflammation-related carcinomas [21-23]. Our findings on the apoptotic effects of two omega-3 components, EPA and DHA with anti-inflammatory potentials, in single treatments or in combination with bortezomib (the most effective drug in MM) in **Chapter 8** further support the involvement of an

inflammatory response in MM. More interestingly, we found that EPA and DHA could inhibit baseline activity of NFκB in MM cells. As a matter of fact, there is not as yet sufficient evidence supporting the potential contribution of TLR signaling to MM pathogenesis. Nonetheless, in **Chapter 2**, inferring from noticeable findings on TLR expression and function in other B-lymphoid cancers and also various findings on TLR activation effects on osteoclasts and endothelial cells biology, we rather speculated the contribution of TLR signaling to MM complications such as bone lesions and angiogenesis. However, whether TLR triggering will actually lead to latter effects in MM is a question which should be addressed by future researches.

The TLR signaling-cell adhesion link in MM cells

The effect of TLR activation on expression of adhesion molecules and adhesion to FN or BMSCs has never been studied before in normal and malignant B lymphocytes. Deciphering this effect of TLRs will certainly obtain a novel insight into how innate immunity could modulate interaction of B cells, which are important cells in both innate and adaptive immune responses, with microenvironment components in such events as inflammation or B cell development. In MM, malignant plasma cells are also entangled with a network of cytokines, adhesion molecules and their cellular/matrix ligands in an inflamed tumor environment [24]. With respect to tumor cells interaction with BMME components, MM is the most extensively studied example among B lymphoid cancers, as interaction of malignant plasma cells with BMME contributes to a large extent to disease pathogenesis [24]. The present research was the first to address through *in vitro* analysis the question whether TLR triggering on human myeloma cells could modulate their surface expression of adhesion molecules and their adhesion to two important BMME components FN and BMSCs. To explore the above interaction in normal human B cells, we stimulated freshly isolated cells with TLR9 ligand (CpG-C), as this receptor is highly expressed in B cells. We found that TLR9 activation upregulated α4 integrin on B cells and increased their adhesion to FN, and inhibiting IκBα phosphorylation attenuated TLR9 triggering effect indicating that NFκB expands the signal to adhesion and integrin modulation. Of note, NFκB controls baseline expression of several adhesion molecules including ICAM-1 and VCAM-1 in human normal B and plasma cells [25]. Interestingly, in separate experiments (**Chapter 6**) we showed that treatment of normal human PBMCs with Pam3CSK4 also increased surface expression of α4 and β7 integrins dose-dependently on T cells, B cells and monocytes, indicating common mechanisms exploited by TLRs in these normal cells to modulate integrin expression. To investigate the situation in MM cells, we stimulated a panel of HMCLs with ligands of TLR1/2 (Pams3CSK4), TLR3 (Poly I:C), TLR4 (LPS) and TLR9 (CpG-C). We found that Pam3CSK4 upregulated α4 and αVβ3 expression in all HMCLs but downregulated β7 expression in some of them, these effects were inhibited following MyD88 siRNA gene

silencing (It should be mentioned that other ligands also yielded more or less the same, although a bit heterogeneous, results as Pam3CSK4, but were not shown in this study). Surprisingly, in spite of absence of TLR2 protein (but presence of mRNA), Pam3CSK4 (TLR1/2 ligand) had a profound stimulatory and modulatory effect in our *in vitro* model system, regarding the fact that TLR1 will be functional only as heterodimerized with TLR2. Although discrepancy between TLR mRNA and protein expression has also been shown in B-chronic lymphocytic leukemia cells [26], one possible explanation in our findings is that the very low TLR2 protein (undetectable by our antibodies) might have been sufficient for making a functional heterodimer with TLR1. Furthermore, Pam3CSK4 increased adhesion of some HMCLs to FN (**Chapter 5**) and BMSCs (**Chapter 6**) and decreased that in some others. In addition, blocking NFκB pathway attenuated TLR1/2 activation-induced upregulation of α4 and αVβ3 but not downregulation of β7, and even it further reduced β7 baseline expression.

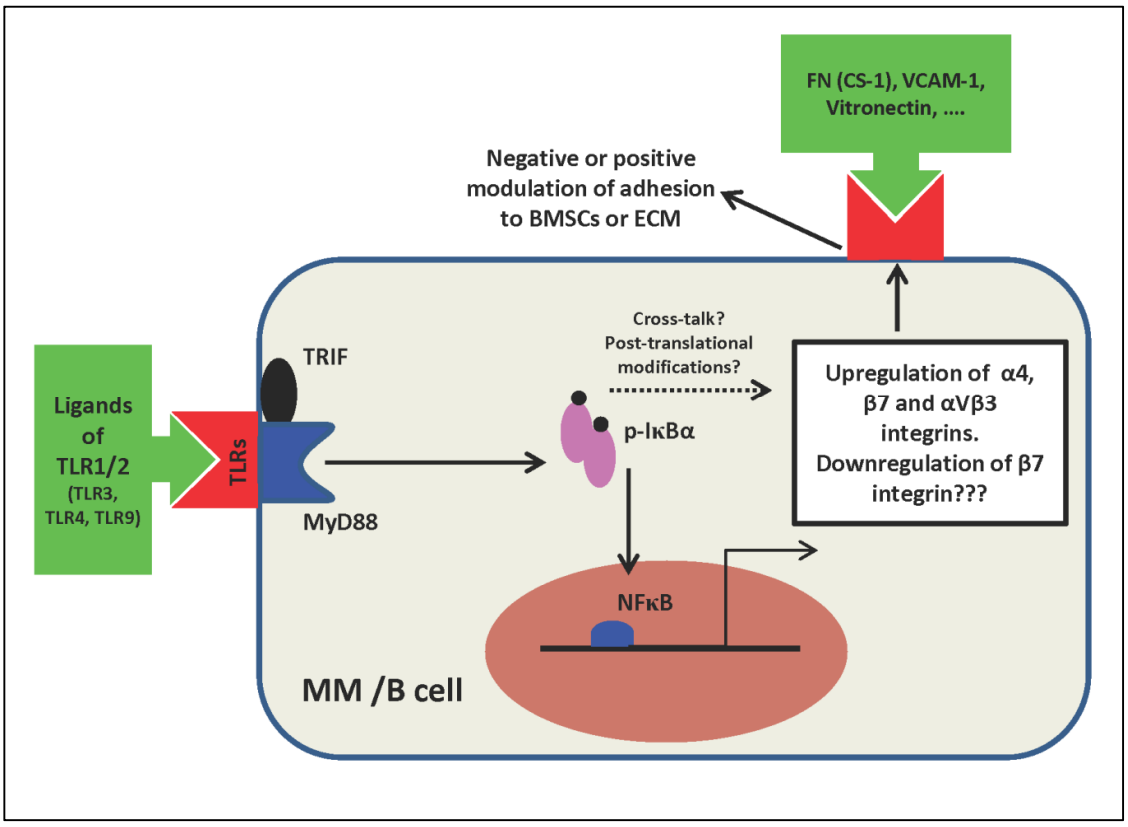

Figure 1. Schematic presentation of postulated mechanisms controlling TLR triggering effects on surface expression of integrin molecules and adhesion to FN and BMSCs in normal B lymphocytes or MM cells. Following a 24-hour treatment of MM cells with TLR ligands, especially TLR1/2 ligand (Pam3CSK4) which had more concrete results in our study; signals are transduced through MyD88 to IκBα phosphorylation culminating in NFκB activation. The latter transcription factor activates genes controlling expression of adhesion molecules such as β1 integrins, α4, αVβ3 and β7, with the outcome being up-regulation of relevant proteins on cell surface. However, this mechanism may not explain down-regulation of β7 in some HMCLs. Also, since TLR1 activation may modify affinity of integrin-ligand binding, adhesion (depending on the cell type) may be down-or up-regulated. Moreover, whether this modulation of adhesion and adhesion molecules is also due to a cross-talk between TLR-and integrin-mediated signaling pathways or post-translational modifications of adhesion molecules is not known to us.

These results suggest that NFκB mediates Pam3CSK4-induced upregulation of α4 and αVβ3 but Pam3CSK4-induced downregulation of β7 is probably controlled by another pathway other than NFκB. All above findings indicate that MM cells may engage specific mechanisms/pathways to relay TLR signaling to **"modulation of α4/αVβ3 and β7 integrin expression".** On the other hand, in adhered cells the mechanisms linking TLR triggering to **"modulation of adhesion"** appear to be more complicated involving more than just one pathway. LPS treatment of monocytes modulated their adhesion and spreading through pathways involving NFκB, MAPK, PI3K, and Rap1GTPase [27-29]. Furthermore, signaling complexes, adapters, cytoskeletal proteins and scaffolds which are engaged downstream of integrin activation could also be involved [30,31]. Interestingly, signal transduction due to cytokine treatment on adhered cells may result in a similar picture. In neutrophils, some cytokines activated NFκB only in adhered cells [32]; in fact the link between cytokine/growth factor signaling and integrin signaling is well established [33-35]. TLR4 activation-induced modulation of adhesion though PI3K [36] and NFκB [37] has been reported in colon cancer cells. Treating MM primary cells and HMCLs with hepatocyte growth factor (HGF) increased their adhesion to FN which was dependent on α4 and β1 integrin subunits and mediated by PI3K and NFκB pathways [38]. However, HGF did not have any effect on surface expression of α4 and β1, in close contrast to Pam3CSK4 in our study which indicates that altering affinity of integrins for their ligands in addition to increasing their surface expression by Pam3CSK4 would be plausible. Taken together, our findings indicate that NFκB in MM cells functions as the master switch relaying TLR signaling to modulation of integrin expression and suggest that other factors and pathways could also be involved in modulation of adhesion following TLR triggering.

TLR activation-adhesion-apoptosis axis in MM cells

Extensive researches in recent years have uncovered the critical role of adhesion of MM cells to BMME components including BMSCs and FN in disease pathogenesis [24,39]. Due to this adhesion, various cytokines and growth factors are induced in both MM cells and BMSCs mostly through autocrine and paracrine systems. IL-6, VEGF and IGF are among the most important cytokines secreted in this interplay and are essential for growth, proliferation and drug resistance of MM cells and also contribute to angiogenesis and osteoclastogenesis (bone resorption)[39]. However, direct physical contact through integrin molecules especially VLA4 (CD49dCD29, α4β1) on MM cells and FN or VCAM-1 on BMSCs plays a striking role in drug resistance of MM cells, a feature recently coined as **"cell adhesion mediated drug resistance (CAMDR)"[40-43]**. CAMDR is basically a form of environment mediated drug resistance (EMDR) which confers transient *de novo* drug resistance to tumor cells while they are in contact with BMSCs or ECM (such as FN, laminin and collagens), however, this can finally contribute to *acquired* drug resistant phenotype [44]. In HMCLs, α4 integrin

subunit plays the key role in CAMDR; other integrins including β1, αV and β7 have also been implicated. Modulation of CAMDR toward increasing drug sensitivity of MM cells *in vitro* and disruption of tumor cells-stroma adhesion *in vivo* (and thus decreasing tumor load) continues to be a top-priority goal in most chemotherapeutic and immunotherapeutic protocols in MM [44]. To this end, bortezomib has been shown to overcome CAMDR through downregulation of VLA4 [45] and also in synergism with Tipifarnib (a farnesyl transferase inhibitor) through induction of endoplasmic reticulum stress pathway [46]. Indeed the key role of VLA4 in CAMDR was supported by the fact that blocking adhesion of MM cells to FN with specific antibodies increased their drug sensitivity [41]. It has also been shown that BMSCs could protect MM cells by secreting some cytokines other than direct contact [47,48]. However, in our unpublished observations, we found that disrupting BMSCs-MM cells adhesion by fixing the former with paraformaldehyde or putting the HMCLs in a transwell insert significantly increased apoptotic response of Pam3CSK4-treated HMCLs to bortezomib compared to same HMCLs adhered to FN or BMSCs. Moreover, incubation of HMCLs with supernatant of BMSCs culture could not restore viability of MM cells back to that in adhered cells. These findings indicate that direct physical contact between MM cells and BMSCs and FN is fundamental in providing drug resistance.

As mentioned above, Pam3CSK4 increased adhesion to FN or BMSCs in some HMCLs and decreased it in others. This led us to hypothesize that Pam3CSK4 should also modulate drug sensitivity of HMCLs in the context of FN or BMSCs in line with their adhesion patterns. However, we found that Pam3CSK4 increased not only toxicity of HMCLs to but also their apoptosis in combination with bortezomib in the presence or absence of FN/BMSCs. This indicates that TLR1 activation in HMCLs enhanced their apoptotic response to bortezomib in an adhesion-independent manner. Notably, some level of CAMDR was obvious in cells adhered to FN or BMSCs, which was completely reversed by combination of Pam3CSK4+bortezomib.

Pam3CSK4 alone had a modest apoptotic effect on most HMCLs. Although the apoptosis-promoting mechanism of Pam3CSK4 has not been clearly defined, TLR1/2 ligand has been associated with induction of apoptosis, activation of NFκB and production of reactive oxygen species (ROS) [49,50]. We rather found that Pam3CSK4 caused a significant level of apoptosis in all HMCLs but had a partial effect on level of cleaved caspase-3 protein and caspase-3 enzymatic activity suggesting it may engage caspase-independent mechanisms. Intriguingly, in our study omega-3 components (EPA and DHA), which seemingly exert their effects through NFκB inhibition, also induced apoptosis of HMCLs and increased cytotoxicity to bortezomib, while Pams3CSK4 which usually drive NFκB activation caused the same effects. These findings might imply that either NFκB will stimulate survival or

apoptotic pathways depending on the type of the stimuli [26], or it drives apoptosis indirectly through induction of apoptosis-inducing mediators (probably in the case of TLR-1/2 triggering), or it directly controls apoptosis (in the case of omega-3) [26]. Of important note, there was a small difference in % specific apoptosis, cleaved caspase-3 protein level, and caspase-3 enzymatic activity between cells adhered to FN or BMSCs and non-adhered cells. This implies that inhibition of caspase cascade might partly explain the mechanism of CAMDR induction in HMCLs, indeed exact mechanisms that HMCLs exploit to provoke CAMDR are not as yet clear. Nevertheless, induction of CAMDR following adhesion of some HMCLs to FN via β1 integrin was reported to be mediated by upregulation of $p27^{Kip1}$ protein and G1 cell cycle arrest [51]. Whether other molecules could also be involved in MM CAMDR has not been clearly explored. However, β1 integrin-mediated adhesion has been shown to protect some HMCLs and leukemia cell lines from Fas-induced apoptosis through cellular re-distribution of CASP8 (an anti-apoptotic protein) and FLIP [52], or through proteasomal degradation of BCL2-interacting mediator of cell death (BIM) in leukemia cell lines [51]. The apoptotic effect of bortezomib on HMCLs has been well documented [53], in our study it resulted in increase in % specific apoptosis, cleaved caspase-3 protein level, and caspase-3 enzymatic activity. In combination with bortezomib, Pam3CSK4 highly increased the above parameters, either in an additive or synergistic manner. In HMCLs adhered to HS-5 or MM primary BMSCs, the above combined treatment synergistically led to a much higher level of apoptosis compared to their single effects, however, in the context of FN the combined effect seemed to be more additive.

From a mechanistical perspective, the link between TLR activation (TLR1 in our system), integrin signaling and apoptosis seems to involve multiple mechanisms, which is schematically proposed in figure 2. To understand how Pam3CSK4 would enhance bortezomib-induced cell death in HMCLs adhered to FN; we measured TNF-α in supernatants from all conditions to see if this cytokine mediated apoptotic effects but it was undetectable (data not shown). In line with this idea, type-I IFNs have been associated with TLR3 activation-induced apoptosis of MM cells (IFNα) [54] and various human tumor cell lines (IFNβ) [55]. Although not tested in our system, involvement of these cytokines in Pam3CSK4-induced apoptosis seems plausible [56,57]. It is well established that signal transduction downstream of integrins following adhesion to FN/BMSCs triggers activation of PI3K/Akt, Raf/MAPK, and NFκB pathways [58]. Hence, at pathway level the protective shield conferred by BMME to MM cells should be a function of activation of or cross-talk between these pathways. To see which pathway (s) might mediate TLR1 activation link to drug-induced apoptosis *in HMCLs adhered to FN*, we blocked above pathways with specific inhibitors and found that NFκB and MAPK pathways apparently control the above link. Furthermore, in our p53-signaling pathway gene expression profiling, we found several genes upregulated in Pam3CSK4-treated FN-adhered cells which were exposed to bortezomib.

These genes included GML, TP63, IFNB1, CDKN1A, EGR1 and FASLG, which are mostly involved in p53 signaling pathway and control cell proliferation, cell cycle or apoptosis [59-62]. On the other hand, we obtained some inconsistent results at protein level for other factors including Bcl2, Bax, p73 and p53, which did not provide a direct involvement of p53 protein and its downstream targets in Pam3CSK4-induced increase in apoptotic response to bortezomib. Based on these findings, we can speculate that p53 pathway might contribute indirectly to above response and partly explain a mechanism which could be associated with CAMDR in HMCLs. However, we still don't know how some genes in p53 signaling pathway are influenced, whether integrin signaling pathways are really involved and how activation of PI3K/Akt, Raf/MAPK, and NFκB pathways might relay TLR1 triggering to p53-related signaling and apoptosis and thus further thorough research should yet disclose the real mediators in our system.

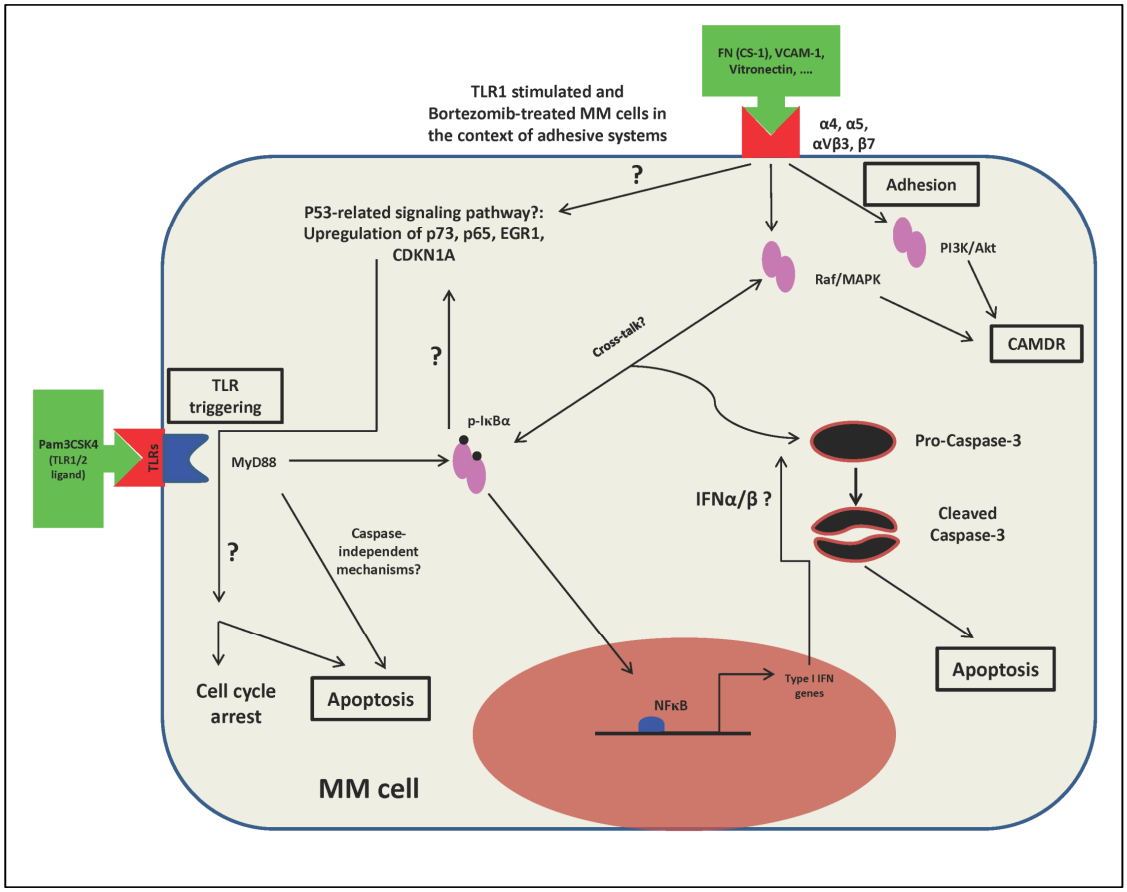

Figure 2. A proposed model illustrating how TLR1 triggering could modulate CAMDR and enhance bortezomib-induced apoptosis in the context of FN or BMSCs. In our system, HMCLs were first stimulated with TLR1 ligand (Pam3CSK4) and then exposed to FN/BMSCs and bortezomib. Pam3CSK4 may promote apoptotic effect of bortezomib in adhered HMCLs through several mechanisms: 1)-NFκB activation induces type I IFNs (IFNα/β) which in turn might trigger apoptosis via caspase-3 activation, 2)-Signal transduction through NFkB, cross-talk with MAPK signaling downstream of integrin activation, which may end up in caspase-3 activation. MAPK and PI3K pathways collaboration seems rather control CAMDR. 3)-In interplay of integrin signaling and TLR triggering (NFκB activation), some genes related to p53 signaling pathway (p65, p73, EGR1 and CDKN1A) are activated which could culminate in apoptosis or cell cycle arrest, 4)-Pam3CSK4 may also induce apoptosis in HMCLs through a caspase-independent mechanism.

Concluding remarks and future perspectives

It goes without saying that TLR biology in MM field is novel enough to warrant more extensive investigation into the mechanisms underlying their heterogeneous expression, activation-induced functional responses and also to assess their therapeutic aspects. To this end, we have convincing scientific proofs to attest that these molecules will have a potential contribution to MM pathogenesis, although only based on *in vitro* findings. However, the highly heterogeneous biology of MM, the positive and negative effects of TLR activation on HMCLs and MM primary cells and lack of established and reliable animal models for MM, will turn decision making on clinical application of TLR ligands in MM into a challenge. Instead, several TLR ligands have already been applied to clinical trials in a variety of human cancers and also in other B-lymphoid malignancies including some lymphomas and B-CLL [63-65]. While some recent studies have shown that TLR triggering in MM cells optimizes their physiology with respect to growth, proliferation and drug resistance, the striking feature of our study was modulation of MM cells adhesion to FN and BMSCs by TLR1/2 activation and also increasing adhered MM cell death in combination with bortezomib and thus complete reversal of any level of CAMDR. Additionally, we demonstrated that TLR1/2 activation in combination with bortezomib would dramatically decrease immunoglobulin free light chain (FLC) (which have detrimental complications in MM patients) production in adhered HMCLs. From a clinical standpoint, these findings suggest a therapeutic application of Pam3CSK4 (TLR1/2 ligand) in MM combined treatment protocols. On the other hand, in our study lack of access to MM primary cells, focus mostly on HMCLs, and also some level of heterogeneity between HMCLs, will make the above suggestion still risky. Indeed, future investigations will uncover by relying on high precision technologies such as oligonucleotide-based DNA microarray and functional genomics the seemingly complicated pathways exploited by TLRs in several aspects of MM cells physiology especially immune escape and drug resistance.

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Appendix

Nederlandse samenvatting

Acknowledgement

Curriculum vitae

List of publications

Nederlandse Samenvatting

Onderzoek aan de biologische functie van toll-like receptoren (TLR) in multipele myeloma (MM) kan nieuw inzicht geven in de pathogenese van deze ziekte m.n. in relatie tot de betrokkenheid van ontstekingsfactoren (**hoofdstuk 1**). De rol van ontstekingsgerelateerde mechanismen in MM worden onder andere geïmpliceerd door onze experimenten met omega-3 componenten EPA en DHA (anti-ontstekingsmiddelen). Deze stoffen verhoogden de gevoeligheid van MM cellen voor cytotoxische geneesmiddelen en versterkten de celdood door apoptose van MM cellen (**hoofdstuk 8**). In **hoofdstuk 2** wordt een samenvatting van de huidige kennis van de functionele expressie van TLRs in normale B-cellen en diverse B-lymfoïde maligniteiten gegeven en hierin wordt een mogelijke rol van TLRs in B celfunctie/ differentiatie en (waarschijnlijk) maligne transformatie nader belicht. In vergelijking met normale B-cellen lijkt het patroon van TLR expressie en functionele responsen in MM meer divers en veelal uniek voor specifieke MM cellen (cellijnen) duidend op mogelijk verschillende factoren die expressie op moleculair niveau beinvloeden. MM cellen hebben over het algemeen een verhoogde TLR expressie en een ander expressiepatroon van de verschillende TLR isoformen in vergelijking met normale plasmacellen, wat mogelijk aangeeft dat MM cellen sterker zullen reageren in een ontstekingsomgeving. In **hoofdstuk 4** hebben we het profiel van TLR-expressie in MM cellen op mRNA- en eiwitniveaus onderzocht. Hierin wordt duidelijk dat MM cellen een breed panel van TLRs tot expressie brengen. In een pilot-studie (**hoofdstuk 3**) onderzochten we de functionele response van normale B-cellen op TLR activatie. TLR9 stimulatie met CpG moduleerde de adhesie van de B cellen aan de extracellulaire matrix component fibronectin (FN). Omdat adhesie invloed heeft op celulaire activatie en celdifferentiatie is dit een eerste aanwijzing dat TLR activatie in normale B cel belangrijk kan zijn in ontstekingsreacties. Als belangrijke spelers in ontstekingsreacties kunnen TLRs zowel positieve als negatieve effecten hebben op tumorcellen, afhankelijk van het type tumor en tumorconditie. In het beenmerg is adhesie van MM cellen aan micro-omgeving componenten (FN en stromale cellen) een cruciale factor in MM pathogenese. Ons onderzoek toont aan dat TLR activatie leidt tot modulatie van adhesie van MM cellen aan FN (**hoofdstuk 5,6**). TLR1/2 stimulatie met Pam3CSK4 leidt tot vermindering en stimulatie van adhesie en een paralelle verandering van expressie van verschillende adhesiemoleculen. Daarnaast is aangetoond dat TLR1/2 stimulatie de gevoeligheid van MM cellen voor de proteasoom remmer bortezomib (Velcade) verhoogd. De verhoogde celdood en apoptose van MM cellen na TLR1/2 stimulatie was niet te verklaren met een verminderde celadhesie en daarmee veranderde “cel-adhesie gemedieerde drug resistentie (CAMDR)”. In verschillende geteste MM cellijnen stimuleerde TLR1/2 activatie echter adhesie terwijl dit toch gepaard ging met een verhoogde gevoeligheid voor bortezomib (**hoofdstuk 5,6**). TLR1/2 stimulatie vermindert ook de productie van

immunoglobuline vrije lichte keten (FLC) door MM cellen en dit effect werd versterkt in combinatie met bortezomib (**hoofdstuk 7**). De sterk verhoogde productie van FLC en precipitatie van deze eiwitten in de niertubuli wordt gezien als belangrijke factor in het veroorzaken van nierfalen in MM patienten. Dit onderzoek suggereert dat stimulatie van TLR1/2 met bijv Pam3CSK4 van therapeutische waarde zou kunnen zijn in MM. Echter nader mechanistisch onderzoek is noodzakelijk.

Samenvattend kan geconcludeerd worden dat dit onderzoek als eerste de rol van TLR (TLR1/2) activatie in MM cellen m.b.t. adhesie aan FN en BMSCs, overleving en CAMDR in kaart gebracht heeft. In de toekomst is het van belang te onderzoeken welke potentiële liganden van TLRs in MM beenmerg aanwezig zijn, of activatie van TLRs *in situ* daadwerkelijk bijdraagt aan MM pathogenese (bijv in preklinische modellen voor MM), en of TLRs een rol in hebben in MM oncogenese (via het onderzoek aan de mogelijke link tussen het aangeboren immuunsysteem en oncogenese).

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Curriculum vitae

Jahangir Abdi was born in May 22, 1969, in Nourabad Delfan Township, Lorestan Province, Iran. He began his academic education in the field of Medical Laboratory Science in 1989 in Shiraz University of Medical Sciences (Shiraz, Iran) and graduated with an Associate Degree in 1991. After several years of work in Medical Diagnostic Settings, he was readmitted into above field in Shahid Beheshti University of Medical Sciences (Tehran, Iran) and received his Bachelor in 1997. After immediate admission into Iranian High Institute for Education and Research in Transfusion Medicine (Tehran, Iran) he graduated in 2000 with a Master in Experimental Hematology & Blood Banking. During mastership, he focused as a thesis topic on mechanisms of phagocytosis and intracellular killing in blood neutrophils of thalassemia major patients. Jahangir conducted quality control and supervision in hematology, immunohematology and blood coagulation laboratories for 8 years but was never persuaded with confinement to Lab. career. So he was prompted to open a new venue in his life: research in biomedical sciences. In October 2008, he could find a research opening in the multidisciplinary environment of Division Immunopharmacology, UIPS, Faculty of Science, Utrecht University, Utrecht, the Netherlands. Under supervision of Prof. Johan Garssen and Dr. Frank Redegeld, Jahangir started a PhD research project on Toll-like receptors in human multiple myeloma cells with a focus on TLR activation link to cell adhesion, apoptosis and drug sensitivity. He has become increasingly more interested in TLR triggering link to proto-oncogene signaling in B cell malignancies, and is now willing to expand his experience at higher steps to delving thoroughly into molecular pathway targeting in multiple myeloma cancer stem cell and other related blood malignancies

List of publications

From this thesis

1. **Jahangir Abdi**, Ferdi Engels, Johan Garssen and Frank Redegeld. Toll-like receptor-9 triggering modulates expression of alpha-4 integrin on human B lymphocytes and their adhesion to extra-cellular matrix proteins. *Experimental Hematology. 39(9):927-33, 2011*

2. **Jahangir Abdi**, Ferdi Engels, Johan Garssen and Frank Redegeld. The role of Toll-like receptor mediated signaling in the pathogenesis of multiple myeloma. *Critical Reviews in Oncology and Hematology 80(2):225-40, 2011*

3. **Jahangir Abdi**, Tuna Mutis, Johan Garssen, Frank Redegeld, Characterization of the Toll-like receptor expression profile in human multiple myeloma cells. *PLoS ONE 8(4): e60671. doi:10.1371/journal.pone.0060671*

4. **Jahangir Abdi**, Tuna Mutis, Johan Garssen and Frank Redegeld, Stimulation of Toll-like receptor-1/2 combined with Velcade increases cytotoxicity to human multiple myeloma cells independent of modulation of adhesion to fibronectin. *Blood Cancer Journal (accepted, May 2013)*

5. **Jahangir Abdi**, Johan Garssen and Frank Redegeld, Combined effect of Toll-like receptor-1 activation and Bortezomib on immunoglobulin free light chain production of human myeloma cell lines in the context of bone marrow stromal cells or fibronectin. *Manuscript in preparation*

6. **Jahangir Abdi**, Tuna Mutis, Johan Garssen and Frank Redegeld, Toll-like receptor (TLR)-1/2 triggering on multiple myeloma cells modulates their adhesion to bone marrow stromal cells and enhances bortezomib-induced apoptosis by an adhesion-independent mechanism. *Manuscript submitted*

7. **J Abdi**, J Garssen, J Faber, F Redegeld, Omega-3 components, EPA and DHA, induce apoptosis in multiple myeloma cells and enhance bortezomib-induced apoptosis, *Manuscript in preparation*

Other publications

1. Shaiegan Mojgan, **Abdi Jahangir**, Zaman-Vaziree Maryam, Khajeian Abdolhamid. Comparison of Neutrophil Function in Patients with Thalassemia Major and Healthy Controls. *Arch Iranian Med (English Journal, ISI) July 2002; 5(3): 175-178*
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3. Kiani. AA**, Abdi. J**. Prevalence of alloimmunization against red blood cell antigens in thalassemia major patients of Lorestan Province in 2004. *Blood (journal of IBTORC in Farsi) 2006, 3(3): 265-271*
4. **Abdi. J**. Prevalence of HBcAb among the HBsAg negative first-time blood donors in Khorramabad and Borujerd blood centers. *Blood (journal of IBTORC in Farsi) 2008; 4(5): 323-329*
5. **Abdi. J,** Kiani.AA. Seroepidemiologic evaluation of Rh blood group system major antigens (D,C,E,c,e) and their phenotypes among the blood donors in Khorramabad, Iran. *Blood (journal of IBTORC in Farsi) 2009; 6(3): 219-226*
6. A. Sepahvand, **J. Abdi,** Y. Shirkhani, Sh. Fallahi, M. Tarrahi, S. Soleimannejad. Dermatophytosis in Western Part of Iran, Khorramabad. *Asian Journal of Biological Sciences. 01/2009; 2(3): 58-65.*
7. Ali Asghar Kiani, **Jahangir Abdi**, Rahele Halabian, Mehryar Habibi Roudkenar, Mohammad Soleiman Soltanpour, Naser Amirizadeh, , Ahmad Kazemi. Over expression of HIF-1α in human mesenchymal stem cells increases their supportive functions for hematopoietic stem cells in an experimental co-culture model. *Hematology; March 2013 (in press)*

Abstracts

During PhD study

1. **Jahangir Abdi**, Tuna Mutis, Ferdi Engels, Johan Garssen, Frank Redegeld. Toll-like receptor 9 triggering on normal B lymphocytes and multiple myeloma cells modulates their interaction with fibronectin. *Ducth Society for Immunology (NVVI) Annual Meeting, DEC 14,15, 2011. P-12*

2. **Abdi, J**, T. Mutis, J. Garssen, F. Engels, F. Redegeld. Toll-like receptor-1 triggering on multiple myeloma cells modulates their adhesion to bone marrow stromal cells. British Society of Haematology, 52nd Annual Scientific Meeting, Glasgow, 16-18 April 2012. (*British Journal of Haematology, April 2012; Vol. 157, Suppl 1: P-108)*
3. **Abdi J**, Mutis T, Garssen J, Engels F, Redegeld F. Adhesion of multiple myeloma cells to fibronectin is influenced by Toll-like receptor-1 triggering on their surface. XXXIV World Congress of the International Society of Hematology (ISH), Cancun, Mexico April 25-28, 2012. (*Revista de Hematología Volumen 13, Suplemento 1, 2012, P-A1011)*
4. **Abdi J**, Mutis T, Garssen J, Redegeld F. Adhesion of multiple myeloma cells to fibronectin is influenced by Toll-like receptor-1 triggering. ISEH 41st. Annual Scientific Meeting 23-26 August, 2012, Hotel Okura Amsterdam, the Netherlands. (*Experimental Hematology 2012; 40 (Suppl 1). P185)*
5. **Abdi J**, Mutis T, Garssen J, Redegeld F. Toll-like receptor-1 ligand and Omega-3 components (EPA and DHA) enhance the anti-myeloma activity of bortezomib in the context of bone marrow stromal cells. *Lymphoma & Myeloma 2012: An International Congress on Hematologic Malignancies, October 25-27, Waldorf-Astoria, New York, New York, USA. P31*

In previous congresses

1. Shahzamani. K, Lashkarian. E, Sabahi. F, **Abdi. J**. Prevalence of HCV in Blood Donors referring to Lorestan Province Blood Center. *3rd Iranian Congress on Virology, 21-23 Jan 2006; Tehran, Iran, 78 (in Persian)*
2. K. Shahzamani, H. Esmail Lashkarian, F. Sabahi, **Abdi. J**. Prevalence of Hepatitis C virus in Blood Donors Referred to Lorestan Blood Transfusion Center. *12th International Congress on Infectious Diseases,15-18 June 2006, Portugal, 02203*
3. A. Kiani, **J. Abdi**, H.R.M. Goodarzi. Prevalence of HBcAb among the HBsAg negative first-time blood donors in Khorramabad and Borujerd blood centers, Lorestan, Iran. 19th Conference of the Asian Pacific Association for the Study of the Liver. *Hepatol Int (2009) 3:86–220, PE202*
4. **Abdi J**, Kiani AA, Engels F. Seroepidemiologic evaluation of Rh blood group system major antigens (D,C,E,c,e) and their phenotypes among the blood donors in Khorramabad, Iran. 42nd Annual Congress of the German Society for Transfusion Medicine and Immunohaematology (DGTI), September 15–18, 2009, Rostock, Germany. (*Transfus Med Hemother 2009;36(Suppl 1):1–70, P 1.19)*

5. A.A. Kiani, **J. Abdi,** Y. Shirkhani, M. Kashi, M.R. Mehrabi. Studying the effect of pre-storage washing (for leukoreduction) on the function of platelets in PLT bags kept for 7 days. 14th congress of the European Hematology Association. Berlin, Germany. June 4-7, 2009. (*Haematologica 2009; 94[Suppl.2]:684 abs. 1806)*
6. Kiani AA, **Abdi J,** Sepahvand A, Shirkhani Y, Kashi M, Negravi S. Studying the effect of pre-storage washing (for leukoreduction) on the function of platelets in PLT bags kept for 7 days. XXXIst International Congress of the ISBT in joint cooperation with the 43rd Congress of the DGTI, Berlin, Germany, June 26th to July 1st, 2010. (*Vox Sanguinis 2010; [Suppl s1]: 1-541, P-0336)*

Table 1,2. Characteristics and functional responses of various human myeloma cells lines (HMCLs) following TLR1 triggering in our *in vitro* model system

HMCLs	Light chain (LC) isotype	TLR expression Profile (protein)	Change in LC secretion following TLR (-1/2) activation	Modulation of integrin molecules expression following TLR (-1/2) activation	Modulation of adhesion to bone marrow stromal cells following TLR (-1/2) activation	Modulation of adhesion to fibronectin following TLR (-1/2) activation	Integrin molecules involved in induced adhesion	Integrin molecules involved in downregulated adhesion
Fravel	Kappa (low)	1,3,4,7,8,9	nd	↑(α4, αVβ3) ↓β7	Upregulation	Downregulation	α4, αVβ3	nd
L363	Lambda	1,3,4,7,8,9	Decreased	↑(α4, αVβ3)	Upregulation	Upregulation	α4, αVβ3	-
UM6	Kappa	1,3,4,7,8,9	nd	↑(α4, αVβ3)	Upregulation	Upregulation	α4, αVβ3	-
UM9	Lambda	1,3,4,7,8,9	nd	nd	Upregulation	nd	-	-
OPM-1	Lambda	1,3,4,7,8,9	nd	↑(α4, αVβ3) ↓β7	Downregulation	Downregulation	-	β7
OPM-2	Lambda	1,3,4,7,8,9	Decreased	↑(α4, αVβ3) ↓β7	Downregulation	Downregulation	-	β7
U266	Lambda	1,3,4,7,8,9	Decreased	↑(α4, αVβ3)	Upregulation	Upregulation	α4, αVβ3	-
RPMI-8226	Lambda	1,3,4,7,8,9	nd	No change	nd	Downregulation	-	-
XG1	Kappa	1,3,4,7,8,9	nd	nd	nd	nd	-	-
NCI-H929	Kappa	1,3,4,7,8,9	Decreased	↑(α4, αVβ3) ↓β7	Downregulation	Downregulation	-	β7

HMCLs	Modulation of survival in fibronectin context following TLR-1/2 activation	Modulation of survival in bone marrow stromal cells context following TLR-1/2 activation	Modulation of drug (botezomib) sensitivity in both contexts	Effect of omega-3 components (EPA/DHA) on survival	Effect of omega-3 components (EPA/DHA) on drug (bortezomib) sensitivity
Fravel	nd	nd	nd	nd	nd
L363	Decreased	Decreased	Increased	Decreased	Increased
UM6	nd	nd	nd	nd	nd
UM9	nd	nd	nd	nd	nd
OPM-1	nd	nd	nd	nd	nd
OPM-2	Decreased	Decreased	Increased	Decreased	Increased
U266	Decreased	Decreased	Increased	Decreased	Increased
RPMI-8226	nd	nd	nd	nd	nd
XG1	nd	nd	nd	nd	nd
NCI-H929	nd	nd	nd	nd	nd