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Review

Toll-like receptors: New targets for multiple myeloma treatment?

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ABSTRACT

Despite recent biomedical improvements in treating multiple myeloma, this disease still remains incurable. Toll-like receptors (TLRs) are key immune receptors that recognize conserved molecular patterns expressed by pathogens and damaged cells. Activation of TLRs can induce several effects including inflammatory responses, modulation of cell cycle, apoptosis, or regulation of cell metabolism. In multiple myeloma there is a dysregulated signalling of TLRs due to an abnormal presence of certain pathogens and release of molecules from damaged cells. Thus, TLRs could be critical players for tumour microenvironment and multiple myeloma progression. This haematological malignancy is characterized by a high percentage of recurrences, where many patients can develop residual drug-resistant malignant cells. Strategic targeting of TLRs might result in novel therapeutic combinations that improve the response to current treatments, reducing relapses. This review examines the potential of TLRs as targets for the treatment of multiple myeloma, making a particular emphasis on their therapeutic applications.

1. Introduction

Multiple myeloma (MM) is the second most prevalent haematological malignancy. Elevated calcium levels, renal failure, anemia and osteolytic bone lesions (CRAB features) clinically define this pathology. In MM, monoclonal plasma cells multiply and secrete large quantities of monoclonal immunoglobulins (M-protein). The clonal expansion of malignant plasma cells is associated with a decreased production of normal immunoglobulins, making MM patients vulnerable to infections and virus reactivation [1]. Chronic infection and inflammation could increase the risk of developing MM, and thus might be involved in its pathogenesis and progression [2]. Myeloma pathogenesis can be broadly explained by two interacting mechanisms, intraclonal evolution of cancer cells and development of an immunosuppressive tumour microenvironment [3]. Actually, MM is associated with increased risk of infections caused by Gram positive bacteria such as *Streptococcus pneumonia, Straphylococcus spp* and Gram-negative bacilli including *Pseudomonas, Haemophilus* or *Escherichia coli*, as well as invasive fungal infections. MM patients are also highly susceptible to viruses, especially Epstein-Barr virus and hepatitis B virus [4]. The increased susceptibility to infection is complicated and multifactorial. However, these pathogens share highly conserved microbial structures that can be detected by Toll-like receptors. Toll-like receptors (TLRs) are immune receptors that link innate and adaptive immune responses. These sentinel receptors are members of the pattern recognition receptor family, a group of proteins that identify preserved pathogen associated molecular patterns (PAMPs). As infections and inflammation are augmented, there is an abnormal chronic presence of PAMPs yielding an altered TLRs signalling [5]. Moreover, TLRs are also activated by DAMPs, (danger associated molecular patterns), released by damaged cells, and those may support MM environment, as well [6]. How dysregulated TLRs signalling can affect to MM progression is a big question to answer.

Dynamic interplay between cancer cells and the immune system is essential for cancer progression. In fact, the immune system can drive both pro-tumour and anti-tumour behaviours [7]. Based on its anti-tumour properties, immunotherapy has radically expanded our toolkit against cancer. Treatments for MM continue to evolve with many emerging immunotherapies such as chimeric antigen receptor T cells, monoclonal antibodies (e.g. Daratumumab), bispecific antibodies, antibody drug conjugates, or checkpoint inhibitors [8,9]. However,

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despite the huge efforts of scientific community, MM remains incurable. Most MM patients can develop residual drug-resistant malignant cells that survive to treatment [10]; thus, novel approaches to defeat residual malignant cells are generating considerable interest. Given the implication of TLRs in the bone marrow environment and their potential effects on cell apoptosis and proliferation, agonists and antagonist of TLRs could improve the current therapies against MM. Here, we examine the potential of TLRs as targets for the treatment of MM, making a particular emphasis on their therapeutic applications.

2. Toll-like receptors

Toll-like receptors (TLRs) are type-I transmembrane glycoproteins expressed in numerous cell types including B and T lymphocytes [11,12]. Myeloma cells also express TLRs, where they may influence its progression and host immune responses [13]. At present, 10 TLRs have been described in humans, which are centrally involved in innate and adaptive immune responses. They are in the cell membrane or anchored in intracellular endosomes where they can recognize a variety of molecular patterns (PAMPs and DAMPs) (Table 1). TLRs have three principal domains: extracellular, single-spanning transmembrane domain, and cytoplasmic. The extracellular domain detects and binds TLRs agonists, and the cytoplasmic domain, or TIR domain, joins to the adaptors to initiate the downstream signalling pathway. TLRs recruit TIR domain-containing adaptor proteins such as MyD88 and TRIF, which initiate signal transduction pathways that culminate in the activation of NF-κB, IRFs, or MAP kinases to regulate the expression of cytokines, and type I interferon (IFN) [14] (Fig. 1). TLRs activation induces proinflammatory factors, including nitric oxide, IL-6 and IL-12, which result in resistance of tumour cells to cytotoxic T lymphocyte and natural killer (NK) cell attack [15]. However, activation of TLRs not only promotes the expression and release of proinflammatory cytokines and mediators [16], but also seems to affect directly the progression of tumour cells. In addition, TLRs as TLR5 and TLR7 appear to increase cell apoptosis [17,18]. By contrast, TLR2 and TLR4 could enhance proliferation and survival of cancer cells, [19,20] promoting metastasis [21]. In fact, some TLRs, as TLR9, can stimulate tumour cell invasion and metastasis by regulating metalloproteinases [22]. Other studies suggest that TLRs can directly regulate cell metabolism, affecting tumour behaviour and function. Actually, TLR3 induces tumour cells to switch from OXPHOS to anaerobic glycolysis, helping cells to adapt to hypoxia in the tumour microenvironment [23].

Table 1
PAMPs and DAMPs for Toll-like receptors.

TLR	PAMP	DAMP
TLR1	Triacyl lipopeptides (with TLR2) [28]	β-defensin-3 (with TLR2) [126]
TLR2	Triacyl lipopeptides (with TLR1) [28] Diacyl lipopeptides (with TLR6) [28]	β-defensin-3 (with TLR1) [126] HMGB1 [35]Heat shock proteins (HSPs) [127] Biglycan [35] Versican [127] Hyaluronan [128,129]
TLR3	Viral dsRNA [53,54]	dsRNA from necrotic cells [53,54]
TLR4	Lipopolysaccharide (LPS) of Gram-negative bacteria [66]	β-defensin-2 [130] HMGB1 [131] HMGN1 [131]Heat shock proteins (HSPs) [131] Biglycan [132] Hyaluronan [128] Peroxiredoxin 1 (Prx 1) [133] S100 proteins [134] CD138 [135] Endoplasmin [134] Fibronectin [136] Heparan sulfate [137] Fibrinogen [138]LDL and beta amyloid (in concert with TLR6) [56,57] Lipid A [56,57] Resistin [56,57] Surfactant protein A [139] Tenascin C [140]
TLR5	Bacterial flagellin from both gram-positive and gram-negative bacteria [55,81]	HMGB1 [56,57]
TLR6	Diacyl lipopeptides (with TLR2) [28]	Versican [56,57,129]
TLR7	Viral ssRNA [30,89]	ssRNA from necrotic cells [88,141]
TLR8	Viral ssRNA [30,89]	ssRNA from necrotic cells [88,141]
TLR9	Bacterial and viral CpG DNA [57,100]	Self DNA [104] HMGB1 [107] mtDNA [105] IgG-chromatin complexes [106]

The pro- or anti-tumour effect of TLR signalling seems to be determined by the specific TLR being stimulated, and the downstream signalling cascade in the activated cells. In addition, chronic low-grade TLR activation favours a tumour-promoting pro-inflammatory state, while high-dose TLR activation induces anti-tumour response [24]. Nonetheless, recent evidences support that targeting TLRs in cancer immunotherapy is a promising strategy to fight against cancer, and the specific TLR ligands, either alone or combination, exhibit anti-tumour potential [25].

MM cell lines usually express a variety of TLRs, being TLR1, TLR4, TLR7, and TLR9 the most frequent detected among the cell lines [5]. From the scarce data available, MM patients show a quite heterogeneous expression pattern of TLRs. Analysis of tumour cells from sorted bone marrow mononuclear cells of MM patients showed high TLR2, TLR4 and TLR9 mRNA levels, and these findings were consistent with the high protein expression of TLR4 and TLR9 [26]. However, other study showed a strong protein expression of TLR1, TLR7, TLR8 and TLR9 in MM patients [13]. Not only, altered TLRs expression is present in MM patients, but also some TLR signalling downstream targets as TRAF6 are changed. TRAF6 protein in bone marrow mononuclear cells from MM patients is significantly elevated as compared with healthy subjects [27].

2.1. Toll-like receptors 1, 2, and 6

The expression of TLR1, TLR2 and TLR6 seem to be higher in MM patients than in healthy donors [26]. TLR2 is anchored to the cell membrane and resides on the cell surface, where heterodimerizes with either TLR1 or TLR6 to form the functional receptor [28]. One of the best characterized agonists of TLR2 are the lipopeptides, ubiquitous to all bacteria and highly expressed in the outer membrane of Gram-positive bacteria; triacyl lipopeptides are recognized by TLR2/TLR1, while diacyl lipopeptides are recognized by TLR2/TLR6. Regardless of which dimer is activated, the classic signalling cascade is the same [29], although the kinetics could be different depending on the ligands and lead to different physiological results [30]. Following ligand stimulation, TLR2 heterodimers generally initiate a MyD88-dependent intracellular signalling pathway, common to all TLRs except TLR3. This MyD88-dependent signalling requires the adaptor molecule TIRAP to activate downstream pathways. Upon TLR activation, the IL1-receptor associated kinase 4 (IRAK4), is then recruited to MyD88. IRAK4 then recruits and phosphorylates IRAK1 and IRAK2, which allows them to interact with the TNF Receptor Associated Factor 6 (TRAF6). TRAF6 activates the TGF-β-activated kinase 1 (TAK1) by forming a complex with its binding partners, TAK1-binding proteins (TAB1, TAB2, and TAB3). TAK 1 has the ability to activate both the nuclear factor kappa B (NF-κB) pathway and the mitogen-activated protein kinase (MAPK)

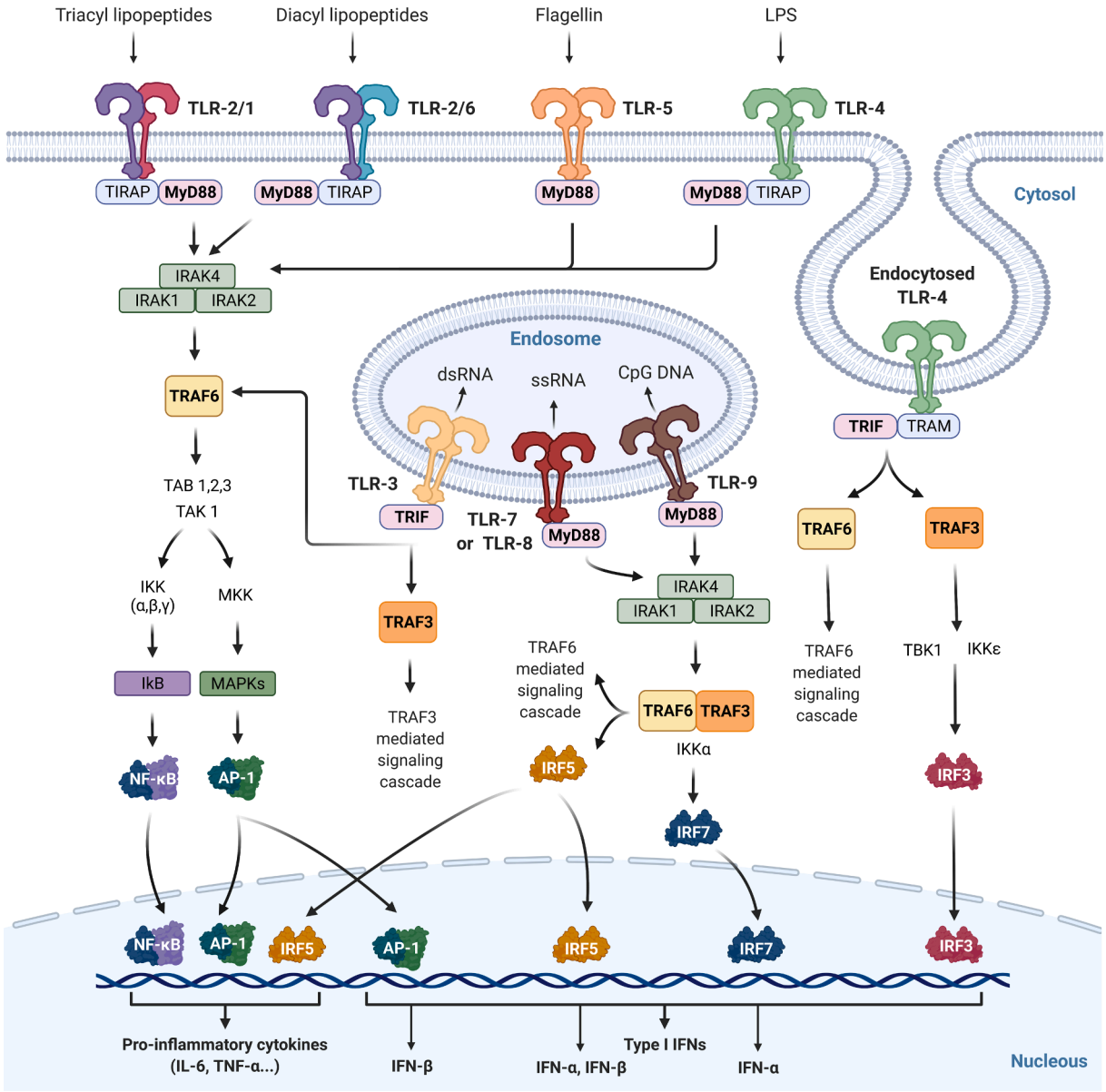

Fig. 1. Signalling pathways of Toll-like receptors (TLRs). TLRs are present on the cell surface and in endosomes, where they bind different ligands. Upon ligand stimulation, adaptor proteins are recruited to the TIR domain of TLRs and initiate intracellular signalling. Four types of adaptor proteins have been identified in mammals: MyD88, TRIF, TRAM, and TIRAP. TLRs activate two types of pathways that involve myeloid differentiation primary response protein 88 (MyD88) and/or TIR domain-containing adaptor protein inducing IFN-β (TRIF). Downstream signals culminate in the activation of transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and interferon-regulatory factors (IRFs), which regulate the production of pro-inflammatory cytokines and type 1 interferon (IFN). Created with BioRender.com.

pathway. During NF-κB activation, TAK1 activates IκB kinase complex (IKK α, β, γ) which catalyses the phosphorylation and degradation of the inhibitory protein IκB. This pathway induces nuclear translocation of NF-κB to modulate gene transcription and consequent inflammatory cytokine production. TAK1 also activates MAP kinase kinases (MKKs), which phosphorylates MAPKs. MAPKs mediate the activation of the activator protein-1 (AP-1) transcription factor, responsible for the expression of pro-inflammatory cytokines and IFN-β [31–33] (Fig. 1). The common immune response induced by TLR2 is to increase IL-6, type I IFN and TNF-α production during acute processes, while IL-10 was consistently found in chronic infections [34,35].

TLR2 is also capable of recognizing endogenous danger signals such as HMGB1 and biglycan [36]. DAMPs released by cancer cells can be detected by TLR2 expressed on innate immune cells and promote anti-tumour immune responses [37]. However, the activation of TLR2 on Treg lymphocytes and macrophages, promotes an immunosuppressive microenvironment driving tumour progression [38]. TLR2 activation on cancer cells may induce cell proliferation and invasion through different cell-intrinsic mechanisms [39]. In fact, TLR2-MyD88 axis seems to have a key role in oncogenesis of intestinal and breast epithelial cells, where the inhibition of TLR2 reduces the growth of tumour cells [40]. Similarly, the activation of TLR2/1 and TLR6/2 increase tumour cell survival in chronic lymphocytic leukaemia cells by triggering NF-κB signalling [41].

In MM cells, ligands for TLR1, TLR2, and TLR6 enhance IL-6 dependent cell proliferation, probably via the induction of NF-κB. Pam3Cys-Ser-Lys4, agonist of TLR2/1, is a potent inducer of IL-6 production that yields cell proliferation of OH-2 cells. Neutralizing IL-6 antibody partially inhibits TLR-induced cell proliferation. Similar results were also obtained in MM patient samples [42]. Additionally, TLR2

ligands seem to promote the immune evasion mechanism in MM cells by upregulated PD-L1 (a known inhibitor of T-cell functions). TLR2 could trigger MyD88 and MEK-dependent pathway and contribute for IFN-γ–induced expression of PD-L1 in malignant plasma cells [43].

MM drug resistance is often associated with adhesion to stromal cells. TLR2/1 activation induces increased adhesion to bone marrow stromal cells in parallel with an increased surface expression of integrin molecules α4 and αVβ3 [44]. However, some MM cell lines as OPM-1, OPM-2 or NCI-H929 show a dose-dependent decrease in adhesion upon TLR2 activation, following a downregulation of β7 integrin expression. Furthermore, TLR2/1 increases cytotoxic and apoptotic effects of broadly used proteasome inhibitor Bortezomib (BTZ) in myeloma cells independent of its effect on stromal cell adhesion [44]. Melphalan, a genotoxic agent used in MM therapy, upregulates cell surface HSP70 expression (detected by TLR2 as a DAMP) in MM cell lines, and induces a significant increase of exosomes release from MM cells. MM cell-derived exosomes are able to induce IFN-γ production, through a mechanism based on the activation of NF-κB pathway in a TLR2/HSP70-dependent manner [45], suggesting a novel mechanism of synergism between chemotherapy and anti-tumour innate immune responses, based on the drug-promotion of exosomes exposing DAMPs.

Given the current evidence, TLR2 could be a crucial target for MM treatment. In fact, some TLR2 agonists are being studied for their anti-tumour responses. CADI-05, a TLR2 agonist, induces a Th1 immune response and has been found useful in management of lung cancer and melanoma [46]. Intradermal CADI-05 administration significantly increases the survival rate on bladder cancer [47]. Other TLR2 ligands are also in phase 2 clinical trials for cancer therapy with promising results, such as CBLB612 (synthetic lipopeptide TLR2 agonist), ISA-201 (peptide agonist for TLR2), or OPN-305 (anti-TLR2 IgG4 monoclonal antibody) [48,49].

Recently, a new TLR belonging to the same gene cluster as TLR1 and TLR6 has been described. TLR10 is found on the cell membrane and probably acts as a TLR2 coreceptor. The heterodimer TLR10/2 can recruit MyD88, however they fail to trigger a TLR-induced signalling such as the activation of NF-κB [50]. Nowadays, TLR10 has been suggested as a MyD88 inhibitor, yielding a reduction in the production of pro-inflammatory cytokines, such as IL-6 and TNF-α [51]. Conversely, Mrna/protein expression levels of TLR2 and TLR10 are both positively correlated with tissue inflammatory grades, although IL-6 and IL-8 secretions are significantly higher in TLR10-knockdown cells [52]. TLR10 expression could be critical in mature B cells. Actually, TLR10 expression is upregulated by activation of B cells in vivo [53]. However, the research about TLR10 and its role in B cells remains scarce. In fact, recent results suggest a loss of TLR10 expression during malignant plasma-cell differentiation, as most of the human myeloma cell lines lack TLR10 [5], but its expression in MM patients is unexplored. Therefore, the identification of TLR10 ligands and intracellular mechanisms present the potential to selectively target B cells during immunotherapy with potential applications in MM.

2.2. Toll-like receptor 3

TLR3 is located on the membrane of endosomes forming a homodimer, and its activation occurs when double-stranded RNA (dsRNA) molecules from viruses and RNA associated or released by necrotic cells bind to the TLR3 leucine-rich repeats (LLR) domain [54,55]. As the aforementioned molecules are the prototypic ligands of this receptor, TLR3 is considered as one of the most important detectors of viral infections and neoplasms [56]. Apart from natural dsRNA, TLR3 also recognises synthetic dsRNA analogues, such as polyriboinosinic:polyribocytidylic acid (Poly I:C), polyadenylic:polyuridylic acid (polyA:U), polyriboinosinic-polyribocytidylic acidpolylysine carboxymethylcellulose (Poly I:CLC or Hiltonol™) and Poly I:C12U (Ampligen™ or rintatolimod), which have been consistently used to stimulate signalling via TLR3 in vitro and in vivo models [55].

Unlike other TLRs, TLR3 works exclusively via TRIF-dependent/MyD88-independent pathway [57,58]. TLR3 interacts with TRIF directly through the TIR domains. Upon ligand binding, TRIF recruits TRAF6 which acts in the MyD88 dependent and independent pathways leading to the subsequent activation of NF-κB and AP-1 transcription factor, responsible for the expression and production of proinflammatory cytokines and IFN-β. Upon the activation of TRIF, TRAF3 is also recruited, which promotes the activation of TANK binding kinase 1 (TBK1) and IKKε, leading to the phosphorylation and activation of the transcription interferon regulatory factor 3 (IRF3) [32,59]. Therefore, TLR3 signalling supports the synthesis and secretion of type I IFNs and a wide variety of proinflammatory cytokines including TNF-α and IL-6 [60] (Fig. 1).

TLR3 is highly expressed in myeloid cells, T cells, NK cells, endothelial cells, epithelial cells, keratinocytes, and neurons [56]. Aberrant TLR3 activity participates in numerous pathologies, like chronic inflammation, sepsis, autoimmune disorders, and cancer. In particular, its defective activity has been correlated with a predisposition to suffer from breast carcinoma, cervical cancer, oral squamous cell carcinoma, hepatocellular carcinoma and colorectal carcinoma, among others [55]. Furthermore, activation of the TLR3 signalling pathway has been described as a promising anti-cancer therapy, in which Th1 immune activation, the consequent secretion of cytokines and chemokines, and apoptosis are the main mechanisms involved [56,61]. In particular, TLR3 has been involved in promoting tumour cell death in several types of cancers, including breast cancer, colon cancer, bladder cancer, head and neck carcinoma, pharynx carcinoma, renal carcinoma, hepatocellular carcinoma, lung cancer and melanoma [61,62].

In this line, signalling through TLR3 has been shown to have heterogeneous effects on human myeloma cell lines, causing an increased proliferation and survival in some MM cells and cell death and apoptosis in other cell lines. Different studies indicate that cell apoptosis is promoted by p38MAPK pathway and mediated by IFN-α, while proliferation is due to NF-κB activation [63,64]. Actually, the association of NF-κB activation with both proliferation and survival of malignant plasma cells and the inhibitory action of IFN-α on growth and proliferation of normal and malignant haematopoiesis are well established [63].

As mentioned above, Poly I:C and its more stable and effective derivative Poly I:CLC are synthetic analogues of viral dsRNA, that stimulate TLR3, cytosolic RIG-I-like receptors (RLR) and melanoma differentiation-associated gene 5 (MDA5), causing a greater production of type I IFN along with a direct activation of apoptosis on tumour cells which express these factors. Poly I:C has been shown to be one of the most potent adjuvants which can be included in tumour vaccines [57,65]. In addition, Poly I:C-induced apoptosis in MM cells is dependent on caspases 3, 8 and 9. The involvement of caspases in Poly I:C/IFN-α -dependent apoptosis agrees with results obtained in melanoma or breast cancer cells [64]. Furthermore, this agonist can also induce tumour regression by converting tumour-supporting macrophages into tumour suppressors, which produces proinflammatory cytokines and promotes M1 polarisation. This action is mediated by TNF-α through a MyD88-independent pathway [24]. It is worth mentioning the importance of TRIM56 like a positive regulator of TLR3 antiviral signalling. TRIM56 overexpression has been described to suppress cell proliferation, induce proinflammatory cytokine production and increase apoptosis in MM cells. This suppressive effect of TRIM56 in MM is due to activation of the TLR3/TRIF signalling pathway [62].

Activated $CD4^{+}$T cells also express TLR3, which stimulation by Poly I:C, increases their proliferation and cell survival via NF-κB pathway. The incremented survival is due to high expression levels of the anti-apoptotic protein Bcl-Xl. On the other hand, Poly I:C has also been shown to induce proliferation of CD8+T cells, as well as enhance their response in a way that obviates the need for CD4+T cell support or co-stimulatory molecule expression in APCs (antigen presenting cells). In addition, TLR3 co-stimulation, along with TCR stimulation, was found to promote the generation of memory T cells, which is partly because

TLR3 is able to prolong T cell survival. The fact that TLR3 activation in T cells renders APC- or CD4-mediated co-stimulation unnecessary and promotes the generation of memory T cells is a factor to consider when designing cancer vaccines, since the tumour microenvironment is devoid of co-stimulatory signals [61].

Several studies have shown that the release of DAMPs from dying cancer cells, either spontaneously or after treatment, initiates an efficient and long-lasting anti-cancer immune response through the activation of TLRs and other receptors in host immune cells [66]. The potential of TLR3 stimulation as a promising strategy to boost cancer immunosurveillance has been demonstrated, especially as an adjuvant of tumour-targeted therapeutic vaccines [55,56]. For this reason, it is currently being used as an adjuvant therapy, in combination with other drugs or vaccines, against a wide range of cancers [58], including MM [62]. Therefore, TLR3 agonists are able to directly induce apoptosis on MM cells as well as to activate the immune host system, making the TLR3 signalling pathway an interesting therapeutic target [64]. However, not all myeloma cell lines express TLR3 [61] and its expression in MM patients is poorly explored. Unfortunately, it has been observed that apart from IFN-α production, TLR3 signalling also induces NF-κB activation, which potential malignant effects. In fact, cells that respond to TLR3 signalling only through NF-κB activation without any INFα production experience an increase in NF-κB-dependent cell proliferation [64].

2.3. Toll-like receptor 4

TLR4 is the one of the most studied TLRs; in fact its presence has been described in numerous cell types including myeloid cells, NK cells, mast cells, T cells, endothelial cells, epithelial cells and keratinocytes [56]. TLR4 is activated by lipopolysaccharide (LPS) from the plasma membrane of Gram-negative bacteria [67], and it also recognises a wide variety of DAMPs, including B-defensin 2, biglycan, CD138, endoplasmin, fibronectin, heat shock proteins, heparan sulfate, hyaluronic acid, fibrinogen, HMGB1, LDL and beta amyloid (in concert with TLR6), lipid A, peroxiredoxin 1, resistin, S100A8, surfactant protein A and tenascin C [57,58] (Table 1). TLR4/LPS binding takes place in complex with lipid A binding protein, cluster of differentiation 14 (CD14), and myeloid differentiation protein 2 (MD2) [65], since CD14 and MD-2 are accessory proteins required for LPS/TLR4 ligation [65,68].

TLR4 can be found in both the plasma membrane and the endosomal membrane carrying out both the MyD88-dependent pathway (like all other TLRs) and the MyD88-independent/TRIF-dependent pathway (like TLR3); TLR4 is the only TLR that can signal both ways [57]. This has led to the evolution of additional precautions, such as an extensive ligand detection mechanism (CD14, lipid binding protein, and MD2) and signal propagation mechanism (requirement of TIRAP for MyD88 and TRAM for TRIF signalling pathways) [58] (Fig. 1).

In the XIX century, William Coley observed that the injection of heat-killed bacterial toxins was able to induce tumour regression, and even cure cancer, demonstrating that microbial products could have anti-tumour effects. The heat-killed bacterial toxins contained LPS, which, as mentioned above, is the main ligand for TLR4 [57,65]. This approach has led to the development of several TLR agonists with the aim of achieving anti-tumour effects. Today, only three TLR agonists are approved by FDA for their use in humans: the bacillus Calmette-Guérin (BCG) which stimulates TLR2 and TLR4, monophosphoryl lipid A (MPLA) which is a TLR4 agonist, and imiquimod, a synthetic imidazoquinoline detected by TLR7 [69]. The most significant anti-tumour effect observed with BCG treatment, which is composed of a mixture of TLR agonists, has been in bladder cancer. BCG produces a strong immunostimulatory effect dependent primarily on the simultaneous activation of TLR2 and TLR4, indicating the advantage of multiple TLR involvement [57,61]. The anti-tumour activity produced by stimulation of these two TLRs has been linked to locally secreted cytokines, especially IFN-γ and IL-2, stimulation by dendritic cells (DCs) and macrophages, and the influx of activated Th1 cells, γδ T cells and NKT cells [57]. Regarding to MPLA, which is less toxic than LPS [70], it specifically activates the TRAM/TRIF pathway in TLR4 signalling, leading to induction of IFN-β and regulation of CD80/86, which is a critical point for coadjuvancy [71]. In fact, MPLA is included in the formulation of Cervarix®, a vaccine against human papillomavirus [72]. MPLA promotes rapid activation of innate immune responses to minimally immunogenic antigens, including tumour-associated antigens. This, in turn, triggers activation of antigen-presenting cells and cytokine cascades leading to anti-tumour helper T-cell responses (Th1 and Th2) [73]. Although MPLA has not been approved for cancer treatment, several MPLA congeners are being tested as adjuvants for cancer vaccines, such as glucopyranosyl lipid A (GLA, G100), a synthetic TLR4 agonist that activates DCs and enhances the Th1 immune response [74], or AS15 (MPLA combined with QS-21 and CpG oligonucleotides) [75]. Nonetheless, TLR4 signalling is considered a double-edged sword. It can induce a T cell-mediated anti-tumour response. However, TLR4 is also expressed by a variety of cancer cell lines, where contributes to the oncogenic expression of NF-κB, EGFR, and PI3K/Akt activation [57]. TLR4 activation in tumour cells has been shown to have pro-tumour rather than anti-tumour effects, such as in liver cancer, stomach cancer, prostate cancer, melanoma and glioma [61].

In MM cells, TLR4 activation has been shown to produce different effects including increased proliferation, immune response evasion and protection against apoptosis. Cell proliferation is induced through the activation of the MAPK and NF-κB pathways, which promote increased levels of IL-6 and IL-18 [76]. Increased IL-18 levels in MM patients have been shown to be a poor prognostic factor [77]. Additionally, TLR2 ligands seem to promote an immune evasion mechanism in MM cells through the IFN-γ –induced expression of PD-L1 in malignant plasma cells by triggering MyD88 and MEK-dependent pathway [43]. Furthermore, overexpression of TLR4 in malignant plasma cells has been reported to be associated with activation of the proto-oncogenes MAFB and c-MAF, indicating poor prognosis [63]. TLR4 signalling, in addition to triggering increased proliferation and immune response evasion in MM cell lines, also leads to decreased endoplasmic reticulum-induced apoptosis. In consequence, TLR4-mediated disruption of ER stress responses appears to contribute to MM cell proliferation and suppress ER-dependent death signalling [78] which can be related to drug resistance. TLR4 activation seems to enhance mitochondrial biogenesis and lead to an increase in mitochondrial mass in human MM cell lines [79]. It has been shown that when MM cells are treated with BTZ, a proteasome inhibitor commonly used in the treatment of MM, TLR4 targeting is activated and increased in BTZ-resistant cells. In this line, the combination of BTZ with TAK-242 (a selective TLR4 inhibitor) has shown to be able to overcome drug resistance by causing increased and prolonged oxidative stress, strong mitochondrial depolarisation and severe deterioration of mitochondrial fitness, which in turn causes a cellular energy crisis and initiates mitophagy and apoptosis [79]. In other words, TLR4 signalling functions as a stress response strategy that protects mitochondria under exposure to BTZ, maintaining mitochondrial metabolism and driving drug resistance. For this reason, TLR4 suppression could be a promising target in patients with refractory MM to overcome BTZ resistance [79]. Similarly, adrographolide, a TLR4 inhibitor, is able to abolish TLR4 protein expression. Such suppression is mediated by inhibition of the TLR4/NF-κB signalling pathway. Thus, andrographolide may be a promising drug candidate for the clinical treatment of MM [80].

Lastly, increased expression of the TLR4 has been observed in MM mesenchymal stromal cells (MSC) in comparison to MSCs from healthy donors. Bone marrow MSCs are abnormal in MM and play a crucial role by enhancing growth, survival and drug resistance of MM cells. At the clinical level, TLR4 expression in MM MSCs progresses in parallel with disease stage, indicating that the TLR4 axis is pivotal in MM by potentiating the pro-tumour activity of MSCs. Furthermore, selective inhibition of TLR4 effectively reduces the ability of MM MSCs to sustain MM

cell growth in an IL-6-dependent way and delayed MM development [81]. Therefore, specific targeting of the pathological microenvironment in the bone marrow through TLR4 targeting could be an innovative approach to intervene the MM development.

2.4. Toll-like receptor 5

TLR5 is a crucial determinant of pathogen-host interaction and essential for immune homeostasis. It is exclusively expressed on the cell surface of numerous cells including B and T cells [57]. TLR5 senses and recognizes flagellin, the major structural protein of bacterial flagella, from both Gram-positive and Gram-negative bacteria [56,82]. Flagellin activates TLR5 mainly through MyD88-dependent pathway, where TLR5 interacts with MyD88 directly through the TIR domains [83] (Fig. 1). However, some studies suggest the formation of a TLR5/TLR4 heterodimeric complex that activates cells through the TRIF-mediated pathway instead of the MyD88 adapter molecule [84,85].

Several studies have shown that the activation of TLR5 has anti-tumour effects in different models, like colon cancer and breast cancer, which has been linked to the expression and function of TLR5 on DCs and their important role in anti-tumour immune responses [24,82,86]. On the other hand, flagellin has also been reported to induce tumour proliferation in gastric cancer and salivary gland adenocarcinoma, as such activation of TLR5 increases IL-8 production, NF-κB activation and tumour cell proliferation mediated by extracellular signal-regulated kinase signalling [87]. Therefore, the disparities in these studies suggest that different cancer cells have particular responses and sensitivities to flagellin-TLR5 activation [82].

Salmonella flagellin-derived Entolimod (CBLB502) is a pharmacologically optimized TLR5 agonist. It has been shown to elicit a strong anti-tumour activity through the CXCR3-dependent NK-DC-CD8+T cell axis [84,88]. In addition, the adenovirus Ad5-based vaccine Mobilan was designed for intra-tumour application with the purpose of extending the potential application of TLR5-targeted anti-cancer immunotherapy to tumours that do not primarily express the TLR5 receptor. Mobilan is capable of supporting strong NF-κB signalling and inducing recruitment of innate immune cells, such as neutrophils and NK cells [88]. Specifically, TLR5 agonists are considerably less toxic when compared to other TLRs agonists because of the absence of induction of auto-amplifying "cytokine storm" inducing cytokines as TNF-α, IL-1β and IL-2, which can cause septic shock. Conversely, TLR5 agonists induce fast and short-lived generation of elevated levels of G-CSF, IL-6, IL-8 and IL-10 [86].

Myeloma cell lines exposed to TLR5 ligand stimulate NF-κB via P38 and PI3K/AkT signalling and exhibit an increase in proliferation, IgG production and IL-6 expression. In addition, such activation has been shown to suppress caspases and PARP activity and make MM cells resistant to apoptosis and doxorubicin [67]. It has been suggested that the enhanced cell viability and chemoresistance of some MM cell lines stimulated by flagellin could be the result of autocrine or paracrine signalling through secreted IL-6 [82]. In fact, IL-6 is the main proliferation and survival promoter of myeloma cells, and the level of circulating IL-6 has been correlated with MM progression; IL-6 is largely produced in the microenvironment. So, triggering TLRs could allow an additional autocrine loop of IL-6 which could be involved to contribute to the pathophysiology of MM by boosting proliferation, survival, drug resistance and immune evasion of MM cells [64].

2.5. Toll-like receptors 7 and 8

TLR7 and TLR8 are intracellular pattern recognition receptors that are located on the endosome membrane, sharing a high degree of sequence homology and function similarity. Currently, it is thought that monocytes, B cells and plasmacytoid dendritic cells (pDCs) are the primary cell types expressing TLR7, while monocytes and myeloid dendritic cells (mDCs) might be the most TLR8 responsive [89]. Although some studies described the murine TLR8 as non-functional due to a five amino acid deletion in the ectodomain, some studies have described that mouse TLR8 is indeed functional, but its function could be different from human TLR8 [90].

TLR7 and TLR8 are activated by single-stranded RNA (ssRNA) molecules and small chemical ligands [91]. These ssRNA molecules are normally originating from viruses, being an important part of the response to viral infections [31]. In addition to RNA from pathogens, it is suggested that endogenous RNA molecules can also activate TLR7/8, so aberrant activation of TLR7 has been implicated as driver of some autoimmune diseases such as lupus [89]. Even more, recent studies have shown that particularly human TLR8 is also an important receptor for the detection of various pathogenic bacteria [91]. Upon ligand stimulation, both receptors initiate the MyD88-dependent intracellular signalling pathway. Activation of MyD88/TRAF6 pathway leads to the activation of NF-κB and MAPK pathways, which results in the production and release of type I IFN and inflammatory cytokines (TNF-α, IL-6, IL-2) [89,92]. However, some studies have shown that TLR7, TLR8 and TLR9 activation also induce antiviral responses through the activation of Interferon regulatory factor 7 and 5 (IRF7 and IRF5), which are responsible for the expression and production of IFN-α and IFN-β. Both factors are activated in a MyD88, TRAF6 and IRAKs dependent manner. However, it is suggested that IKKα and TRAF3 are also essentials for activation of IRF7 [31] (Fig. 1).

In addition to their expression in immune cells, these receptors are also present in cancer cells, where TLR7/8 signalling is not very clear and has been correlated with either beneficial or harmful outcomes. On the one hand, TLR7/8 stimulation can lead to tumour progression through the production of immunosuppressive cytokines, increased cell proliferation and resistance to apoptosis. On the other hand, the activation of these two TLRs in both immune cells and cancer cells (often combined with chemo/immunotherapy) can also inhibit tumour growth via different pathways [37]. These positive or negative effects derived from the activation of TLR7/8, depend on each type of cancer cell, since each one may have different TLR expression and, as a result, respond differently to TLR stimulation. For example, TLR7 and 8 are highly expressed and stage-dependent in human pancreatic cancer compared to normal pancreas, and some studies describe that R848 stimulation of TLR7/8 in these cells showed increased NF-κB and cyclooxygenase-2 expression that has been previously linked to increased cancer cell proliferation, reduced chemosensitivity [93], immune evasion and immunotherapy resistance [94]. Otherwise, many other studies have demonstrated the potential anti-tumour effect of this widely known TLR7/8 agonist which might confirm the cell-type-dependent effect mentioned above [95,96]. Other authors suggest a higher expression of TLR7/8 in human colorectal cancer [97] and lung cancer than in normal tissue [98], and an overexpressed TLR7 in oesophageal cancer [99]. In addition, a high expression of both is associated with high expression of immune cell markers and predicts longer overall survival of patients with melanoma [100].

In MM cells, previous studies reported that TLR7 is one of the most frequently expressed TLRs by human myeloma cell lines [5]. Furthermore, it has been shown that some TLR7/8 ligands, such as loxoribine or R848, inhibit apoptosis and promote proliferation and cell survival. These effects may be partially mediated by the autocrine secretion of IL-6 [101]. Therefore, targeting of inhibiting TLR7/8 signalling might be a potential mechanism to abrogate this inflammation-mediated effect in MM progression.

2.6. Toll-like receptor 9

B cells express TLR9 and its expression in malignant B cells is heterogeneous in each cancer subtype, even in individual patients. TLR9 is highly expressed in myeloma cells [5]. It is primarily located in intracellular vesicles within the ER and translocate to endosomes upon stimulation by ligands. TLR9 preferentially detects unmethylated CpG oligodinucleotides (ODN) with a species-specific preference for hexamer

CpG motifs that are less common in vertebrates [102]. Activation of TLR9 requires two CpG ODN binding symmetrically to the C-terminal fragment of one TLR9 and the CpG-binding groove in the N-terminal fragment of another TLR9, creating a homodimer [103]. Methylated single stranded DNA (ssDNA) and doubled stranded DNA (dsDNA) have lower TLR9 affinity and induce less TLR9 dimerization [104]. Moreover, CpG ODN that only bind to the N-terminal fragment have an inhibitory effect on TLR9 [105]. Self-derived DNA fragments as DNA released from damaged cells or necrotic cells could trigger sterile inflammation via TLR9, acting as a DAMP [106]. Similarly, mtDNA [107] and IgG-chromatin complexes [108] can also be detected as DAMPs by TLR9. In fact, HMGB1 mediates the activation of DCs and B cells through TLR9 by DNA-containing immune complexes through a mechanism involving the immunoglobulin superfamily member RAGE [109]. Upon ligand stimulation, TLR9 initiates the same MyD88-dependent intracellular signalling pathway as TLR7 and TLR8 [31,32] (Fig. 1).

High TLR9 expression is found in bone marrow mononuclear cells from MM patients and its stimulation by CpG DNA can promote MM cells proliferation and protect these cells from serum-deprivation apoptosis [26]. TLR9 oncogenic effect has been reported in myeloma cells, where the aberrant expression of such receptors prevents chemotherapy-induced apoptosis and enhance cell proliferation through promoting the autocrine secretion of IL-6 probably via the induction of NF-κB [54]. Actually, triggering of TLR9 in B-cells results in the upregulation of several B-cell activation markers, including HLA-DR, CD25, CD80 and CD86, as well as the production of several cytokines and chemokines [110]. Additionally, TLR9 ligands seem to promote an immune evasion mechanism in MM cells through the IFN-γ–induced expression of PD-L1 in malignant plasma cells by triggering MyD88- and MEK-dependent pathway [43]. Despite these effects, TLR9 agonists are being explored as both monotherapy and in combination with chemotherapy, radiotherapy, or immunotherapy. In multiple tumours including breast cancer, lung cancer, melanoma, colon, cervical, pancreatic cancer and lymphoma, TLR9 agonists seem to inhibit tumour growth [111,112], may be due to its effects on B-cells. In combination, TLR9 activation and radiotherapy led to a more potent tumour-specific humoral response compared to single treatment [113]. Similarly, current evidence suggest that TLR9 agonists plus immunotherapy may enhance anti-tumour T cell responses and augment clinical benefits [114,115]. Actually, CpG ODN enhances the efficacy of immune checkpoint inhibitors in several types of cancer [116]. Moreover, the combination of CpG ODN and Poly I:C (TLR3 agonist) with dacarbazine (a common chemotherapy treatment) led to a significant increase in the inhibition of B16 melanoma lung metastases [117]. TLR9 activation can also modulate tumour microenvironment through DCs. CpG ODN in combination with a TLR2 agonist activate conventional DCs to augment IL-12 production promoting anti-tumour responses. This combination also reduces the number of tumour-infiltrating regulatory T cells dramatically due to downregulation of IL-10 production in a mouse epithelial lung cancer model [118]. In a MM environment, DCs activated by CpG inhibit growth and induce apoptosis in myeloma cells via secreted IFN-α [119]. Supporting this, C792, a TLR9 ligand, inhibits dendritic cell-induced MM cell growth and triggers apoptosis in both, in vivo and in vitro MM models. Moreover, C792 enhances the anti-MM activity of bortezomib, lenalidomide, SAHA and melphalan [120].

In addition, TLR9 ligands are also promising adjuvants for vaccines against infectious diseases and cancer. Actually, CpG ODN increase the retention of antigens in early endosomes, which is important for eliciting anti-tumour immunity [121]. Nowadays, some TLR9 ligands are being used as adjuvants in phase 2 clinical trials (NCT02254772, NCT00185965, NCT02115126, and NCT02927964) for lymphoma treatment.

3. New perspectives

The landscape of MM treatment continues to change as we develop a deeper understanding of its biology and its tumour microenvironment, including host immunity. In this review, we have explored the potential TLR-targeting approaches. Among massive cancer immunotherapy strategies available nowadays, Toll-like receptors highlight due to their effective activation of innate and adaptive immune cells, such as DCs, T cells, or macrophages. However, TLRs represent a double-edged sword with both anti-tumour and pro-tumour consequences in MM (Table 2). On one hand, TLR1/2/6 display pro-tumour effects; they can induce IL-6 dependent MM cell proliferation, increase adhesion to bone marrow stromal cells and promote the immune response evasion in MM cells. Similarly, TLR5 ligand stimulates NF-κB signalling and exhibits an increase in proliferation, IgG production and IL-6 expression, suppressing caspases and making MM cells resistance to apoptosis. TLR7 and TLR8 also inhibit apoptosis and promote MM proliferation and cell survival. Furthermore, TLR4 activation offers protection against apoptosis and immune system response and increases MM cell proliferation through the activation of the MAPK and NF-κB signalling pathways, and increased levels of IL-6 and IL-18. TLR4-mediated disruption of ER stress responses appears to contribute to MM cell proliferation and suppress ER-dependent death signalling which can be related to drug resistance. Therefore, the inhibition of these TLRs signalling might be a potential mechanism to abrogate inflammation-mediated effect in MM progression.

On the other hand, TLR9 and TLR3 could have key beneficial roles. TLR9 stimulation by CpG DNA promotes the immune evasion mechanism in MM cells, MM cells proliferation and protects these cells from serum-deprivation apoptosis. However, in a MM environment, dendritic cells activated by CpG inhibit MM growth and induce apoptosis in myeloma cells via secreted IFN-α. Interesting consequences can be also obtained with TLR3. This activation induces MM apoptosis by caspases activation and inhibits cell growth and proliferation. Additionally, TLR3 agonists stimulate immune system, boosting anti-tumour responses (see Fig. 2).

With the fast development of TLR-based agonist and antagonists targeted for therapeutic applications, examining TLR signalling within MM models may reveal novel therapeutic approaches. In fact, preclinical studies are showing promising results of TLRs agonists in several cancer models; nowadays there are more than one hundred clinical trials testing modulation of TLR in cancers, but none in relation to MM. The identification of the endogenous TLRs ligands in MM context would further make us to understand the mechanisms and effects of TLRs. In addition, a deeper knowledge about synergies and crosstalk between several TLRs would help us to explain MM context and develop novel therapeutic

Table 2
TLR-related molecules and their described effects on multiple myeloma.

Molecule	TLR	Effects on multiple myeloma
Pam3Cys-Ser-Lys4	TLR1/2 agonist	cell proliferation [41] increased cytotoxic and apoptotic effects of bortezomib [43]
Peptidoglycan	TLR2 ligand	upregulated PD-L1 expression [42]
HSP70	TLR2 ligand	IFNγ production [44]
Poly I:C	TLR3 agonist	apoptosis [63] apoptosis and reduction of inflammatory factors [62]
LPS	TLR4 agonist	cell proliferation, protection against apoptosis, and increased levels of IL-6 and IL-18 [75] cell proliferation and decreased apoptosis [77]
TAK-242	TLR4 inhibitor	mitophagy and apoptosis [78]
C34	TLR4 antagonist	cell growth reduction [80]
Flagellin	TLR5 agonist	enhanced cell viability and chemoresistance [81] cell proliferation and apoptosis resistance [66]
Loxoribine R848	TLR7/8 agonist	apoptosis inhibition and promotion of proliferation and cell survival [99]
CpG ODN	TLR9 agonist	cell growth inhibition and apoptosis [117]

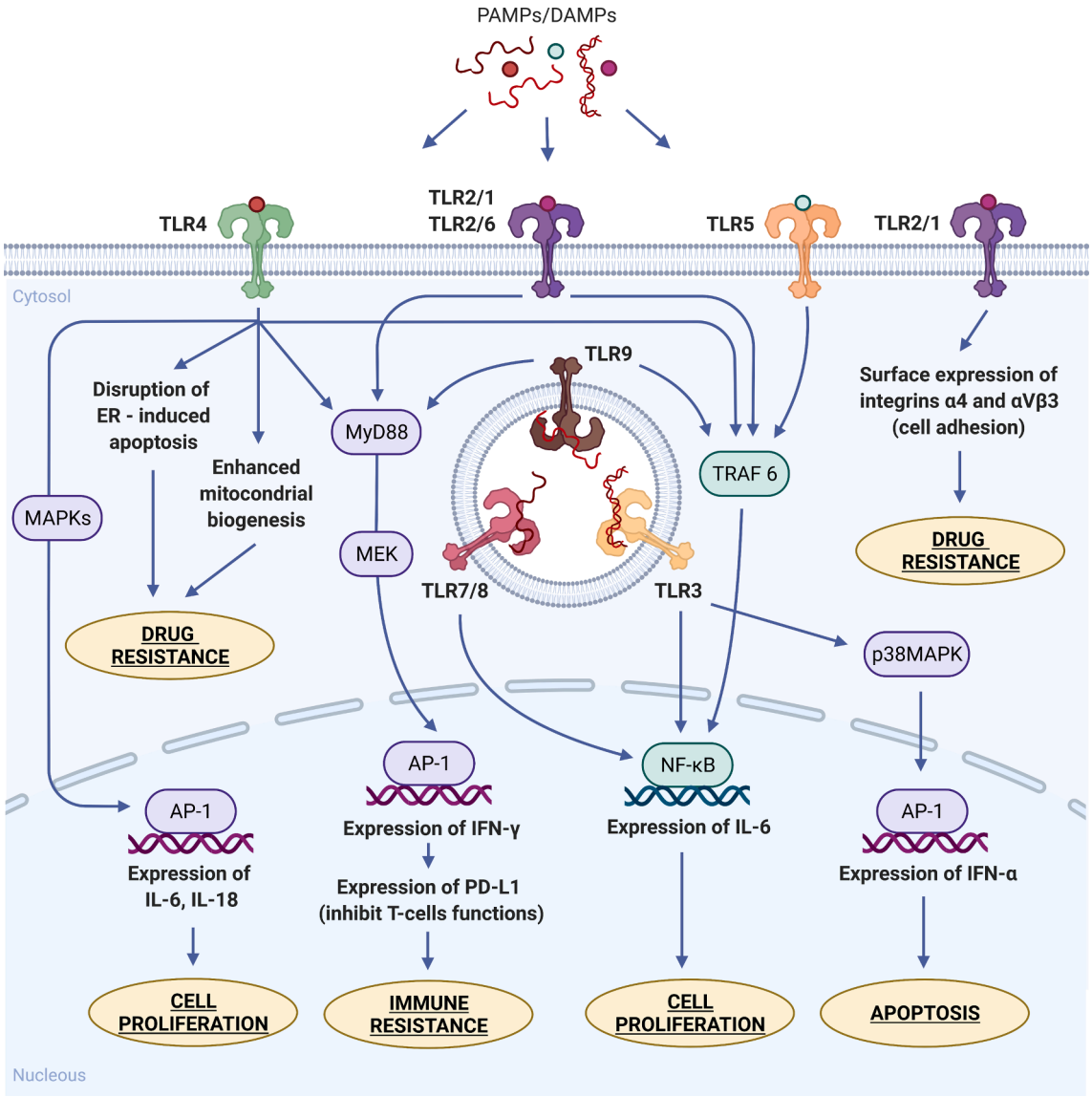

Fig. 2. Squematic direct effects of TLRs activation in multiple myeloma (MM) cells. MM cells present an aberrant expression pattern of TLRs, together with a chronic presence of PAMPs and DAMPs in the tumour microenvironment; this situation would lead to inadequate signalling in the cells. Here we represent the main consequences of the individual activation of each TLR in MM. The result of these activations has mainly pro- tumorigenic effects due to an autocrine stimulation, and these effects might be different when the TLRs are activated in other cells and/or several ligands are combined at the same time. The combination of TLR agonists with other treatments such as chemotherapy or radiotherapy could induce opposite responses.

approaches.

MM is associated with immune dysfunction. Defects in T-cell responses have been reported and regulatory T cell populations are significantly modified during MM transition, suggesting that immune dysfunction could be an early event in the malignant transformation process of plasma cells. However, the possible TLRs dysregulation during plasma cells transformation is still unknown and the potential role of altered TLRs signalling in MM progression remains unexplored. Tumour cells may escape from T cell surveillance by altering the balance of co-stimulatory and co-inhibitory molecular interactions. The PD-1/PD-L1 axis is the most studied pathway in MM. PD-L1 expressing cells may evade T cell attack via several mechanisms, including induction of apoptosis, anergy or exhaustion of T cells, increased production of immunosuppressive IL-10, and stimulation of Treg cell-mediated suppression. Malignant plasma cells express PD-L1 through a common pathway involving MEK/ERK and MyD88, which could be targeted therapeutically using TLR ligands to reduce PD-L1 expression, making MM cells more sensitive to T-cell attack [43].

Considering the essential role of bone marrow microenvironment components in myeloma tumour expansion, survival, invasion and drug resistance, TLR triggering may also contribute to adhesion-induced or *de novo* drug resistance of MM cells. In fact, TLR2/1 activation enhances adhesion to bone marrow stromal cells in parallel with an increased surface expression of integrins, and TLR4 signalling functions as a stress response strategy that protects mitochondria under exposure to BTZ, maintaining mitochondrial metabolism and driving drug resistance. Future preclinical and clinical studies are needed to address if TLRs can be exploited as novel therapeutic targets for MM. MM microenvironment encompasses a wide spectrum of cell types and extracellular matrix proteins, including fibronectin, collagen, laminin, and osteopontin, that can be detected as DAMPs.

Angiogenesis is considered a hallmark of MM progression. In MM patients syndecan-1, a heparan sulphate proteoglycan is overexpressed by myeloma cells in the bone marrow and peripheral blood [122]. The high levels of heparan sulfate in the tumour microenvironment resulting from syndecan-1 shedding also act as positive regulators that condition

the microenvironment for robust tumour growth. TLR4 activation seem to increase syndecan-1 expression in some immune cells as macrophages and dendritic cells [123,124]. Moreover, TLR signalling regulates the complex process of angiogenesis and control metalloproteinases expression [125]. In fact, TLR9 can stimulate tumour cell invasion and metastasis by regulating metalloproteinases.

Nowadays, new strategies for TLR agonist delivery to promote anti-tumour responses are being developed [126]. Engineered bacterial therapies that target the tumour immune landscape can offer a new class of cancer immunotherapy. Bacterial therapies can stimulate both innate and adaptive immune responses, change the immune dynamics of the tumour microenvironment, and offer unique strategies for targeting tumours cells by TLRs [127]. TLRs-based immunotherapy may be a beneficial approach for MM patients at both as adjuvants in vaccines and in treatments. Furthermore, TLRs agonists could play a fundamental role in the treatment of drug resistant cells.

TLRs could play critical modulatory effects in MM. Further research is essential to increase our understanding of the function and diversity of TLRs in MM. Although the activation of TLRs has key consequences for MM cells, they have not been sufficiently addressed to date and, in fact, delving into the mechanisms and relationship between different TLRs could hugely help to identify and exploit new therapeutic targets against multiple myeloma.

CRediT authorship contribution statement

Olaia Akesolo: Conceptualization, Resources, Writing – original draft. **Berta Buey:** Conceptualization, Resources, Writing – original draft. **Manuel Beltrán-Visiedo:** Writing – review & editing. **David Giraldos:** Writing – review & editing. **Isabel Marzo:** Writing – review & editing. **Eva Latorre:** Conceptualization, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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