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Modulation of normal and malignant plasma cells function by toll-like receptors

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1. ABSTRACT

Toll-like receptors (TLRs) are well known activators of immune responses, but their involvement in the plasma cell (PC) differentiation process remains mostly unknown. This review is focused on the expression and function of TLRs on normal PCs and their malignant counterpart, Multiple Myeloma cells. We report studies that suggest a role for TLR ligands as adjuvants of the humoral immune response through the survival of newly generated immature PCs. On the contrary, TLRs do not seem to be involved in the long-term maintenance of PCs in the bone marrow. Multiple Myeloma cells express a broad range of TLRs, and show heterogeneous responses to different ligands. These double-edged-sword effects are presented and discussed in the context of tumor progression, and as putative therapeutic targets.

2. INTRODUCTION

Vertebrates are frequently attacked by multiple infectious agents throughout their life. To counteract this danger, evolution has selected a panel of receptors that recognize these agents and alert the immune system. Among these receptors, Toll-like receptors (TLRs) play a major role (1). TLRs are type-I transmembrane proteins bearing a cytoplasm Toll/IL-1R homology domain (TIR). They recognize highly conserved specific structures from pathogens (bacteria and viruses) that are named pathogen-associated molecular patterns (PAMPs). So far, 10 and 13 TLRs have been identified in humans and mice respectively. For each TLR, one or several corresponding ligands have been identified, except for TLR10, which is not functional in the mouse. Each TLR has a different specificity in terms of PAMP recognition (2-7). In addition

to responding to PAMPs, TLRs also respond to endogenous molecules, which are mostly produced during cellular stress or death as heat shock proteins, products of extracellular matrix degradation, and endogenous RNA or DNA (8-10). They are named danger-associated molecular patterns (DAMP). The engagement of TLRs by their ligands induces various signalling pathways (11). TLRs first dimerize and recruit several TIR-domain-containing adaptor molecules: MYD88, TIRAP, TRIF, and TRAM. MyD88 is used by all of the TLRs except for TLR3, whereas TRIF is used only by TLR3 and TLR4. Activated signaling pathways involve NF-kB, mitogen-activated protein kinases (MAPKs) and PI3k/Akt that lead to cell activation, maturation, type I interferon and inflammatory cytokine production.

Murine and human B cells express several TLRs whose level of expression and responsiveness varies depending on the B-cell subsets (12, 13). *In vitro*, TLR activation of B cells results in the activation, proliferation and differentiation into immunoglobulin-secreting plasma-cells (PCs) (12). Memory B cells have a greater capacity to proliferate and differentiate into PCs after TLR stimulation than do naïve B cells. This has been supported by a study showing that polyclonal activation of human memory B cells through TLRs is essential for the maintenance of serological memory (14). Other *in vivo* experiments support the notion that TLRs are adjuvants that accelerate antibody responses, but are not essential for long-tem humoral responses (15-17). Altogether, these studies shed light on the importance of TLRs in the initial steps of B cell activation. However, B cell differentiation into immature PCs and then matured long-lived PCs is a multistep process that could be influenced by many cytokines and cell-cell interactions (18). A growing body of literature suggests that PAMPs could also target PCs on their way toward terminal differentiation, and their malignant counterpart that is Multiple Myeloma (MM). The scope of this review is therefore to provide an overview of the literature addressing TLR expression and function in PCs from normal and malignant origin.

3. PLASMA CELLS ARE EFFECTORS OF HUMORAL RESPONSES

3.1. Plasma cell differentiation is a multistep process

Protective humoral immunity is provided by PCs through their production of antibodies (19, 20). PCs arise by two different routes: one occurring inside the germinal center of secondary lymphoid organs, and one occurring outside the follicle. Several parameters influence the decision to follow one route or the other, including the nature of B cells that are activated (naïve versus memory), the nature of the antigen, and the affinity of the B cell receptor (BCR) for the antigen. For example, in the absence of T cell signaling, memory B cells are the only cells to differentiate into PC upon TLR activation. PCs arising from the germinal center route are generally considered immature and are precursors of long-lived PCs that are able to migrate within the bone marrow and produce a high amount of highly specific antibody. On the contrary, PCs of extrafollicular origin are short-lived, as most of them do not migrate to the bone marrow and these produce only a small amount of antibody. The immature PCs exit the lymphoid organs into the peripheral blood and continue to differentiate into fully mature PCs upon recruitment into an appropriate site of survival (mucosal tissues, bone-marrow or inflamed tissues) (21-23). The differentiation of B cells into immature PCs and then into fully mature antibody secreting cells involves profound molecular changes. Several transcription factors that negatively feed back onto each other are involved in this process (Pax5, Blimp-1, XBP-1 for example) (24, 25). Profound morphological and phenotypical changes also occur during this differentiation. Immature PCs are also called plasmablasts, as they are proliferating cells with low Ig secreting capacities. They are detected in the blood six to eight days after an antigenic challenge. They are also found in secondary lymphoid organs, such as tonsils, but probably at a later stage of maturation (26). Thanks to the study of reactive plasmacytoses that are expansions of plasmablasts retaining the capacity to differentiate into mature PCs, the phenotype of these circulating immature cells has been characterised (CD19+, CD45++, HLA-DR++, CD38++, CD138-). This phenotype switches to CD19+/-, CD45+, HLA-DR+/-, CD38+++, CD138+ upon differentiation into mature PCs (27). Of note, tonsillar plasmablasts have a low expression of CD138. Plasmablasts are short-lived but can be transiently rescued from apoptosis by IL-6, IFN-alpha or plasmacytoid dendritic cells (28, 29).

3.2. Rescuing immature plasma cells from apoptosis in cellular niches

Although long-lived PCs are responsible for sustaining serological memory, they represent a minor fraction of cells within the bone marrow in humans and mice (0.1 to 1% of bone marrow cells) (30). Interestingly this pool of PC is quite constant over a lifespan, despite infections that each potentially generates 104 to 105 new cells (31). The size of the PC population must therefore be tightly regulated. One hypothesis is that newly generated PCs must compete with previously formed PC to access and occupy a limited number of survival environmental niches (32-34). In other words, following infection or vaccination, newly generated PCs are prone to die if they do not find an appropriate niche. At the same time, existing PCs from previous infections are displaced from the bone marrow by these new PCs, circulate in the blood where survival factors are limited, and eventually die. Evidently, this replacement would not result in the eradication of older PCs but would probably affect a minor portion of the PC pool as the repertoire of antigen specificity is maintained over years (20).

What is meant by “long-lived” with regards to PCs? Recent studies have tried to evaluate the duration of the humoral response after the first antigen encounter. It has been shown that long-lived antibody responses after diphtheria and tetanus toxin vaccination can last from ten to twenty years, whereas a response against a live viral infection could last much longer (35, 36). Furthermore, an earlier study estimated the half-live of virus-specific PC in mice as 172 days in the spleen and 94 days in the bone marrow. Both half-lives are quite long compared with the lifespan of the mouse itself (37).

Many soluble factors and cell-cell interactions constitute the bone marrow niche. Stromal cells play an initial role through stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4, as PCs express CXCR4 and are attracted by SDF-1 (38). Furthermore, CXCR4 deficient mice have a marked decrease in the bone marrow homing of PCs (39). In addition to bone marrow stromal cells, osteoclasts support the survival of PCs from immature to mature stages *in vitro* (40). Recently, neutrophils have been shown to co-localize with PCs in the bone marrow niche, and strongly support PC survival through the production of IL-6 and APRIL (41). This last report confirms that members of the tumor necrosis factor (TNF) family such as BAFF, APRIL, TACI and BCMA, seem to be involved in the maintenance of long-lived PCs (42, 43). Regardless of the cell to cell interactions, or the cytokine cocktail present in the niche, signalling probably converges on the same target gene, i.e, prdm-1, the gene encoding Blimp-1 (B-lymphocyte-induced maturation protein-1). Blimp-1 is instrumental in the survival of PCs in the bone marrow, as conditional deletion of prdm-1 induces a disappearance of long-lived PC from the bone marrow (44). Of note, besides the many cytokines found in the bone marrow, LPS and CpG-ODNs are also able to induce Blimp-1 expression *in vitro* (45).

PCs therefore appear as a heterogeneous population when characterized over the differentiation process, from their site of origin in secondary lymphoid organs to their site of long-term survival. They depend on microenvironmental niches for their survival and final differentiation. It could therefore be hypothesized that TLR signaling could aid these “PCs-to be” during the course of the infection.

4. MULTIPLE MYELOMA : A MALIGNANT PLASMACYTOSIS

Multiple myeloma (MM) is a fatal plasma cell malignancy that is characterized by excess monoclonal bone marrow plasma cells (PC), osteolytic bone lesions, renal failure and immunodeficiency (46, 47). Malignant PCs secrete monoclonal immunoglobulin (IgG and IgA). In the USA, MM is the second most frequent blood malignancy after non-Hodgkin lymphoma. The disease accounts for about 1 % of all neoplastic diseases and 10% of all hematological malignancies.

In recent years, considerable advances in the genetic and phenotypic characterization of MM have been made (48-51). MM appears as a heterogeneous disease in terms of phenotype, genotype, growth factors and drug responses. The cellular origin of MM PCs is still a matter of debate (52) but the disease always emerges from a state of medullar plasmacytosis often seen in the elderly population, characterized by the presence of serum monoclonal IgG or IgA with no sign of organ failure or bone resorption (53). This premalignant state is known as Monoclonal Gammopathy of Undetermined Significance (MGUS). Numerous kariotic and genetic abnormalities are seen in MM PCs at an early state of disease progression (54). MM is associated with translocations of the heavy chain immunoglobulin gene (14q32) with different oncogenic partners that could be CCND1 (t (11;14)), CCND3 (t (6;14)), MAF (t (14;16)), FGFR3 (t (4;14)) or MAFB t (14;20)). A partial or complete loss of chromosome 13 can also be observed in MM (55).

Thanks to array comparative genomic hybridization, the genetic heterogeneity of MM patients has been classified into 7 groups (56). CD-1 and CD-2 groups are characterized by overexpression of cyclin D1 and D3. The MS and the MAF groups have an overexpression of FGFR3 or MAF respectively. The HY group represents 50% of patients with a hyperdiploid profile. The LB group has a low level of osteolytic lesions and, finally, the PR group is characterized by overexpression of genes associated with proliferation. Secondary late onset translocations and gene mutations can be implicated in the progression from MGUS to overt MM such as MYC (57), NRAS, KRAS and FGFR3 mutations (58).

The bone marrow microenvironnement of MM PC constitutes a cellular niche as it has an essential role in the development, maintenance and progression of the disease (59). Direct interactions between MM cells and bone marrow stromal cells or extracellular matrix proteins promote and sustain MM growth factor secretion together with cell survival signaling pathways. Among many other bone marrow cytokines, IL-6, IGF1, and members of the TNF superfamily such as BAFF and APRIL play a major role (60, 61). In addition to soluble factors, receptors involved in the adherence of MM to the bone marrow niche such as integrins, cadherins, selectins, syndecans also play a major role in the medullar niche (62).

5. TLR EXPRESSION AND SIGNALING IN NORMAL AND MM PLASMA-CELLS

Tonsillar PCs express all TLR (except 10) at levels comparable to tonsillar naïve and memory B cells (63). This expression does not seem to be altered after exiting secondary lymphoid organs as a similar pattern has been found in peripheral blood plasma cells. PCs finally mature in the bone marrow where they become long-lived PCs (37). It has been shown that they express either only significant levels of TLR1, RP105, MD-1 and MD-2 (64) or of TLR1, 5 and 9 (65). These studies (as for most studies on TLR expression) suffer from a lack of reliable protein detection. Indeed, commercially available TLR antibodies fail to give satisfactory results in human cells. Therefore, once available, reevaluation of TLR expression by other means than RT-PCR might help to definitively address the question of the TLR expression profile during PC differentiation. Fortunately, a recent study addressed the question of the level of TLR9 mRNA that correlates with a significant cellular response. This quantitative RNA approach circumvents the problems of protein detection (66). However, the authors show that, above the defined threshold of TLR9 expression, the magnitude of response to CpG-ODN is dependent on tumor cell type.

MM primary cells and human myeloma cell lines (HMCLs) express a broad range of TLR (64, 65, 67-70). A study of primary MM cells from 414 patients at diagnosis

Table 1. Heterogeneous expression of TLR3, 4, 7 and 9 on primary myeloma cells

	TLR3				TLR4				TLR7				TLR9			
	pos % (n)	p value	median (range)	p value	pos % (n)	p value	median (range)	p value	pos % (n)	p value	median (range)	p value	pos % (n)	p value	median (range)	p value
PR (47)	15 (7)	NS	223 (137-1654)	NS	17 (8)	NS	203 (77-742)	NS	47 (22)	NS	923 (212-4590)	0.018	79 (37)	NS	706 (241-2454)	NS
LB (58)	12 (7)	0.014	273 (137-556)	NS	12 (7)	0.002	98 (53-339)	0.007	14 (8)	<0.001	433 (102-1493)	NS	74 (43)	NS	724 (302-1902)	NS
MS (68)	32 (22)	NS	259 (76-751)	NS	42 (29)	0.024	161 (61-837)	NS	15 (10)	<0.001	194 (121-1320)	0.009	87 (59)	NS	1228 (360-5422)	0.003
HY(116)	38 (44)	<0.001	311 (84-1566)	NS	16 (19)	<0.001	103 (53-888)	<0.001	46 (54)	0.001	613 (68-6167)	0.042	77 (90)	NS	587 (228-2071)	<0.001
CD (88)	19 (17)	NS	233 (107-904)	NS	31 (27)	NS	172 (44-419)	NS	39 (35)	NS	375 (99-7584)	NS	86 (76)	NS	797 (319-5050)	NS
MF (37)	30 (11)	NS	465 (168-834)	NS	97(36)	<0.001	1447(195-9266)	<0.001	32 (12)	NS	232 (92-2436)	0.036	65 (24)	0.031	665 (299-1632)	NS
All (414)	26(108)		296 (76-1654)		30(126)		201 (44-9266)		34(141)		450 (68-7584)		79(329)		744 (228-5422)	

Expression of TLR3, 4, 7 and 9 on MM primary cells with respect to molecular classification as defined by Zhan et al.: PR: proliferation, LB: low bone disease, MS: MMSET, HY: hyperdiploid, CD: cyclinD, MF: MAF. Median and range represent TLR mRNA expression in relative units. p values are determined using CHI2 test for positive samples expression and using Wilcoxon test for expression level. Significant over-expressions and under-expressions are represented in bold and italic respectively. NS = non-significant. Data can be accessed on the website http://amazonia.transcriptome.eu/. Reprinted with permission from Elsevier (110).

revealed an almost universal expression of TLR9, and around 30% expressed TLR3, 5 and 7. A comparison of TLR levels of expression by real-time PCR showed that TLR2, 4 and 9 were significantly higher expressed in bone marrow mononuclear cells from MM patients than in control donors (65). Even if the expression on PCs was not directly addressed, this increase suggests that cells of MM bone marrow have good potential to respond to PAMPs. In addition to TLRs, their signaling proteins seem to be expressed in the majority of HMCLs, ie, MYD88, TRIF, and TRAF6 (personal data and (68, 71). TRAF3 expression, however, seems less frequent as it is mutated or deleted in 12,3% of primary MM cells and 17% of HMCLs (72). This lack of expression could impact the growth of MM cells as TRAF3 is a known inhibitor of the non-canonical NF-kB pathway, and could participate to sustain NF-kB signaling. Furthermore, TRAF3 negative MM cells could be more sensitive to TLR ligands. This is supported by the finding that TRAF3 transgenic mice show an increased response to antigen and TLR agonists, which evolves over time into plasmacytosis and hypergammaglobulinemia (73). Distribution of the TLR expression pattern appears heterogeneous when the molecular classification of patients defined by Zhan described above is addressed (Table 1). Indeed, patients with a favorable outcome, such as hyperdiploïd patients, express TLR3 and TLR7 more frequently compared to other groups, but lower levels of TLR4 and 9. Inversely, TLR9 and 4 are overexpressed by MS and MAF groups that both have a poorer prognosis. As Maf is a transcription factor, it could be of interest to determine its involvement in TLR4 expression. On HMCLs, a pattern similar to primary MM cells is observed. Indeed, TLR1, 7, and 9 are the most strongly expressed (96%, 68% and 64% of cell lines respectively). TLR3 and 4 are expressed in nearly half of the HMCLs, whereas TLR2 and 8 are only detected in 28% and 24% of the cell lines, respectively (personal data, n=25).

6. COULD MM PLASMA CELLS ENCOUNTER TLR LIGANDS ?

In MM, normal PC differentiation and survival is blunted due to competition with MM PC for the medullar niche. As a consequence, the level of normal IgG is low and patients are highly susceptible to recurrent and persistent bacterial, fungal, and viral infections. Next to general organ failure, infections are a main cause of death for MM patients (46). A retrospective epidemiological study based on 4641 MM and 2046 MGUS patients has shown that the risk of developing the disease is significantly increased when patients have a history of multiple infections such as pneumonia, hepatitis, meningitis, septicemia, herpes zoster, and poliomyelitis. Risk was also associated with influenza infection, but only for white men (74). Furthermore, a history of pneumonia in the 5 years preceding MGUS appearance has been shown to be a predictor of MGUS risk (75). This finding suggests that infectious agents by themselves, or infection-associated inflammation could be potential triggers for MGUS and/or MM development.

During the course of the disease, several pathogens are associated with MM, such as Gram-positive bacteria (*Streptococcus pneumonia, Staphylococcal* infections) and Gram-negative bacteria (*Pseudomonas, Haemophilus influenzae, Escherichia coli*) as well as invasive fungal infections. Numerous sources of PAMP are therefore present in or even associated with MM. One should not forget, however, that even in the absence of a clear correlation of MM progression with a particular infectious agent, the bone marrow environment is still a soup of DAMP. Indeed, MM cells deregulate the balance of bone resorption/formation by inhibiting osteoblastogenesis and promoting osteoclastogenesis. The consequence is a dramatic bone resorption that impairs the patient's quality of life, increasing the release of extracellular matrix proteins, fibronectin, collagen and inflammatory mediators

(76). Many of these microenvironment components are described as TLR agonists (77).

High Mobility Group Bex1 (HMGB1) protein is the most abundant chromatin-associated non-histone protein expressed in all nucleated eukaryotic cells. It is also released in the extracellular medium by macrophages after TLR stimulation or during cell death (78). HMGB1 is described as a TLR4 agonist and a TLR3, 7 and 9 co-ligand (79, 80). HMGB1 accelerates the delivery of CpG-ODNs to its receptor (81). Interestingly it is released by bone cells such as osteoblasts and osteoclasts in the bone marrow environment. HMGB1 could therefore act as a co-factor for TLR ligand recognition in the MM niche (82). Furthermore, soluble CD138, a molecule belonging to the heparan sulfate family, is a potent TLR4 agonist, and is highly expressed and released by MM PCs. Of note, the soluble CD138 level correlates with poor prognosis (83).

In conclusion, despite the heterogeneous expression of TLR on MM cells, it is highly probably they will encounter a PAMP or DAMP for which they bear the corresponding TLR.

7. FUNCTION OF TLR IN NORMAL PLASMA CELLS

The strong expression of multiple TLR observed on tonsillar and peripheral PCs suggests that they could respond to a broad range of ligands. However, only a few studies have addressed this question, and it remains crucial to have a complete understanding of the mechanism of humoral responses for vaccine design. PCs located in the tonsils or plasmablasts circulating in the peripheral blood are phenotypically and functionally different from the long-lived bone marrow PCs. This discrepancy is also observed for TLR responses. Indeed, PCs from tonsils and peripheral blood have an increase of total Ig synthesis and secretion upon TLR triggering (63). TLR1/2, 3, 5 and 9 are mainly involved in Ig secretion in tonsillar PCs. TLR1/2 ligands exclusively increase intracellular IgM expression and secretion. TLR3 (poly (I:C)) and 9 ligands (CpG-ODNs) increase IgG expression from tonsillar PCs, whereas TLR7 ligand increases IgG expression only in peripheral blood PCs (63). In addition, our personal data suggest that plasmablasts have increased survival and total immunoglobulin secretion in the presence of TLR9 ligands (unpublished data). On the contrary, a study of normal bone marrow mononuclear cells (as opposed to purified PCs) has shown a lack of response (65). Neither an increase of CD138+ cells (a marker of mature PCs), nor a decrease in apoptosis was detected in the presence of TLR1/2 ligand (Pam3), TLR4 ligand (LPS) or TLR9 ligand (CpG-ODNs). These results are in agreement with an absence or a low TLR expression on mature PCs.

Therefore, TLR expression and functions seem to differ accordingly to their developmental stage. Fully matured and long-lived PCs are quiescent cells that do not seem to be affected by infections or PAMPs as plasmablasts are. They are either less sensitive, or completely insensitive to pathogen-derived signals of activation, and their continuous antibody secretion is not altered. This lack of sensitivity is not a hallmark of bone marrow cells, as TLR ligands directly induce human and mouse hematopoietic stem cells to differentiate towards myelopoïesis (84-86).

8. FUNCTION OF TLR IN MULTIPLE MYELOMA CELLS

8.1. TLR on MM cells as protumoral effectors

Can we consider TLRs as receptors for protumoral ligands (either PAMPs or DAMPs) present in the bone marrow microenvironment? In other words, can MM cells hijack the TLR machinery for their own benefit? This question is worth addressing due to the significant expression of TLR and their signaling molecules found in MM cells. This question can be considered on three different levels: first cell growth, second apoptosis, and finally immune escape.

Proliferation of several HMCLs and primary MM cells can be increased by most of the TLR ligands when receptors are present (64, 67). Most studies, however, focus only on ligands of TLR4 and 9. The increase in proliferation is mainly mediated by an autocrine IL-6 loop that can substitute paracrine IL-6. Indeed, IL-6 is a major proliferation and survival factor for MM cells (87, 88) and circulating IL-6, as well as circulating soluble IL-6R, was found to increase during MM progression (89). In MM, IL-6 originates from the bone marrow microenvironnement. Therefore, this supplementary contribution could enhance the pathogenic potential of the milieu where MM cells emerge, and facilitate their growth or maintenance in the case of an altered niche (as a treatment consequence for example). Autocrine TLR-induced or paracrine IL-6 is not the only cytokine that stimulates growth, as Insulin growth factor -1 (IGF-1) besides IL-6 (either autocrine or paracrine), is also a major cytokine stimulating MM cell growth. However, so far no increase in expression of IGF-1 has been reported after TLR triggering. This could be worth investigating, as TLR2 and 4 agonists can induce an IL-6 independent proliferation of the RPMI-8226 HMCL (64).

Protection against various apoptotic conditions is also a feature of TLR stimulation. Serum or IL-6 deprivation is, accordingly to the IL-6 induction, compensated by TLR4 and 9 (67). Drug-induced apoptosis by dexamethasone or adriamycin (both effective drugs for MM) is partially or totally inhibited by pretreatment of HMCLs by LPS or CpG-ODNs (67, 90). In addition to the action of chemotherapy several effectors of the immune system (CD8+ T cells, gamma-delta T cells and NK cells) have the potential to kill primary MM cells or HMCLs in both antigen-dependent or independent mechanisms (91-93). However, due to the age of MM patients (the median age at diagnosis is 65) and their immune deficiency (for example MM dendritic cell dysfunction (94), and regulatory T cell accumulation (95)) that is somehow common to most cancer patients, immune effectors from MM patients have limited action towards malignant cells. To boost vaccination strategies with myeloma-derived antigen, adjuvant therapies using bacterial extracts, such as

BCG (TLR2, and 4 ligands) streptococcus preparation (TLR4 ligand), imiquimod (TLR7 ligand), or CpG-ODNs (TLR9 ligand) have been proposed. Synthetic TLR9 ligands CpG-ODNs are currently the most used adjuvants in cancer therapy. To enhance their half-life from a few minutes to two days (96) and to enhance affinity to TLR9, CpG-ODNs are chemically modified to replace the native phosphodiester backbone by a phosphorothioate (PS) backbone. However, this modification has significant drawbacks, because it induces unexpected TLR9-independent side effects (69, 97, 98). Indeed, synthetic PS-modified CpG-ODNs inhibit the killing of various TRAIL-R2 (DR5) sensitive tumor cells (MM, breast carcinoma and colon carcinoma) through a TLR9-independent, but PS-dependent binding to TRAIL-R2 ligand. Moreover, this inhibition reduces the TRAIL-mediated cytotoxicity of NK cells, and could therefore interfere with the clinical efficiency of a TLR9 agonist based adjuvant. Similarly, the apoptosis induced by the bone marrow-originated bone morphogenetic protein-2 (BMP-2) is inhibited by PS-modified CpG-ODNs in a TLR9 independent manner (98). Encapsulation of CpG-ODNs, which avoids unspecific binding with unexpected consequences, could be used, especially in clinical trials.

The proliferation and cytotoxicity of NK cells and T lymphocytes can be inhibited by immunoregulatory molecules expressed on or released by tumor cells such as B7-H1 and B7-H2 (99). B7-H1 is a ligand for PD-1, and is mainly induced by IFNg in normal cells. Both molecules are overexpressed in MM cells, but not in plasma cells from MGUS patients. TLR4 agonists significantly enhance B7-H1 and B7-H2 expression through a MyD88/TRAF6-dependent pathway involving MAPKs ERK1, 2 (68). Accordingly, LPS and IFN-gamma inhibit both CTL generation and T lymphocyte proliferation with the same capacity, suggesting that B7-H1/2 expression could be a responsible for the effect. Therefore TLR4 and 9 signaling, in addition to their classical adjuvant function on the immune system, could have an unwanted effect on tumors leading to immune evasion.

Several signaling pathways could mediate the proliferative/survival signals triggered by TLR ligands. MM cells have a strong NF-kB activity, owing to activating mutations of NIK, NF-kB1, NF-kB2, TRAF2, TRAF3, or CYLD in 15-20% of the myeloma cells (72), and to NF-kB-inducing cytokines produced by the microenvironement, such as TNF-alpha, Baff or RANKL (100, 101). TLR ligands could be added to this list, acting through either the MYD88 or TRIF-dependent pathway. Indeed, NF-kB activation has been shown in several HMCLs in response to TLR3, TLR4 or TLR9 ligands (65, 66, 70). IL-6 production and IL-6-induced proliferation depend on NF-kB in most HMCLs (65, 70). MAP kinases Erk1, 2 and JNK have also been implicated in MM proliferation (90), or in the overexpression of B7-H1 (68).

8.2. TLR on MM cells as therapeutic targets

We have learned from seminal works on dendritic cells that responses to TLRs are heterogeneous, depending not only on TLR structure and signaling adaptators diversity, but also on the subsets of dendritic cells (102). A similar model can be applied to non immune cells and to cancer cells. In addition to pro-survival signals, which reflect mainly NF-kB and MAP kinase-dependent transcription of genes, apoptotic messages can be generated upon TLR2, 3, 4, 7 or 9 triggering in various cancer cell types (103). TLR3, which signals through a MYD88-independent/TRIF dependent pathway, is the best characterized. The synthetic TLR3 agonist (poly (I:C)) is known to directly induce apoptosis in several solid cancer cells and has been used in clinical trials as an immune activator. This dual anti-tumoral characteristic turns it into a particularly interesting therapeutic target (104). TLR3 is expressed in nearly 50% of HMCLs, and 25% of primary myeloma cells. We found that poly (IC) induces growth inhibition or apoptosis in three out of six TLR3 positive HMCLs studied. In accordance with the results highlighted in HMCLs (64, 70), a decrease of primary myeloma cell viability was observed in two out of four patient samples after *in vitro* poly (IC) stimulation (personal data). TLR3-dependent apoptotic process in MM was found to be linked to the production of an autocrine IFN-alpha loop, controlled by early p38 MAPK activation (70). Involvement of p38 MAPK activation in IFN-alpha secretion induced by TLRs seemed to be a conserved pathway in MM and pDCs at least (105). Moreover, poly (IC)-induced apoptosis in MM is dependent on caspases 3, 8, and 9. Implication of caspases in poly (IC)/IFN-alpha-dependent apoptosis is in accordance with the results described in melanoma or breast cancer cells (104). TRAF3, a major regulator of type I IFN, does not seem to be involved in the poly (IC) effect as it is mutated and non-functional in some HMCLs induced in apoptosis.

However, we observed that poly (IC) also induced NF-kB activation in all TLR3 positive cell lines, independently of IFN-alpha secretion. In the presence of IFN-alpha, NF-kB could not overcome the apoptosis signal. This suggests that MM cell fate after TLR triggering will depend on the balance of survival versus apoptotic signals. Therefore, to shift the balance in favor of apoptosis, a possible therapeutic approach would be the use of a TLR3 ligand to stimulate the immune system, together with inducers of MM cell death. Activation of the immune system could also be achieved by targeting TLR9 on the surface of plasmacytoid dendritic cells from patients. Indeed, MM-plasmacytoid dendritic cells exhibit altered function and reduced ability to induce T-cell proliferation, which can be reversed by stimulation with CpG-ODNs (106).

9. CONCLUSION

At any one time, a body's population of PCs is heterogeneous, with regard to their localization (mucosa, secondary lymphoid organs, bone marrow), their origin (follicular or extrafollicular), their half-life, and their stage of maturation (plasmablasts, or mature PCs). Similarly, TLR expression and function seem to follow the differentiation process as plasmablasts or PCs from tonsils, but not mature bone marrow PCs, respond to TLR stimulation. Immature PCs and PCs located within sites of

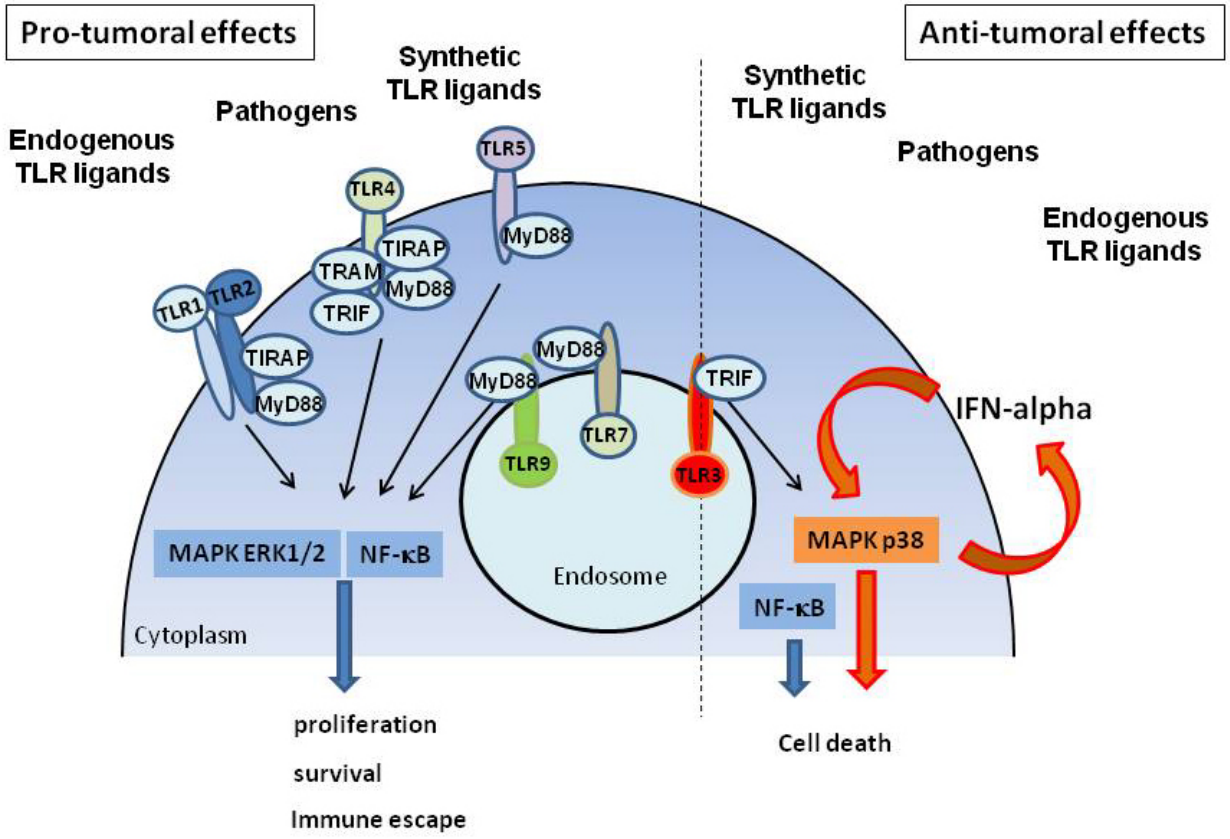

Figure 1. Overview of the consequences of TLR activation on MM cells. Protumoral effects on MM cells have been observed with ligands of TLR2/6, TLR4, TLR5, TLR7/8, and TLR9. These ligands trigger signaling pathways as MAPK ERK1/2 and NF-kB. Cellular outcome are proliferation, resistance to apoptosis and immune escape. On the contrary, on some primary MM cells or HMCLs, TLR3 ligand induces IFN-alpha secretion through MAPK p38 signaling pathway and induces cell death. The apoptosis is observed despite NF-kB activation. This finding suggests that IFN-alpha production is a molecular determinant of the TLR triggering outcome.

B cell activation or pathogen encounter could be therefore still be activated by surrounding molecules from the pathogen during the course of an infection. Later on, once the immune system has been fully mobilized and PCs have found a molecular niche to persist for years in the absence of antigen, the effect of TLRs is no longer needed for the persistence of the serological memory. This review of the literature suggests that TLRs act as an adjuvant of humoral immune responses not only by targeting B cells, but also plasmablasts and immature PCs.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the expansion of autoreactive plasmablasts and PCs (107, 108). Interestingly, many DAMP are present in SLE patients, and endogenous TLR7 and TLR9 ligands have been shown to promote the disease through dendritic cell stimulation (109). IFN-alpha is a major cytokine in SLE physiopathology and a survival factor for PCs. In this context it is of interest that TLR3 is expressed on peripheral blood PCs, and that its stimulation can initiate the production of IFN-alpha by MM PCs. This raises the question if this cytokine production is also a characteristic of normal and SLE plasmablasts. Given this review of the literature, TLRs ligands might participate directly in SLE, contributing to the survival and expansion of the effectors of the disease.

The capacity to respond to TLRs ligands is conserved by MM cells, the malignant counterpart of PCs. MM cells express a broad and heterogeneous range of TLRs associated with various responses *in vitro* (Figure 1). Heterogeneous responses to TLR ligands are also observed in other hematological and solid malignancies, resulting in either pro-tumoral effects or induction of apoptosis. Thus, cancer therapies using TLRs ligands should first identify predictive biomarkers of apoptotic responses to TLR ligands. Furthermore, the uncoupling of the pro-tumoral effects of TLR ligands from their adjuvant effects on the immune system is mandatory. This could be achieved by a simultaneous neutralization of MM growth factors or survival pathways. Is there a role for PAMP or DAMP in the emergence of MM? Due to the lack of *in vivo* data available in animal models, we are restricted only to

speculate on this. A select few epidemiological studies, together with the appearance of strong TLR expression and co-receptors during the MGUS to MM transition, support this hypothesis. Given their immune adjuvant function during infection or vaccination, TLR ligands could therefore perhaps also act as "tumoral adjuvants" in the context of malignant clone emergence. In this scope, the study of TLR expression and function in MM stem cells is worth being investigated.

10. ACKNOWLEDGEMENTS

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Abbreviations: TLRs: Toll-like receptors. MM: Multiple Myeloma. PC: Plasma-cell. TIR : Toll/IL-1R homology domain. PAMP : pathogen-associated molecular patterns. dsRNA : double stranded RNA. LPS : lipopolysachcaride. CpG : unmethylated 2'-deoxyribo (cytidine-phosphtae-guanosine) . DAMP: danger-associated molecular patterns. MAPKs: mitogen-activated protein kinases. SDF-1: stromal cell-derived factor-1. TNF: tumor necrosis factor. Blimp-1: B-lymphocyte-induced maturation protein-1. MGUS: Monoclonal Gammopathy of Undetermined Significance. HMCLs: human Myeloma cell lines. HMGB1: High Mobility Group Box1.

Key Words: Toll-like receptor, Plasma-cell, Plasmablast, Bone-marrow, Multiple myeloma, Humoral, Review

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