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Immunostimulatory Effects of Triggering TLR3 Signaling Pathway — Implication for Cancer Immunotherapy

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1. Introduction

Immunostimulation based therapies hold promise of disease-specific interventions without the toxicity and other side effects, which are associated with traditional therapeutic modalities. Unfortunately, the exact manifestation of their therapeutic benefits and the mechanisms of their action are still pending for most immunotherapeutic strategies. Virtually, such kinds of treatments are ideal to establish standards of care and secure, as well as improve therapeutics outcomes. A growing interest for studying and understanding immunostimulation based therapeutics' strategies has increased since, it could stimulate different components of innate immunity and consequently stimulation of adaptive immunity [1-3].

These immunostimulation based therapies can be divided into two categories, nonspecific and specific immunotherapies. Nonspecific immunotherapy describes therapies that are designed to enhance the immune response without deliberately seeking to modulate the response to a particular antigen. These therapies include three main categories. The first depends on administration of certain agents that induces secretion of inflammatory cytokines that nonspecifically activate the immune system [4]. The second depends on administration of certain type of cytokine that can activate specific populations of immune cells. The third category include the administration of antibodies that block immune checkpoints or other suppressive pathways that are known to shut down the activity of immune system [5]. These nonspecific activation pathways are crucial for the initiation of fully functional immune responses encompassing both the innate and adaptive immune compartments [4].

Toll like receptors (TLRs) are a group of receptors that are expressed mainly in innate immune cells, including macrophages, monocytes, dendritic cells, natural killer cells and mast cells [6]. These TLRs specifically recognize microbes as well as different microbial components called TLR ligands. Interaction of TLR and TLR ligands trigger signaling pathways that lead to activation of innate immune cells through secretion of a plethora of inflammatory mediators including cytokines and chemokines [7, 8]. As such, different TLR ligands have been designed to be used as adjuvant system,

The inclusion of TLR ligands as a potential new class of adjuvants candidates has enabled the development of clinical effective vaccination strategies against many diseases [9, 10]. Polyinosinic-polycytidylic acid [Poly(I:C)] and its clinical grade poly-L-lysine (Poly-ICLC; Hiltonol®) represent important members of these immunostimulatory vaccine adjuvants as has been shown in several preclinical and clinical studies [11, 12]. In this review, we will introduce a brief description of the TLR family followed by a description of the signaling pathway of TLR and its role as a linker between innate and adaptive immunity. The structure, expression and function of Poly(I:C) will be reviewed along with its potential application in cancer vaccination, adoptive immunotherapy and chemo-immunotherapy in preclinical and clinical trials.

2. Toll-like receptors (TLRs) and their agonists

TLRs are a class of transmembrane signaling proteins that play a critical role in initiation and acceleration of innate and adaptive immune responses against different pathogen by recognizing these pathogens themselves or their products, including proteins, carbohydrates, lipids, and nucleic acids (single-and double-stranded RNA and DNA) [13]. TLRs were discovered in 1985 by Christiane Nüsslein-Volhard as factors involved in the embryonic development and resistance of the fly *Drosophila* to bacterial and fungal infection [14-16]. TLRs are patternrecognition receptors (PRR) with an extracellular leucine-rich repeats (LRRs) domain and a conserved cytoplasmic domain homologous to that of the interleukin-1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain [17, 18]. Such structure of TLRs recognizes pathogen-associated microbial patterns (PAMPs) encoded in various pathogens. These products include lipopolysaccharide (LPS), peptidoglycan (PGN), flagellin, bacterial DNA, viral single and double stranded RNA and synthetic double-stranded RNA such as poly(I:C). TLRs also recognize danger-associated molecular patterns (DAMPs) that are endogenous molecules including intracellular proteins such as heat shock proteins (HSPs) and protein fragments from the extracellular matrix (HMGB1) released from necrotic or dead cells [10, 19]. Given this capability of TLRs to recognize a wide range of microbial products and their synthetic mimic, modulation of these receptors can significantly play a central role in shaping the outcomes of the anti-microbial immunity. Since cancer originates from self-cells resulting in a poor anti-tumor immunity if not tolerance, triggering TLR signaling pathways during cancer immunotherapy by their specific agonists can accentuate the resultant anti-tumor immunity.

TLRs expression profiles differ among tissues and cell types of the innate and adaptive immune system. TLRs are predominantly expressed on T cells, B cells, neutrophils, monocytes, NK

cells, mast cells, and antigen-presenting cells (APCs), such as macrophages (MΦ) or dendritic cells (DCs). As such induction of TLR signaling activates APCs to provoke innate immunity and to induce adaptive immunity [6, 7, 20]. More recently, TLR have been found to be expressed on endothelial, epithelial cells and tumor cells, including melanoma [21]. TLRs are fundamentally located on the plasma membrane except TLR3, TLR7 and TLR9 that are located in the endoplasmic reticulum (ER) [22-24]. Mammalian TLRs include a large family consisting of ten to thirteen different types of TLRs that named simply TLR1 to TLR13 and each one of them recognize specific microbial components [25].

To date, ten human and thirteen murine TLRs have been identified; TLR1 to TLR9 are conserved between the human and mice. However, there are TLRs found in humans and not present in all mammals. For example, TLR10 in humans is present in mice. On the other hand, TLR11, TLR12 and TLR13 in mice are functional, but there is a stop codon in the human TLR11 TLR12 and TLR13 genes, which results in a lack of production of human TLR11 TLR12 and TLR13 [26, 27]. Signaling pathways of these TLRs can be triggered by their specific ligands. TLR ligands (TLRLs) are agents that bind to and activate TLRs. They are encoded in different types of organisms at the cell surface or at the internal cell compartments and they are expressed by different types of leucocytes and other cell types [28, 29].

TLRs/TLRLs bind results in a cascade of intra-cellular signaling pathways that induce the production of inflammatory cytokines and immune response [7, 8]. TLRLs are recognized by specific TLRs that are expressed on the surface of cells, for example, TLR2 is essential for the recognition of bacterial lipoproteins, lipomannans and lipoteichoic acids [30], while TLR5 detects bacterial flagellin [31]. TLR4 has been reported to recognize bacterial cell wall component lipopolysaccharide (LPS) [30], and TLR11 which recognize a profilin-like protein of *Toxoplasma gondii* [32] and uropathogenic *E.coli* [33]. In contrast, TLRs located within the endoplasmic reticulum (ER) can detect microbial nucleic acids, for example, TLR3 is required for response to virus-derived double-stranded RNA [34] and TLR9 recognize unmethylated CpG motifs [35]. Whereas, TLR7 and TLR8 could recognize small synthetic antiviral molecules [36], and single-stranded RNA [37].

3. TLRs Signaling pathways

Recent accumulating studies showed that TLRs signaling pathways can be divided into two signaling pathways, the MyD88-dependent pathway which is common to all the TLRs that resulted in the secretion of inflammatory cytokines, and TRIF-dependent pathway that is specific for TLR3 and TLR4 which associated with the stimulation of IFN-β and the maturation of dendritic cells [38-40].

3.1. MyD88-dependent pathway

The myeloid differentiation factor 88 (MyD88) dependent responses utilized by all TLRs except TLR3 and its occurring on hetero-or homo-dimerization of the TLRs receptor, upon activation by PAMPs or DAMPs. This dimerization induces the recruitment of adaptor proteins via the

cytoplasmic TIR domain. These adaptor proteins include the TIR-domain containing proteins, TIRAP (TIR-associated protein), TRIF (TIR domain-containing adaptor protein-inducing IFN-β) and TRAM (TRIF-related adaptor molecule), MyD88, Mal (MyD88 adaptor-like protein), which triggers the TLR-mediated signaling pathways [41-45].

The transcription factor IRF7 is critical regulator for the production of IFN-α/β via MyD88 pathway. IRF7 presents in DCs is very low, and therefore TLR7, TLR8 and TLR9 operate mainly through the NF-κB–IRF5 pathway [10, 46, 47]. It is worth noting that the transducing ability of TLR7, TLR8 and TLR9 may change in favor of the IRF7 pathway. Whereas, TLR4 is located on the cell membrane, activation of TLR4 signal transduction through MyD88/TIRAP and TRAM/TRIF pathways leads to activation of innate immune responses, primarily through IRS serine phosphorylationI

3.2. TRIF-dependent pathway

The TLR3 ligand (dsRNA) and the TLR4 ligand (LPS) activate the TRIF-dependent pathway in which IRF-3 plays a key role. For TLR3 (dsRNA), activation of MyD88-independent pathways occurs via TRIF (TIR domain-containing adaptor protein-inducing IFN-β). Upon dsRNA binds within endosomes, TLR3 recruits the adaptor TRIF through a TIR–TIR interaction. TRIF, in turn, recruits RIP1 to activate NF-κB, via TRAFs and the IKK (IκB kinase) complex. TRIF also recruits protein kinases TBK1/IKKε and TRAF3 to activate IRF3/7. Since, IRF-3 can phosphorylate by TBK-1 and IKKε on C-terminal serines, leading to its dimerization and translocation into the nucleus. Active IRF-3 mediates transcription of genes from the IFN-β promoter. The secreted IFN-β binds to the IFN receptor (IFNR), and thus activates transcription of ISGs, such as IRF-7 [9, 48]. On the other hand, the intracellular dsRNA is recognized by the RNA helicase RIG-I (or MDA-5). Activation of the latter activates TBK-1 and IKKε via its CARD domain and activates IRF-3 as well. IRF-7 further stimulates transcription from the IFN-α and-β promoters in a positive feedback loop. Type I IFNs bind also to their receptors to trigger the JAK–STAT signaling pathway. Whereas, to induce inflammatory cytokines production. TLR4 activation is requires both the MyD88-dependent and independent pathways. This occurs via TIR domain containing adaptors TIRAP, MyD88, TRAM and TRIF, which in turn initiates activation of NFκB, MAPK, and IRF3 allowing its nuclear localization and production of IFN-β [40, 49].

The signaling pathway of TLRs is summarized in Figure 1. TLR7, TLR8 and TLR9 are present in endosomes, which are stimulated by viral ssRNA. Upon activation, TLR7, TLR8 and TLR9 signal through the adaptor MyD88, which in turn leads to phosphorylation and activation of IRF3. Following its activation, TLR3 signals through its adaptor TRIF, and thus activates non-canonical IKK kinases (TBK1/IKKε), which subsequently phosphorylate both IRF3 and IRF7. Active IRF-3 induces transcription from the IFN-β promoter. NF-κ β is also activated by TRIF mediated signaling through canonical IKK kinases (IKKα, β, and γ). RIG-I and MDA5 are located in cytoplasm and can recognize both ssRNA and dsRNA. Intracellular ssRNA and dsRNA are recognized by the RNA helicase RIG-I (or mda-5), which are expressed in most cells, activates TBK-1 and IKKε via its CARD domain through interaction with the mitochondrially located adaptor MAVS.

Different signal transducer and activator of transcription (STAT) family members can be activated by interferons (IFNs). As a response to both type I IFNs and type II IFN (IFNγ) stimulation, STAT1-STAT1 homodimers can be formed. These homodimers bind to IFNγ-activated site (GAS) enhancer elements in the promoters of IFN-stimulated genes, and these results in the induction of genes encoding pro-inflammatory cytokines and apoptotic factors. STAT1 and STAT2 heterodimers, which are activated by binding with IRF9, which in turn migrates to the nucleus to bind to IFN-stimulated response elements (ISREs) and activate antiviral and antibacterial genes.

4. Polyinosinic-polycytidylic acid [Poly(I:C)]

Although, several previous studies have been extensively studied poly(I:C) for over 35 years in humans [50, 51], it is receiving new interest as a crucial component in many new immu-nostimulatory combination therapeutic strategies. Poly(I:C) is a synthetic double-stranded RNA (dsRNA) that has recently been identified as a specific ligand for TLR3 [52-55]. dsRNA is a viral product produced by most viruses during their replication cycle, Both viral dsRNA and its artificial mimic, poly(I:C), are potent inducers of type I interferons (IFN-α/β) [56, 57], which is a crucial cytokines that exert anti-viral and immunostimulatory activities for both T and B lymphocytes [58, 59], DCs [11, 60] and activate monocytes to produce CSF, IL-1β, IL-12, and PGE$_2$ [61, 62].

Several studies have been shown that Poly(I:C) induces a strong innate immune response initiated by two types of PRRs; the TLRs and the RIG-I-like receptors (RLRs) which is a family of cytoplasmic RNA helicases that includes RIG-I and MDA-5 [63-67]. In addition, several studies have shown that Poly(I:C) transduces signals which activate the NF-κB and the IFN-β promoter [53, 54] and activates several nuclear and cytoplasmic enzyme systems such as oligoadenylate synthetase (OAS), the dsRNA dependent protein kinase R (PKR), RIG-I Helicase, and MDA5 (melanoma differentiation associated gene) which are implicated in antiviral and antitumor host defenses [7].

Poly(I:C) interact with TLR3, that is the specific intracellular recognition system that responds and signals to the intracellular presence of dsRNA and RNA virus infection [68]. TLR3 is mainly expressed on a broad range of antigen-processing cells (APCs), dendritic cell (DC) subsets, fibroblasts, intestinal epithelial [54, 69, 70] monocytes, macrophage (MΦ), mast cells, NK cells [71, 72], CD4+and CD8+T cells [6, 73]. Several prior studies have shown that poly(I:C) has potent pleiotropic immunostimulatory effects on several kinds of immune cells [74-76].

Poly(I:C), a synthetic dsRNA mimic copolymer, is a specific TLR3 agonist [13, 77]. Recently, we and others have reported that in vivo administration of the TLR3 agonist poly(I:C) into naïve mice induced rapid increases in the frequencies of NK cells mainly in the liver and that these effects associate with better antigen specific responses and antitumor efficacy [6, 78-81]. Consistent with earlier studies [81], we have demonstrated that the adjuvant effects of poly(I:C) to the antigen-specific CD8+T cell responses are partially dependent on NK cells through creation of a rapid beneficial cytokine milieu [82, 83]. Furthermore, in vitro treatment of highly

purified NK cells with poly(I:C) significantly augmented NK cell-mediated cytotoxicity and up-regulated their expression of the activation marker CD69 [71]. Of particular interest, similar to viral infection, poly(I:C) treatment has been found to preferentially induce recruitment and activation of hepatic NK cells [79] and their trafficking to spleen coincided with enhanced cytokine expression [80]. These studies may explain the increased antitumor effects against metastasis observed in tumor models after poly(I:C) treatment [84].

5. Poly(I:C) and anti-tumor Immunity

A fundamental difference between tumor and microbes is that only the latter encode products (signatures) that are recognized as "danger signals" by pathogen recognition receptors (PPRs) expressed in the innate immune cells. [85] In most cases, immune system can mount vigorous immune responses against microbes, but not against cancer. Therefore, the challenge in cancer immunotherapy is how to manipulate the body's own immune system to fight cancer. [86] Mimicking the anti-microbial immunity, recent preclinical and clinical studies have established that provision of TLRL adjuvant system systemically or into tumor environment itself profoundly awaken the cross talk between innate and adaptive immunity, driving generation of efficacious anti-tumor immunity. [87] For instance, the addition of CpG DNA (typical TLR9L) to a melanoma vaccine resulted in effective cytolytic responses. [88] Imiquimod, a synthetic TLR7/8L, has been successfully used in the treatment of basal cell carcinoma [89, 90] and to enhance the immunogenicity of vaccine containing Flt3 ligand and a melanoma peptide. [91] MPL, a TLR4L, has been used as an adjuvant in clinical trials of vaccines against melanoma, glioma and pancreatic and colorectal carcinoma, inducing substantial tumor-specific immunity in response to vaccination [92, 93]. We have reported recently that co-administration of the TLR3L poly(I:C) in vivo with peptide vaccination established functional effector/memory responses [94] by induction of NK-driven beneficial cytokine milieu and DC activation. [81] Although the impact of both of TLRLs at the time of Ag priming has been the focus of several studies, their impacts at the time of Ag recall on the tumor-specific memory responses have not been actively investigated.

Since, cancer cells do not encode danger signals like virus, generation of anti-tumor memory T cells in the tumor setting would require a large pool of highly activated DCs. In response to TLRLs, resident immature DCs at the site of vaccination undergo a maturation program and migrate to the draining lymph nodes (LNs) [72, 95, 96]. Therefore, utilizing TLRLs is a potential approach to induce activation of immature DCs and maximize their contribution to memory cell responses. In line with this notion, recent studies have established the adjuvant effects of several TLRLs, in particular, TLR3L (poly(I:C), TLR7/8L (Imiquimod), and TLR9L (CpG) to the anti-tumor CD8+T cell responses [81, 94, 97-101]. However, most of these studies tested the adjuvant effects of TLRLs in lymphodepleted hosts, and those few studies that utilized lymphodepleted hosts did not use adoptive cell therapy (ACT) system [98, 102-107].

Because the frequency of DCs in steady state condition is low, growth factors in particular Flt3L and G-CSF have been used to mobilize DCs in vivo [108-110]. With this regard, we have

recently reported a rapid induction of type I IFNs and activation of DCs with decreases in the levels of T_{reg} cells after CTX treatment; resulting in augmented post vaccination responses which were further improved by addition of poly(I:C) to vaccination [111]. We have found also a marked expansion of immature DCs during the recovery phase after treatment with the anticancer drug cyclophosphamide (CTX), indicating to the mobilizing effect of CTX for DCs. Since DCs are equipped with TLRs that sense different microbial products such as poly(I:C), activation of these immature DCs in vivo may augment memory T cell responses. [85]. Recent studies, including ours have demonstrated the adjuvant effects of TLRLs to ACT in lympho-depleted hosts [112-114]. These studies suggest that combinatorial treatments with chemo-therapy/immunotherapy and ACT can markedly improve memory T cell responses. Our observation of expansion of DCs post CTX therapy is a suitable model to dissect the optimal timing of the adjuvant effects of TLRLs to post vaccination responses of ACT in a lymphode-pleted host.

Specifically, our recent studies showed that treatment of a recipient host with the non mye-loablative dose of CTX augments post vaccination CD8$^+$T cell responses, which were associated with a quick activation of DCs in the lymphopenic phase [114]. We also found that CTX induces increases in the numbers of immature DCs during the recovery phase from days 9-16, peaking on day 12 [115]. These DCs demonstrated normal phagocytic ability in vitro and antigen uptake in vivo. Administration of poly(I:C) at the peak of DC expansion resulted in induction of rapid inflammatory milieu which associated with significant increases in the numbers of activated (CCR7^{high}CD40^{high}) DCs in lymph nodes.

Using the pmel-1 TCR transgenic mouse model, in which CD8$^+$T cells can recognize the natural tumor gp100 antigen on B16 melanoma, we demonstrated that vaccination of CTX-lympho-depleted mice with the MHC class-I melanoma gp100 peptide and poly(I:C) during the lymphopenic phase (day 2) and at the peak of DC expansion (day 12) resulted in significant augmentation in the number of activated DCs in lymph nodes with a temporal increase in the expansion of pmel-1 cells. Conditional depletion studies of DCs at day 12 before revaccination of CTX-treated recipient mice revealed that DCs are required for the optimal pmel-1 cell responses. Importantly, the therapeutic anti-tumor effects of the enhanced pmel-1 cell responses were demonstrated toward an advanced tumor of the poorly immunogenic B16 melanoma, indicating the clinical significance of our observation. Furthermore, we have found improvement in DCs expansion when CTX treatment was followed by a daily treatment with 5μg/mouse G-CSF for 5 days [6], without altering the efficacy of our prime-boost vaccination with hgp100/poly(I:C) [116].

T_{reg} cells express different TLRs [117] and can be activated by certain TLR agonists such as TLR4 [118-120], and TLR5 [121], and can be inactivated by TLR2 and TLR7/8 agonists [122, 123]. Indeed, total body irradiation and CTX-preconditioned hosts demonstrated that inter-ference with T_{reg} cell activities can enhance tumor-specific T cell responses and anti-tumor immunity [124]' [125, 126]. Our preliminary studies and previous studies [105, 125-130], however, showed that although T_{reg} cells decrease in numbers during the lymphopenic phase after CTX treatment they recover to their normal levels at the recovery phase. These recovered T_{reg} cells may interfere with the efficacy of vaccination at recovery phase unless the vaccination

is co-administered with a potent adjuvant that can block T_{reg} cell function. Indeed, induction of maturation of DCs by certain TLR agonists has been found to control T_{reg} cell function in vivo [122, 131, 132]. Therefore, it is possible that the enhanced pmel-1 cell expansion and anti-tumor effects of pmel-1 cells to peptide/poly(I:C) revaccination on day 12 post CTX treatment involve blocking of T_{reg} cell.

The potent anti-tumor efficacy of prime-boost vaccination with tumor antigen/poly(I:C) at precise time points post CTX therapy would lead to its potential application in the clinical setting. We would envision two treatment protocols for the clinical application of this prime-boost vaccination with tumor peptide and poly(I:C) on cancer (e.g. melanoma) patients. 1) Patients can be with a lymphodepletion dose of CTX followed by G-CSF to correct leucopenia, and vaccinate the patient with a candidate tumor Ag (gp100, MART, or TRP2) with co-administration of the clinical grade form of poly(I:C) such as Hiltonol®. Vaccination and Hiltonol® treatments can be repeated during the recovery from leucopenia (after 10-15 days of chemotherapy) when the numbers of DCs are increased. 2) The same as above but with adoptive transfer of peripheral blood mononuclear cells harvested from the cancer patient prior chemotherapy. These cells can be stimulated in vitro with 1ng/mL of the tumor antigens in the presence of 10ng/mL IL-12 for 3-5 days as we recently described in our preclinical model [133].

6. Poly(I:C) and antiviral immune response

The innate immune response is the first barrier against the invading pathogens and viruses and it responds through activating inflammatory and antiviral defense mechanisms by inducing IFNs-α/β against the viruses [134]. Several in vitro and in vivo studies have demonstrated that the main effects of poly(I:C) is the induction of IFNs-α/β, which play a crucial role in innate anti-viral response [135, 136].Several studies have been extensively demonstrated that poly(I:C) triggers the activation of PKR and other kinases which followed by the phosphorylation of the substrates of these enzymes that results in the subsequent translocation of transcription factors, NF-ĸB and IRF-3, to the nucleus where then they bind to the IFN-β promoter to form a transcription complex that induce IFN-β production [137-140]. It has been thought that the proximal inducer of IFNs-α/β is intracellular dsRNA generated as an intermediate during viral replication [53].

Recently, it has been shown that dsRNA and ssRNA molecules are recognized as intermediate by TLRs that is expressed on DCs, NK cells, MΦ and epithelium during virus replication [141]. Several studies have been extensively demonstrated that Poly(I:C) plays a critical role in inducing innate immune response against many viruses, such as influenza virus [142], human respiratory syncytial virus (RSV) [143], herpes simplex virus 2 (HSV-2) [144], and murine cytomegalovirus [25]. Several in vitro and in vivo studies have been shown that retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5), are the key in the detection of viral dsRNA and Poly(I:C) in the cytosol and subsequent eradication of the replicating viral genomes [66, 145, 146]. Treatment of animals with poly(I:C) results inactiva-

tion of the TLR3 receptor which in turn induces the activation of NF-κB and the production of type I interferons that induces strong antiviral and antineoplastic effects accompanied by activation of CD8+T and NK cells [53, 147]. In addition, poly(I:C) is shown to serve as an adjuvant to induce protective CD4+T cell responses against HIV [148].

7. Poly(I:C) and clinical trials

7.1. Hiltonol®

Hiltonol® or Poly-ICLC is a synthetic, nuclease-resistant, hydrophilic complex of polyinosinic and polycytidylic acid, stabilized with poly-L-lysine and carboxymethyl cellulose. Poly-ICLC is IFN-β inducer, which is known to steady the blood-brain-barrier (BBB) and minimize cellular infiltration into damaged brain regions following stroke [149, 150]. Several clinical trials were conducted to utilize poly-ICLC as an IFN inducer in cancer patients, the first clinical trial (phase 1) were conducted to determine the maximum tolerated dose (MTD) and it found that the MTD was (12 mg/m^2), which induced effective response in cancer patients [51]. Another study was conducted to determine the immunomodulatory effect of Poly-ICLC in cancer patients and it showed that there was no detectable serum IFN in patients that received 1 mg/m2 Poly-ICLC by IM injection [151]. In contrast, IFN was detectable in the serum of patients that received 4 mg/m^2 Poly-ICLC by IV injection. Then, phase I and II clinical trials were conducted in patients with many types of cancers including leukemia, lymphoma, brain tumors, myeloma, juvenile laryngeal papillomatosis, renal cell carcinoma, breast cancer, ovarian cancer, and melanoma [152-160].

In most of these early clinical trials, about 6 mg/m^2 Poly-ICLC was generally used intravenously. Fever, often with temperatures greater than 40°C, was a universal adverse event in the trials and was the primary dose-limiting factor. Other common adverse events reported in these trials included nausea, vomiting, hypotension, thrombocytopenia, leukopenia, arthralgia, myalgia, and fatigue. Additionally, very few objective responses were reported in these clinical trials. Due to its toxicity and relative ineffectiveness, utilizing Poly-ICLC was deserted after the availability of recombinant IFN. It was subsequently determined that lower doses (10 to 50 mg/kg) of Poly-ICLC resulted in a broader host defense stimulation, a potent adjuvant effect, and a specific antiretroviral effect mediated by the 2'5'OAS and PKR nuclear enzyme systems [161]. Consequently, Poly-ICLC is currently being developed for use only at doses up to 50 mg/kg. The poly-ICLC treatment prior to exposure to oxygen-glucose deprivation maintained the paracellular and transcellular transport across the endothelium and attenuated the drop in transendothelial electric resistance by enhancing IFN-β mRNA expression in astrocytes and microglia [162]. Recent study utilized poly-ICLC in activation of TLR3 signaling, which exerts a beneficial effect on NK cells, resulting in the increased cetuximab-dependent lysis of head and neck cancer (HNC) cells [163].

7.2. Ampligen®

Ampligen® is known as [poly(I)-poly(C12U)] that is composed of poly(IC) with a U mismatch at every 12th base of the C strand. The main effect of Ampligen® is the induction of Th1 response. This has been demonstrated in delayed-type hypersensitivity reactions and in current clinical studies with human immunodeficiency virus-infected patients. Other cells targeted are NK cells, cytotoxic CD8+T cells, and LAK-NK cells. Despite initial trials in the 1990s, little is known about Ampligen®, since; it is not routinely used in clinical practice. However, due to its notable and potent antiviral effects, Ampligen® has been generally well tolerated in randomized clinical trials [164, 165]. Since, Ampligen® did not consistently produce antiviral effects, but its antiviral activity was always seen after therapeutic treatment. Therefore, it was assumed that Ampligen have exquisite specificity for TLR3 [166, 167]. Early studies showed that treatment of chronically HBV-infected chimpanzees with polyICLC transiently reduced the levels of serum viral DNA, HBsAg and HBeAg [168]. In two independent studies, transient suppression of DHBV in ducks by Ampligen® was also observed [169, 170]. In vitro studies, Ampligen and zidovudine have combined: to act in synergy against HIV-1-infected cell lines [171, 172] and enabling restoration of the immune function in HIV-infected individuals. Wither through maintaining a stable or raising CD4 count, increasing delayed-type hypersensitivity reaction, and reducing rate of progression to AIDS [173].

More recently, Nicodemus et al (2010) evaluated polyIC12U and confirmed the potential of TLR3 stimulation with Ampligen® in enhancement bioactivity of cancer immunotherapies [166]. Their results revealed that Ampligen® is a potent inducer for the dendritic cell maturation and local cytokines producing in culture systems including IL-4, IL-6, IL-12p70, IFN g, MIP-1a and TNF α. Interestingly, treatment of wild-type mice with Ampligen® 24 hours following infection with a lethal viral inoculation was fully protective, whereas dosing 4 h prior to inoculation is ineffective in preventing mortality [167]. Intranasal Ampligen® administered to mice in conjunction with prototype avian flu vaccines, greatly enhances the cellular and humoral immunity achievable with the vaccine and also protects mice from lethal infection [174].

8. Conclusion

Since the discovery of the important role of TLRs in innate immunity and the initiation of an immune response that follows the activation of antigen-specific acquired immunity, rapid progress has been made on our understanding of the molecular mechanisms of TLRs. The inclusion of TLR ligands as a potential new class of adjuvants candidates has enabled the development of clinical effective vaccination strategies against many diseases. Poly(I:C) and Poly-ICLC represent two important member of these immunostimulatory vaccine adjuvants in mice, nonhuman primates and in human. TLR3 is specifically expressed in quite cell-type and species; it is expressed in specific myeloid cells, vascular endothelial cells and airway epithelial cells among others. Human TLR3 is highly expressed in immature DCs whereas

mouse TLR3 is highly expressed in macrophages, but in both species, its expression is induced by IFN.

Unlike other TLRs, TLR3 is unique, since, it is not required MyD88 for signaling, and instead, TRIF is the critical adaptor protein for its signaling. Whereas TLR3 displays a distinct intra-cellular localization compared with other TLRs, TLR7 and TLR9 trigger a quick, but short IFN response, whereas TLR3 might be more important for a prolonged response and the initiation of the adaptive immune response. Poly(I:C) interact with TLR3 that responds and signals to the intracellular presence of dsRNA and RNA virus infection. Poly(I:C), is strong inducers of IFN-α/β, which is a crucial cytokines that exert anti-viral and immunostimulatory activities for both T and B lymphocytes, DCs and activate monocytes to produce CSF, IL-1β, IL-12, and PGE2. It's also transduces signals which activate the NF-κB and the IFN-β promoter and activates several nuclear and cytoplasmic enzyme systems such as OAS, PKR, RIG-I Helicase, and MDA5 which are implicated in antiviral and antitumor host defenses.

The above mentioned pleotropic effects of the TLR3 agonist Poly(I:C) makes it as a candidate adjuvant system for anti-viral and anti-tumor immune responses, in particular its clinical forms Hiltonol® and Ampligen® induce similar immunomodulatory effects with accepted side effects.

Acknowledgements

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