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Toll Like Receptors Play a Role in General Immunity, Eye Infection and Inflammation: TLRs for Nanodelivery

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Abstract

Dendritic cells [DCs] are potent antigen presenting cells [APC], which plays a vital role in immune system by detecting and capturing pathogens in the body. DCs perform a pivotal role in induction of T cell response. Regulation of immune response can be achieved by specific antigen [Ag] delivery to DCs. A delivery system that can efficiently target and present Ags to DCs for the purpose of anti-tumour activity is currently a topic of significant research interest. DCs are receiving attention due to their key role in anti cancer host response and due to their adjuvanic property in tumour vaccines. Role of toll like receptors [TLR] in innate immune system and their part in eventual stimulation of adaptive immunity is exploited to develop vaccines. TLR agonists in conjugation with vaccines are shown to increase therapeutic efficacy in some cases. TLRs also play a vital role in protecting the cornea from invading pathogens. Due to adverse effects in the treatment of ocular inflammations, cancer and in viral infections, an alternate approach such as the use of TLRs will solve the inquisitive question regarding side effects. The intended delivery is attained by the use of nanoparticles which in turn leads to prolonged half-life in the body. Co-delivery of Ags, TLRs and immunomodulators using nanoparticles has been demonstrated to elicit potent cellular immune responses and are currently under development of clinically applicable immunisations and vaccines.

Introduction

In spite of all the efforts to suppress the spread of infectious diseases and cancer by the biomedical sector, they still represent the major cause of death. According to world health organisation [WHO], cancer is responsible for one third of lost years of life [1]. Most cancers are treated by combination of standard therapies like surgery and chemotherapy. Conventional therapies not only target tumors cells but also healthy cells as the drug does not discriminate the cell types. To overcome these obstacles, targeted nanomedicine is designed for the delivery of drugs selectively to tumor cells. Therapeutic vaccination against tumors proved far more stringent than prevention. The goldilocks approach of establishing tumor immunity with the molecularly designed nanoweapons would aid in the homeostatic induction of immune cells and other host effector molecules. Toll like receptors [TLRs] are one such crucial sensors of innate immune system identified as key targets in tumor immunology. TLRs are pattern recognition receptors [PRR] which recognise antigens [Ag] and initiate the immune cell cascade. The use of TLRs as molecular targets for intensifying immune response provides a substantial advance in the field of therapeutics. Pathogen mimicking nanoparticles capable of interacting with dendritic cells [DCs] in conjugation with TLR regulates the immune system. Amalgamating nanocarriers with the agonists increases the levels of proinflammatory cytokines, chemotactic factors and type 1 Interferon [IFN]. This response eventually elevates adaptive immunity and further vaccine responses, to combat malignant cells. On the basis of pharmacokinetics profile, either agonist or antagonists are used therapeutically for various diseases. Clinical trials are imperative in using these agents to collect safety and efficacious data for health interventions. This paper will explore the horizons of TLR utilisation in cancer biology, corneal inflammations and in viral infections to up-regulate immunosuppressive state of humans.

TLR and immune system

The innate immune system relies on the recognition of simple molecules and regular patterns displayed by pathogens and many microorganisms which are not seen on the body's own cells [3]. These structures, known as pathogen associated molecular patterns (PAMPs), are recognised by receptors known as PRRs. The innate immune system as well as the adaptive immune system relies heavily on the function of PRRs. However, recognition of non-pathogenic and commensal microflora by PRR is largely unknown [4]. Presumably, compartmentalization (confinement of commensal microflora to luminal side of intestinal epithelium), transforming growth factor (TGF-β) and interleukin (IL-10) play an important role in this process. PAMP's are highly conserved throughout the evolution and are less destined for mutation since they are vital for survival of microbes. Three main features of PAMPs make them ideal targets for innate immune system. First, they are produced only by microbes and not by host cells. Second, they are invariant between microorganisms of a given

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class and thirdly, PAMPs are essential for survival of the microbial flora [5]. There are a variety of different types of PRRs, where TLRs are one major group. TLRs belong to type I transmembrane receptors characterised by the presence of leucine-rich repeat domain (LRR) and an intracellular Toll/IL-1 receptor domain (TIR). They appear to be the one of the most conserved components of the immune system with basic signaling receptors for the innate immune stimulation. Its expression is ubiquitous from epithelial to immunocompetent cells [6,7]. Associated with the TIR domain are the presence of five adaptors myeloid differentiation MyD88, MyD88-adaptor like (Mal), TIR-domain containing adaptor protein inducing IFN-β (TRIF), TRIF related adaptor molecule (TRAM) and sterile α- and armadillo motif containing protein (SARM) [8]. These adaptor molecules activate intracellular molecules including protein kinases to orchestrate the inflammatory response [9]. The human TLR family is known to consist of 10 members with this number likely to increase as relatively little attention has been given to them in the past [3,10]. TLR 1 and TLR 2 dimerization responds to triacyl peptides [11] whereas, TLR 2/TLR 6 recognizes diacyl peptides [12]. TLR 2 in combination with lectin receptors enables the recognition of fungal cell wall component zymosan [14]. Double stranded RNA and some synthetic RNA moieties are ligands for TLR 3 [13]. TLR 4 serves as a specific receptor for lipopolysaccharide (LPS), the most powerful microbial stimulant of innate immunity. By promoting the release of inflammatory cytokines, LPS also mobilize the innate and adaptive immune response [15]. TLR 5 has been shown to recognise conserved domains of flagellin (a monomeric constituent of flagella). TLR 5 is reported to be present on basolateral side of intestinal epithelial cells and in sub epithelial compartment necessating the balance of homeostasis at the mucosal layer [16]. Down the lane, TLR 7, TLR 8 and TLR 9 are reported for recognition of ssRNA from viruses. The host ssRNA are not recognised as foreign by TLR 7/8 due to the subcellular expression of these TLRs in the endosomal compartment. TLR7/8 also recognises synthetic compounds: imidazoquinolines used for treating genital warts and loxoribine as an immunostimulant [17]. TLR 9 is a receptor of ummethylated CpG DNA motifs which are the characteristics of a bacterial genome [18]. CpG motifs with GACGTT sequence stimulate mouse TLR 9 while with the core sequence GTCGTT optimally induce human TLR 9 [19]. TLR 11 gene present in animals are resistant to uropathogenic strains of *E.Coli*. Human kidney also appears to contain TLR 11 gene yet the protein is not expressed [20]. Animals are shown to explicit TLR 12 and TLR 13 with no counterpart expression in humans [21]. TLRs efficiently converts immature DCs into mature DCs. Maturation of DCs are marked by the increased expression of cytokines, major histocompatibility complex [MHC] class I and class II, tumour necrosis factor [TNF-α, IL-1, IL-6, IL-8, IL-12 and co-stimulatory molecules like CD40, CD80 and CD86. Once the maturation is complete DCs migrate to secondary lymphoid organs (lymph node) for presentation of Ag to T cells. Chemokine CCR7 is responsible for migration of DCs to lymph node and also for pinocytic activity. Immature DCs are adherent to actin cytoskeleton and once they encounter Ag, actin gets polarized and so DCs detach from actin fibres. The increased phagocytic activity of DC is regulated by extracellular signal regulated protein kinase (ERK1/2) and p38α mitogen activated kinase. Ag coupled with TLR is much preferred to be endocytosed by APCs. In addition to that cross presentation of Ag is more when it has been coupled with TLRs [9]. When an Ag is encountered by DC it migrates to lymph node and present to naive CD4+ T cells and CD8+ cytotoxic cells. Following this, naive CD4+ T cells differentiate into memory helper T cells which in turn are responsible for differentiation and expansion of CD8+, cytotoxic T lymphocytes [CTL] and B cells [8]. Other immune cells such as macrophages, mast cells, eosinophils, neutrophils etc also recognise Ag through TLRs.

TLRs and adjuvanticity

Adjuvants are compounds known to enhance the magnitude and quality of immune response to antigens with minimal toxic profile. The reduction in the amount of Ag with increased immunogenicity makes them ideal for a successful vaccination strategy. Interest in vaccine adjuvants has been growing rapidly due to the presence of only few FDA approved adjuvants in the market. Alum was the first adjuvant approved by FDA. Recently MF59 has been approved in Europe for flu vaccine (Fluad, Novartis vaccine) and ASO4 for viral vaccines [22,23]. Addition of adjuvants to vaccines may substantially decrease the amount of Ag to be administered. While there have been spectacular successes in tumor targeted immunotherapy, the need for tumor vaccines top the list in clinical oncology. There is still a degree of pessimism for the use of adjuvants for treating cancer. To augment this, multiple adjuvants have been developed ranging from live-attenuated strains to molecularly defined compositions to elicit receptor associated immune response. Cell wall skeleton from *Mycobacterium bovis* (Bacille Calmette-Guerin vaccine) has been used as an adjuvant since, the cell wall of this bacterium comprises of NOD-2 agonist muramyl dipeptide. The inflammatory response to BCG antigens is processed via TLR 2 and TLR 4. Lipoarabinomannan and lipomannan from this bacterium activates the immune cells for the production of TNF and interleukin (IL-12) [24]. Though the use of BCG stands as an age old concept, its clinical usage is limited since the synthetic production is highly complex. Hence the focus is diverted towards development of synthetic agonists. The synthetic component derived from *Staphylococcus aureus,* 16 S-[2, 3-bis (palmitoyl)propyl] cysteine (Pam2) lipopeptides is shown to activate DCs and natural killer [NK] cells for the production of interferon [IFN-γ] [25]. With the incorporation of RGDS peptide for the effective cell penetration, their use as an adjuvant has surpassed the disadvantages of commercial adjuvants in use. The Pam2 lipopeptides have two palmitoyl bases attached to two separate peptide sequences for the determination of TLR 2 agonist activity [26]. The mucosal adjuvant effect of TLR 3 dsRNA agonist produces high concentration of IgA and also protects from the recurrent viral infections. Due to the toxicity profile of poly (I: C), a chemically modified form [PolyI:C_{12}U] of the above formulation was synthesized. Additionally, PolyI:C_{12}U [AMPLIGEN] is used as an adjuvant for mucosal vaccine for avian H5N1 virus [27]. In regard to tumor therapy, poly (I: C) has been used as a cancer adjuvant vaccine since they can effectively activate DCs and NK cells. The main function of poly (I: C) is to boost the maturation of DCs for further priming and clonal expansion of antigen specific T cell response. Interestingly, the TLR 3 agonist acts through diverse accessory molecules rather than acting via DCs alone [28]. A recent study by McCartney showed the involvement of NK cells, IFN-α and IL-12 associated with the TLR 3 signaling pathway activation through poly (I: C) agonist [29]. Cervarix, a vaccine against human papilloma virus is formulated with monophosphoryl lipid A [MPL], a TLR 4 targeted adjuvant for promoting the immune response. The clinical grade form of MPLA has low toxic profile of about 0.1%. [30]. TLR 5 agonist, flagellin is used as an adjuvant for the delivery of recombinant hemagglutinin. The use of flagellin to adjuvant influenza vaccines via TLR 5 innate immune pathway trigger a cascade of multitudinal transcriptional events responsible for adaptive immunity. [31,32]. Commonly used agonists for TLR 7 are imiquimoid and resiquimod. Very recently, a

guanosine analog 7-allyl-7,8-dihydro-8-oxo-guanosine (loxoribine) has been identified as a TLR 7 agonist. They activate plasmacytoid DCs and B cells for the induction of IFN-α and IFN-regulated cytokines [33]. A very similar pattern of activating myeloid DCs, monocytes and monocytes derived DCs for the release of TNF-α, IL-12 and macrophage inflammatory protein – 1 alpha is through TLR 8 agonist – VTX-2337 which is in phase I study. Due to its high adjuvanting property, it has been used to treat solid tumors or lymphoma [34]. Synthetic and natural oligodeoxynucleotides containing specific motifs of CpG dinucleotide are potent immunostimulatory agents for activation of TLR 9 pathway [35]. They directly induce the differentiation of B cells into antibody secreting plasma cells. On the other hand, release of IFN-α from immature DCs activates NK cells for the action of tumor cell lysis. Mature DCs capture the released tumor Ags and process it through major histocompatibility complex [MHC] which in turn leads to the migration of DCs to lymph nodes for the delivery of processed Ags to lymphocytes initiating Th1 response [36]. The activity and safety of these molecularly defined adjuvants and formulations has provided support for the concept of antitumor and antiviral immunity with reduced adverse effects.

Sorting of toll signals via TLR adaptors

The downstream pathways of TLR signaling require two major adaptor proteins MyD88 and TRIF. The utilisation of TIR domain containing adapter protein or Mal [TIRAP] and TRIF related adaptor molecule [TRAM] has been associated with TLR 2 and TLR 4. Several transcriptional regulations involving nuclear factor [NF-kB], activator protein-1 [AP-1], interferon regulatory factor 3 and 7 [IRF] constitute pleiotropic effects with tightly regulated gateways for gene modulation [37]. In regard to MyD88 dependent pathway, the adaptor molecule MyD88 possesses TIR domain in C terminal and death domain in N-terminal portion. Associating with TIR domains of TLRs, MyD88 recruits IL-1 receptor associated kinase (IRAK). After phosphorylation it interacts with TNF receptor associated factor [TRAF 6] for the activation of two crucial signaling pathways JNK and NF-kB [38]. In the case of MyD88 independent pathway, LPS induces the activation of NF-kB and JNK through TLR 4 stimulation. Precisely, IFN-induced protein 10 [IP-10] and glucocorticoid attenuated response gene [GARG 16] were induced for the initiation of immune cascades. They also augment surface activation markers like CD40, CD80 or CD 86 in dendritic cells [39]. The molecular stack of 232 aminoacid cytoplasmic protein is attributed to TIRAP adaptor molecule which strongly influences the NF-κB promoter equivalent to MyD88 [40]. MicroRNAs [miRNA] have recently been identified as important regulators of TLRs. The miR155 is known to target TLR 2, TLR 3, TLR 4 and TLR 9 through MyD88 and adaptor molecules in monocytes, DCs and macrophages. Similarly, miR125b, miR147, miR223 and miR27b regulate TLR 4 by activating NF-κB in cholangiocytes, alveolar macrophages, inflamed lung tissues and in peritoneal macrophages respectively [41]. Despite the wealth of information regarding miRNA in manipulation of TLR signaling is known, complete pathway of TLR signaling remains unexplored indicating the involvement of additional gene products for cell specific expression.

Corneal immunity

The delicate visual organ that is responsible for vision is highly vulnerable to immunological inflammation and microbes. The features that account for the constructive action of vision are 1) ocular barriers 2) absence of lymphatic drainage pathways 3) presence of immunomodulatory factors and 4) presence of APCs. Presence and utilisation of TLRs for improving ocular immunity will quench the thirst for corneal haze.

Corneal TLR

Cornea is an ideal model for evaluating *in vivo* angiogenesis in cancer. The introduction of targets initiates angiogenesis, thus providing a platform for tumour study [42]. Rabbit eyes are excellent prototype for such studies. Recently research on mice eyes has also been initiated. Basically, cornea is a transparent, dome-shaped surface, with two specialized function i) protective barrier between the external environment and internal milieu, ii) constitutes the main refractive element of visual system [42,43]. It is made of three major layers: epithelial, stromal and endothelial cells. Corneal epithelium extends a barrier, and restricts entry of pathogens into eye [43]. It has an ability to detect and fight microbial pathogens [44-46]. Nonetheless, corneal innate immunity involves many other components such as keratocytes, corneal fibroblasts, langerhans cells (dendritic cells) and immunoglobulins (IgG and IgA) [45]. It has been demonstrated that injury or infections to the cornea triggers an immune reaction which leads to recruitment of polymorphonuclear cells, lymphocytes, and fibroblasts following the release of chemotactic factors such as IL-8 and cationic antimicrobial protein of 37 kD from corneal epithelium (Figure 1). Expression of TLR 1 to 10 is observed in corneal epithelium from patients with ocular diseases and also from human cadavers. However, not all subjects express all the TLRs. TLR 7 and TLR 8 expression is very low in some cases [46]. The constitutive as well as inducible expression of various TLRs such as TLR2, TLR3, TLR4, TLR5, TLR9 and TLR10 have been seen in corneal epithelium *in vitro* and *in vivo* [44-46]. Corneal expression of TLRs is mediated during inflammatory disorders such as atopic keratoconjuctivitis, sjogren's syndrome as well as during bacterial, viral and fungal infections [47]. TLR2 and TLR4 expression in cornea functions as a gram-positive and gram-negative bacteria [*Pseudomonas aeruginosa*] sensor [48]. Flagellin protein present in flagella [*Pseudomonas*] is a ligand for TLR 5 [49]. Lipoprotein also binds the TLR2/1 dimer albeit from mycoplasma [50]. TLR7 has been shown to recognize Herpes Simplex Virus [HSV-1] infection and likely involved in corneal innate immune responses in herpetic keratitis [43]. TLR9 recognises CpG rich DNA viruses as well as gram negative and gram positive bacteria [51]. Upregulation of defensins, small peptide that target bacterial cell wall has been recognised after activation by TLR, thus solidifying the importance of TLR [43]. Although the corneal inflammatory response via TLR signalling is vital for efficient removal of pathogenic microbial agents, an inadequately regulated proinflammatory cytokine production may result in inflammatory responses which would lead to corneal scarring and loss of vision altogether. In some cases, constant activation of TLR leads to other corneal inflammatory conditions such as dry eye syndrome and allergy. Thus a TLR decelerator is necessary to curtail the proinflammatory state. Although information regarding this indispensable process is scarce, negative regulators of TLRs induce single Ig IL-1 related molecule [SIGIRR] and ST2 [member of TIR family which do not activate NF-kB] [52]. These two molecules act by impounding on MyD88 and IRAK [IL-1R associated kinases] [47]. SIGIRR or ST2 blockade leads to serious bacterial corneal diseases. This strongly suggests that even though TLR signaling is necessary to fight against pathogens, if the signaling activity is unchecked by SIGIRR or ST2 serious corneal injury develops. This poses a most formidable conundrum of how to modulate TLR signaling in such a manner so as to maximize surveillance and disposal of foreign invaders while curbing potential damage to self. Ocular epithelial cells are very selective in

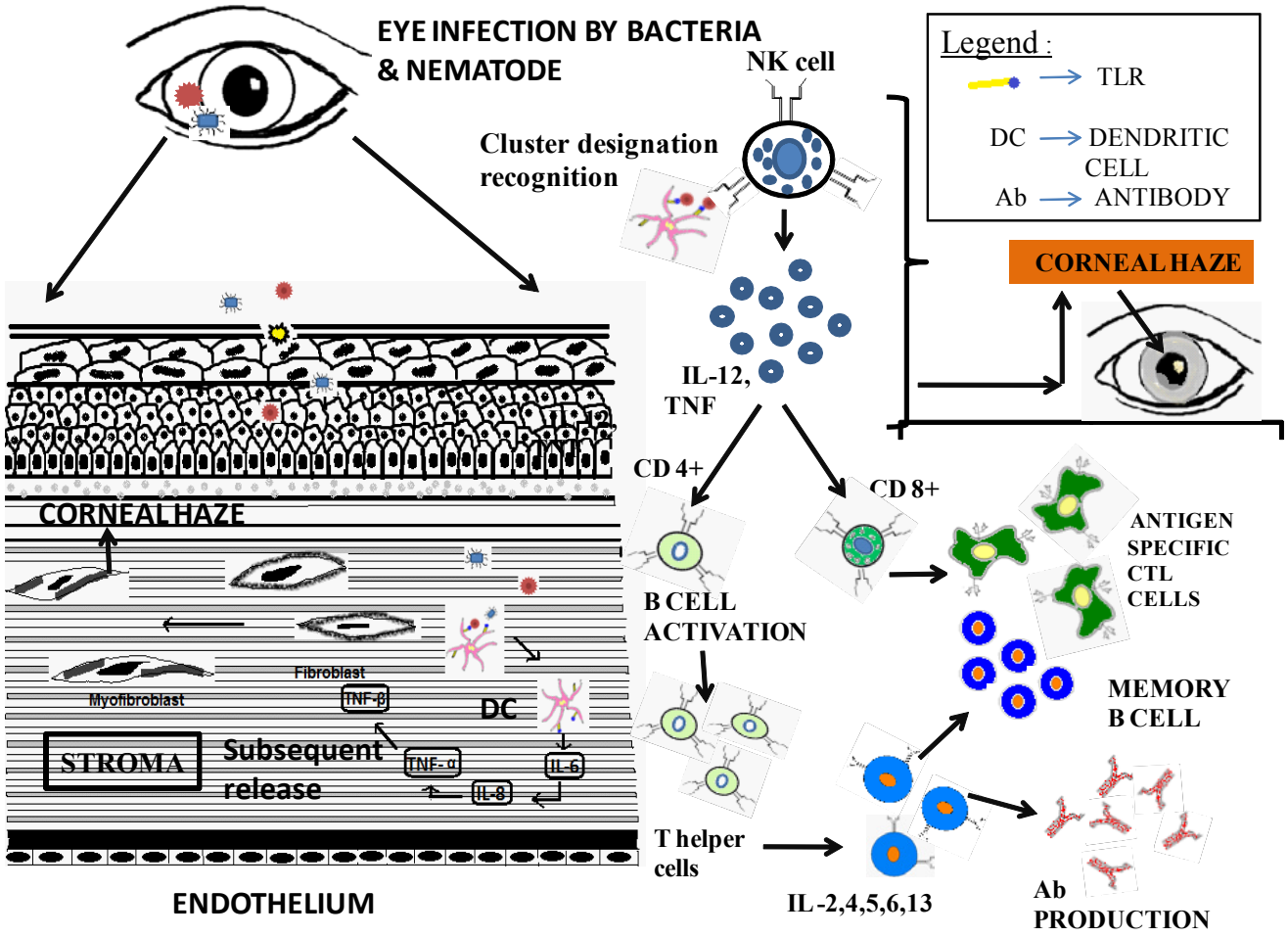

Figure 1: Cornea is made of three layers such as outer epithelium, middle stromal cells and inner endothelium cells. Inflammation of eye can lead to serious loss of vision and is a leading cause of blindness. Immune responses in the eye are modulated by tissue resident cells. Cornea is exposed to microbes constantly. Entry of bacteria into the eye results in rapid proliferation since vitreous acts an excellent medium. Ocular barrier breakdown and inflammatory cell recruitment is expected due to initiation of inflammatory products cascade. Though lysozyme secretion catalyzes the hydrolysis of cell membrane, some pathogenic bacteria possess high *in vitro* resistance to lysozyme. When they breach the epithelial layer, TLRs of ocular cells recognise the Ag and secrete proinflammatory cytokines like IL-8, IL-1 and TNF- α. These chemokines attract DCs to the infection site. Release of TGF-β, IL and CXC-2 recruits neutrophils to the infected site. This process results in transformation of normal fibroblast to myofibroblast, thus reducing corneal transparency. Once the sentinels take up the Ag, they present it to NK cells which then activate T-helper cells and indirectly the B cells. As a result antibodies are raised against the Ag.

responding to microbial products by discriminating the pathogenic bacteria from non-pathogenic ones. Corneal opaqueness which is often referred as corneal haze results in patients who are treated for myopia, hyperopia and astigmatism. Sometimes it disappears within 6-9 months but not in all cases. *Wolbachia,* an endosymbiotic bacteria lives in a parasite *Onchocerca volvulus* which mainly infects Africans [53]. During these infestations, the parasite resides in subcutaneous nodules and releases millions of microfilariae which migrate to anterior chamber of eye. As a result of immune defense, several chemokines and cytokines are released to combat the infection. As a result of the release of CXCR2, a chemokine produced by stromal cells, neutrophils are recruited to the site [54]. This phenomenon results in the transformation of fibroblast present in stromal layer to myofibroblast with high expression of smooth muscle actin. These myofibroblasts accumulate extracellular matrix and matrix metalloproteins in the infection site to alter biophysical environment of the infectious site. Corneal opacity is also caused by decreased expression of corneal crystallins by activated fibroblasts [55]. A Major threat to aboriginals in Australia is Trachoma infection which leads to morbidity and blindness (Figure 1). In most cases, active trachoma in the conjunctiva heals without any loss of vision. On the contrary, chronic infection leads to block of lachrymal ductules leading to loss of tears thus creating a platform for secondary infections. Apparently vaccines produced only short term immunity and hence TLR agonist can be used against this infection. No particular synthetic TLR agonist has been synthesized till now for Trachoma yet LPS can be an alternate agonist to some extent [56-58]. Approach to corneal haze with nanotechnology is in its infancy. Various nanoformulations comprising TLR agonist and antagonists have been used. Studies by Ueta clearly show that corneal epithelial cells engage TLR 3 to produce proinflammatory factors in response to poly (I: C), a viral mimic [59].

Enhancement of tumor immunity by augmenting DCs

Current cancer vaccine suffers from poorly quantifiable T-cell response and immunosuppressive tumour microenvironment. So there is a need to improvise the vaccine engineering such that lasting

immune response is acquired with reversal of immunosuppression in tumour environment. Customized nanoparticles are designed to meet the expectations of targeted delivery with prolonged half-life in the body [55]. Nanotechnology based drug delivery uses a variety of nanovectors including liposomes, micelles, dendrimers, protein nanoparticles, ceramic nanoparticles, polymeric nanoparticles and metallic nanoparticles [60,61]. These are used to carry and deliver drugs specifically to the site of tumour. These drug delivery systems generally consist of three parts; the core material, a therapeutic drug and surface modifiers [62]. The surface engineering involves functionalisation with ligands such as peptides, aptamer, antibodies, nucleic acids or small molecules in order to increase specificity and efficacy of treatments [63]. Anti tumour drugs with low molecular weight tend to distribute throughout the body by systemic circulation, yet diffuse from the cancerous tissue easily reducing the residence time and eventually get eliminated by renal filtration. Therefore, high molecular weight polymer like polyethylene glycol [PEG] is conjugated to drug molecules, so that the drug tends to stay within the leaky vasculature of tumour cells. Non PEGlyated particles have short retention time in the systemic circulation [64,65]. Tumour targeting can be passive or active [66]. Passive targeting takes advantage of leaky endothelial cells, which are from fast growing cancerous tissue, so that nanoparticles can enter and accumulate in the interstitial space. This method is more primitive as it relies on diffusion [62]. A more favourable approach can be achieved if the nanoparticles specifically target tumour cells and are activated once the target is reached. Active targeting involves the conjugation of drug to the nanoparticle of a targeting constituent to provide specific accumulation of nanoparticle at the tumour site [67,68]. Tumor cells overexpress receptors or Ags, which can be helpful for the efficient uptake of drugs via receptor-mediated endocytosis. Various ligands such as proteins, antibodies, carbohydrates, nucleic acids and aptamers are used for targeted delivery [69]. Imaging the nanoparticles is the next hurdle in the field of therapeutics. The development of multifunctional nanoparticles in which drug delivery, sensing and imaging capabilities are integrated is known to be a powerful system. Nanoparticles are targeted to APCs for enhancement of tumor immunity. Specific targeting of DCs through nanoparticles enhances the proliferation of Ag specific CD8 and CD4 cells [70,71].

After phagocytosis, when nanoformulation gets dislodged from endosomes, Ag or drug is released. The cytosolic Ag is then recognised by MHC class I molecule on DCs leading to cross presentation. Hamdy et al. examined that co-delivery of ovalbumin and 7 acyl lipid A in PLGA-nanoparticle augmented DC maturation and ovalbumin-specific CD4+ and CD8+ T-cell proliferation responses. Following up this study, encapsulated ovalbumin and TLR 9 in PLGA showed tumor regression of about 80% in animals with ovalbumin expressing B16 melanoma. Mutual activation of DC and NK cells in the presence of TLR agonist forms the basis for tumour suppression [72]. Pham et al experimented in TLR 4 agonist to enhance the maturation of DCs by NK cells. Lower doses of cyclophosphamide and IL-12 promoted the articulation of tumor specific CTL and several co-stimulatory molecules. This vaccine model was found to inhibit mouse colon cancer [CT-26] [71]. A research group headed by Conforti et al. [50] revealed that TLR 3 stimulates the epithelial cells of tumor to intiate chemokine cascade. This fundamental knowledge gave rise to use of poly (A:U) to activate CCL5 and CXCL10 [Chemotactic cytokines]. Systemic administration of poly (A:U) displayed immunoadjuvant effect through TLR3/TLR7. Both these TLRs are essential for clonal selection of Ag specific CD8+ T cells. When poly (A:U) is combined with vaccine or chemotherapy the anti tumour titre was elevated [73].

Cell specific targeting via mannose receptors

In an early study, cell specific ligands for targeted drug delivery were investigated using mannan coated nanoparticles [74]. DCs and macrophages both express mannose receptors (ManR). ManR are PRRs which recognise glycoproteins present in fungal cell walls, viruses and in prokaryotes. ManR is a scavenging receptor which recycles back to the cell surface where it can be employed in consecutive cycles for internalisation. The nanoparticles coated with mannan ligands can be used for targeting APCs to enhance the particle uptake for increasing the anti tumor titre [74]. Without addition of the mannan receptor ligands on the nanoparticles, Ag recognition is limited with low T cell responses. Binding and cellular uptake was improved from 5% of the uncoated nanoparticles to about 53% with mannan coated nanoparticles, again demonstrating the advantage of cell specific binding in targeted delivery systems [75]. To augment anti tumor response through IL-12 gene therapy, mannosylated chitosan nanoparticles are employed for effective endocytosis [76]. Additionally, mannosylated chitosan greatly increase the Ag availability to the macrophages and DCs for better induction of IFN-γ release [77]. Similiar to TLRs, ManR also provide a link between innate and adaptive immunity.

Multifactorial nanoparticles used in various studies

Biodegradable nanoparticles are engineered to imitate the surface properties of pathogens to enhance the property of phagocytosis [2,78]. Diwan et al. fabricated nanoparticles from a biodegradable copolymer (PLGA) to deliver cancer Ags to DCs for promoting strong anticancer T cell response [78]. Two Ags, tetanus toxoid and MUC1 [mucin peptide], along with immunomodulators were studied. The Ag was capable of binding to both MHC class I and class II molecules, for activating $CD4^+$ and $CD8^+$ T cells. Two other immunomodulators like MPLA and CpG were studied for effective tumor immunity. MPLA, a ligand for TLR4, was found to have the strongest effect on T cell activation. CpG oligonucleotides following phagocytosis activated TLR9 pathway [78]. Conclusively, Ags delivered in the form of nanoparticles are very effective in T cell activation when compared to soluble Ag [60]. It was noted that Ag presentation by both MHC molecules as well as TLR co stimulatory signaling are required for the potent activation of Ag specific primary T cells, underlining the significance of co-delivery of immunostimulatory molecules [79,80].

Ideally, tumour associated Ag [TSA] specific cytotoxic T cells are generated after the efficient delivery of TSA to APCs, but effective delivery methods are not yet available. Triggering of antitumor immune responses can be achieved using Freund's incomplete adjuvant, but due to poor tumour specific cytotoxic T cell induction and adverse side effects, a superior method is desirable. A report by Yoshikawa et al. showed that use of biodegradable poly (γ-glutamic acid)-based (γ-PGA) nanoparticles greatly elicited a potent immune response without adverse side effects [81]. The γ-PGA nanoparticles efficiently translocate from endosome into the cytosol for efficient MHC class I Ag presentation. As γ-PGA nanoparticles enhance Ag presentation, $CD8^+$ T cells are promoted to differentiate into cytotoxic T cells, displaying antitumor activity. Hence γ-PGA nanoparticles are used widely in Ag delivery based tumor vaccines [81]. Though the mechanism of immune response has been extensively researched, further investigation regarding the intricate mechanism of γ-PGA nanoparticles in activating the TLR and adaptive immunity is required.

From the age old concept of immunology, both CTLs and NK cells are employed for eliciting antitumor immunity [79]. Exogenous

administration of poly (I:C), a TLR 3 agonist resulted in tumor regression with the activation of NK and CTLs [82]. This method of TLR 3 agonist targeting has been used in a number of different studies for triggering of apoptosis in tumor cells [83,73].

An interesting study by Stone et al. showed that when CD40 and TLR agonists were combined on a single platform they generated a much stronger immune response. Other notable effects of this combinatory approach resulted in elicitation of potent tumour-specific $CD8^+$ T cells These findings highlighted the concept of long lasting immunological memory against tumor through elicitation of effector T cells [84].

Transition of DCs from immunosuppressive stage to immunostimulatory state by immunostimulants is also necessary for effective immunological response. The production of IL-12p70 (IL-12 in a biologically active form with two subunits p35 and p40) is accomplished by synergistic use of two TLR agonists viz poly (I:C) and R-848 for the activation of TLR 3 and TLR 7/8 respectively. 10-100 fold increase in IL-12p70 was noticed upon addition of CD40L. CD40L is used additionally since they enhance CD+8 T-cell responses

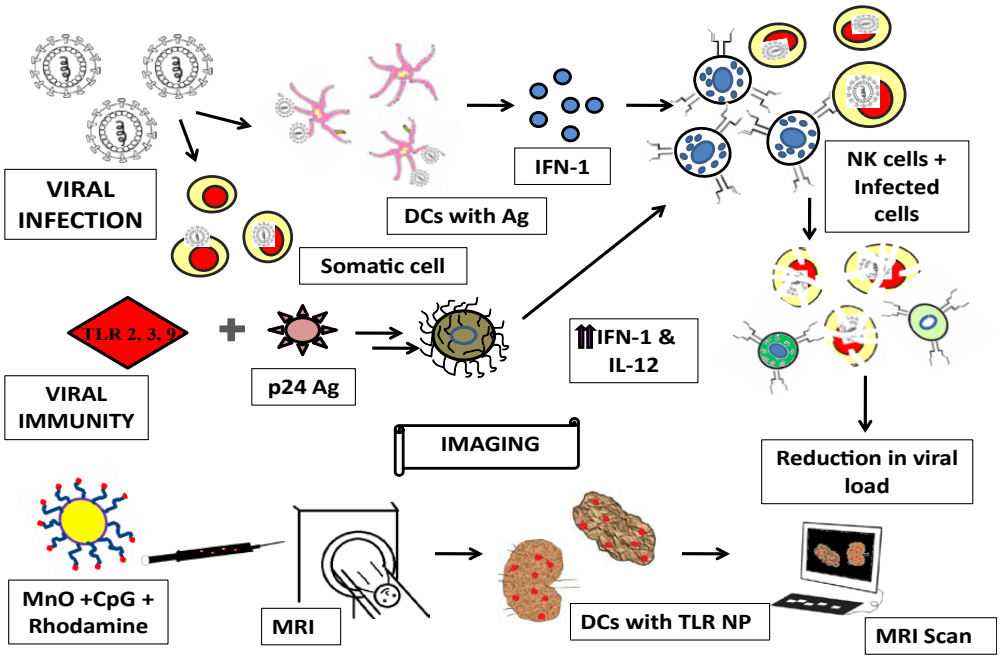

Figure 2: Highly active retroviral therapy (HAART) of p^{24} Ag from HIV virus is engineered with TLR 3 and encapsulated to form microcapsule. This formulation results in increased production of chemokines. When this preparation is supplemented along with HAART, the fold of T cells doubled which is the positive response in HIV patients. When HIV epitope peptide is coupled with TLR2/6, TLR 3, TLR 9 the avidity of Ag is increased. Viral infection leads to IFN-1 release by APCs. As a result NK cells get activated to lyse the viral infected cells. Mean while clonal expansion of CD4+ and CD8+ cells takes place to combat the infection. Advantage of this TLR mediated vaccine is the level of IFN-1 which is shown to be elevated after the treatment. Use of magnetic nanoparticle core [MnO] with incorporation of CpG ODN using phosphoramidite enables trafficking of TLR nanoparticles. Photodection is enabled by introducing rhodamine into polymer chain. Location of magnetic nanoparticle with rhodamine can be visualised by MRI. The dual role of imaging and eliciting immunity is the catch of this technique.

TLR type	Function
TLR 1+ TLR 6	Recognises triacyl peptides [11]. Decreases infection related morbidity and mortality. Necessary for good vaccine response [92].
TLR2 in complex with TLR1 or TLR6	Lipoproteins and lipopeptides recognition [12,25]
TLR3	Viral double-stranded RNA recognition and trigger apoptosis directly [13].
TLR4	Lipopolysaccharide recognition [15] Germinal centre formation, Ig maturation [93], Neuroinflammation and brain damage [94].
TLR5	Flagellin recognition [30] Protect the intestine from commensal bacteria [95]
TLR7 or TLR8	Single-stranded RNA recognition [17] Chemoresistance [96].
TLR9	Microbial DNA recognition [18]
TLR 10	Only human TLR without any agonist or function [97].
TLR 11	Preventing infection to internal organs of urinogenital system [20].
TLR 12	Unknown [21]
TLR 13	Unknown [21]

Table 1: Functions of various TLRs.

[85]. Though many successful vaccines induce persistent antibody responses, the above strategy holds best for the induction of robust immunity.

TLR immunity against viruses

Viral infections are often not cleared entirely from the body unlike bacterial infections. There is a major global threat from viral infections such as Human Immuno deficiency Virus [HIV], Ebola, Avian Influenza Virus and West Nile Virus. Prevention using vaccination is critical for such infections, since only a few viral medicines are available. TLR responsiveness to viral glycoproteins has been reported in many cases. Viral nucleic acid sensing TLRs are endosomal and hence viral nucleic acid has to be taken up by APC's for processing and signalling. TLR 3 and TLR 9 play the most important role in viral immunity. TLR 3 recognises the viral double stranded RNA and TLR 9 perceives the non-methylated CpG dinucleotides present both in viral and bacterial genome [43]. Other TLRs such as TLR 7 and 8 also plays a crucial role in viral immunity. They initiate IFN–α and IFN-β production in DCs and monocytes through IRAK-4 TLR adaptor [86]. Studies show that virus can elicit both innate and adaptive immunity by TLR dependent and independent pathways. Delivery of appropriate TLR agonist for viral immunity through a nanovehicle is a challenging task. Several nanovehicles have been studied extensively. Nanocarrier systems made of calcium phosphate are preferred as a biomedical carrier due to the wide use of this material in biomedical application. It has been shown that calcium phosphate with CpG coated by a viral peptide Influenza A virus hemagglutinin [HA] serve as a potent antiviral vaccine. In nanoformulations these calcium phosphate particles are readily taken by cells and rapidly driven out without altering the intracellular levels of calcium [87].

Haes et al. researched on synergising p^{24} Ag (HIV) with TLR 3 ligand poly (I:C) on monocyte derived DC cells. This showed an increased response of CCR7, CD80, CD83, CD86 and IL-12 (Figure 2). This study described the use of biodegradable microcapsule composed of poly electrolytes. Ovalbumin was used as an exemplary Ag for encapsulation into these microparticles to promote Ag presentation by DCs to CD4+ and CD8+ cells. Electrostatic interactions between microcapsules and TLRs stimulated the production of IL-12 and IFN-1 from DCs]. IFN helps to keep the HIV viral load down by inducing T helper and cytotoxic T lymphocytes. Thus microcapsules act as an efficacious Ag delivery vehicle in the field of HIV vaccination. Use of HIV envelope protein and ligands for TLR2/6, TLR 3, TLR 9 increased the functional avidity of Ags compared with using ligands for only two of above mentioned TLR for clearance of virus from the host [88].

The majority of vaccines against sexually transmitted disease are given parentally, eliciting a high immune response. Vaccine against genital herpes is designed with the knowledge that DCs, epithelial cells of vagina and cervical mucosa express TLR2. Delivering TLR 2 agonist induces protective immunity against this disease. Newly designed vaccine contains Herpes Simplex Virus type 2 (HSV2), $CD8^+$ T cell peptide epitope with palmitic acid (TLR agonist). When the above formulation was administered to wild type B6 which is TLR 2 /MD88 deficient, the mice produced HSV 2 specific memory $CD8^+$ cytotoxic T cell in genital tract and in spleen [89,90]. This self adjuvanting vaccine can be improved by the employment of nanoparticle to enhance prolonged circulation time in the blood. Advances in nanotechnology have pushed forwarded the synthesis of magnetic nanoparticles for the theragnostics.

The core of the magnetic nanoparticle is commonly made of MnO to which ssDNA is incorporated by phospharamidite. The chromophore [rhodamine] is attached to the polymer chain for imaging. The incorporated ssDNA efficiently elevates TLR 9 pathway for enhancing the antiviral immunity. This novel approach is gaining importance for its optical and magnetic resonance properties [91].

Conclusions

Though targeting DCs using TLRs signalling is still in its infancy, it is considered as a very promising technology. Nanodelivery is a suitable method due to rapid and efficient uptake by DCs, and subsequently potent immune responses can be achieved. Co-delivery of cancer associated Ags and TLR agonist loaded nanoparticle has been shown to generate potent immune response. Understanding how these delivery systems affects the mechanism of DCs in stimulating naive T cells is limited and needs further research. In addition, cellular and molecular mechanisms behind these immune responses are not completely understood, and some immunotherapy studies have led to development of autoimmune conditions in patients. Therefore identification of those molecular triggers involved in innate and adaptive immune systems may revolutionise cancer treatments and vaccine technologies. Once a greater understanding of these signaling pathways has been gained, more effective treatments could include combinations of agents that trigger multiple immunologic pathways.

Future Perspectives

Prevention is better than cure, yet it is difficult to prevent certain disease to which our body is prone. Considerable attention has been given to TLRs over the last decade, furthering our theoretical and mechanistic understanding of innate and adaptive immunity. A current need is to produce safe, immune potentiators or adjuvants using TLRs. The cornea serve as an ideal model for TLR exploration and experimentation as it is easily accessible and enjoys relative immune privilege. Opportunistic diseases like HIV can be addressed through TLRs with the hope of potential treatment development. Further studies on this molecular target will increase our understanding of TLRs. In the future we may see clinicians employing selective TLR ligands as an adjuvant to generate tolerance or utilizing small molecules to modulate TLR signaling in the prevention or treatment of various disorders.

Executive Summary

- Dendritic cells induce T cell response and indirectly elicit adaptive immunity.
- Toll like receptor recognise pathogens through pathogen associated molecular patterns.
- IL-1 receptor studies led to the insight of TLR.
- The three phase in which tumour escapes: elimination phase, equilibrium phase and escape phase.
- Human's express TLR 1-10 gene, TLR 11, TLR 12, TLR 13 gene has been found to explicit in animals.
- TLRs are used as adjuvants to trigger dendritic cell maturation, leukocyte migration, release of chemokines and cytokines for enhanced immunity.
- Corneal angiogenesis studies are conducted in rabbit eyes. Cornea express most of the TLRs.

- Corneal haziness can be sorted out in humans by use of TLRs in combination with nanoparticles.
- Nanoparticle drugs conjugated with PEG follows EPR effect and found to be efficient in combating tumour.
- Viral immunity is attained by employing TLRs. Even the dreadful virus HIV is combated using TLR ligands.

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