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Dendritic Cells for Active Anti-Cancer Immunotherapy: Targeting Activation Pathways Through Genetic Modification

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Abstract

Tumour immunotherapy has become a treatment modality for cancer, harnessing the immune system to recognize and eradicate tumour cells specifically. It is based on the expression of tumour associated antigens (TAA) by the tumour cells and aims at the induction of TAA-specific effector T cell responses, whilst overruling various mechanisms that can hamper the anti-tumour immune response, e.g. regulatory T cells (Treg). (Re-) activation of effector T cells requires the completion of a carefully orchestrated series of specific steps. Particularly important is the provision of TAA presentation and strong stimulatory signals, delivered by co-stimulatory surface molecules and cytokines. These can only be delivered by professional antigen-presenting cells, in particular dendritic cells (DC). Therefore, DC need to be loaded with TAA and appropriately activated. It is not surprising that an extensive part of DC research has focused on the delivery of both TAA and activation signals to DC, developing a one step approach to obtain potent stimulatory DC. The simultaneous delivery of TAA and activation signals is therefore the topic of this review, emphasizing the role of DC in mediating T cell activation and how we can manipulate DC for the pill-pose of enhancing tumour immunotherapy. As we gain a better understanding of the molecular and cellular mechanisms that mediate induction of TAA-specific T cells, rational approaches for the activation of T cell responses can be developed for the treatment of cancer.

Keywords

Dendritic cell; toll like receptor; transcription factor; cytotoxic T lymphocytes; gene therapy; cancer

GENERAL INTRODUCTION

The concept of a central role for the immune system in the treatment of diseases is longstanding. Already a century ago George Bernard Shaw postulated that the stimulation of phagocytes is at bottom the only genuinely scientific treatment for all diseases [1]. At the same time, Paul Ehrlich formulated his immune surveillance hypothesis, stating that the immune system can distinguish self from non-self and that the latter can be eliminated without damaging the former [2]. He put forward the fundamental immunological idea of unique receptors on immune cells. It took another 70 years to identify – what we now know

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as - the T cell receptor (TCR) (reviewed by [3]). Originally these concepts were devised for explaining immune responses against foreign invaders. However, Burnet and Thomas, translated these in view of anti-tumour immune responses in their so-called tumour surveillance hypothesis, proposing a model in which tumour cells are recognized as foreign by the immune system and subsequently specifically eliminated without damaging their healthy counterparts, much in the same way as for virus infected cells, thus avoiding auto-immunity [4].

The identification of antigens that are specifically expressed by the tumour, but not by normal tissues offered a rationale for this hypothesis. These tumour-associated antigens (TAA) can be divided in several groups according to their expression pattern [5]. As melanoma constitutes the most immunogenic cancer, most of the TAA known to date were identified from melanoma tumours. It has been demonstrated that each of these antigens can give rise to a number of epitopes that will be presented in major histocompatibility (MHC) class I or class II molecules and that these MHC/peptide complexes can be recognized by the TCR of $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ T cells, respectively [6]. The former are able to kill tumour cells by secreting cytolytic granules or activating death receptor-associated apoptosis pathways, while $CD4^+$ Th 1 cells secrete cytokines that assist in the activation and maintenance of CTL, as well as in inhibition of tumour cell proliferation [7]. Extensive research into these TAA and immune responses directed against them has instigated attempts for the manipulation of the anti-tumour immune response by actively trying to stimulate immunity - CTL and Th 1 cells - against these TAA and consequently against the tumour itself. These therapeutic vaccination approaches have been developed as an alternative or additional treatment regimen to classic therapies, such as radiotherapy, chemotherapy and surgical removal of tumours.

It has to be mentioned that although cancer cells can potentially be distinguished from normal cells through this arsenal of TAA, most of these TAA are still considered as auto-antigens by the immune system. As a consequence, several tolerogenic mechanisms as well as different modes of active inhibition will impede the induction of a productive anti-tumour immune response. A manifestation of this phenomenon is the presence of high numbers of CTL in the tumour and the periphery without measurable tumour regression [8]. Taken together, for any therapeutic vaccination strategy to be successful, it is crucial not only to induce a specific immune response against the tumour, but also to overcome intrinsic tolerogenic and suppressive mechanisms.

DC-BASED THERAPY OF CANCER

Immunotherapy seeks to mobilize a patient's immune system for therapeutic benefit. It can be passive, i.e. transfer of immune effector cells (T cells) or proteins (antibodies), or active through vaccination. In cancer, passive immunotherapy can lead to some objective clinical responses, thus demonstrating that the immune system can reject tumours [9]. However, passive immunotherapy is not expected to yield long-lived memory T cells that might control tumour outgrowth.

Active immunotherapy using TAA and adjuvant has the potential to induce both tumour-specific effector and memory T cells. In this regard DC, known as the professional antigen-presenting cells of our immune system, are considered as nature's adjuvant and are therefore well studied as a cellular anti-cancer vaccine [10].

DC Subsets

DC were first visualized as Langerhans cells in the skin in 1868 by Paul Langerhans, then Ralph Steinman and Cohn identified DC from mouse spleen in 1973 [11]. They constitute a

distinct lineage of white blood cell development, which was originally thought to share a progenitor with other phagocytes, such as macrophages and granulocytes. Although granulocyte-macrophage colony stimulating factor (GM-CSF) was recognized as a key stimulating cytokine for these populations, DC were regarded as a separate cell lineage, because of their unique properties [12]. The intricate development and life cycle of DC is reviewed in detail in numerous reviews [13-15]. In summary, DC can originate from both lymphoid and myeloid progenitors, giving rise to several distinct subsets, which are defined by the expression of surface markers and by their function [16]. Although the definition of DC subsets in mouse is much more complicated than in human, they can be broadly divided into myeloid DC and plasmacytoid DC. These DC subsets differ in their life span, cytokine production, receptors for antigen uptake, receptors for microbial ligands, which are essentially pathogen recognition receptors, such as toll like receptors (TLR), as well as receptors for cytokines and chemokines and linked herewith their function. Plasmacytoid DC, characterized by the expression of BDCA-2 and a relative long life span are able to make high amounts of interferon-α (IFN-α) in response to viral infection [17, 18]. These DC can – although very weakly - capture soluble and particulate antigens [19], respond to IL-3 [20] and express TLR9, which engages bacterial DNA and demethylated CpG DNA [21]. In contrast to plasmacytoid DC, myeloid DC respond to GM-CSF rather than IL-3 [22] and are characterized by the high expression of CD11c and low expression of CD123 [23]. They express a number of endocytic receptors that are not found on plasmacytoid DC and that enable them to efficiently sample the environment [15]. They are further characterized by their ability to make IL-12 in response to several stimuli, amongst which ligands of TLR2 (lipopeptides), TLR3 (dsRNA) and TLR4 (bacterial cell wall components) [24].

It has to be mentioned that although production of IFN-α is the major function of plasmacytoid DC and production of IL-12 is the major function of myeloid DC, both can be stimulated to produce IL-12 or IFN-α, respectively [25-27]. This cytokine pattern induced upon interaction of pathogen associated patterns with pathogen recognition receptors is part of an important final stage of DC differentiation, i.e. maturation, which comprises more than up-regulation of cytokines and which is pivotal for determining the subsequent immune response. The maturation of DC and the induction of immunity versus tolerance are discussed in more detail in the following section.

Regulation of T Cell Immunity by DC

DC have a remarkable functional plasticity, inducing either tolerance or immunity. The hypothesis is that distinct DC developmental and activation stages play a role in the induction of tolerance versus immunity. This is correlated with the two-signal model of T cell stimulation, in which it is proposed that a productive T cell immune response requires specific recognition of MHC/peptide complexes by the TCR (signal 1) along with signalling through co-stimulatory molecules (signal 2). Indeed both immature myeloid and plasmacytoid DC, have been described to induce T cell tolerance [28-30], whereas both mature DC types have shown to induce immunity [31-33].

Immature DC are specialized in capturing antigens. They efficiently take up pathogens, apoptotic cells and antigens from the environment by phagocytosis, macropinocytosis, or endocytosis, process these, but are considered to be unable to present these antigens efficiently to T cells [34]. Therefore, immature DC are believed to induce T cell anergy and/or Treg [35, 36]. Indeed, in steady-state conditions, DC remain immature and tissue-resident, expressing small amounts of MHC molecules as well as co-stimulatory molecules, hence inducing T cell anergy instead of T cell activation upon DC-T cell interaction [37]. Furthermore, injection of immature DC in humans can be tolerogenic [28, 38, 39]. On the other hand, mature DC are considered to be immunogenic. Maturation of DC can be induced by a variety of signals, such as microorganisms [40, 41], cytokines [42], interaction with

$CD4^+$ Th cells [43-45] and chemicals like haptens [46-48]. DC maturation is associated with several coordinated events, including: (a) loss of endocytic and/or phagocytic receptors; (b) changes in morphology; (c) up-regulation of MHC molecules, co-stimulatory molecules such as CD40, CD80, CD83 and CD86, adhesion molecules and chemokine receptors, such as CCR7 [10]. The latter is one of the first changes and enables the DC to migrate from the peripheral tissue to the T cell areas of the draining lymphoid organs [49], where DC present peptides, derived from antigens acquired in the periphery, in the context of MHC to passing T cells. The phenotypic changes – high expression of antigen presenting, co-stimulatory and adhesion molecules - make mature DC potent inducers of T cell immunity.

However, the view that immature DC induce tolerance and mature DC induce immunity is simplified. It has been demonstrated that mature DC can contribute to T cell tolerance through the induction of Treg [50]. It was suggested that it is the maturation trigger that dictates the DC's T cell polarizing or tolerating immune functions. Some triggers have been demonstrated to promote production of cytokines, such as IL-12 and IFN-α, hence inducing Th 1 responses. These include molecules derived from bacterial or viral products, such as LPS, CpG motifs and dsRNA, pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and IFN-α, as well as T cell signals, mainly the interaction of CD40Ligand expressed on $CD4^+$ Th cells with CD40 expressed on DC [51]. By contrast, anti-inflammatory molecules, such as IL-10 and prostaglandin E_2 (PGE2) hamper full DC maturation and cytokine production, thus resulting in the stimulation of Treg responses [28].

In summary, instruction of adaptive immunity occurs largely through triggering the maturation of DC. When appropriately activated immature DC transform from highly phagocytic, tissue-resident cells into highly immunogenic, lymph node-homing cells that are competent to induce T cell responses to non-self antigens acquired in the periphery.

DC Activation in View Of Tumour Immunology: A Role for TLR

As mentioned in the general introduction, immune tolerance represents a major barrier in the induction of effective cellular anti-tumour immune responses. This can be explained by the fact that tumour cells arise endogenously and that most TAA are therefore recognized as self. As a consequence, many T cells of the tumour-specific T cell repertoire have been eliminated centrally or peripherally [52]. Additionally, tumour cells have acquired several mechanisms to suppress T cells that have escaped this selection. These include antigen presentation in the absence of co-stimulation, expression of inhibitory/death inducing signals (e.g. PD-L1, FasL), expression of immunomodulatory enzymes (e.g. indoleamine-2,3-deoxygenase) and secretion of immune suppressive cytokines and chemokines (e.g. IL-10, transforming growth factor-β (TGF-β) [53].

Moreover, these secreted inhibitory factors not only suppress effector T cells, but also promote the existence of immune cells with suppressive functions, such as Treg, regulatory DC (DCreg) and myeloid-derived suppressor cells [54, 55]. In Fig. (**1**), the different mechanisms of anti-tumour immunity and tumour-induced suppression mentioned throughout the introduction are summarized (Fig. **1**).

One of the paradigms of immunology is that a productive T cell immune response requires specific recognition of an MHC/peptide complex by the TCR (signal 1) along with adequate signalling through co-stimulatory molecules (signal 2). Recently, data indicate that to establish strong anti-tumour responses, an inflammatory environment (signal 3) is required alongside functional recognition of T cells by antigen-presenting cells. This can be achieved by strong activation of the innate arm of the immune system, in particular through TLR. The critical roles of TLR in activation of adaptive immune responses have been well documented and extensively reviewed by [24, 56-58]. In summary, TLR have been shown to control

adaptive immune responses at multiple levels, including the control of antigen uptake, through the remodelling of the actin cytoskeleton, resulting in uptake of antigens *via* macropinocytosis [59].

Furthermore, it was demonstrated that the efficiency of presenting antigens from phagocytosed cargo is dependent on the presence of TLR ligands within the cargo. Blander *et al*. demonstrated that the generation of peptide/MHC class II complexes is controlled by TLR in a strictly phagosome-autonomous manner. The enhanced rate of phagolysosomal maturation in the presence of signals from TLR2 and TLR4 has been clearly demonstrated [60-62]. Thus, antigen sampling by DC is controlled by TLR and the engagement of TLR signalling helps DC to distinguish between cargo containing self or non-self antigens, a property that can be exploited to induce anti-tumour immune responses. These TLR further control the maturation of DC, including their capacity to migrate, the up-regulation of the co-stimulatory molecules CD40, CD80, CD86 and CD70 and production of primarily Th 1 cytokines like IL-1, IL-6, TNF-α and IL-12. The very fact that protective Th 1 cytokine response can be achieved by using TLR signalling is another interesting property in terms of anti-tumour immune responses [24, 56, 63]. Yang *et al*. showed that in order to break tolerance of tumour-specific CTL, persistent TLR ligation should be provided alongside antigen-loaded mature DC [64]. Similarly, Lang *et al*. showed that even in a transgenic mouse model where the antigen is highly expressed in the target organ (in this case the pancreas) adoptively transferred CTL were not able to actually lyse the target cells. Only concomitant TLR ligand administration induced frank diabetes [65]. Besides a lack of sufficient stimulation to break tolerance, several inhibitory mechanisms, such as the presence of Treg need to be overcome. In mouse studies, it has been shown that depletion of these Treg greatly enhances the anti-tumour response [66, 67]. Again, signalling through certain combinations of TLR on DC not only provided a synergy with respect to the production of cytokines such as IL-12 [68, 69], but also offers protection from Treg inhibition [70]. From these data it is clear that the engagement of TLR signalling pathways is a promising mechanism for boosting DC-based vaccine responses.

MECHANISMS OF TLR-SIGNALLING

Understanding the mechanism of TLR signalling and its correlated feedback mechanisms is pivotal to the exploitation of these pathways for the pill-pose of enhancing DC-based vaccines. Binding of TLR to their ligands, usually bacterial or viral-derived proteins and nucleic acids trigger a complex network of signalling molecules and adaptor proteins that will ultimately modulate DC responses in innate and adaptive immunity [24, 71, 72]. All these signalling networks cooperate, integrate and finally converge into a few pathways, such as that of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) [72-76]. In addition, these pathways interact with each other [77, 78]. It is this crosstalk together with the strength, timing and context of stimulation that determines the type and duration of immune responses [79-81]. The MAPK and NF-κB pathway are schematically represented in Fig. **2** and will be discussed in more detail in the following sections.

Activation of MAPK Pathways in DC

MAPK are a diverse group of intracellular serine/threonine kinases firstly discovered in yeast. They are phylogenetically conserved and regulate a wide range of cellular processes ranging from cell growth, differentiation and survival to cellular stress and immune responses [82]. Three groups of MAPK have been identified: the extracellular signal-regulated protein kinases (ERK) [83, 84], the c-Jun N-terminal kinases (JNK) [85, 86] and the p38 stress-activated protein kinases (p38) [87, 88]. They are structurally related and their catalytic core is blocked by a conserved activation loop [89]. Phosphorylation of the activation loop unblocks the catalytic site leading to phosphorylation of specific targets.

Generally, MAPK activation starts at cellular membranes (plasma membrane or in intracellular organelles) after the engagement of receptors to their ligands. MAPK pathways consist on a three-modular cascade involving activating phosphorylations of downstream kinases by upstream kinases. In this way, MAPK kinase kinases (MAPKKK) are firstly activated by phosphorylation after their recruitment to the cytoplasmic domain of receptors through multi-protein complexes. These phosphorylated MAPKKK subsequently phosphorylate MAPK kinases (MAPKK), which in turn will phosphorylate MAPK.

In DC, MAPK are always activated after signal transduction from TLR. TLR are integral membrane proteins with an extracellular domain that binds to molecular patterns, a single transmembrane domain and a cytoplasmic domain. The TLR cytoplasmic tail contains the Toll/IL-1 receptor (TIR) domain, essential for signal transduction [90]. Simplifying, mainly (but not exclusively) two TIR-domain adaptor molecules, MyD88 (myeloid differentiation factor 88) and TRIF (Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β) are recruited to the TLR TIR domain. Then, MAPKKK are activated through further recruitment of various protein kinases and scaffold proteins, such as ubiquitin ligases TNFR-associated factor 6 (TRAF6) and TRAF3 [91]. MAPK activation has been shown to depend on MyD88-TRAF6-mediated recruitment of a complex containing MAPKKK TAK1 (transforming growth factor-beta-activated kinase-1) [92], but also in an alternative pathway involving TRIF-TRAF3 [91]. However, there might be other pathways leading to MAPK activation depending on the cell type or the type of stimulus. As an example, Ubc13 E2 ubiquitin-conjugated protein has been shown to regulate MAPK signalling through IKKγ binding in B cells, independently of TRAF6-TAK1 [93].

The MAPK ERK Pathway

The extracellular signal-related kinase (ERK) comprises at least 8 isoforms from which ERK1 and ERK2 are the most extensively studied. The classical ERK activation pathway starts with the binding of growth factors to cell membrane receptors such as epidermal growth factor receptor (EGFR) [94]. This results in Ras activation and association to the MAPKKK Raf-1. Raf-1 subsequently phosphorylates MAPKK, MEK1 and MEK2 (MEK1/2), which in turn phosphorylate ERK1/2 threonine and tyrosine residues at its activation loop. In fact, there is evidence that Raf-1 plays an important role in LPS-dependent ERK activation during DC maturation [95]. However, this study was performed in an immortalised murine DC2.4 cell line and it does not rule out the possibility of Raf-1 phosphorylation by MEK1 through a positive feedback mechanism [96]. In the context of TLR-dependent ERK activation, TAK1 plays a prominent role [97]. However, ERK phosphorylation was not completely abrogated in cells lacking TAK1 [92], suggesting additional TLR-associated pathways leading to ERK phosphorylation. Classical ERK targets range from kinases such as 40S ribosomal protein S6 kinase (RSK) to transcription factors such as c-myc, cFos, ATF-2, Elk-1, Ets-1 and c-Jun just to name a few [98].

In DC, ERK is involved in cell survival [73], in regulation of inflammation and immune suppression [99, 100]. In fact, ERK negatively regulates DC maturation through secretion of immunosuppressive cytokines such as IL-10 and TGF-β [76, 77, 99-105]. As already mentioned above, ERK is virtually activated by signalling through all TLR through TRAF6-TAK1 and TRAF3, although it is preferentially activated by specific TLR such as TLR2 which promotes immune suppression and tolerance [75, 103, 105] or by other TLR depending on the cell type and stimulation context [71, 106, 107].

Actually, inhibition of ERK phosphorylation with specific MEK inhibitors PD98059 and U0126 enhances DC maturation, increases pro-inflammatory cytokine secretion and counteracts tumour-induced immune suppression [101, 108-110]. Consequently, it is tempting to speculate that some kinase inhibitors used in cancer chemotherapy [111] could

be effective immune stimulators by ERK inhibition in antigen-presenting cells. In fact, the tyrosine kinase inhibitor sunitinib, widely used in the treatment of metastatic renal cancer among others, enhances anti-tumour immune responses by decreasing numbers of myeloid-derived suppressor cells and Treg [112]. However, in this particular case, immune activation was linked to inhibition of STAT3 phosphorylation, although some ERK inhibition was observed [113].

The MAPK p38 Pathway

MAPK p38 comprises at least four splice isoforms from which p38α and p38β are ubiquitously expressed. In the context of TLR activation, p38 phosphorylation is completely dependent on TAK1 activity as shown in embryonic fibroblasts and B cells [92]. In fact, it has been shown that TAK1 phosphorylates MKK3 and MKK6, the two main p38 MAPKK [114, 115]. In turn, MKK3 and MKK6 phosphorylate p38 within its activation loop. Classical p38 targets range from kinases such as MK2 and transcription factors such as ATF, p53, c/EBPβ and NFAT just to mention a few [116].

MAPK p38 plays an important part in DC maturation and secretion of pro-inflammatory cytokines. Therefore, it is not surprising that p38 inhibition is one of the most frequent strategies for the treatment of auto-immune and inflammatory diseases [111]. In fact, LPS and TNF-α-dependent up-regulation of DC co-stimulatory molecules and maturation markers such as CD40, CD80, CD86, CD83 and MHC class II is inhibited by SB203580, a specific p38 inhibitor [117]. Combined evidence from p38 specific inhibitors and KO mice has shown that the p38 pathway is involved in secretion of IL-1β, TNF-α, IL-6 and IL-12 [117-119]. Accordingly, LPS-induced p38 activity could up-regulate transcription of cytokine genes by ATF2 and CREB phosphorylation [82, 120]. However, there is evidence that p38 activity on its own does not up-regulate pro-inflammatory cytokine genes [100, 121]. In fact, p38 induces changes in chromatin structure that allow NF-κB to bind to cytokine promoters leading to their transcriptional transactivation [121].

Remarkably, MAPK p38 and ERK play antagonistic effects in many cell types, including antigen-presenting cells such as macrophages and DC [101]. There is growing evidence that ERK and p38 regulation is interconnected by several mechanisms. In macrophages, TGF-β-activated ERK up-regulates MAPK phosphatase 1 (MKP1) that eventually dephosphorylates p38 [122]. In some cases, p38 stimulates phosphatase PP2A activity, leading to MEK1/2 dephosphorylation [98, 123]. In zymosan-stimulated splenic DC, ERK leads to p38 dephosphorylation through up-regulation of suppressor of cytokine signalling 3 (SOCS3) [105]. Moreover, direct interactions between ERK and p38 have been reported, adding a potential regulatory level in both MAPK pathways [124].

The MAPK JNK Pathway

c-Jun N-terminal kinase (JNK) proteins are encoded by 3 genes with 10 or more alternative splice forms [125]. From these, the JNK1 and JNK2 isoforms are the most extensively studied. JNK is activated by UV irradiation, environmental and chemical stress, pro-inflammatory cytokines and TLR signalling [91, 97, 114, 125]. JNK activation relies on its dual phosphorylation of threonine and tyrosine residues by MAPKK, MKK4 and MKK7. The classical JNK targets are c-Jun, from which it receives its name, ATF2, p53, Elk-1 and c-Myc amongst others [98, 120, 125, 126].

ATF2 and c-Jun are components of AP-1, a transcription factor that transactivates many pro-inflammatory genes. Consequently, JNK is involved in inflammatory responses and inhibitors are being evaluated in clinical trials for the treatment of autoimmune diseases such

as rheumatoid arthritis as well as asthma, inflammatory diseases and some types of leukaemia [111].

In the context of TLR signalling, TAK1 is involved in JNK activation through MKK3 and MKK7 phosphorylation [92, 97, 114, 127]. In general, JNK activity enhances DC maturation and pro-inflammatory cytokine secretion, although at lower levels compared to p38 [100, 128]. Treatment of DC with the specific JNK inhibitor SP600125 decreased LPS-induced c-Jun phosphorylation, inhibited TNF-α and IL-12 secretion and slightly inhibited up-regulation of co-stimulatory molecules CD80, CD86, CD83 and ICAM-I [128]. Therefore, JNK has been considered as a cooperative pathway together with NF-κB and p38, rather than playing a central role in DC maturation.

The NF-κB Pathway in DC

NF-κB was first identified in 1986 as a transcription factor that binds to a 10 base pair DNA element, consensus sequence 5′-GGGAATTTCC-3′ and its many variations, in the κ immunoglobulin light-chain enhancer in B cells, hence its name [129].

The molecules that comprise the NF-κB family have been shown to be central to immunity and inflammation [130]. Their importance in DC biology has been demonstrated by the many DC-related defects in NF-κB KO mice [131-136].

The NF-κB family members exist as homo- or heterodimeric complexes formed by combinations of five distinct DNA binding subunits, p65/RelA, RelB, c-Rel, p50 and p52. Of these, RelA, RelB and c-Rel are synthesized in their mature forms and contain a transactivation domain that interacts with the transcriptional apparatus. On the other hand, p50 and p52 are synthesized in precursor forms, which contain C-terminal ankyrin repeats that are proteolysed by the proteasome resulting in the production of mature proteins. Both p50 and p52 contain a DNA binding domain, but lack a transactivation domain. It is noteworthy that in particular RelB has been implicated in the differentiation and maturation of DC. RelB-deficient mice lack mature myeloid DC [132, 137]. Furthermore, DC in which RelB expression is inhibited retain an immature phenotype and are associated with induction of immune tolerance *in vivo* [138].

All the NF-κB proteins are characterized by the presence of a highly conserved 300 amino acid Rel homology domain that is located at the N-terminus of the protein. It is this domain that is responsible for the dimerization and subsequent DNA binding. Furthermore, this Rel homology domain interacts with specific inhibitory factors known as inhibitors of κB (IκB) proteins. A number of different IκB proteins exist. These are IκBα, IκBβ, IκBγ, IκB and Bcl-3. It is generally believed that the different isoforms are associated with particular Rel protein dimers, bound *via* their Rel homology domain. For example IκBα and IκBβ associate with RelA/p50 and p50/c-Rel, whereas IκB only binds to RelA and c-Rel hetero- and homodimers. The binding of IκB to these dimers keeps these NF-κB dimers in an inactive state, mainly in the cytosol. The NF-κB/IκB complex can shuttle between the cytoplasm and the nucleus in unstimulated cells, but nuclear export of the complex is inefficient and therefore, the NF-κB/IκB complex is mainly cytoplasmic in resting cells.

NF-κB can be activated by many divergent stimuli, including pro-inflammatory cytokines (such as TNF-α, IL-1), T cell delivered signalling (CD40L), bacteria, viruses and cellular stress (such as UV, ionizing radiation and chemotherapeutic agents), signals that mediate DC maturation [139]. Different NF-κB activation pathways have been described [140, 141]. Essentially, these pathways share adaptor molecules with the above-described MAPK pathways and can basically be divided into a classical (canonical) and non-classical pathway [140, 142, 143]. Both pathways start a chain reaction of events resulting in the activation of

IκB kinases (IKK), which stimulate the phosphorylation and ubiquitination-induced degradation of IκB, as such releasing an active form of NF-κB.

IκB kinase (IKK) is a multi-subunit protein kinase consisting of two highly homologous catalytic subunits, IKKα and IKKβ, which phosphorylate IκB and a non-enzymatic regulatory subunit, IKKγ (also called NEMO, NF-κB essential modulator), which is required for the activation of IKKα/IKKβ heterodimers in response to pro-inflammatory cytokines, such as TNF-α and IL-1 [144-146]. Phosphorylation of IκB at two critical serine residues (Ser32/Ser36 in IκBα and Ser19/Ser23 in IκBβ) in their N-terminal regulatory domain by the IKK complex targets them for rapid polyubiquitination and subsequent degradation by the 26S proteasome [147]. This IκB isoform phosphorylation is stimulus specific, for example IκBβ is only phosphorylated by certain stimuli including LPS and IL-1β, whereas most NF-κB activators trigger IκBα phosphorylation. This level of control is also thought to impact on the cell type specificity and kinetics of the response, which in turn can influence the duration of transcription.

In the classical pathway, it has been shown that IKK®, but not IKKα, is important in NF-κB activation. Furthermore, it has been demonstrated that these two kinases have distinct rather than overlapping functions [148-151]. The classical pathway includes signalling from TLR/IL-1R family members, intracellular pattern recognition receptors including retinoic acid inducible gene (RIG-I), melanoma differentiation associated factor-5 (MDA-5) and protein kinase R (PKR), as well as signalling from the TNFR (reviewed by [152]). Mediators such as lymphotoxin-β, CD40L and receptor activator of NF-κB ligand (RANKL) activate the non-classical pathway [153, 154]. This pathway involves IKKα phosphorylation, processing of the p52 precursor p100 and nuclear translocation of the heterodimer p52/RelB and is believed to play a key role in adaptive immunity [155, 156].

The NF-κB pathway is further controlled by post-translation modifications, i.e. phosphorylation and acetylation. These modulate the interaction of Rel proteins with other components of the transcriptional machinery and alter their kinetics in and out of the nucleus. The phosphorylation status of NF-κB can influence activation, e.g. phosphorylation of RelA may enhance transcriptional activation. On the other hand phosphorylation of the p50 precursor p105 can reduce its processing into p50 [157]. NF-κB can also associate with other transcriptional proteins such as histone acetyltransferase and histone deacetylase (reviewed by [158]). Acetylation of the Rel proteins can increase the time it is located in the nucleus [159]. Furthermore recent data suggest that the phosphorylation and acetylation steps occur stepwise, phosphorylation of RelA is required prior to and enhances acetylation of Rel A in order to improve transcriptional activity of NF-κB [160].

As mentioned above, NF-κB targets different genes, encoding proteins that are associated with mature DC, amongst these are cytokines (e.g. IL-6, IL-12, TNF-α), chemokines (e.g. MIP-1α, MCP1), adhesion molecules (e.g. ICAM-1), inducible effector enzymes (e.g. COX-2), as well as regulators of apoptosis (e.g. c-IAP, XIAP, Bcl-xL). Since, NF- κB regulates so many different immune activation-related genes and hence controls the properties of DC, tight regulation by negative feedback is required to avoid the generation of chronic inflammatory milieu that can lead to autoimmunity. Several feedback molecules have been described, including IκB, A20 (or TNF-α inducible protein 3, TNFAIP3), tripartite-motif protein (TRIM) 30α and phosphatase PP2A, to name a few [161-166].

The remainder of this section will introduce A20, since A20 has been recently studied and shown to be potentially interesting in view of enhancing tumour antigen-specific immune responses. A20 was originally identified as a TNF-inducible gene in human umbilical vein endothelial cells [167]. Subsequent research demonstrated that A20 is also induced in many

other cell types, amongst which mouse and human DC [161, 168, 169]. A20 expression is under the immediate control of NF-κB. Therefore, it is not surprising that A20 expression is observed in response to various stimuli including TNF, IL-1 and TLR ligands [170-173]. Recently, it has been demonstrated that A20 is an ubiquitin-editing enzyme with de-ubiquitinase activity in the N-terminal region and ubiquitinase activity in the zinc finger domain of the C-terminal region. It is through this dual ubiquitin-editing function of A20, that it can modulate down-regulation of NF-κB signalling, this through interaction with several proteins of the TNF-, IL-1/TLR-signaling pathways, amongst which TRIF, RIP, IRAK1, TRAF6, NEMO/IKKγ and IκB-kinases [169, 170, 172, 174-177]. Similar to NF-κB, A20 can be further regulated by post-translational modification. An IKK-dependent phosphorylation of A20 at Ser381 has been shown to increase the ability of A20 to inhibit NF-κB activation. The mechanism, however, has yet to be elucidated [178]. The role of A20 in DC will be discussed in the section ‘down-regulation of feedback mechanisms’.

MODIFICATION OF DC IN ORDER TO MIMIC PERSISTENT TLR SIGNALLING

DC are considered to be the most potent antigen-presenting cells of the immune system, one of the most well-studied vaccination approaches is the use of DC loaded *ex vivo* or *in vivo* with TAA in one form or another to boost/induce anti-tumour immune responses in cancer patients. Several approaches have been studied for loading DC with antigen, the description of which is outside the scope of this review. Of these the most promising strategies introduce the genetic information of the entire tumour antigen fused to MHC class II targeting signals, hence obtaining antigen-presentation to both $CD8^+$ and $CD4^+$ T cells, respectively [179, 180]. Broadly speaking, these genetic modification methods can be divided in viral and non-viral methods, which have been reviewed in [14, 181-184]. In recent years, these strategies have been employed to not only deliver TAA, but simultaneously deliver signals that result in functional modification of DC, be it the introduction of activation-inducing molecules for anti-tumour therapy or the introduction of activation inhibitors, with the purpose of inducing antigen-specific tolerance.

CO-DELIVERY OF TUMOUR ANTIGEN AND CONSTITUTIVE ACTIVE TLR4 TO DC

Both mouse and human DC express TLR4, which binds LPS (aided by CD14 and MD-2), resulting in DC maturation [185]. This is mediated by both NF-κB and MAPK [82, 117]. LPS-mediated activation has been shown to remarkably enhance the ability of DC to stimulate antigen-specific immune responses *in vitro*. Moreover, it was reported that LPS-activated DC could overcome suppression by Treg, a critical factor in anti-tumour immunology [186]. However, the clinical use of LPS for immunotherapy is prohibited due to cytotoxicity concerns as well as batch-to-batch variability. Therefore, several groups have focused on the generation and evaluation of a constitutive active TLR4 (caTLR4) for DC maturation [187-190]. Therefore, the TLR was truncated between positions M620 - P621 and linked to the nerve growth factor leader sequence [187, 189] or the leader sequence of LAMP1 [188]. Alternatively the cytoplasmic domain of TLR4 was linked to the extracellular single-chain immunoglobulin anti-erbB2 [190]. These caTLR4 molecules were subsequently delivered to DC under the form of mRNA, either by transfection [187, 189] or electroporation [188] or through retroviral transduction [190]. Evidence for NF-κB activation upon introduction of caTLR4 was delivered and it was shown that DC modified with this caTLR4, either [188] or not [187, 189] in combination with CD40L, become mature. Moreover, these DC were able to stimulate CTL, specific for both the strong immunogen MelanA and the weak immunogen Trp2 [187-189].

Co-delivery of Tumour Antigen and Activation Signals to DC

With regard to activation of DC *via* TLR4, Hamdy *et al.* have shown that vaccination of B16 tumour bearing mice with PLGA nanoparticles co-encapsulating the poorly immunogenic melanoma antigen, TRP2, along with the TLR4 ligand, 7-acyl lipid A, has therapeutic effects [191]. With regard to co-delivery of TAA and TLR ligands in a one step modification, we have demonstrated that electroporation can be applied for the simultaneous delivery of TAA encoding mRNA and dsRNA, in particular Ampligen or poly(I:C_{12}U), which is a GMP-grade synthetic analogue of the TLR3 ligand, poly(I:C) and therefore suitable for human and more importantly clinical use [26, 161]. This strategy resulted in up-regulation of A20, thus suggesting activation of NF-κB [161]. Moreover, small amounts of Ampligen were sufficient to induce fully mature DC, capable of efficiently stimulating MelanA-specific immune responses [26].

Besides the delivery of these activation stimuli using non-viral strategies, it has been repeatedly shown that many of the viral vector preparations used for delivery of antigens are inherently activating DC [192-197]. It has been demonstrated that different virus types, amongst which adenovirus, lentivirus (human immunodeficiency virus and equine anaemia virus), as well as Moloney murine leukaemia virus, up-regulate co-stimulatory and antigen-presenting molecules on DC. In case of adenoviral and lentiviral vectors, it has been further shown that these induce production of Th 1 and pro-inflammatory cytokines, resulting in DC with strong T cell stimulatory capacity. This can be explained by the triggering of intracellular signalling pathways, such as those initiated by 2′,5′-oligoadenylate synthetases (2′,5′-OAS) and PKR. Furthermore, it has been demonstrated in a 293T reporter assay that lentiviruses can activate several TLR [192, 193]. Additionally, it has been shown that direct administration of lentiviral vectors results in transduction of cells at the periphery, that these cells migrate to lymphoid organs [198] and are able to stimulate antigen-specific immune responses [199-204]. The latter two observations further deliver circumstantial evidence for the capacity of lentiviral vectors to activate DC.

Delivery of Signal Adaptor Molecules to DC (Fig. 3)

DC function can effectively be modulated using gene therapy techniques by the introduction of genes that control DC differentiation and maturation. NF-κB has been one of the first pathways to be targeted in DC as an adjuvant strategy. In this case, NF-κB inducing kinase (NIK) was over-expressed using adenovirus vectors. NIK over-expression increased DC maturation as assessed by up-regulation of co-stimulatory molecules, MHC molecules and secretion of pro-inflammatory cytokines [205]. In this context, co-expression of NIK with GFP significantly increased Th1 GFP-specific immune responses, although the relevance of this strategy in anti-viral or anti-tumour immunity was not assessed [205]. Another strategy has been the introduction of MyD88, TRIF or IRAK-1, all major TLR adaptor molecules, which when over-expressed were shown to transduce downstream signals without TLR stimuli. Akazawa *et al.* [206] successfully introduced MyD88 and TRIF in mouse DC using lentiviral vectors, resulting in DC with different properties. MyD88-modified DC produced IL-6 and IL-12p40, but showed no up-regulation of phenotypic markers, whereas TICAM-1 introduction stimulated interferon IFN-α production and had enhanced levels of CD86. Both MyD88 and TRIF augmented the allo-stimulatory capacity of the DC. Moreover, antigen-specific responses could be efficiently induced and tumour outgrowth was delayed by immunization with these MyD88- and TRIF-modified DC [207]. Xu *et al.* generated retroviral vectors encoding the chimeric proteins consisting of the extracellular single-chain immunoglobulin anti-erbB2 linked to an intracellular TLR-signalling component composed of either MyD88 or IRAK-1. Their experiments were performed in the DC line, JAWS II, in which only the IRAK-1 chimera was able to mediate IL-12 and TNF-α secretion. The latter was subsequently, evaluated for its capacity to enhance the T cell stimulatory capacity of

DC, demonstrating enhanced ovalbumin-specific OT-II $CD4^+$ T cell responses [190]. A fourth study targeted NF-κB activation in DC using lentiviral vectors, by expressing Kaposi's sarcoma associated human herpes virus (KSHV) vFLIP [208]. In this case, DC maturation was enhanced by up-regulation of MHC I and II, co-stimulatory molecules CD80, CD86, CD40 and ICAM-I. In addition, increased antigen-specific $CD8^+$ T cell responses and expression of TNF-α and IL-12 was achieved and an enhanced anti-tumour and anti-parasite immunity were demonstrated [208, 209].

As already discussed, another group of signalling pathways with a critical role in DC function is the MAPK family. MAPK pathways regulate DC maturation and modulate the type of immune response in concert with NF-κB and other pathways. Therefore, the specific delivery of antigen with modulators of MAPK pathways to DC could enhance antigen-specific immune responses or induce antigen-specific immune suppression. Additionally, activation of individual MAPK pathways in the absence of TLR stimulation could dissect the specific role of each individual pathway in DC function. This could potentially lead to a fine-tuning of DC-dependent immune responses. MAPK have been recently targeted in DC using LV, by expression of selected constitutive activated mutants of the three main MAPK pathways [100].

MAPK ERK and p38 were activated by expressing MEK1 and MKK6 mutants containing glutamate and aspartate residues in their activation loop, mimicking activating phosphorylated serine or threonine residues [210]. A fusion protein between MKK7 and JNK1 was expressed to achieve constitutive JNK1 phosphorylation [211]. In addition, expression of constitutive activators prevents inactivation by negative feedback mechanisms such as MAPK phosphatase activities, which may be important to counteract tolerogenic mechanisms in anti-tumour immunity. In the absence of TLR stimulation, p38 activation resulted in a DC maturation phenotype different from full maturation as achieved by LPS treatment [100]. No specific effects on MHC levels or CD86 were observed. On the other hand, there was specific up-regulation of CD80, CD40 and ICAM-I. Additionally, no significant secretion of pro-inflammatory cytokines IL-12, TNF-α, IL-1β or IL-23 was detected [100], in contrast to studies using p38 inhibitors in the context of TLR stimulation [117-119].

Therefore, p38 activation would not be directly involved in transcriptional up-regulation of cytokine genes, as suggested by other studies [121]. Interestingly, co-expression of OVA with the p38 activator in DC significantly increased antigen-specific $CD4^+$ and $CD8^+$ T cell responses leading to increased anti-tumour immunity [100, 209]. MAPK p38 constitutive activation also increased $CD8^+$ T cell responses to human tumour antigens NY-ESO in an HLA-A2 humanised transgenic mouse model, and MelanA/MART-1 in a human DC-T cell culture [100].

Specific activation of JNK1 in DC showed only a moderate up-regulation of CD80 and ICAM-I and no significant secretion of pro-inflammatory cytokines. These results confirmed previous studies with inhibitors showing that in comparison with p38, JNK marginally control DC maturation [100, 128]. Nevertheless, increased antigen-specific $CD8^+$ T cell responses were detected after subcutaneous vaccination with LV expressing MKK7-JNK1, suggesting that JNK1 may play a subtle but still important role in DC *in vivo* [100].

In the absence of TLR stimulation, constitutive ERK activation in DC resulted in an immature phenotype with down-regulated CD40 and increased TGF-β expression [100]. Curiously, in contrast to abundant published literature on the subject, ERK constitutive activation did not induce IL-10 secretion [76, 77, 99-105]. This situation may be analogous to that of p38 and IL-12 secretion. ERK activation may be important but not sufficient for

high-level secretion of IL-10. Interestingly, co-expression of the ERK activator with an OVA transgene in DC led to impaired OVA-specific $CD4^+$ and $CD8^+$ T cell responses and systemic increase in Foxp3 Treg [100]. Therefore, constitutive activation of ERK in the absence of TLR stimulation can effectively suppress antigen-specific immune responses.

DOWN-REGULATION OF FEEDBACK MECHANISMS

As mentioned above, A20 is one of the feedback regulators of NF-κB. A20 shares some similarities with IκBα, another feedback regulator. For one, the expression of both is controlled by NF-κB and shows similar temporal profiles [212]. However, IκBα directly inhibits NF-κB, while A20 functions upstream to deactivate several adaptor molecules of the TNFR, IL-1/TLR signalling through its ubiquitinating/de-ubiquitinating properties. In this way, A20 controls IKK and thus the degradation of IκBα. Recent studies demonstrate that A20 is expressed in mouse DC activated with LPS (TLR4) [168], as well as in human DC activated with poly(I:C) or through electroporation with its clinically acceptable analogue, Ampligen [161]. This led to the hypothesis that down-regulation of A20 would result in prolonged NF-κB activation, hence mimicking persistent TLR ligation and as such resulting in DC with enhanced stimulatory capacity. RNA interference through introduction of a lentivirally delivered shRNA or through introduction of siRNA, respectively, was applied to down-regulate A20 [161, 168]. Both Song *et al.* and we demonstrated that A20 plays a crucial part in controlling the maturation, cytokine production and immunostimulatory potency of the DC [168]. While, we focused on the expression pattern of NF-κB, the secretion of cytokines, in particular IL-10 and IL-12 and their role in stimulation of MelanA-specific T cells, Song *et al.* focused on the ability of the DC to stimulate antigen-specific CTL and the effect of Treg on both DC and effector T cells.

In summary, human DC with down-regulated A20 expression have higher levels of NF-κB and show enhanced and sustained secretion of both IL-10 and IL-12. IL-10 was shown to negatively influence T cell proliferation, whereas IL-12 had an impact on Th 1 cell induction. In general these DC were more potent in the stimulation of MelanA-specific $CD8^+$ T cells [161]. Mouse DC with down-regulated A20 expression showed spontaneous and enhanced expression of co-stimulatory molecules and pro-inflammatory cytokines, where refractory to Treg inhibition and activated tumour-infiltrating CTL and Th cells that in turn were unresponsive to Treg mediated suppression [168]. These studies identify A20 as an antigen-presentation attenuator and an ideal target in view of anti-tumour immunotherapy, since it not only enables the DC to induce strong effector T cell responses and it suppresses the suppressors, the Treg.

FUTURE PERSPECTIVES

DC play a crucial role in regulating both innate and adaptive immunity. Accordingly, DC present many pathogen recognition receptors such as TLR that enable them to detect potential threats such as viruses and bacteria. TLR engagement leads to the activation of NF-κB and MAPK through MyD88 and TRIF-dependent pathways in a series of down-stream kinase cascades. The implication of each MAPK pathway in DC function and immunity has been extensively characterised by the use of specific MAPK inhibitors and by single activation of MAPK pathways. In this way, MAPK p38 plays a major role in regulating inflammatory immune responses, leading to increased immunisation, while JNK pathways play a secondary role (although probably important *in vivo*). ERK activity is linked to secretion of suppressive cytokines IL-10 and TGF-β and its activity is clearly linked to immune suppression and tolerance. On the other hand NF-κB, a transcription factor that can be induced upon stimulation with a variety of different stimuli, has always been associated with maturation of DC and their capacity to stimulate potent T cell responses, as well as to

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overcome Treg, hence with immune activation. Finally, NF-κB and MAPK pathways can be modulated in DC by gene therapy techniques, leading to either immune activation and increased immunity, or immune suppression and tolerance.

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ABBREVIATIONS

DC	Dendritic cell
TAA	Tumour associated antigen
Th	T helper
IL	Interleukin
Treg	Regulatory T cell
DCreg	Regulatory DC
CTL	Cytolytic T lymphocyte
TLR	Toll like receptor

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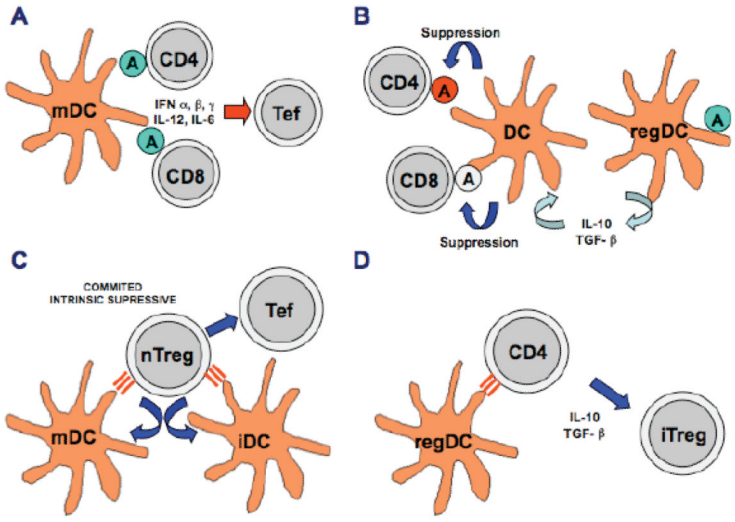

Fig. (1).

Anti-tumour immunity and tumour-induced suppression. In this figure, the four main mechanisms involving DC-T cell interaction in the regulation of tumour immunity are shown. (**A**) In an effective anti-tumour immunotherapy, mature DC (mDC) present TAA on their surface, represented as circled As. These antigens are recognised by specific CD4 and CD8 T cells in a pro-inflammatory setting (in the presence of the depicted cytokines), leading to differentiation of Th cells and CTL (T effector cells, Tef) from CD4 and CD8 T cells, respectively. (**B**) Antigen presentation also takes place in the tumour environment. In this setting, antigen-presenting cells such as DCreg induce antigen-specific and polyclonal tolerance leading to tumour-induced immune suppression. In the figure, a tumour-associated DCreg secretes tolerogenic cytokines IL-10 and TGF-β and converts other non-commited DC into tolerogenic DC. In an analogous manner as in A, antigens are presented to CD4 and CD8 T cells, which do not further differentiate into effector T cells. (**C**) Most TAA are either auto-antigens or mutated auto-antigens (quasi-auto-antigens). Therefore, natural T regulatory cells (nTreg) recognise these TAA both in the context of mature DC and immature DC (iDC) by high-affinity binding of their TCR (depicted in the picture as parallel bars between DC and nTreg). These natural Treg actively suppress DC and T effector cells by several mechanisms not yet well characterised. (**D**) Tumour-associated DCreg present TAA to naive and memory CD4 T cells in the context of a suppressive environment, in the presence of cytokines such as IL-10 and TGF-β, as in (**B**). In this situation, CD4 T cells differentiate into induced Treg (iTreg). There is increasing evidence that induced Treg can suppress immune responses in similar ways to natural Treg, as in (**C**).

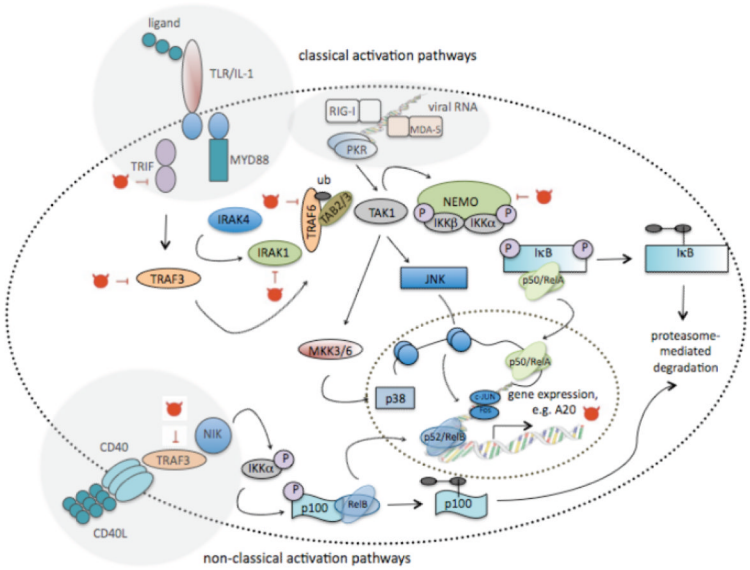

Fig. (2).

Schematic representation of the MAPK and NF-κB pathway that are reported to enhance DC maturation. The MAPK pathway is activated upon TLR stimulation, whereas activation of the transcription factor NF-κB can be initiated by a variety of stimuli, resulting in the so-called canonical or classical pathway, initiated upon ligation of TLR, RNA helicase and PKR versus the non-classical pathway, initiated by stimuli, such as CD40L, lymphotoxin-β or RANKL. The MAPK p38 aids the binding of NF-κB to its consensus sequence by altering the chromatin structure of the DNA. Both the MAPK JNK and NF-κB pathway are important in DC maturation. They drive the expression of co-stimulatory, adhesion and antigen-presenting molecules, as well as pro-inflammatory cytokines. Moreover, NF-κB drives the expression of several feedback proteins that attenuate activation, hence avoiding chronic inflammation. One of these, the zinc finger protein A20, which has ubiquitinating and de-ubiquitinating enzymatic activity, interacts with several adaptor proteins and has been studied as a mode to mimic persistent DC activation, as such enhancing the DC's potency to stimulate tumour antigen-specific T cells.

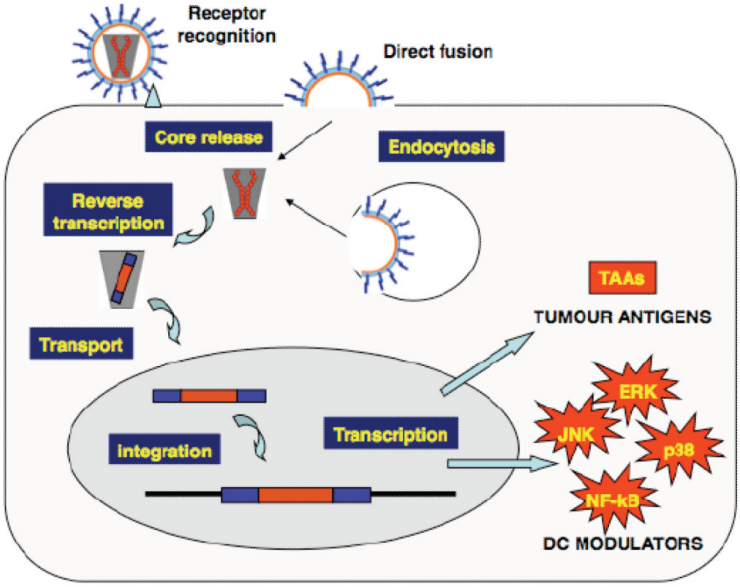

Fig. (3).

Targeting intracellular signalling pathways by genetic modification of DC. Gene therapy techniques allow the modification of DC function by introducing specific genes that modify intracellular signalling pathways. These signalling pathways will ultimately modify DC function by either increasing or suppressing their immunogenicity. In the figure, a viral vector carrying the therapeutic genes binds to the cell membrane (receptor recognition) and it fuses directly to the cell membrane leading to the core release. As it is the case of other vectors either based on retroviruses or not, virus-like particles can also be internalised by endocytosis. In the case of lentivectors (and other retrovirus-based vectors), through a process of reverse transcription, the therapeutic genetic information encoded as RNA is copied into a DNA version that is transported to the cell nucleus. Then, the therapeutic DNA genes are incorporated into the host genome, and they will lead to transcription and translation of both TAA and modulators of DC signalling (DC modulators). In this case, activation of ERK, JNK, p38 and NF-κB is depicted. Activation of these pathways will regulate DC maturation phenotype and cytokine secretion, and ultimately will enhance presentation of TAA to specific CD4 and CD8 T cells as shown in Fig. (**1**).

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