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Enhancement of Antitumor Vaccination by Targeting Dendritic Cell-Related IL-10

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Understanding mechanisms associated to dendritic cell (DC) functions has allowed developing new antitumor therapeutic vaccination strategies. However, these vaccines have demonstrated limited clinical results. Although the low immunogenicity of tumor antigens used and the presence of tumor-associated suppressive factors may in part account for these results, intrinsic vaccine-related factors may also be involved. Vaccines modulate DC functions by inducing activating and inhibitory signals that determine ensuing T cell responses. In this mini review, we focus on IL-10, inhibitory cytokine induced in DC upon vaccination, which defines a suppressive cell subset, discussing its implications as a potential target in combined vaccination immunotherapies.

Keywords: antitumor therapeutic vaccination, dendritic cells, interleukin 10, immunosuppressive cells, PD-L1, type I IFN

DENDRITIC CELLS IN THERAPEUTIC VACCINATION

Since William Coley treated cancer patients with bacterial extracts to activate immunity, therapeutic vaccination has been considered a promising immunotherapeutic approach (1). During the last decades, we have witnessed the identification of dendritic cells (DC) as professional antigen presenting cells (2), characterization of their biological properties (3) and subsets (4), as well as the development of new techniques and tools to directly purify them (5) or differentiate from peripheral blood precursors (6). Therefore, a plethora of vaccination clinical trials has been carried out, either through *in vivo* administration of antigens and adjuvants, or *ex vivo*-prepared antigen-loaded DC (7, 8). Advances related to understanding those receptors and biological pathways involved in antigen capture and DC activation have allowed developing new vaccines, in terms of improving antigen targeting (9) or vaccine formulation (10), as well as improving direct DC isolation or differentiation from precursors, antigen loading and maturation (11). Despite these efforts in improving vaccine immunogenicity, those strategies reaching clinical phases, have provided limited clinical results (7). Accordingly, there is only a single licensed therapeutic cancer vaccine, Provenge, approved for castration resistant prostate cancer (12).

Characterization of the tumor microenvironment has clearly demonstrated the presence of immunosuppressive mechanisms which render T-cells dysfunctional (13, 14), partially accounting for vaccine failure. However, there are vaccine intrinsic factors which have not been fully elucidated and whose characterization may also explain in part these results. A variety of protocols have been used in vaccination clinical trials, with differences in parameters such as the type of antigens, its loading method in case of DC vaccines, the adjuvant or maturation protocol and the type/stage of disease and patients vaccinated, among others (15). This heterogeneity has made difficult to draw solid conclusions to identify those factors linking properties of vaccines with the ensuing immunological and clinical results (16).

Thus, although characterization of the vaccine product is usually a requisite for its release, commonly analyzed parameters, usually related to pro-immunogenic vaccine properties, have not completely revealed the relevant clues on vaccine immunogenicity (17).

Activation of naive T-cells by DC requires antigen recognition on MHC molecules, co-stimulatory signals and polarizing cytokines, according to the three-signal model (18). Although immature, resting DC usually lack these molecules, in the case of infections, contact with microbial pathogens leads to antigen capture and simultaneous sensing of pathogen associated molecular patterns (PAMPs), inducing thus the upregulation of genes involved in eliciting immunogenic responses (3). Similarly, in the tumor setting, danger associated molecular patterns (DAMPs) and other signals released by dying tumor cells are known to promote DC activation (19, 20). However, as in many biological processes, recognition of these signals by DC may also lead to upregulation of genes associated with negative feedback mechanisms, regulating thus immune activation. These include expression of membrane-bound co-inhibitory ligands (21, 22) that modulate signal 2, together with the release of soluble molecules (cytokines and metabolites) (23, 24) modulating signal 3. Vaccines rely either on the administration of antigens and immunostimulatory molecules (adjuvants) which will reach DC *in vivo* (25), or on administering DC that have been antigen-loaded and stimulated *ex vivo* (15). Similarly to infectious processes, DC may upregulate these control elements upon vaccine administration or during the DC preparation process (in the case of DC vaccines). Therefore, understanding these feedback mechanisms and delineation of optimized strategies to block them may allow developing more immunogenic vaccines. In this Minireview we will focus on IL-10, a cytokine regulating many functions, describing those mechanisms that control their induction on DC, its effect on these cells during vaccination as well as the rationale to best block their inhibitory effect with therapeutic vaccination purposes.

IL-10: AN INHIBITORY MOLECULE IN VACCINATION

Among cytokines reported to down-regulate the activation of antitumor immune responses, IL-10 plays a prominent role. IL-10 is a pleiotropic cytokine traditionally considered as immunosuppressive for antigen presenting cell functions and concomitant priming of T-cells (26). Although initially considered a cytokine produced by Th2 cells (27) or Tregs, it is now known that it is produced not only by other lymphocytic subsets, but also by cells of innate immunity, including DC and macrophages (28). Stimuli such as Toll-like receptor (TLR) ligands or CD40 ligand (CD40L), usually present in microbial pathogens or induced because of inflammation, have been included as adjuvants in different vaccination strategies (25). Although they have a high capacity to promote DC maturation and release pro-immunogenic cytokines like IL-12 (29), they may also induce IL-10, even with synergistic effects in some

cases (30–32). There is an inverse relationship between IL-10 and IL-12 production by DC, which has been attributed to different mechanisms, including MAP kinase activation (33–35) and transcription factors NFIL3 (36) and DC-SCRIPT (37). Interestingly, DC-activating adjuvants have different cytokine-producing profiles, which may vary depending on the cytokine considered. Therefore, not all ligands have the same capacity to induce IL-10 (38), depending on the receptor involved and its associated signaling pathway. Indeed, although there are differences between murine and human studies because of the type of DC subsets and the corresponding TLR repertoire (39), some stimuli like TLR molecules (TLR2, TLR4, TLR7, or TLR9, among others) strongly induce IL-10 production (32, 40, 41). However, others like TLR3 ligand poly(I:C) or CD40 agonists (CD40L or antiCD40 antibodies) are considered poorer IL-10 inducers, mainly when used alone (42, 43). DC receptors responsible for sensing microbial or endogenous inflammatory/danger signals can be grouped according to the mediator molecules and the corresponding signaling pathways used to induce DC activation (44). Most TLR ligands signal through MyD88, with the exception of TLR3, which relies on TRIF for signal transduction, and TLR4, which depends on MyD88- and TRIF-dependent pathways (45). Other non-TLR DC receptors, such as lectins receptors, Nod-like receptors or RIG-like receptors, use other sets of signaling molecules, including Syk, ASC and MAVS (46). Thus, signaling through MyD88 or Syk vs. signaling through TRIF (47) may explain the distinct capacity to produce IL-10 by different ligands. Furthermore, some of these pathways lead to the activation of different MAP kinases (p38, Jun, ERK, among others) that promote or inhibit IL-10 and IL-12 production, depending on the relative activation balance between kinases. In this respect, signaling pathways resulting in the activation of ERK (33–35) and MK2 (48) have been shown to induce IL-10. In addition, the receptor and signaling pathway not only determine IL-10 production, but also the susceptibility of DC to autocrine effects mediated by its own IL-10. It has been demonstrated that IL-10 induced in DC after stimulation by ligands that signal through the MyD88 pathway (e.g., TLR4 or TLR9 ligands), inhibits DC functions, such as the secretion of cytokines IL-6 and IL-1β and expression of IL-12 p35, IL-23 p19, TNF-α, and IFN-β mRNA (49). In contrast, IL-10 induced by curdlan (a Dectin-1 ligand) does not affect DC functions. Finally, IL-10 receptor expression may also represent an additional control mechanism. Surface expression of IL-10 receptor is downregulated upon LPS-induced maturation (50), potentially explaining the increased susceptibility of immature vs. mature DC to IL-10 inhibitory effects. All these results suggest that there is an intricate network of interactions involving pathways regulating IL-10 production as well as those implicated in the susceptibility to its effects, which has to be considered when analyzing the role of IL-10 during DC activation.

IL-10 has been described in many cancer patients as a poor prognostic factor (51–54). It can be detected in serum (52, 53) and in the tumor (51), produced by tumor cells (55, 56) as well as by infiltrating hematopoietic cells, including myeloid (57) and lymphocytic subsets (58–61). Although IL-10 has an inhibitory
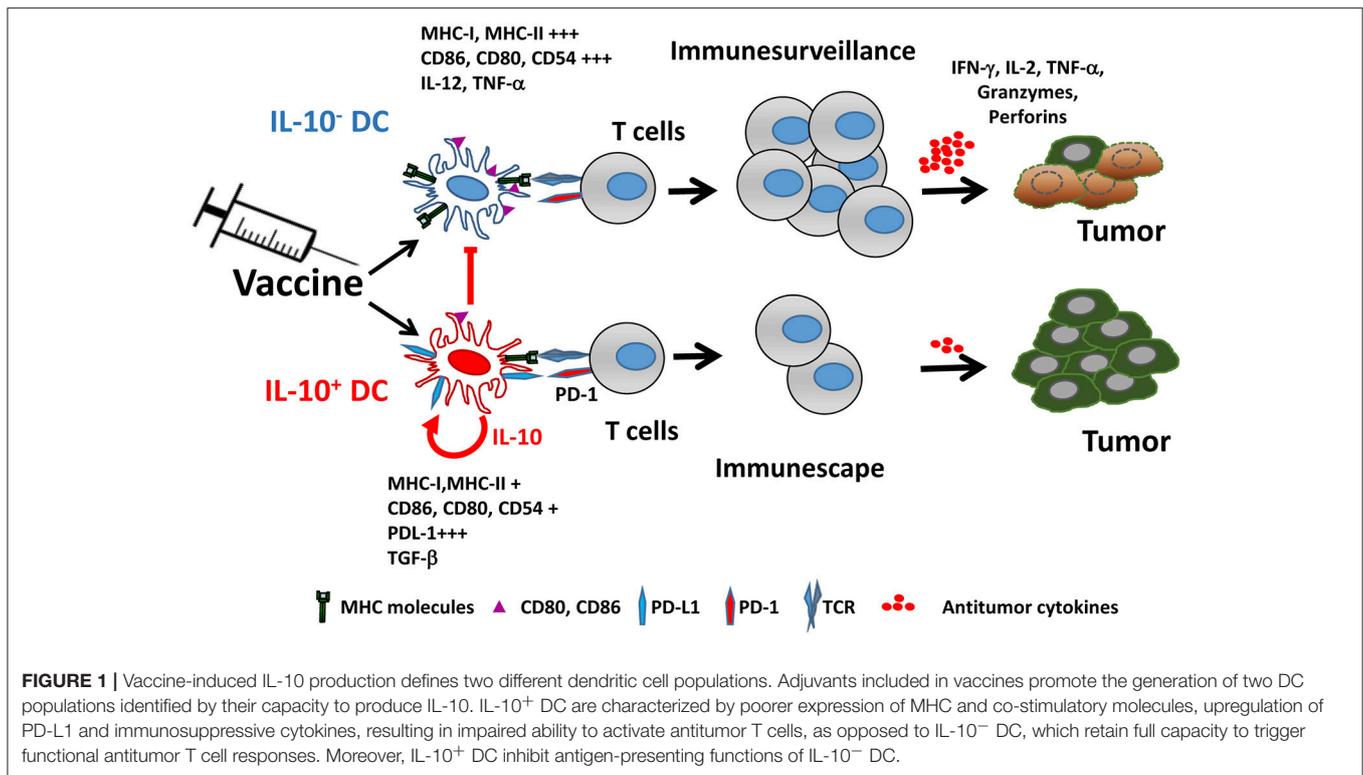
impact for antigen presenting cells, contradictory effects have been reported in the case of antitumor T-cells (62–64). In this regard, it has been demonstrated that IL-10 increases functional properties of already activated effector CD8 T-cells. In fact, in different murine tumor models, including mammary carcinoma, skin squamous carcinoma, and several transplantable models, such as breast, colon, and melanoma tumors, administration of IL-10 inhibits tumor growth, promoting antitumor functions of effector T-cells, increasing tumor infiltration, IFN-gamma production and lytic molecules (65, 66). However, regarding priming of naive T-cells during initial stages, IL-10 has been considered detrimental. This is due to its inhibitory role on antigen presenting cells at different levels, such as migration (67), expression of co-stimulatory molecules (68), production of Th1 polarizing cytokines (57, 69) and blocking cross-priming and priming with low-affinity peptides of a self/tumor-antigen and concomitant T-cell activation (70). Therefore, to consider IL-10 as a target in tumor immunotherapy, the immunological context has to be taken into account. In those settings where already primed effector cells exist, IL-10 may potentiate their properties (65, 66). However, if T-cell priming or generation of an antitumor immune response is needed, as in vaccination, therapeutic benefit would be achieved by IL-10 blockade. Indeed, preclinical data regarding IL-10 blockade has demonstrated an enhancement of vaccine immunogenicity, not only in the cancer setting (43, 71, 72) but also in other models (41, 73, 74). Although, as previously mentioned, IL-10 can be expressed by different cell subsets, we and others (43, 75) have demonstrated that antigen presenting cell-derived IL-10 down-regulates the elicitation of Th1 responses. Indeed, vaccine-dependent induction of IFN-gamma-producing T-cells is greatly enhanced by IL-10 blockade when using adjuvants promoting IL-10 production by DC (75), which in the case of tumors results in a stronger therapeutic effect (43). Thus, although different IL-10 sources coexist in the tumor setting, DC-derived IL-10 seems to determine vaccination efficacy.

IL-10 AS A MARKER OF SUPPRESSIVE DC

IL-10 production by DC not only affects their functional properties, but also identifies a subpopulation characterized by many immunosuppressive features, both at the phenotypical and functional levels. Mice vaccinated with IL-10-inducing adjuvants have an IL-10-producing DC subset (from now on IL-10+ DC), which is almost absent in unvaccinated mice. IL-10+ DC are characterized by poorer expression of co-stimulatory molecules and inflammatory cytokines, as well as by upregulation of co-inhibitory ligands such as PD-L1, resulting thus in a cell population with much lower T-cell stimulatory capability (76) (**Figure 1**). Interestingly, in addition to their low antigen presenting capacity, IL-10+ DC also suppress antigen presentation by third-party cells, reinforcing their inhibitory role. Some of these features of IL-10+ DC have been also observed in persistent viral infection models (77), suggesting that generation of this DC subset may not be specific of vaccines and operates in other settings.

There are no mechanistic experiments in the vaccination setting explaining the presence of different DC populations according to their IL-10 production. Indeed, it is not known whether the vaccine generates both types of DC from different precursors or the same DC subset may behave differently. However, in the viral model it has been demonstrated that, whereas IL-10- DC are originated from conventional DC precursors, the source of IL-10+ DC are monocytes differentiated as a consequence of inflammatory mediators, which are subsequently endowed with suppressive properties by type I IFN (78). This association between IL-10 production by DC and the presence of type I IFN, is in agreement with the vaccine setting, where many adjuvants known to induce IL-10 (ligands of TLR4, TLR7, and TLR9) also induce type I IFN (79), a cytokine that determines IL-10 levels (80). Moreover, IL-10+ DC have higher ISG expression, indicating a stronger response to type I IFN. IL-10 and type I IFN production have been linked as regulatory mechanisms, and blockade of IFN signal leads to a diminished IL-10 expression (81, 82). However, there may be additional factors behind IL-10 induction, since potent type I IFN inducers, such as the TLR3 ligand poly(I:C), are poor at inducing IL-10. Similarly, expression of other inhibitory molecules, such as PD-L1, also depends on type I IFN, in agreement with the pivotal role of this cytokine in generating the immunosuppressive effect on this DC subpopulation (81, 82). Interestingly, in addition to these immunoregulatory effects, it has been reported that type I IFN is required by DC to induce rejection of immunogenic tumors, supporting the induction of adaptive responses (83). Moreover, efficacy of therapeutic strategies such as vaccines (84) or other approaches relying on cross-presentation of tumor antigens by DC (85) also depends on type I IFN. Overall, these results suggest that type I IFN plays a dual role on DC-mediated tumor immunity, by promoting the generation of antitumor responses, but at the same time inducing regulatory mechanisms -including IL-10- to balance the magnitude of these responses (86).

In addition to the murine setting, there are several examples of suppressive DC in human studies, having in common the production of IL-10. Besides those protocols aimed at preparing tolerogenic DC by incubation with IL-10 in the therapy of autoimmune diseases, which are not the scope of this Minireview, there are also inflammatory conditions where induction of IL-10 takes place and results in similar suppressive functions. As an example, *in vitro* differentiation of monocytes in an inflammatory environment, such as the presence of TLR ligands (e.g., LPS or R848), led to generation of tolerogenic CD14+ DC expressing high IL-10 and PD-L1 levels (87) in a STAT3-dependent manner. Moreover, combined DC stimulation with potent TLR ligands results in partial inhibition, due to IL-10 induced by some of these compounds (42). More interestingly, the relevance of vaccine-derived IL-10 has been also demonstrated in the clinical setting. A recent paper (88) analyzing properties of monocyte-derived DC used as vaccines in prostate cancer patients reported that IL-10, in addition to CD14, and MCP-1 and MDC chemokines, identified a gene signature that could discriminate between patients responding or not to the vaccine. Authors found that

FIGURE 1 | Vaccine-induced IL-10 production defines two different dendritic cell populations. Adjuvants included in vaccines promote the generation of two DC populations identified by their capacity to produce IL-10. IL-10$^+$ DC are characterized by poorer expression of MHC and co-stimulatory molecules, upregulation of PD-L1 and immunosuppressive cytokines, resulting in impaired ability to activate antitumor T cells, as opposed to IL-10$^-$ DC, which retain full capacity to trigger functional antitumor T cell responses. Moreover, IL-10$^+$ DC inhibit antigen-presenting functions of IL-10$^-$ DC.

clinical and strong immunological responses correlated with low expression of molecules belonging to this gene signature, some of them, like CD14 and IL-10, already described as tolerogenic markers of DC. In addition to monocyte-derived DC, vaccines based on DC directly isolated from blood have been also used. When analyzing the cell composition of these vaccines, Bakdash et al. found a BDCA1+ DC population positive for CD14, which is elevated in patients as compared with healthy donors (89). These cells are a DC subset that, although overlaps in many factors with monocytes and classical BDCA1+ DC, can be considered as a distinct population, characterized by displaying suppressive properties. Therefore, they have been suggested as responsible for hampering vaccine efficacy in patients. Interestingly, besides CD14 expression [as in DC previously mentioned (88)], these DC are characterized by stronger IL-10 secretion than monocytes or classical BDCA1+ DC upon LPS stimulation, reagent also used to induce DC maturation in (88). Also in the clinical setting, we reported results from a vaccination pilot clinical trial of patients suffering from chronic hepatitis C by using a DC vaccine (90). Poor Th1 immune responses were detected in vaccinated patients. Although disease status could have played a role in these results, we detected higher levels of CD14+ DC and a stronger IL-10 production by the DC vaccine prepared from patients, as opposed to DC obtained from healthy individuals, in agreement with aforementioned clinical studies. Interestingly, blockade of IL-10 during vaccine preparation restored *in vitro* production of Th1 responses in these patients, suggesting again a relevant role of IL-10 in vaccine efficacy.

Therefore, considering the relevance of IL-10 during therapeutic vaccination, different blockade strategies have been used, depending on the type of vaccine. As summarized in **Table 1**, for those vaccines relying on direct antigen administration, antibodies blocking IL-10 or IL-10R have been used in most cases. When using DC administration, in addition to direct IL-10/IL-10R blockade, genetic ablation of IL-10/IL-10R expression and pharmacological inhibition of pathways leading to IL-10 production have been also used. Despite these different options in preclinical studies, only antibodies against IL-10, in combination with a TLR9 ligand, have advanced to clinical phases for the treatment of patients with advanced malignancies.

In addition to IL-10 as a target, description of this IL-10+ DC subset and its immunosuppressive properties allows the identification of other relevant molecular mechanisms involved in their inhibitory effect. Therefore, these molecules could be potentially amenable to modulation as a strategy to enhance vaccine potency. Among them, PD-L1 is an interesting upregulated target, since there are already approved therapies directed at this pathway (102). By using samples from patients with different types of tumors (hepatocellular carcinoma and glioblastoma, among others), it has been demonstrated that PD-L1 expression is regulated by IL-10 (103, 104). However, in our hands in vaccination experiments in murine models, combination of vaccine with IL-10 blockade did not modify PD-L1 expression on DC, despite a decrease in the percentage of IL-10+ DC (76), pointing at PD-L1 as an independent target and providing an additional opportunity

TABLE 1 | Strategies aimed at blocking IL-10 for combination with antitumor vaccines.

IL-10 blocking strategy	Vaccine	Tumor	Reference
PRECLINICAL			
Anti-IL-10 antibodies	Plasmid encoding MIP3α-hgp100	B16F10	(91)
	DC pulsed with gp100 peptide	B16F10	(92)
	DC pulsed with tumor cells	MC38	(93)
Anti-IL-10R antibodies	OVA + Imiquimod	B16-OVA	(43)
	HPV E7 peptide + LPS	TC-1	(72)
Soluble IL-10R	DC pulsed with tumor cells	B16F10	(94)
siRNA targeting IL-10	DC pulsed with E7 peptide	TC-1	(95)
siRNA targeting IL-10R	DC pulsed with E7 peptide	TC-1	(96)
	DC pulsed with MART-1 peptide	Human melanoma (in vitro)	(97)
shRNA targeting IL-10	DC pulsed with tumor cells	MC38	(98)
Aptamer targeting IL-10R	Monotherapy (no vaccine)	CT26	(99)
Inhibitors of IL-10-inducing pathways: p38 MAPK inhibitor	DC pulsed with tumor cells	B16F10	(100)
COX2 inhibitor	DC pulsed with tumor cells	B16F10	(100)
Retinoic acid receptor alpha inhibitor	DC pulsed with tumor cells	B16F10	(101)
CLINICAL			
Anti-IL-10 antibodies	TLR9 ligand	Advanced tumors	clinicaltrials.gov NCT02731742

for DC modulation. Accordingly, combined blockade of IL-10 and PD1/PD-L1 clearly potentiated vaccine immunogenicity, resulting in a greater therapeutic antitumor effect (76, 84). These results are in agreement with equivalent experiments carried out in viral models *in vitro* (105) and *in vivo* (106). In addition to these two important suppressive pathways, other inhibitory molecules, including enzymes (IDO) (77), cytokines (TGF-beta), ligands for receptors found on T-cells (HVEM) or inhibitory intracellular molecules (IRAK-3) (78), have been described in this IL-10+ DC subset. Co-expression of these negative factors has been already reported in other examples of DC with poor stimulatory capacity (107, 108), indicating that they are commonly operating in settings where T-cell responses are not fully activated, and suggesting that combined blockade of these mechanisms may improve DC functions, with special relevance in vaccination protocols.

CONCLUSION AND FUTURE DIRECTIONS

Different immunosuppressive elements present at the tumor microenvironment have been described, demonstrating that they may hamper effector functions of tumor-infiltrating lymphocytes. These mechanisms would partially account for the limited effect of therapeutic vaccines, suggesting that combination therapies that include vaccines plus blockade of these elements may increase their efficacy. However, together with these elements, additional inhibitory pathways induced by the vaccine are triggered. Many of them, such as IL-10 production, exert their effects at the level of DC, by

impairing their antigen presenting functions and negatively regulating T-cell activation. Some of the already identified vaccine-induced suppressive elements present in IL-10+ DC, are common to those operating at the tumor level, allowing the design of new combinatorial vaccination strategies based on drugs currently approved or in development. Therefore, future vaccination strategies, besides highly immunogenic and properly formulated and adjuvanted antigens, should incorporate blockade of IL-10 and additional inhibitory elements, enhancing thus vaccine potency and associated therapeutic efficacy.

AUTHOR CONTRIBUTIONS

DL and PS conceived, wrote the manuscript and did the figures. MR and LS contributed to manuscript editing and final revision.

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