



Clinical implications of antigen transfer mechanisms from malignant to dendritic cells: Exploiting cross-priming

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Expansion and activation of cytolytic T lymphocytes bearing high-affinity T-cell receptors specific for tumor antigens is a major goal of active cancer immunotherapy. Physiologically, T cells receive promitotic and activating signals from endogenous professional antigen-presenting cells (APC) rather than directly from malignant cells. This phenomenon fits with the broader concept of cross-presentation that earlier was demonstrated for minor histocompatibility and viral antigens. Many mechanisms have been found to be capable of transferring antigenic material from malignant cells to APC so that it can be processed and subsequently presented by MHC class I molecules expressed on APC. Dendritic cells (DC) are believed to be the most relevant APC mediating cross-presentation because they can take up antigens from apoptotic, necrotic, and even intact tumor cells. There exist specific molecular mechanisms that ensure this transfer of antigenic material: 1) opsonization of apoptotic bodies; 2) receptors for released heat shock proteins carrying peptides processed intracellularly; 3) Fc receptors that uptake immunocomplexes and immunoglobulins; and 4) pinocytosis. DC have the peculiar capability of reentering the exogenously captured material into the MHC class I pathway. Exploitation of these pieces of knowledge is achieved by providing DC with complex mixtures of tumor antigens *ex vivo* and by agents and procedures that promote infiltration of malignant tissue by DC. The final outcome of DC cross-presentation could be T-cell activation (cross-priming) but also, and importantly, T-cell tolerance contingent upon the activation/maturation status of DC. Artificial enhancement of tumor antigen cross-presentation and control of the immune-promoting status of the antigen-presenting DC will have important therapeutic implications in the near future. © 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Cross-presentation: cross-priming and cross-tolerance

In the late 1970s, Bevan and coworkers using congenic mouse strains made the unexpected observation that endogenous antigen-presenting cells (APC) were necessary for cytotoxic T-lymphocyte (CTL) priming against minor histocompatibility antigens [1,2]. The system required the interplay of professional APC, eventually discovered to be of he-

matopoietic origin [3], whose functions were to capture and process antigens in such a way that they would be presented to T cells. This function was called cross-priming because it was the result of antigens being cross-presented by APC, as opposed to direct presentation by other somatic cells, which also has been proven true in some experimental systems [4]. Although formal definitive proof of the identity of the *in vivo* APC [5] is lacking, the best candidate are cells, or better a class of cells, called dendritic cells (DC) [6–8], which are sufficient to mediate cross-priming [9].

DC are a complex network of cells probably derived from both myeloid and lymphoid precursors [10], which give rise to an array of differentiated cells that colonize peripheral tissues and lymphoid organs including the thymus

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[10]. Their mission is believed to include the following: 1) internalization and processing of multiple moieties and particles present in their tissular environment; 2) migration from peripheral to lymphoid tissues; 3) antigen presentation on MHC class I and class II molecules; and 4) expression of cytokines and costimulatory ligands critical for activation of T cells [6,7].

Recent evidence strongly suggests that the result of antigen presentation by DC in lymphoid organs could be T-cell clonal anergy or deletion. The recently coined term for this tolerizing result of cross-presentation is “cross-tolerance” [11]. It is conceivable that the most frequent outcome of the interaction of antigen-presenting DC with specific T cells might be tolerogenic [12,13]. According to this model [13], DC would be in charge of constantly capturing, transporting, and presenting harmless antigens to T cells in order to impose tolerance [12,13]. In concordance with this notion are the observations of DC migrating from peripheral tissues carrying self-apoptotic bodies [14].

In transgenic mice models of cross-presentation in which ovalbumin is expressed only in insulin-secreting cells of the pancreas, cross-tolerance only occurs beyond a certain threshold of protein expression [15]. Therefore, peripheral tolerance probably is imposed upon highly expressed antigens. In contrast, antigens below these thresholds are ignored by the immune system for either T-cell priming or tolerization [15,16].

To achieve cross-presentation, the antigen-presenting machinery of DC is highly active and regulated [17,18]. DC have the peculiar ability to transfer exogenously captured antigens to the endogenous pathway of MHC class I presentation [17–19]. This may be due to a yet unidentified molecular mechanism that shuttles antigenic proteins from endosomes into the cytoplasm [19]. The class II presentation pathway also is prominent in these cells [17,18]. Empty (peptideless) molecules are expressed such that they can be easily loaded with antigenic determinants on the plasma membrane [20]. As mentioned earlier, the physiology of cross-presentation can be exploited for either priming or tolerization. Evidence that tumor antigens can be cross-presented came primarily from observations with granulocyte-macrophage colony-stimulating factor (GM-CSF) transfected tumor cells that can act as vaccines [3,21]. Thus, transfected tumor cells locally attract and differentiate DC, and accordingly a crucial role of bone marrow-derived APC has been demonstrated in immunity with these GM-CSF transfectants [3]. Direct injection of DC into tumor tissue is another way to exploit the ability to cross-present tumor antigens [22–24]. In fact, coculture of DC and tumor cells yields DC that can activate tumor-specific T cells *in vitro*, indicating the ability of DC to take up antigens from “intact” tumor cells [25]. On the other side of immunotherapy, it has been shown that cross-tolerance can be imposed by DC under certain conditions, such as culture with tumor necrosis factor [26,27] or genetic modification to express Fas-L

[28], transforming growth factor- β [29], or interleukin (IL)-10 [30]. Thus, the induced deletion or inactivation of damaging T cells could ameliorate the course of autoimmune conditions, hypersensitivity reactions, or graft rejection [31].

Control of DC maturation and the outcome of cross-presentation

Peripherally deployed DC have a complex system of sensors that enable them to detect 1) nonphysiologic cell destruction or damage [32]; 2) presence of microorganisms [33–35], or 3) tissular inflammation. In order to do so, they bear receptors for proinflammatory cytokines [7], for materials released by cells dying under stressful conditions [36], and for molecules that are the hallmark of microbial invasion (such as prokaryotic deoxyribonucleic acid, lipopolysaccharide, lipoteichoic acid, dsRNA, and others) [35,37]. Upon conditions of stimulation of these receptors, DC rapidly migrate to lymphoid tissue due to a change in the pattern of the chemokine receptors that they express [38], while they both up-regulate antigen presentation and down-regulate antigen capture [17,18]. As they migrate into lymphoid tissue, they up-regulate the expression of costimulatory molecules that provide signals to T cells that, upon antigen recognition, are complementary for T-cell receptor (TCR)-mediated activation [7]. These molecular changes are collectively termed DC maturation and are driven by multiple signaling cascades [39] that involve NF- κ B transcription factors as key players [40]. With respect to antigen-presenting molecules, they mobilize onto the plasma membrane MHC class II molecules that had been retained in antigen-loading compartments [41,42].

The current paradigm is that antigen presentation by immature DC leads to T-cell anergy or deletion [43], whereas presentation by mature DC leads to T-cell priming and subsequent effector T-cell response [44]. However, this likely is an oversimplification because DC maturation is the result of a very complex set of gene expression programs. For example, CTL tolerization requires a certain degree of maturation, described as partial maturation changes, that can be achieved, for instance, after exposure to tumor necrosis factor- α as a single maturation-inducing factor [26,27].

Overall, DC maturation status should be envisioned as a condition determined by a gene expression pattern that results from the type of maturation stimulus and its own intensity [45]. Each gene expression profile shares many common molecules, but some critical differences in the up- or down-regulated genes could be crucial to understanding contrary outcomes of the immune response. For instance, genes such as those encoding IL-12 [45] are tightly regulated and clearly induced only in the presence of a combination of potent stimuli [46,47]. The “two-signal model” is a theory proposing that TCR signals (signal 1), in the absence of costimulatory interactions (signal 2), result in T-cell inactivation or deletion [48–50]. In contrast, according to this

model, the interplay of signal 1 + signal 2 is the driving force of T-cell activation [48–50]. It has been proposed that other types of signals (signal 3) are critical for T-cell priming [51], mainly in the case of CTL induction. Signal 3 should be “turned on” only on licensed DC that can prime CTL effector function [52–54]. IL-12 is a good candidate to be a signal 3 [51], and this cytokine is able to shape the immune response toward a CTL/T_H1 type of cellular immune response [55]. Other molecules such as 4-1BBL might have a crucial role [56]. Signal 3, regardless of its molecular nature, is conceived to bias the cytokine pattern of T helper cells toward T_H1 [51]. Artificial means to provide IL-12 achieve antitumor effects either as a recombinant protein or when transferred in gene therapy approaches [57]. There is interest in determining which DC subtype mediates cross-priming in vivo [58]. In mouse spleen, two populations can be distinguished based on CD8 α expression. The CD8 α subset readily secretes IL-12 upon stimulation and is believed to be essential for cross-priming. However, recent evidence indicates that CD8⁺ spleen DC only represent a more mature status of the same lineage rather than a population of a different ontogeny [59].

CD40 is a key signaling molecule at licensing DC for CTL activation [52,54], providing signals for maturation that are synergistic with other stimuli (such as LPS) [47,60]. CD40 binds a cognate ligand expressed on activated T helper cells [61]. Therefore, MHC class II-restricted antigens recognized by CD4⁺ T cells determine the outcome of cross-presentation [52–54]. This is being exploited in experimental antitumor immunotherapy by either artificial ligation of CD40 with monoclonal antibodies [62,63] or ectopically expressed CD40L [64,65]. In addition, DC vaccines set to elicit antitumor CTL are known to benefit from the inclusion of MHC class II-restricted determinants [66].

All of these therapeutic approaches that provide artificial costimulation without antigen rely on a certain degree of basal cross-priming against tumor antigens that can be amplified. Evidence for this basal cross-priming has been obtained in mouse tumors, but it is clear that it is not sufficient to induce effective immunity [67,68].

Heat shock proteins chaperoning antigen and providing maturation stimulus

Two intracellular chaperones, gp96 and HSP-70, have been found to display important immunologic functions at cross-priming [69,70]. HSP-70 and gp96 have binding activity for short peptides undergoing the MHC class I-presenting pathway. In a sense, the array of peptides bound to these moieties in a given cell is a reflection of the proteins being translated in it at a given time point. Both molecules are expressed intracellularly, gp96 in the rough endoplasmic reticulum and HSP-70 in the cytosol. They are released only when cells die and pores are formed across the plasma membrane.

Both heat shock proteins share the interesting properties of being avidly internalized by APC [71–73] and of providing strong DC maturation signals by interacting with surface receptors [74,75]. The receptor for internalization is CD91 [72,76]. The identity of the maturing receptor has not yet been established, but it could be TLR2/4 receptors previously identified by their property of conferring responsiveness to microbe components [77]. Existence of endogenous ligands for these and other danger signal detectors has been predicted by Polly Matzinger in her danger theory [32,78].

HSP-70 and gp96 derived from tumors have been used to specifically vaccinate against these tumors [69,79]. The procedures require obtaining autologous tumor mass that is lysed in order to purify gp96 or HSP-70 by various chromatographic techniques. There is evidence that HSP-70, when induced under stress conditions, is involved in endogenous cross-priming against antigens that are released after necrosis of malignant cells [22,80]. Other members of the mammalian heat shock protein family are proposed to share similar functions [69,70].

As mentioned earlier, it has been observed in mouse tumor models that affinity-purified heat shock proteins from tumors vaccinate specifically against inoculation of the corresponding viable cell line [69,79]. The vaccine also increases therapeutic activity against established transplanted tumors that is mediated by CTLs. Fueled by these observations, a number of pilot clinical trials are being conducted [81,82]. Information released from such trials is scanty but indicates that the procedures are safe and well tolerated [69,81,82]. To date, clinical efficacy appears to be limited. As often is the case in early clinical experimentation, only cancer patients with heavy disease burden were eligible for study inclusion, but augmentation of the cellular immune response occurred even in these patients.

Apoptotic bodies and antigen transfer

Malignant cells undergo programmed cell death [83]. Their remains as apoptotic bodies are opsonized by thrombospondin and rapidly internalized by macrophages [84]. Phagocytosis of apoptotic bodies is thought to be immunosuppressive [85], but if mediated by DC it can result in immunity against the antigens contained by apoptotic bodies [84,86,87], provided the engulfing DC experiences maturation [86]. In this regard, apoptotic bodies have been confirmed to transfer viral antigens and labeled apoptotic bodies have been found inside tumor-associated DC [88]. In addition, incubation of ex vivo cultured DC with tumor apoptotic bodies has been found to provide efficient means to load DC with tumor antigens [84,89–91]. There is no definitive proof that such DC cross-presented tumor antigens are taken up via this mechanism in tumor-bearing mice. It should be kept in mind that, in general, apoptotic death in surrounding cells does not induce maturation of DC; there-

fore, to achieve immunity a maturation-inducing stimulus should reach the DC loaded with apoptotic bodies [85,86,92]. It is important to note that there is experimental proof that DC internalize antigenic material through pinocytosis of soluble proteins that could come from malignant cell debris [93].

Because maturation induction signals are believed to be critical for cross-priming, the most relevant factor must be whether the tumor environment displays proinflammatory factors or the cells are dying under stress conditions [86,92]. Necrosis is thought to induce immunity, whereas “normal” apoptosis would be involved in tolerance induction or in allowing tissular antigens to remain ignored by the immune system [12,13]. Stress-induced molecules in cells undergoing either apoptosis or necrosis are capable of activating DC maturation. Heat shock proteins are one example of this type of molecule, but other endogenous danger signals probably will be identified in the future [94]. Local application in the tumor nodules of proinflammatory cytokines/chemokines, stress molecules, or microbial components are interesting approaches to obtaining clinical benefits from these mechanisms.

Cellular exosomes, membrane cooption, and Fc receptors

Tumor cell lines constantly release vesicles (nanometers in diameter) coated by plasma membrane [95]. They contain MHC class I molecules and heat shock proteins involved in MHC class I antigen presentation [96]. Such vesicles can be internalized and processed by DC *in vitro*, but exosome capture does not induce DC maturation [95]. Continuous release of these exosomes is believed to occur *in vivo*. The observation that coculture of viable tumor cells and DC can result in antigen transfer [25] probably is related to these structures.

A nonmutually exclusive mechanism that can explain this observation is the proposed ability of DC to exchange membrane patches with cells they interact with [97,98]. During this cooption of patches of membrane, they can gain surface expression of antigen-presenting molecules that originally were attached to the plasma membrane of the donor cells [97,98]. Mechanisms of this cooption are not defined yet and could involve fusion with exosomes released by tumor cells. Importantly, MHC class I molecules carrying melanoma tumor antigens can be transferred through this mechanism to DC in order to elicit CTL *in vitro*.

It was found recently that antigen coating of apoptotic bodies with antibodies enhances cross-priming [99,100]. Intriguingly, the mechanism was not a simple increase in opsonization/internalization by Fc receptors, but instead was related to a property of the internalized immunocomplexes that rendered the material more prone to be presented [99,100]. There is hope that this property can be exploited in cross-priming against tumor antigens. The first observations were made in experimental myelomas, and it has been

speculated that these types of mechanisms can be important in understanding the therapeutic effects of anti-idiotypic humoral responses in malignancies of lymphoid origin. Moreover, it has been repeatedly noted, even in clinical trials, that *in vitro* DC pulsing with either immunoglobulin alone or tumor idiotype conjugated with keyhole limpet hemocyanin (KLH) can induce cellular immune responses *in vivo* upon reinfusion of the pulsed DC into the patient [101–104]. These results suggest that the Fc receptors expressed by DC may internalize tumor-specific idiotype-bearing immunoglobulins alone or as part of immunconjugates (immunoglobulin chemically complexed to KLH). Soluble proteins, including pathologic immunoglobulins, can be taken up by DC through macropinocytosis in an Fc-receptor-independent fashion [93,105].

Tumor cell RNA and tumor-DC hybridomas in cross-priming

mRNA normally is confined to the cell interior. Its release as translatable genetic material could provide excellent means of antigen transfer if captured in its integrity by DC. DC can be loaded with RNA encoding for tumor antigens or with total tumor RNA for immunotherapy of cancer [106–108]. Those DC are useful for treating experimental malignancies, and clinical trials have reported discrete biologic effects in prostate cancer patients [109]. In those techniques, cationic lipids usually assist RNA entry into DC, but in some cases it has been proved that it enters DC efficiently without artificial help [108,109]. On the other hand, double-stranded RNA can stimulate the DC receptor TLR-3 [110] and provide stress signals to DC [111]. It would not be surprising that RNA forming secondary structures would be a maturing signal for DC, whereas TLR-3 might be involved in internalization. Whether RNA is an important messenger for antigen transfer awaits the results of clear-cut experimentation.

One of the best ways to generate antitumor vaccines might be to fuse DC and tumor cells to generate hybrid cells [112,113]. In this strategy, malignant cells provide antigenicity, whereas DC provide immunogenicity. In mouse tumor models the strategy can break tolerance against self-antigens [113]. The only clinical trial reported to date disclosed extremely encouraging efficacy results [114]. However, caution still is necessary until the issue of data reproducibility is addressed successfully [115]. Artificial means to generate these syncytia *in vivo* are being attempted in mouse models by gene transfer into tumor cells of fusogenic retroviral proteins [116,117] and subsequent intratumor injection of DC.

Intratumor injection of DC

The simplest approach to take advantage of these concepts is to release *in vitro*-cultured DC inside malignant tissue by direct injection [22–24]. Alternatively, it is possible to at-

tract DC to tumors by transferring into cancer cells constructions of genes expressing GM-CSF [21,118] or DC-attracting chemokines, such as MIP3 α [119].

DC injection into tumor masses has some antitumor activity against micrometastasis [22]. However, if artificially injected DC are transfected with genes to express IL-12 [23,24], IL-7 [120], CD40L [121], or IL-2 [122] to a lesser extent, they are highly efficacious against malignant tumors by eliciting specific CTLs. It is not yet clear what kind of antigen transfer mechanism shuttles tumor antigens into DC in vivo, as they are artificially released into the tumor. It could be any of those described in this review (Fig. 1) or several operating in combination. Injection damage, caused by hydrostatic pressure and puncture, could help to create some local stress [123]. In this regard, it is worth noting that local radiotherapy that destroys tumor cells enhances the efficacy of DC vaccination and adoptive T-cell therapy [124]. Moreover, according to a recent report, frozen/thawed tumor cells are less efficient than irradiated tumor cells at transferring immunogenic antigen to DC [90]. Nonetheless, other authors report similar antigen transfer efficacy of necrotic and apoptotic cells, at least for CTL priming in vitro [125].

In all of these approaches, DC are allowed to capture and present every available antigen in malignant tissue (cancer

cells + stroma), not only tumor antigens. Accordingly, damaging immunity could be induced to self-components. In transgenic mice with surrogate self-antigens shared by vital tissues and transplanted/eradicated tumors, lethal anti-self-reactions have been observed [126]. However, the real risk is believed to be small in light of clinical trials that were almost uneventful in this regard [8,127], with the exception of vitiligo in several melanoma patients [128]. After DC reach lymphoid tissue, they die within 24 to 48 hours [129]. Their death is induced by activated T cells [130], thus providing a negative feedback mechanism that regulates the immune response. There is evidence that antigen released by dying DC can be taken up by resident sister DC that can perform second-hand antigen cross-presentation [131], but the real importance of this mechanism currently is unknown. From a practical point of view, discovery of a means to extend the lifespan of DC in lymphoid organs can be important [132,133]. In the context of intratumor injection, it is interesting that DC can mediate a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent killing of tumor cells, thus creating apoptotic bodies available for uptake by the killer DC [134]. Cytotoxicity as mediated by natural killer cells, CTLs, or macrophages could have similar consequences in terms of generating antigenic material for cross-presentation [135].

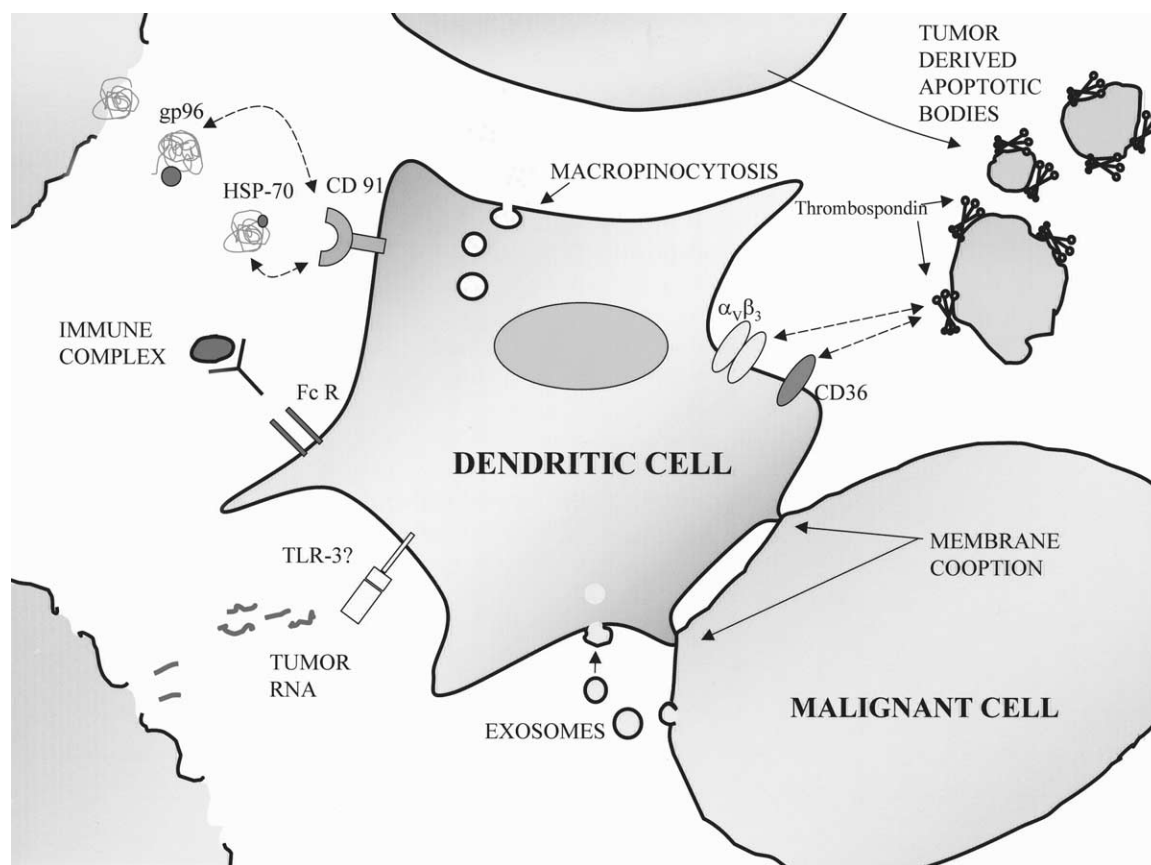


Figure 1. Antigen transfer pathways from cancer to dendritic cells.

CTL cross-priming and active immunization against cancer: what to expect in the near future?

From our point of view, strategies based on increasing the amount and quality of antigen transferred from cancer cells to DC will be major priming procedures against cancer. It is likely that they will be accompanied by a means to increase the maturation status of DC in a manner that renders them more capable to prime CTLs (licensed). Figure 1 shows a summary of the diverse antigen transfer pathways that conceivably are involved in cross-priming.

To be successful, it is wise to use a combination of antigens for priming [123]. We favor approaches in which DC are allowed to serve themselves from the buffet of tumor components rather than from a fixed antigenic menu, thus avoiding excessive antigen focusing with propensity to select out antigen-loss variants. Combination therapies including effector T-cell in vitro culture for adoptive T-cell therapy will benefit from the success of efficacious priming procedures [136,137]. Apart from adoptive T-cell therapy, other strategies that are likely to be combined in order to increase efficacy are as follows:

1. *Repeated boosting* [138]. There is room for improvement with this strategy because in some acute viral infections a large number of specific T-cells are expanded, whereas the expansion reached by the best current active antitumor immunotherapy regimes is about one order of magnitude less [139,140].
2. *Exogenous administration of agents that inhibit regulatory T cells [141] and/or interfere with molecules involved in down-regulating T-cell responses.* Means to counteract the function of transforming growth factor- β [142], CTLA-4 [143], and/or PD-1 [144,145] are the best candidates so far, but similar possibilities are to be explored with blocking strategies for the functions of IL-10 and IL-13 [146], which are immunosuppressive in some experimental tumor models.
3. *Combination with immunostimulatory monoclonal antibodies or cytokines that enhance the cellular immune response.* Among the antibodies that hopefully have clinical potential are anti-CD137 (4-1BB) [147,148], anti-CD40 [62,63], and anti-CTLA-4 [143]. Among the cytokines we would highlight are IL-12, IL-2, IL-15, and GM-CSF, for all of which we predict a role in combination immunotherapy. Recent evidence showing that soluble Flt-3L has antitumor effects [149] and increases DC recovery in cancer patients [94] is likely to be important for artificial cross-priming [94].

Development of combination strategies has a serious difficulty in that safety studies must be performed first with single agents and a long period of time is needed before combinations can be tested, even though excellent preclinical efficacy and safety data have been obtained.

In addition, translational research review and approval processes are a limiting bottleneck to faster development. In

some countries up to three levels of regulatory authorities and committees are involved in a sequential decision. Pilot clinical experimentation and safety studies involving a small number of cancer patients without expectations of curative treatment should be simplified in terms of bureaucracy. In some cases direct testing of combination strategies should be allowed, at least for clinical investigation groups with records of excellent practice standards.

There is some controversy regarding these opinions, because others argue that no definitive statistic data exist on the clinical efficacy of DC-mediated immunotherapy against cancer. In their opinion, given that the field in its infancy, it would be wise to test separately in patients the many variables of the technology that should be optimized (route, dose, source of DC, antigen loading strategy) before beginning combined treatments in clinical trials. In any case, regulatory authorities should place emphasis on the expert peer-review of the preclinical safety profiles of new therapeutic agents to be tested as a single agent or in combination, as well as on the balance of risk/potential benefit to the patients.

Careful follow-up of the clinical effects and assessment of specific immune responses will teach us the best and safest approaches. In our opinion, the next decade will witness a major impact of immunotherapy on the management of malignancies. In particular, the prognosis of minimal residual disease cases is expected to improve significantly. New ways for using cross-priming soon will be in the arsenal against cancer.

Acknowledgments

The authors thank Drs. L. Chen, J.J. Lasarte, C. Qian, C. Smerdou, G. Gonzalez-Aseguinolaza, and J. Prieto for discussion and critical comments. The authors are grateful for long-term financial support by the Spanish CICYT, FIS, Gobierno de Navarra, and Fundación Ramón Areces. Maurizio Bendandi is a Leukemia and Lymphoma Society Scholar in Clinical Research.

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