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The role of dendritic cells in cytotoxic immune response regulation in ovarian cancer micro-environment

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1. ABSTRACT

Ovarian cancer is the most lethal gynecological malignancy. At the time of diagnosis most patients present with an advanced stage of the disease and require multidisciplinary systemic treatment, including surgery and adjuvant chemotherapy. Despite good initial response to cytostatics, the vast majority of patients develops a recurrence and will need novel therapeutic strategies, as relapsed ovarian cancer is still incurable. One promising treatment option is the use of dendritic cells (DCs) which might induce effective anti-tumor immunity. The ability of DCs to generate an anti-cancer response has been documented in various kinds of human tumors, including malignant melanoma, renal cell carcinoma, and breast cancer tumors. Although DCs were identified in the micro-environment of ovarian cancer, lack of clearly defined ovarian-specific tumor antigens capable of being recognized by T cells is considered the major prohibiting factor in ovarian cancer vaccine development. There is therefore a strong need to identify and employ attractive candidates for tumor-specific antigens. In this review we will focus on current knowledge of the influence of DC mechanisms of cytotoxic T-cell responses and recent advances in DC identification in ovarian cancer patients, in addition to summarizing the data on DC vaccinations in these patients.

2. INTRODUCTION

Ovarian cancer belongs to the most lethal class of gynecological malignancies and remains the fifth most common cause of cancer-related deaths among women after lung, breast, colorectal, and pancreatic cancer (1). The chance of surviving ovarian cancer dramatically decreases if a woman presents with advanced International Federation of Gynecologists and Obstetricians (FIGO) stage III/IV as compared with earlier (FIGO stage I/II) stages of the disease. Unfortunately, due to the absence of early symptoms, more than two-thirds of patients are diagnosed in the III or IV stage of the disease (2). Standard treatment includes surgery, combined chemotherapy or/and radiation, with 90% of the patients developing a recurrence (3). The 5-year survival rate in these patients, according to the different data, does not exceed 15% (2). Despite progress in chemotherapy and multidisciplinary management, the outcomes of ovarian cancer treatment still remain unsatisfactory, and there is a strong need to develop and improve new and alternative treatment strategies, *i.e.*, gene therapy or immunotherapy.

It has been well documented that multiple mechanisms may be involved in the development and progression of a tumor, including a defect in the immune system at the induction and/or effector phases of antitumor

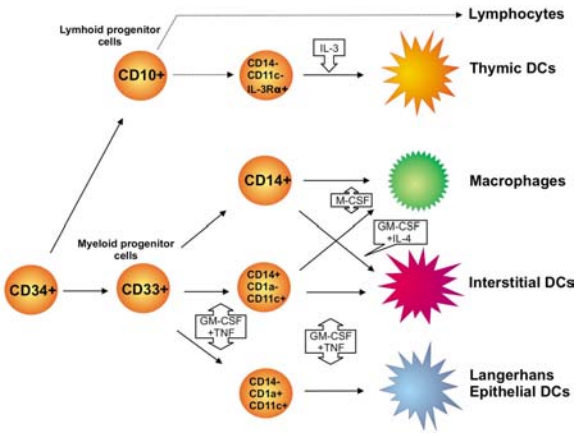

Figure 1. Current proposals of human DCs differentiation

immune responses, such as antigen presentation in the absence of major histocompatibility complex (MHC) Class II molecules or co-stimulatory molecules. The majority of tumor antigens (Ags) are nonmutated self-antigens to which the immune system is tolerant. As a result, tumor Ags may not be effectively presented to the immune system, or T cells responding to tumor Ags may be anergized (4, 5).

One of the most promising strategies to correct such defects is the enhancement of tumor antigen presentation with the help of dendritic cells, the most powerful inducers of tumor-specific ($CD8^+$) cytotoxic T-lymphocyte (CTL) responses. Unlike other antigen presenting cells (APC), such as B cells and macrophages, DCs are capable of initiating not only secondary immune responses, but also primary immune responses (such as activation of the naïve T cells) that are directed against specific antigens (6, 7, 8, 9).

The DC system represents a heterogeneous group of APC differing at the level of precursor cells, factors influencing growth and maturation, phenotype and APC function (6, 7, 9). Morphologically and phenotypically distinct DCs, which are present at many different anatomical sites, are derived from two lineages: myeloid or lymphoid (10, 11). Moreover, DC phenotypic and functional characteristics are closely linked to their stage of maturation. Circulating DC precursors home to lymphoid and non- lymphoid tissues where they reside as immature cells. At this stage DCs are well equipped to acquire antigens but nevertheless express low levels of the requisite MHC and co-stimulatory molecules needed for T-lymphocyte stimulation. Following antigen engulfment and processing, DCs migrate to secondary lymphoid organs where they mature, becoming APCs able to select and activate naïve Ag-specific T cells and induce an Ag-specific immune response (6, 9).

These unique properties, coupled with the fact that it is now possible to generate large numbers of functional dendritic cells *in vitro*, make DCs very attractive vectors for cancer immunotherapy.

In this review we focus on the biology, development, and interaction of DCs with other immune system cells. We also cover the problems of DC identification in ovarian cancer patients and the sources of DCs for clinical immunotherapy. Additionally, we try to present reasons for the poor effectiveness of antitumor responses and difficulties in the development of ovarian cancer vaccines.

3. BIOLOGY OF DENDRITIC CELLS (DCS)

3.1. Dendritic cell development

Dendritic cells originate from the bone marrow and their precursors' move via the bloodstream to almost all organs, where they can be found as sentinels in an immature state with high endocytic and phagocytic capacity (12, 13). At least three different subsets of circulating DC precursors have been identified in humans: $CD14^+CD11c^+$ monocytes, $CD14^-$ $CD11c^+$ circulating DCs, and $CD14^-$ $CD11c^-$ IL-3 receptor (R) $alpha^+$ plasmacytoid cells (Figure 1).

The $CD14^+CD11c^+$ monocytes (14, 15) give rise to immature DCs under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 or tumor necrosis factor (TNF)-alpha. Furthermore, in the presence of transforming growth factor (TGF)-beta (16), $CD11c^+$ blood DCs can differentiate into skin epidermal Langerhans cells (LCs). If cultured with GM-CSF or macrophage colony stimulating factor (M-CSF), both monocytes and $CD11c^+$ blood circulating DCs can give rise to macrophages, suggesting the plasticity of the DC system (7). The $CD14^-$ $CD11c^-$ $IL\text{-}3R^+$ precursors differentiate into plasmacytoid DCs that die rapidly after isolation and are critically dependent on IL-3 for survival and CD40-ligand (L) for maturation (17, 18) (Figure 1).

3.2. Dendritic cell subsets

Myeloid dendritic cells (MDCs, DC1) and plasmacytoid/lymphoid DCs (PDCs, DC2) are considered to be the principal subpopulations of human DCs that differ in morphology, expression of markers, and function.

MDCs are characterized by monocytoid morphology, possessing an irregular outline and a hyperlobulated nucleus (11). They express myeloid markers such as CD13, CD33, the β_2 integrin CD11c, and low levels of the IL-3 alpha-chain CD123 (19). Myeloid DCs also express blood dendritic cell antigen-BDCA-1 (CD1c) that is specific for peripheral blood (PB) myeloid DCs (20).

The myeloid DCs are professional antigen-presenting cells, highly specialized in the presentation of antigen and initiation of antigen-specific immune responses. They tend to produce a Th1 polarized response when used to stimulate CD4 cells (21, 22). Myeloid DCs have recently been documented as involved in supporting innate immunity and in promoting the production of cytokines and cytotoxicity of NK cells as well as enhancing their tumoricidal activity (23). Table 1 shows surface marker profiles for human DC subsets.

Table 1. Expression of surface markers on human DC subsets

Myeloid DCs		Lymphoid/plasmacytoid DCs
$CD1c^{+}$(BDCA-1^{+})	$CD1c^{-}$(BDCA-1^{-})	$CD1c^{-}$(BDCA-1^{-})
$CD4^{+/-}$	$CD4^{+/-}$	$CD4^{+}$
$CD11c^{++}$	$CD11c^{+/-}$	$CD11c^{-}$
$CD33^{+}$	$CD33^{+/-}$	$CD33^{-}$
$CD45RA^{-}$	$CD45RA^{-}$	$CD45RA^{+}$
$CD123^{-}$	$CD123^{-}$	$CD123^{++}$
BDCA-2^{-}	BDCA-2^{-}	BDCA-2^{+}
BDCA-4^{-}	BDCA-4^{-}	BDCA-4^{+}
BDCA-3^{-}	BDCA-3^{+}	BDCA-3^{-}
$CMRF58^{-}$	$CMRF58^{+}$	$CMRF58^{-}$

Expression levels are indicated as follows: - = lack of expression; +/- = weak expression; + = intermediate expression; ++ = strong expression.

Plasmacytoid DCs have a morphology resembling that of plasma cells which possess a rounded morphology with an oval or indented nucleus and a prominent perinuclear pale zone (11, 24). PDCs have been characterized primarily in the peripheral blood and are identified as leukocyte lineage (Lin)-human leukocyte antigen ($HLA\text{-}DR^{+}$ $CD123^{+}$ $CD11c^{-}$ cells (10). They also show positive expression of two recently identified PDCs-selective antigens (BDCA-2, a C-type lectin) and BDCA-4 (neuropilin-1) (25, 26). Blood PDCs are relatively rare blood leukocytes, comprising <0.4% of the total number of peripheral blood mononuclear cells (PBMC) and declining in abundance by 1% per year in adults (27). In the steady state, PDCs have also been reported in the thymus (28) and peritoneal fluid (29, 30, 31). PDCs are still relatively poorly characterized as they form a rare blood population and are difficult to cultivate *in vitro* (17). They display low phagocytic activity and are the major source of IFN-alpha production in response to viral infection (32). PDCs can differentiate into antigen-presenting cells capable of triggering T-effector or -suppressor responses (33). The effector functions of PDCs depend on the secondary signals they receive after recruitment to a tissue site. On the one hand, when they encounter toll-receptor ligands, PDCs develop into pro-inflammatory interferon alpha-secreting cells that are also capable of acquiring and processing antigens. On the other hand, PDCs stimulated by CD40 ligand in the absence of toll-receptor ligands develop into suppressor-type PDCs that induce the development of $CD8^{+}$ T regulatory cells (34, 35, 36). Curiel *et al.* (31) show that numerous functional lymphoid DCs accumulate in tumor-associated ascites and inhibit anti-tumor immunity. The same authors found that *in vivo* lymphoid DCs produce high levels of the angiogenic cytokines (TNF-alpha and IL-8) and induce potent neovascularization. Human studies have suggested that plasmacytoid DCs mainly generate a Th2 response; however, recent work has highlighted the plasticity of DC-stimulated $CD4^{+}$ T-cell responses (37). Additionally, natural interferon-producing cells/plasmacytoid DCs (IpCs/PDCs) play an important role in NK-cell activation (23).

3.3. Immature, semi-mature and mature dendritic cells in T cell tolerance and immunity

DCs exist in at least three functionally and phenotypically distinct stages: immature, semi-mature, and mature (38).

Immature DCs are characterized by high antigen uptake ability with low T-cell stimulatory capacity. Clinical studies have proven that immature DCs induce tolerance by the induction of regulatory T cells (39). Immature DCs express several receptors that facilitate antigen recognition and uptake, including CD36 and αγβ5–receptors for apoptotic bodies (40), mannose C-type lectin receptors (41) and DEC205 receptors (42), (hsp)-70, and glycoprotein (gp) 96–heat shock proteins receptors (7) as well as specific receptors for the fragment crystallizable (Fc) domain of immunoglobulins (FcγR for the Fc domain of IgG and FcεR for the Fc domain of IgE) (43, 44). Dendritic cells also express CD1a molecule that belongs to the group of complement-regulatory CD1 proteins (CD1a, b, c, d, e) and presents predominantly non-peptide molecules originating from lipids and glycolipids. CD1a could represent an important mechanism for the presentation of tumor-derived glycolipid antigens to T cells and the subsequent production of an effective anti-tumor response (45). Immature DCs express only low levels of CD40, CD80, CD83, and CD86 and moderate levels of MHC Class II molecules. Immature DC-induced T-cell anergy results from low MHC receptors expression, absent or low co-stimulatory molecules expression, and also the insufficient secretion of pro-inflammatory cytokines (38).

Semi-mature DCs are characterized by a high expression of MHC II and co-stimulatory molecules, but a low or absent production of pro-inflammatory cytokines, such as IL-1 beta, IL-6, TNF-alpha, IL-12p40, or IL-12p70. Inducers of DC semi-maturation can include lactobacilli from the gut flora (46), apoptotic cells (47), or TNF-alpha (48). *In vivo*, these semi-mature DCs are actively tolerogenic by inducing $IL\text{-}10^{+}$ $CD4^{+}$ regulatory T cells (Tregs) in an antigen-specific manner (38).

The process of DC maturation may be induced by numerous factors, including pathogen-related molecules such as lipopolysaccharide (LPS) (49), bacterial DNA, double-stranded RNA (dsRNA) (24), and the balance between pro-inflammatory and anti-inflammatory signals in the local micro-environment, which includes TNF-alpha, IL-1, IL-6, IL-10, TGF-beta, and prostaglandins (7, 24). Moreover, DC maturation can be induced by T cell-derived signals, such as CD40 ligand (50, 51). Upon exposure to these factors, DCs lose their phagocytic activity and migrate to draining lymph nodes where they become mature DCs (mDCs). Human mDCs possess a high antigen-presenting capability and T-cell stimulatory capacity due to the expression of high levels of antigen-presenting, adhesion, and co-stimulatory molecules as well as other DC-specific markers, such as CD83 and DC-lysosome-associated membrane protein (DC-LAMP) (7). On maturation, DCs develop more prominent projections (dendrites) that extend into the surrounding environment. It has been suggested that the extraordinary length and number of projections may facilitate interaction with lymphocytes (37) (Figure 2).

Full DC maturation has been shown to be required for T-cell priming and can, for example, be triggered by toll-like receptors (TLRs) [*e.g.,* LPS], either

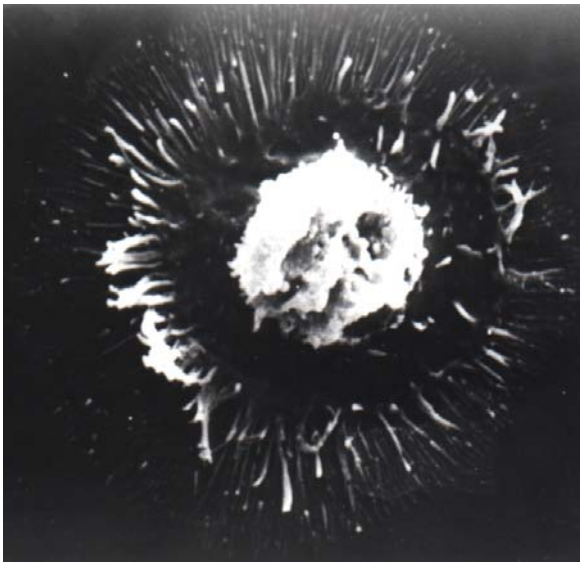

Figure 2. Mature dendritic cell with length projections.

alone or in combination with an enhancing CD40 signal, or after subcutaneous injections of semi-mature DCs by the triggering of endogenous ligands for TLRs (38).

After Ag-uptake and processing, DCs leave peripheral tissues and travel to the lymphoid organs, such as the spleen and lymph nodes. Next, DCs attract T and B cells by releasing chemokines and maintain the viability of re-circulating T lymphocytes (7, 52). Immature and mature DCs are recruited by the expression of different chemokines and chemokine receptors. Immature DCs can produce inflammatory chemokines, such as macrophage inflammatory protein (MIP)-1 alpha, monocyte chemo-attractant protein (MCP)-1, MCP-2, and MCP-4 (53) and express receptors for inflammatory chemokines, such as CCR1, CCR2, CCR5, CCR6, and CXCR1. In contrast, mature DCs have lost their responsiveness to most of these chemokines and their receptors and up-regulate the synthesis of constitutive chemokines and the CCR7 receptor. Consequently, the process of DC maturation responds to Epstein-Barr virus-induced ligand chemokine ELC (MIP-3 beta) and secondary lymphoid tissue chemokine SLC (6Ckine), chemokines that drive DC migration to the lymphoid vessels and T-cell areas of secondary lymphoid organs (54). In T-cell areas, DCs produce chemokines such as DC-CK-1 and MDC that chemo-attract naïve and memory T cells (55, 53).

3.4. Dendritic cell activation

DC activation is a multi-level process that consists of morphologic, immuno-phenotypic, and functional changes that correspond to the DC differentiation pathway and migratory capacity, their antigen uptake capacity and potential to induce cytokine production, immunity, or tolerance (37).

Effective T-cell activation involves two sets of signals. The first is derived from the T-cell receptor (TCR) after being triggered by the antigenic peptides presented by MHC molecules on the surfaces of DCs. The other is mediated by the interaction between co-stimulatory receptors on DCs with their respective ligands on T lymphocytes (56). The second co-stimulatory signal is mediated, in part, by the interaction of CD80 (B7-1) and CD86 (B7-2), both present on DC surfaces, with CD28 on T cells (57). Moreover, two families of receptors appear to be critical for DC activation: the TLRs and the TNF family receptors, amongst which CD40 appears to be a major factor. It has been shown that the CD40-CD40L interaction between DCs and CD4 T lymphocytes induces maturation, reflected in the production of high levels of IL-12, enhanced T-cell stimulatory capacity, and enhanced DC survival (58). IL-12 skews the Th response towards a Th1 response, leading to IFN-gamma production that augments the synthesis of IL-12 by DCs (59). It has been suggested that IL-12 secretion links innate and adaptive immunity as it enhances natural killer (NK), NKT, and T- lymphocyte cytotoxicity and anti-tumor responses (60). Upon contact with CD40-CD40L, DCs receive additional signals provided by the interaction with RANK/TRANCE, 4-IBB/4-IBBL or OX40/OX40L, which help DCs in terminal maturation. After interaction with T cells, DCs are believed to die by the process of apoptosis (53).

4. IDENTIFICATION OF DCS IN OVARIAN CANCER PATIENTS

DCs have been identified in ovarian cancer patients by using flow cytometry and immunohistochemistry. Flow cytometry identified DCs in the peripheral blood and ascites of these patients (61, 31, 30). Immunohistochemistry technique showed the presence of DCs in tissue specimens (62). Methods for the detection and isolation of DCs are typically based on a multitude of immunophenotypic criteria, such as the absence of a panel of leukocyte lineage specific antigens (e.g. CD3, CD14, CD19, CD56) and the presence of human leukocyte antigen HLA-DR, CD4, or CD33 (63, 20). In previous papers, DCs were identified in the ascites of patients with ovarian cancer as Lin-HLA-DR$^+$ on subjected cells (61) or in tissue samples using immunohistochemistry method as S100$^+$, HLA-DR$^+$ CD1a$^+$ cells (62). Betjes *et al.* (64) identified DCs as large cells with an irregular outline, eccentric nucleus, acid phosphatase staining or CD68 reactivity in a spot juxtanuclear, and strong MHC Class II expression on their cell surfaces. Using monoclonal antibodies anti-BDCA-1 and anti-BDCA-2, Wertel *et al.* (29, 30) identified immature myeloid and lymphoid DCs in the peripheral blood and ascites from ovarian cancer patients. Studies showed that, although the percentage of DCs from the ascites of patients with ovarian carcinoma was significantly higher than that in peripheral blood, DCs in ascites have undifferentiated cell surface characteristics, suggesting their low level of maturity. They have an immature phenotype and co-stimulatory antigens, such as CD80 and CD86, are either completely absent or present only in low numbers on their surface (61, 65). These findings hint that certain factors associated with the tumor local micro-environment might influence the differentiation process of these DCs and their expression of function-associated markers (66). DCs from the ascites are able to secrete high

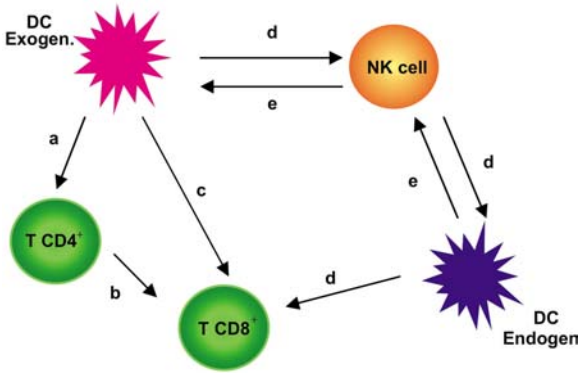

Figure 3. Pathways of CTL induction by DCs. a-b-presentation of peptides by DCs to $CD4^+$ T cells and then to $CD8^+$ lymphocytes. c-the presentation of exogenous antigens via cross-priming/presentation (*i.e.,* from proteins that are loaded onto DCs). d-e-NK cells activated after transfer of DCs secrete IFN-gamma which stimulate DCs to produce IL-12, leading to induction of $CD8^+$ lymphocytes. "Reproduced with permission from Blood. C. Adam, S. King, T. Allgieier, H. Braummuller, C. Luking, J. Mysliwietz, A. Kriegeskorte, D. H. Busch, M. Rocken, R. Mocikat: DC-NK cell cross talk as a novel $CD4^+$ T-cell-independent pathway for antitumor CTL induction. Reproduced with permission from 78.

levels of immunosuppressive cytokines such as IL-10 and TGF-beta (61).

The percentages of dendritic cells (defined as leukocyte lineage negative, HLA-DR positive leukocytes) were found to be substantially higher in ascitic fluid compared to washings. As was the case with the observations performed in normal subjects, the percentage of dendritic cells was significantly higher in the ascitic fluid than in the peripheral blood (61). In our study we found that lymphoid DCs markedly outnumbered myeloid DCs in the peritoneal fluid (PF) of patients with ovarian cancer. The myeloid to lymphoid DC ratio was significantly lower in women with ovarian cancer in comparison to patients with benign disease (30). This data may suggest that lymphoid DC subsets have an influence on the local immune response in the PF of patients with malignant disease. This may be important for the understanding of the mechanism of tumor immune escape as lymphoid DCs are expected to induce tolerance rather than immunity (67, 68). In a study conducted by Zou *et al.* (34), it was documented that lymphoid DC subsets accumulate in tumor ascites and subsequently inhibit anti-tumor immunity (34, 31).

5. DENDRITIC CELLS REGULATION $CD8^+$ CYTOTOXIC T LYMPHOCYTES RESPONSES

DCs possess various functional properties that distinguish them from other antigen-presenting cells. The ability to present antigens to naïve T cells represents one of the most crucial functions of DCs. In this respect, they are 10 to 100 times more potent than monocytes or B cells at stimulating allogeneic T cells to proliferate (69). DCs can also stimulate autologous T cells, potentially presenting either self- or exogenous antigens (70, 71). T cells respond to an immunogenic stimulus by the proliferation, production, and secretion of cytokines, or by exhibiting cytotoxic T- lymphocyte activity as a MHC-restricted phenomenon (72).

Cytotoxic T lymphocytes are one type of critical effector cell that is able to lyse tumor cells. Two activation pathways are likely to give rise to CTL priming. The classic pathway involves presentation of peptides by DCs to $CD4^+$ T cells, provision of T-cell help, and cross-presentation of Ag to $CD8^+$ lymphocytes (Figure 3) (73, 74, 75). Antigen processing occurs first in the cytosol through an ATP-dependent proteolytic system that starts by ubiquitin conjugation. DCs constitutively express di-ubiquitin, which may permit more efficient Ag processing. The ubiquitinylated proteins are directed to the proteasome that cleaves the protein into peptides. The peptides are then translocated into the endoplasmic reticulum (ER) via ATP-dependent TAP1/2 transmembrane transporters and are trimmed into 8-10 mers that accommodate the MHC Class I-binding groove (76, 7). The efficient generation of CTL from naïve $CD8^+$ T cells in the classic pathway requires help from $CD4^+$ T cells. This help involves the secretion of cytokines and CD40/CD40L interactions that lead to increased expression of co-stimulatory molecules on DCs and to the induction of interleukin 12 (51). $CD4^+$ T-helper cells must also be present to recognize a separate collection of peptides displayed on the Class II-MHC molecules of the DCs. This results in the secretion of cytokines that promote the expansion and maturation of the CTLs by the helper T cells (77). In the absence of this, T cells become tolerant to the antigen.

Recently, Adam *et al.* (78) proposed the novel mechanism of CTL priming mediated by NK cells that are activated after DC transfer. They presented evidence that the interplay between DCs and NK cells can completely replace $CD4^+$ T-cell help in the induction of $CD8^+$ CTL. By the secretion of IFN-gamma, the activated NK cells stimulate DCs, including endogenous DCs, to produce IL-12, leading to the induction of $CD8^+$ lymphocytes (Figure 3). This conclusion is supported by experiments whereby neutralization of IFN-gamma inhibited IL-12 production by DCs as well as CTL induction. Thus IFN-gamma and IL-12 are instrumental in the T-helper cell-independent pathway that links DC-NK cell cross-talk to CTL immunity (78).

The reciprocal stimulation of DCs and NK cells has been well documented. In 1987, Doherty *et al.* (79) suggested that NK cells play a positive role in the induction of CTL. In the next two sets of *in vivo* experiments, murine NK cells were shown to be critical for the induction of tumor-specific CTL (80) and for a Th1 bias associated with acute allograft rejection (81), indicating that NK cells play a supportive rather than an inhibitory role in the induction of Ag-specific type1 immunity. In 2003, Mailliard *et al.* (82) have demonstrated that "helper activity" of NK cells may support the development of Th1- and CTL-dominated type1 immunity, which may in turn have important implications for cancer immunotherapy. Both Mailliard *et*

Table 2. Delivery of antigens into dendritic cells

Known tumor-associated antigens [MAGE-1 and MAGE-3, MUC1, Her-2/neu, tyrosinase, carcinoembryonic antigen (CEA), Melan-A/MART, PMSA]
Synthetic or eluted peptides
Soluble protein
Transfection with cDNA or RNA encoding known tumor-associated antigens
Recombinant viruses (adenoviruses, vaccinia, or retroviruses)
Whole tumor vaccine
Tumor lysates
Apoptotic bodies, necrotic cells
Tumor RNA
Hybrid cell
Heat shock proteins
DC-derived exosomes

al. (82) and Kaliński *et al.* (23) have reported that NK cells are capable of inducing stable type1-polarized DCs (DC1) that act as carriers of NK cell-derived helper signals for the development of type1 immune responses. NK cell-induced DC1 show a strongly elevated ability to produce IL-12p70 after subsequent CD40-ligand stimulation. DC1 induction depends on NK cell-produced IFN-gamma and TNF-alpha, with the possible involvement of additional factors. DC1 induced by NK cells or by NK cell-related soluble factors, are stable, resistant to tumor-related suppressive factors and show a strongly enhanced ability to induce Th1 and CTL responses (23). In analogy to resting T cells, the induction of the "helper" function of NK cells, relies on a two-signal activation paradigm. While NKG2D-dependent tumor cell recognition is sufficient to induce the cytotoxic "effector" function of NK cells, the induction of “NK cell help” requires an additional co-stimulatory signal. Such second signal for the induction of NK cell “helper” activity can be provided by type-1 interferons (IFN-alpha or IFN-beta), products of virally-infected cells, or by IL-2 produced by activated $CD4^+$ Th cells (82, 23). Both IL-2 and IFN-alpha/IFN-beta can also synergize with IL-18 in the induction of NK-cell helper activity, but none of these factors can by itself induce “NK cell help” (23). This data may contribute to a better understanding of the poor effectiveness of immune responses against cancer, as opposed to effective anti-viral immunity. During an early phase of tumor growth, NK cells can eliminate transformed tumor cells, but due to the absence of a second, e.g., IFN-alpha-dependent signal, NK cells are not induced to exert any “helper activity” and thus neither activate nor polarize local DCs. Finally, despite the ability of NK cells to recognize the transformed cells and control their initial growth, they cannot support the development of tumor-specific type1 immunity, resulting in the eventual loss of control of tumor growth. This is in contrast to viral infections, where efficiently activated local NK cells contribute to the induction of adaptive Ag-specific responses, resulting in effective virus elimination (82).

6. SOURCES OF DCS FOR CLINICAL USE

Human DCs can be generated *in vitro* from purified $CD34^+$ cells using appropriate cytokine combinations. Besides the well-known cytokines GM-CSF, IL-4, TNF-alpha, and FLT-3 ligand (L), TGF-beta seems to play a key role in the generation of DCs from $CD34^+$ cells under serum-free conditions. Sources of $CD34^+$ cells include bone marrow, cord blood, and granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood (83). Alternatively, when cultured for 6 to 7 days in the presence of GM-CSF and IL-4, human DCs also develop from peripheral blood $CD14^+$ monocytes. These cells have the characteristics of immature DCs and can be further induced to mature by inflammatory stimuli, including TNF-alpha, IL-1, LPS, CD40 ligation, or monocyte-conditioned medium (84, 85, 15). Recently, an alternative procedure for generating monocyte-derived DCs based on an *in vitro* culture with IFN-alpha and GM-CSF has been described (86). DCs differentiated in this setting (IFN-DCs) show increased expression of co-stimulatory molecules and more potent functional activities when compared with DCs cultured with GM-CSF and IL-4. IFN-DCs generated from adherent monocyte-enriched PBMC are characterized by a semi-mature phenotype, but exert efficient T- cell priming activity towards $CD8^+$ T cells recognizing different human tumor antigens (87). Tosi *et al.* (87) have reported that the ability of adherent PBMCs to differentiate into IFN-DCs expressing higher levels of co-stimulatory molecules and exerting efficient T-cell priming capacity was associated with the presence of contaminating NK cells that have undergone phenotypic and functional activation upon IFN-alpha treatment.

6.1. Tumor antigens and antigen-loading onto DCs

The specific immunotherapy of cancer uses tumor-directed $CD8^+$ CTLs that lyse tumor cells presenting MHC Class I-associated peptides derived from tumor-associated proteins. There are different strategies now being applied in clinical trials to deliver antigens to DCs (Table 2).

The ability of DCs to generate anti-tumor immune responses *in vivo* has been documented in many animal models. DCs loaded with tumor lysates, tumor-antigen-derived peptides, synthetic MHC Class I-restricted peptides, and whole protein, have all been demonstrated to generate tumor-specific immune responses and anti-tumor activity (88, 89). The ability of tumor-antigen-pulsed mature DCs to elicit protective immunity against cancer has been reported in various kinds of human tumors such as malignant melanoma (90), leukemia (91), renal cell carcinoma (92), prostate cancer (93), and breast cancer (94); however, the number of studies investigating DC immunotherapy for ovarian cancer is limited. The major prohibiting factor in the development of ovarian cancer vaccines with DCs is the lack of clearly defined ovarian-specific tumor antigens capable of being recognized by T cells. Most known tumor antigens are expressed to some degree on normal tissues and are therefore tumor-associated (TAA) rather than tumor-specific (95).

In the treatment of ovarian cancer, only a few T-cell epitopes have been identified, including those derived from the HER-2/neu proto-oncogene and the epithelial mucin MUC1. The HER-2/neu proto-oncogene is over-expressed in approximately 20% to 30% of patients with breast and ovarian cancer and may correlate with poor prognosis (96).

A number of reports have characterized HLA-A2 restricted epitopes from HER-2 (E75 and GP2) that can be recognized by peptide-specific T cells (97, 98). The MUC1 protein is over-expressed in more than 90% of breast and ovarian cancer cells and would thus seem to be an attractive and broadly applicable target for cancer vaccination therapies (99). Recently, two novel 9-mer peptides, M1.1 and M1.2, with a high-binding probability to HLA-A2 were identified (100).

There are a number of protein candidates reported to be over-expressed in ovarian tumors that may represent attractive candidates for tumor antigens. Ovarian carcinoma cells over-express the cancer antigen (CA)-125, which secreted form has long been recognized as the gold standard for monitoring patients with ovarian cancer (101). CA-125 has been used as a target antigen for antibody-based therapy (102); however, it has not yet been considered as a target for cellular immunotherapy (103). The major reason for this has been the lack of information on the primary sequence of CA-125 that may allow identification of epitopes recognized by $CD8^+$ CTL and helper $CD4^+$ T cells. Another potential limitation of CA-125 as an immunotherapeutic target is that it is not uniquely expressed by ovarian carcinoma and can be found in other normal tissues (104). Two antigens, folate-binding protein (105) and mesothelin (106, 107) are surface proteins that have been identified as over-expressed in ovarian cancers. Although there are many other antigens identified that may be expressed by ovarian tumor cells, e. g., MAGE-1 (108), EGF receptor (109), the frequency of their expression is often variable, and it remains unknown whether these antigens can serve as tumor-specific CTL targets (104). Additionally, several serine proteases have been identified, including hepsin, stratum corneum chymotryptic enzyme (SCCE), protease M, and the tumor-associated differentially-expressed gene (TADG) 12, TADG-14, TADG-15, TADG-16 (testisin). All of these proteins are highly expressed in ovarian carcinomas, but are not expressed in normal ovaries and only rarely expressed in other adult tissues. Tumor-associated proteases may represent attractive candidates for tumor antigens. They are involved in many biological functions of cancer cells, such as the activation of growth and angiogenic factors as well as the activation of other proteases responsible for invasion and metastasis (104, 110). Recently, the use of NY-ESO-1, "cancer-testis" antigen, recognized by $CD4^+$ and $CD8^+$ T cells, which is thought to be one of the most immunogenic tumor antigens in epithelial ovarian cancer, showed promising results in phase I clinical trial of immunotherapy in cancer patients (111).

7. MAJOR BARRIERS TO SUCCESSFUL DENDRITIC CELL IMMUNOTHERAPY IN OVARIAN CANCER

Neoplastic cells have developed a variety of cellular and molecular mechanisms to evade detection and elimination by the immune system (4, 5, 95, 112). Recently, regulatory T cells (Tregs) have also been shown to contribute to cancer-related immunosuppression (113). Treg cells represent 5% to 10% of thymus-derived $CD4^+$ cells (114). They are identified by their high expression of the IL-2 receptor alpha chain (CD25) and the T cell co-receptor CD4 (115). Most Tregs express the forkhead transcription factor forkhead box protein (FoxP3) (116, 117). Apart from thymic-derived natural Tregs, peripherally induced TR1 (118) and TR3 cells (119) have been described that achieve suppressive effects by the secretion of IL-10 or TGF-beta, which blocks the maturation of dendritic cells (120). Tissue from normal ovaries as well as ovarian neoplasms exhibit intense immunogenicity. In physiology, immune homeostatic mechanisms are capable of blocking immune reactivity. It is Treg cells that play the crucial role in controlling autoimmune pathology through maintenance of peripheral tolerance to self-antigens (115).

In humans, high levels of $CD4^+CD25^+FoxP3^+$ T cells have been detected in non-small cell lung cancer, breast cancer, colorectal cancer, pancreatic cancer, leukemia, and melanoma (117, 121). In 2001 Woo *et al.* (121) detected an increased percentage of Treg cells in patients with late-stage ovarian cancer. Similarly, Curiel *et al.* (122) found large numbers of Treg cells in malignant ascites and in the tumor mass of patients with untreated epithelial ovarian cancers (EOC). Moreover, the authors detected that these cells migrate into the tumor microenvironment in a process mediated by the chemokine CCL22 (predominantly expressed by ovarian tumors) and are capable of suppressing antitumor responses. Curiel *et al.* (122) also demonstrated an inverse correlation between tumor Tregs content and patient survival. These observations are confirmed by a study by Wolf *et al.* (113), who detected that expression of FoxP3 is a negative prognostic factor in patients with EOC.

It has been well documented that immature DCs, that express low surface levels of MHC Class II and co-stimulatory molecules induce Tregs and peripheral tolerance (39, 67). However, there are also reports that demonstrate that mature DCs can also expand $CD4^+CD25^+FoxP3^+$ T cells (123, 124). Yamazaki *et al.* (125) detected that mouse DCs expand alloantigen-specific Tregs from polyclonal populations of T cells in the mixed leukocyte reaction (MLR). Similar findings also were reported recently in human by Banerjee *et al.* (126), who detected that an injection of DCs matured with inflammatory cytokine expanded Tregs *in vivo* in 3 out of 3 myeloma patients. DC-induced Tregs from both healthy donors and patients with myeloma were functional and effectively suppressed T-cell responses (126).

These observations reveal a specific role for DCs in increasing the percentage of functional $CD4^+CD25^+FoxP3^+$ T cells in humans and highlight the need for additional therapeutic options.

8. CLINICAL TRIALS OF DENDRITIC CELLS VACCINATION IN OVARIAN CANCER

In gynecologic oncology, monocyte-derived DCs loaded with tumor lysate antigen efficiently induce $CD8^+$ CTL capable of lysing autologous tumor cells from patients

with endometrial cancer (127), uterine serous papillary carcinoma (128), or ovarian cancer (129). Recently, Tobiasova *et al.* (130) generated the DC vaccine pulsed with apoptotic tumor cells from patients with ovarian cancer. They have shown that DCs pulsed with apoptotic tumor cells and subsequently matured can efficiently prime autologous lymphocytes and induce IFN-gamma secretion *in vitro*. Similarly, Schlienger *et al.* (131) have reported that dendritic cells pulsed with killed autologous primary ovarian tumor cells and matured with CD40 ligand and tumor necrosis factor-related activation-induced cytokine (TRANCE) were capable of inducing antigen-specific T cells that secreted IFN-gamma upon stimulation with autologous tumor cells. These observations are supported by prior studies showing that DCs loaded with peptides, acid-eluted from HLA Class I on the surface of ovarian tumor cells (132), and tumor lysate-pulsed DCs (129, 133) can induce $CD8^+$ CTL that kill autologus tumor cells of women with ovarian cancer. Brossart *et al.* (53) demonstrated that patients with advanced ovarian cancer could be efficiently vaccinated with autologous mature monocyte-derived DCs produced *in vitro* with GM-CSF, IL-4, and TNF-alpha and subsequently pulsed with MUC-1 and HER2/neu-derived peptides even after high dose chemotherapy. The DC vaccinations were performed subcutaneously. The injections were well tolerated, showing no adverse effects. In 5 out of 10 patients, antigen-specific CTLs were detected in the peripheral blood using both intracellular IFN-gamma staining and ^{51}Cr-release assay. In addition, in one patient vaccinated with the MUC-1-derived peptides, CEA-and MAGE-3 peptide-specific T-cell responses were detected after several vaccinations. In a second patient immunized with the HER-2/neu peptides, MUC1-specific T lymphocytes were induced after seven immunizations. It has been suggested that epitope spreading *in vivo* might occur after successful immunization with a single tumor antigen (94).

Furthermore, DCs fused with ovarian cancer cells can stimulate CTL activity against autologous tumor cells. In this case, immunogenic hybrid cells are created with the properties required for the initiation of primary anti-tumor immune responses. Gong *et al.* (134) showed that the human ovarian/DC fusions express both ovarian carcinoma-associated Ags and DC-derived MHC Class II and co-stimulatory molecules. Ovarian carcinoma cells (OVCA) fused to either autologous or allogeneic DCs were effective in inducing anti-tumor CTL that lyse autologous OVCA by a MHC Class I-restricted mechanism. The autologous hybrid cells can present tumor Ags by OVCA- or DC-derived MHC Class I molecules. Moreover, autologous hybrid cells can present tumor Ags by DC-derived MHC Class II molecules and thereby stimulate helper $CD4^+$ cells. By contrast, presentation of tumor Ags by the allogeneic hybrid cells was dependent on OVCA-derived MHC molecules. The allogenic hybrid cells can also stimulate alloreactive T cells and thereby the release of cytokines that contribute to the activation of tumor-specific CTL (135). In studies of Koido *et al.* (136), OVCA cells derived from 22 patients were successfully fused with autologous DCs. The created heterokaryons expressed tumor-associated antigens, such as MUC1 and CA-125, and DC-derived MHC Class II and co-stimulatory molecules. The fusion cells were functional in stimulating the proliferation of autologous T cells. In addition, fusion cells stimulated both CD4 and CD8 T-cell responses (136).

9. PERSPECTIVE

There is a need to better characterize and define the functional roles of DC subsets in ovarian cancer. It would certainly be interesting to investigate how different DC subsets in epithelial ovarian cancer influence the effectiveness of antigen-specific T cells. Detailed investigation into the role of defective DCs in anti-tumor immunity may provide new insights into the development of new therapeutic strategies for malignancies.

Other important aspects include the preparation of DC vaccines and surmounting of barriers to successful immunotherapy. Obtaining “good quality” full blood that is reach in mononuclear cells, from patients who underwent myelosuppressive chemotherapy treatment, may be technically difficult. Efforts of leukapheresis should be conducted several weeks from the completion of the last cytostatics treatment course.

Most significant problems, however, seem to be connected with overcoming mechanisms that enable cancer cells to evade immune elimination and enhance the persistence of the vaccine-induced cellular response. One possible solutions is to enhance T cell co-stimulation by administering agonistic antibodies (*i.e.*, OX40) or antagonistic co-inhibitory receptor antibodies (*i.e.*, CTLA-4) (137, 138). Diminishing Treg cell expansion may possibly be achieved by DC cultures supplementation with specific cytokines (*i.e.*, IL-15) (139, 140). Another controversial mechanism of Treg suppression may be competition with the IL-2 cytokine which is critical for activation of the suppressor function of Treg cells *in vitro* (139, 125, 141). Enhancement of vaccine-mediated antitumor immunity in cancer patients with an accompanying reduction of Tregs was also achieved by administering a cytotoxic hybrid protein, consisting of the diphtheria toxin and IL-2 binding domain as a carrier (denileukin difitox, ONTAK) (141). However, ONTAK failed to reduce Treg cell numbers in patients with metastatic melanoma (142). Interestingly, low doses of cyclophpsphamide, an alkylating cytostatics drug, may also selectively reduce Treg cell counts (117). Although many questions still remain unanswered, current research suggest that Treg cell activity is one of the main barriers to successful immunotherapy.

Finally, future studies should focus as well on the antigenicity of ovarian cancer and the identification of antigens expressed in the majority of epithelial ovarian cancers, especially those that are presented on the cell surface in the context of MHC. Most ovarian tumors are intensively heterogenous and insofar as no highly specific ovarian cancer antigens have been identified, immunotherapeutic vaccinations should target multiple cancer antigens, characteristic for each individual patient as obtained from her own tumor lysates. Once these antigens

are clearly defined, ovarian cancer vaccine trials should be conducted, initially as adjuvant treatment for metastatic FIGO IV patients. There is no doubt that the treatment of these patients with new therapeutic strategies is extremely difficult as they usually present poor general health. They are also immunodeficient as they have overcome not only surgical procedures, but also multiple courses of first-, second-, and third-line chemotherapies. In fact, it is enormously difficult to obtain results that could statistically confirm the benefits of any adoptive treatment applied. As we are all aware, immunotherapy would show more benefits if it were applied immediately after initial debulking surgery, in patients with early stages of the disease, *i.e.,* FIGO stage I or II. Such patients are usually in relatively good health, have minimal residual tumor and DC-based immunotherapy is directed against the minimum number of malignant cells. The fewer cancer cells, the more chance of tumor-antigen specific vaccination success. On the other hand, it is for now almost impossible to obtain the ethical committee permission necessary for a pilot study project in such a group of patients unless the specific mode of action has proven benefits and lack of serious adverse effects.

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Abbreviations: DCs: dendritic cells, FIGO:International Federation of Gynecologists and Obstetricians, MHC: major histocompatibility complex, Ags: antigens, CTL: cytotoxic T lymphocyte, APC: antigen presenting cells, GM-CSF: granulocyte-macrophage colony-stimulating factor, R: receptor, IL: interleukin, L: ligand, TNF: tumor necrosis factor, TGF: transforming growth factor, M-CSF: macrophage-colony stimulating factor, G-CSF: granulocyte-colony stimulating factor, Lin: leukocyte lineage, HLA: human leukocyte antigen, PBMC: peripheral blood mononuclear cells, hsp: heat shock proteins, gp: glycoprotein, LPS: lipopolysaccharide, dsRNA: double-stranded RNA, DC-LAMP: DC-lysosome-associated membrane protein, TLRs: toll-like receptors, MIP: macrophage inflammatory protein, MCP: monocyte chemo-attractant protein, Fc: fragment crystallizable, ELC (MIP-3 beta): Epstein-Barr virus-induced ligand chemokine, SLC (6Ckine): secondary lymphoid tissue chemokine, PF: peritoneal fluid, TCR: T-cell receptor, NK: natural killer, TAA: tumor-associated, CA: cancer antigen, Tregs: regulatory T cells, FoxP3: forkhead transcription factor forkhead box protein, MLR: mixed leukocyte reaction, EOC: epithelial ovarian cancer, TRANCE: tumor necrosis factor-related activation-induced cytokine, OVCA: ovarian carcinoma cells

Key words: Dendritic Cells, Ovarian Cancer, Cytotoxic Immune Response, Ascites, Immunotherapy, Review

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