in vivo *18*: 81-100 (2004)

Antigen Presentation by Dendritic Cells and their Significance in Antineoplastic Immunotherapy

BELA BODEY[1,2], STUART E. SIEGEL[1,2] and HANS E. KAISER[3]

[1]*Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA;*
[2]*Childrens' Center for Cancer and Blood Diseases, Childrens' Hospital Los Angeles, Los Angeles, CA;*
[3]*Department of Pathology, School of Medicine, University of Maryland, Baltimore, MD, U.S.A. and Department of General and Experimental Pathology, University of Vienna, Vienna, Austria*

Abstract. *Dendritic cells (DCs) are present in essentially every mammalian tissue, where they operate at the interface of innate and acquired immunity by recognizing pathogens and presenting pathogen-derived peptides to T lymphocytes. According to the research group of Shortman (1-9), experimental results suggest a "dual" DC differentiation model, demonstrating the existence of both myeloid-derived (with characteristic IF: CD11b$^+$, CD11c$^+$, CD8^{alpha-} and DEC205$^+$) and lymphoid-derived DCs (showing CD11b$^-$, CD11c$^-$, CD8^{alpha+} and DEC205$^+$ IF). DCs, including interdigitating cells (IDCs) and Langerhans cells (LCs), are characterized by dendritic morphology, high migratory mobility and are the most effective, "professional" cells for antigen presentation in primary immune responses. Most of the DCs express immunocytochemically detectable antigens like: S-100, CD1a, CD40 receptor, adhesion molecules (ICAM-1 or CD54, LFA-1 and LFA-3), integrins (CD11a, CD11c and CD18), CD45, CD54, co-stimulatory molecules (B7-1 or CD80, B7-2 or CD86), F418, MHC class I and II and DEC-205, multilectin receptor, immunostimulatory cytokine (IL-12) and, of course, Fc and complement receptors. Following recognition and uptake of antigens, mature dendritic cells (DCs) migrate to the T lymphocyte rich area of draining lymph nodes, display an array of antigen-derived peptides on the surface of major histocompatibility complex (MHC) molecules and acquire the cellular specialization to select and activate naive antigen-specific T lymphocytes. Immunotherapeutic ideas are based on the ability of the mammalian immune system to recognize neoplastically transformed cells. Immunotherapy of human neoplasms has always represented a very attractive fourth-modality therapeutic approach, especially in light of the many shortcomings of conventional surgical, radiation and chemotherapies in the management of neoplastically transformed cells. The cancer vaccine approach to therapy is based on the notion that the immune system could possibly mount a rejection strength response against the neoplastic cell conglomerate. The efficiency of DCs for T lymphocyte stimulation moved a number of research groups to develop DC- based immunotherapy approaches (10). The failure of cancer vaccines may be attributed to the relationship between host and neoplasm: through a natural selection process, the host facilitates the selective enrichment of clones with highly aggressive neoplastically transformed cells, being in various stages of differentiation and only during certain stages express neoplastic cell specific molecules.*

Correspondence to: Bela Bodey, M.D., D.Sc., 8000-1 Canby Avenue, Reseda, CA 91335, U.S.A. Tel: (818) 886-1082, e-mail: Bodey18@aol.com.

Key Words: DC, dendritic cell, CD34$^+$ hematopoietic stem cell, APC, antigen presenting cell, IDC, interdigitating cell, LCs, Langerhans cells, Birbeck granules, Langerin (CD207), folliculo-stellate cells (FSCs), cytokines, interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-12 (IL-12), AP, anterior pituitary, TAA, tumor associated antigen, neuroendocrine immunoregulation, major histocompatibility complex (MHC) class II molecules, MIIC (MHC class II-enriched compartment, exosomes), adhesion molecules, granule-associated marker (Lag), IP, immunophenotype, MoAb, monoclonal antibody, GM-CSF, granulocyte-macrophage colony stimulating factor.

The dendritic cell network

Immunophenotypically and functionally heterogeneous DCs are distributed throughout the human lymphatic and the majority of non-lymphatic tissues and represent the most potent, "professional" antigen presenting cell meshwork (APCs), functioning as an integral part of the immune system (1-9). Folliculo-stellate cells (FSC) in the anterior pituitary (AP), Langerhans cells (LCs) in stratified epithelia such as the skin, oral cavity, upper airways, urethra, female reproductive tissues and lymphatic system (11), "veiled" cells, lympho-dendritic and interdigitating cells (IDCs) in a number of tissues comprising the lymphatic system are the cell types of the DC meshwork. Most of these cells express the immunocytochemical markers S-100, CD1, CD45, CD54, F418, MHC class I and II antigens, Fc and complement receptors.

Immunophenotype (IP) differences among DCs have been detected employing immunocytochemistry. Bednar (12) studied the immunoreactivity of resident DCs in twenty tissues, including lymphatic, skin, bone, soft, nervous, myocardial, lung, esophagus, stomach and intestinal, among others, employing antibodies against CD34, F XIIIa, F VIII, actin, CD68, S-100 protein, HLA-DR, CD3 and OPD4. Three characteristic immunoreactivities of resident DCs were established: a $CD34^+$ subset of cells, a $CD68^+$ subset of cells which were phagocytic, and a $S\text{-}100^+$ subset of cells. The ability of dendritic cells (DCs and LCs) in epithelial tissue structures to interact on a cell to cell level with epithelial cells was also noted (in the thymic microenvironment), which may provide these cells with increased regulatory functions. DCs can also provide all of the known co-stimulatory signals required for activation of unprimed T lymphocytes and are the most effective, specialized APCs in the induction of primary T lymphocyte-mediated immune responses (13,14).

Interdigitating cells (IDCs). Interdigitating cells (IDCs) are specialized, bone marrow derived DCs, located in T cell domains of various human tissues (15-18). Von Gaudecker and Müller-Hermelink (19) morphologically identified the IDC precursors, originally discovered in the human thymus by Kaiserling and co-workers (20) (first appearance during the 10th week of intrauterine life). The same authors described the TEM characteristics of IDCs in an 85 mm long (3rd lunar month) human fetus:

"Large electronlucent cells with irregularly shaped nuclei are found in both the mesenchymal septa and in the presumptive medullary regions of the thymus primordium. These cells appear to be precursors of the IDCs, which have been described in the medulla of the prenatal thymus."

When the already committed, lympho-hematopoietic progenitors come into contact with these IDC precursors, the IDCs develop small finger-like protrusions to assure more efficient contact between these cell types.

A histochemical osmium-zinc iodide impregnation procedure has been reported for the identification of IDCs on semithin sections (21). IDCs in such a preparation appear as large dendritic elements containing numerous cytoplasmic protrusions with established structural contacts with lymphatic cells. Differentiating thymocytes have also been observed to be in intimate contact with IDCs. Kaiserling (20) was the first to describe this significant intercellular contact: "The surfaces of the IDC were in close contact with those of small lymphocytes, sometimes polysomal lymphatic cells, epithelial cells, and occasionally with those of lymphatic cells containing ergastoplasm." As stated above, IDCs are large cells with several cytoplasmic processes, which form an extensive three-dimensional network that envelopes maturating thymocytes (22). Aggregates between IDCs, differentiating thymocytes and functionally complete T lymphocytes represent the specialized microanatomical and functional units within the thymic microenvironment that are crucial for T lymphocyte maturation and education (22).

The most significant TEM features of IDCs are an irregularly-shaped, euchromatic nucleus, with very loosely arranged chromatin, except for a small rim of heterochromatin along the inner surface of the nuclear envelope and a nucleolus (not readily visible in all cases), often in an eccentric position. The cytoplasm contains single or groups of flat cisternae of the rough endoplasmic reticulum. Secretory vesicles are always present near the tubules of the Golgi complex. A number of mitochondria are distributed throughout the cytoplasm. Their characteristic cytoplasmic projections regularly "*interdigitate*" with other IDCs and several other cell types in the developing thymic medulla. One of the most important characteristics of IDCs remains that they are never connected to each other or to other cells by desmosomes (23-25). Immunocytochemical observations established the expression of MHC class I and II molecules, as well as several adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), and lymphocyte function-associated molecules (LFA-3) on the surface of IDCs (26,27).

The well determined localization of IDCs within the thymus provides significant insight into their function. IDCs have been described, in all of the many observations (including our material in dogs), to be located predominantly at the level of the thymic cortico-medullary junction, as well as in the deep cortex, but never in the subcapsular region. In guinea pig thymuses, Klug and Mager (28) found IDCs located mostly in the inner cortex, with kidney-shaped nuclei (with finely dispersed chromatin) and a thin layer of marginally located heterochromatin. The nucleolus in these cells was usually eccentric and small. These IDCs also contained all of the organelles in their cytoplasm, including lysosome-like bodies, and few ergastoplasmic and tubulovesicular structures. The most interesting cytoplasmic features were large electron-dense bodies (phagosomes) containing fragments of picnotic lymphocytes. Many finger-like processes, for contact with other thymic cell types, were also observed. Glycoprotein synthesis by IDCs has been discussed, but contact with thymocytes has always been described as their main physiological function.

The phagocytic activity of IDCs is not of great importance when compared to macrophages, but still numerous ingested cells can be present in their cytoplasm. Klug (29) described such findings following irradiation of the rat thymus. Duijvestijn and co-workers (30-32) observed

the phagocytic activity and the population development of medullary IDC and cortical macrophages following irradiation-induced acute tissue necrosis in the thymus. IDCs clearly demonstrated phagocytic activity sixteen hours after treatment, but this activity could be attributed to the fact that, at this stage, the number of necrotic thymocytes was maximal and the total number of phagocytic cells was few and, thus, IDCs were recruited to phagocytize the necrotic material. In discussing their findings, the authors speculated on the possible similarities between IDCs and epidermal Langerhans cells. Miyazawa and co-workers (33) reported a twelve-fold increase in the thymic macrophage population in mice eight hours after 3 Gy irradiation. The mammalian cell surface is rich in acidic sugars, such as sialic, hyaluronic and chondroitic acids. The surface charges caused by these and similar molecules play an important role in cell to cell interactions, such as phagocytosis. The negative surface charge of irradiated thymocytes was found to be enhanced within a few hours, as illustrated by the attachment of these cells to esterase positive thymic phagocytic cells through the reduction of the electrostatic repulsive forces. The irradiated thymocytes may thus be recognized as foreign proteins (antigens) and phagocytized by thymic phagocytic cells. Higley and O'Morchoe (34) published a morphometric analysis of the thymic non-lymphoid cells within the medulla of rats, between the ages of one and 65 days. They found that the largest depot of thymic non-lymphoid cells is within the medulla or the so-called central part. They described the presence of macrophages, IDCs and LCs at this location.

Von Gaudecker and Müller-Hermelink (19) mentioned diapedesis from the mesenchymal septa and perivascular spaces as a possible mechanism of IDC entrance into the thymus. Once inside the thymus, these IDCs construct a cellular microenvironment at the cortico-medullary junction that is necessary for the migration pattern, differentiation and maturation of thymocytes into T lymphocytes (19,35,36). Monoclonal antibodies (MoABs) reactive with thymic macrophages and IDCs have been developed using these particular cell types isolated from peripheral lymphatic organs (Mac-1, Mac-2 and ER-BMDMl for both macrophages and IDCs; F4/80, BM8, MOMA-2 exclusively for macrophages; and NLDC-145, MIDC-8, M1-8 and ER-TR6 only for IDCs). IDCs have, however, never been identified in B lymphocyte regions.

Langerhans cells (LCs). Another type of DC within the developing cellular microenvironment of various tissues are the so-called Langerhans cells (LCs). Thoughts concerning the origin and function of LCs have historically been based on two differing hypotheses. One group of investigators maintained that LCs perform neural functions (37-44), while others followed Masson's (45) theory that regarded LCs as the last stage in the life cycle of an active melanocyte (the theory of the "*worn out effect*" melanocyte) (46-48). LCs have also been described as lymphatic cells that are capable of forming antibodies (49,50). The limited degree to which LCs phagocytize foreign particles suggests that, under normal conditions, this does not represent their primary function. Tissue culture experiments established that murine epidermal LCs can mature into potent immunostimulatory DCs (51). The necessity of GM-CSF to ensure the *in vitro* viability and function of epidermal LCs has also been described (52). Novel experimental evidence identified that the LCs originate from the $CD34^+$ hematopoietic stem cells located in the yolk sac, liver and later in bone marrow (53). *In vitro* stimulation of $CD34^+$ cells with GM-CSF and TNF-α led to their rapid proliferation and differentiation into a cell clone with the following IP: $CD45+/CD68^+/CD3^-/CD19^-/CD56^-$ and also expressing CD1a, CD4 and MHC class II. The $CD1a^+$ cells included three cell populations: 1) LCs identified by the presence of Birbeck granules; 2) Birbeck granule negative DCs; and 3) $CD14^+$ monocytes. The addition of IL-4 interfered with the generation of monocytes, but also resulted in an increased percentage of $CD1a^+$ LCs (<24%) that are potent stimulators of the primary mixed leukocyte reaction and, as such, are promising candidates for the generation or augmentation of host responses against different pathogens following vaccination. Studies in the last decade have identified the lymphoid origin of LCs (11,54-57). Lymphoid committed, $CD4^{low}$ stem cells, as well as $CD25^+$ and $CD44^+$ pro-T lymphocyte progenitors are capable of differentiating into T lymphocyte, B lymphocyte, natural killer (NK), $CD8^+$ DC and LC lineages in many mammalian tissues, including the epidermis.

The presence of these specialized DCs in the epidermis was first described in 1868 by Paul Langerhans following gold chloride staining of skin sections (58). It was not until 1973 that the first experimental evidence for antigen presentation by LCs was reported (59). Today it is well known that human LCs are $CD1a^+$ DCs that function as very potent APCs for primary and secondary immune responses (53). TEM observations of LCs were first reported by Birbeck and co-workers (60) who described these cells as DCs that can be distinguished by a relatively clear cytoplasm, a lobulated nucleus, cored tubules and unique organelles, and the so-called Birbeck or LC granules. Although the LC granule is the ultimate marker for these cells, the same granules have occasionally been detected inside phagocytic vacuoles within activated macrophages (61-64). In addition, IDCs also occassionally possess Birbeck granules, which serves as the basis for the similarity between LCs and IDCs. Birbeck granules originate from the cell membrane and play a role in receptor-mediated endocytosis, intracellular processing and presentation of CD1a, MHC class II, granule-associated

marker (Lag) and other antigens (65-68). The notion that LCs may represent epidermal macrophages has also been expressed (69,70).

LCs have also been reported within the rat thymic microenvironment (71-74). Olah and co-workers (71) described them as cells of a special type within the thymic medulla:

"...new kind of cells, found in the medulla of the rat thymus is described. The special structure of their cytoplasm which points to intensive cellular activity, as well as the characteristic granules contained in these cells, justify their classification as a separate cell type. The cells in question should be distinguished from the cells contained in the epithelial reticulum of the thymus, from the macrophages containing phagocyted fragments and lysosomes as also from those cells whose granules include a rod-shaped dark structure. It is, on the other hand, possible that the electron-microscopically observed cells of the present study are identical with Ito's inclusion cells (1959)."

As noted previously, the presence of these same cytoplasmic granules has been a defining characteristic of LCs of the skin (75,76).

In routine tissue sections stained with hematoxylin-eosin (HE), LCs cannot be identified. However, they can be easily demonstrated by impregnation with gold salts (74,77,78). Histochemically, the ATPase method has proved most useful for the detection of LCs, provided that proper fixation and cutting techniques are employed. This reaction seems to be specific for LCs.

There are questions concerning the specificity and significance of the LC granules. Hashimoto (70) in his histoenzymatic study described that the granules, not only those attached to the plasma membrane, but also those entirely enclosed within the cytoplasm, were permeated passively by the lanthaum complex during a post-vital incubation procedure. These results suggest that these intracytoplasmic granules have a direct connection with the extracellular spaces. In his opinion, the granules are endocytic in nature, but he discussed whether these typical organelles may also be involved in the secretory functions of LCs. The extrusion of specific molecules from the interior of the LCs into the intercellular space within the thymic microenvironment may be the mechanism by which these DCs are involved in the regulation of thymocyte differentiation and also provides a way for interaction induction between DCs and RE cells located in the medulla.

Just last year, Plzak and collegues (79) defined the molecular structure of the Birbeck granules in LCs: "In LCs langerin (CD207), a type II transmembrane protein with a single C type carbohydrate recognition domain attached to a heptad repeat in the neck region, which is likely to establish oligomers with an alpha-coiled-coil stalk, has been implicated in endocytosis and the formation of Birbeck granules. The langerin structure harbours essential motifs for Ca2+-binding and sugar accomodation". It is significant that the access to the carbohydrate recognition domain of langerin is impaired in tissues showing high cell proliferative activity (*i.e.* neoplasms).

Rowden (80-82) described the expression of Ia-like antigens on the surface of LCs. The relationship between LCs and IDCs in the T lymphocyte areas of lymph nodes and spleen (83), the dendritic reticulum cells of germinal centers (84) and other areas of the spleen (85) appears to lie in their common function in antigen presentation rather than in antigen processing because when trypsin-treated LC suspensions were observed, C3 receptors were detected on LCs in humans (75,76). Trypsin has been reported to destroy C3 receptors on B lymphocyte surfaces, but Berman and Gigli (86) have found that, as in macrophages, this receptor is not sensitive to enzyme digestion in LCs.

It is known that, in mice, the H-2 system is on chromosome 17 and has two parts: an H-2K and an H-2D region, comparable to human HLA-A and HLA-B. These regions code for a set of serologically detectable cell surface alloantigens, known as the Ia antigens. These have α and β polypeptide subunits of approximately 33kD and 28kD, respectively, and they lack a common β_2-microglobulin subunit (on primate B cells, HLA-DR antigens). In mice, the distribution of Ia antigens is as follows: I-A, I-E and I-C region products are present on B cells, macrophages, sperm and fetal liver cells, but not on T cells (87). Certain subsets of T cells, however, appear to possess excess products of the I-J region (suppressor cells) and it has also been shown that a high percentage of the cells in the epidermis express Ia antigen (30-90%) (88). Quantitative absorption studies have found that these cells express 2-4 times less Ia antigen than B lymphocytes (88,89). The investigations of Rowden (80,81) demonstrated that only LCs express HLA-DR antigens on their surface in human skin. Naturally, the expression of MHC (Ia) antigens on the LCs have a physiological role, but it has yet to be studied in great detail. Perhaps in skin, LCs play a role in the recognition of viral and microbial intrusions into the body. Viruses have been found within LCs (90), but it is not known whether viral antigens are displayed in association with the Ia antigens. In recent experiments it was demonstrated that a single ultraviolet solar-simulated radiation (UV-SSR) exposure of mammalian skin induced a dose-dependent reduction in LC density with only slight morphological alterations of the other skin cells (91). The location of LCs was modified; they were present in the spinous rather than in the usual suprabasal layer. Morphologically a cell body rounding and significant

reduction of dendricity was identified. All these alterations at cell and tissue level in the skin may impair the antigen presenting function of LCs and the efficacy of immune responses.

The classical experiments of Katz and co-workers (92) on radiation chimeras and the investigations by Frelinger and co-workers (93) show that bone marrow grafting with appropriate stain differences in the Ir gene region permit the tracing of the arrival of Ia+ cells in the epidermis. Since bone marrow transplantation is now a clinical procedure in the treatment of patients with various pathologic hematologic conditions, there must be many examples of the repopulation of LCs in the organism. A detailed study of these transplantations should prove illuminating since graft-*versus*-host (GvH) disease is a common complication in such procedures. Although LCs may arise from bone marrow monoblasts, nothing is known concerning the relative pool sizes of other possible precursor cells. Evidence is accumulating of the existence of cells within the lymph nodes and spleen with structural, antigenic and functional characteristics similar to LCs (35,94-96). These localizations of LCs also represent a symbiotic union of epithelial (in origin) and mesenchymal cells, but the intimate role played in T cell maturation and in phagocytosis is not yet clear.

Folliculo-stellate cells (FSCs). Folliculo-stellate cells (FSCs) are a key set of DCs in the AP that are involved in cell to cell interactions and regulations between the endocrine and immune systems (97-99). The stellate-shaped FSCs are organized in a cellular network in the AP and are positive for S-100, produce interleukin-6 (IL-6) and are in intercellular contact with hormone producing cells, their stimulation generally resulting in an increase in secretory responses (98,100-103). The presence of a network of lymphoid DCs, expressing a lymphoid DC specific aminopeptidase and MHC class II determinants, has been reported in mouse, rat and human pituitaries (104). Since S-100 immunoreactivity is also typical of lymphoid DCs (105), the subpopulation of S-100-positive pituitary FSCs may represent members of this class of DCs. This would mean that the pituitary FSCs derive from three distinct anlagen: neuroectodermal, ectodermal (oral anlage) and mesenchymal (lymphoid anlage). The multiple ontogenetic origin of the pituitary FSCs and their intermingling with lymphoid DCs allows us to distinguish a DC-FSC cell population at the level of the AP. Since these cells form a morphologically distinct cellular network and since a close functional inter-relationship between the cell groups has been documented (*i.e.* the synchronized increase of S-100 protein and MHC class II determinants during ontogenesis), it is probably better to consider them as a distinguishable cell clone. The increased levels of S-100 and MHC class II antigen expression in the pituitaries of autoimmune-prone BB/R rats raises further questions concerning a possible involvement of DC-FSCs in the development of autoimmune endocrine disorders (106). Several cytokines are now known to influence the release of AP hormones by acting on the hypothalamus and on the pituitary gland. IL-1, IL-2, IL-6, TNF-α and IFN-tau are the most important cytokines involved in the stimulation of the hypothalamic-pituitary-adrenal axis and the suppression of the hypothalamic-pituitary-thyroid and gonadal axes, as well as the release of growth hormone (107). The effects of acute and chronic (systemic) diseases on growth regulation, thyroid, adrenal and reproductive functions may, at least partially, be explained by the numerous important interactions between the neuroendocrine and immune systems.

Antigen presentation by DCs

Denritic cells patrol throughout the peripheral blood, lymph and peripheral tissues, including secondary lymphatic organs (108). Processing exogenous and endogenous proteins for presentation by MHC molecules to T lymphocyte's receptor repertoire is the defining function of "professional" antigen-presenting cells (APC) as major regulatory cells in antigen-specific immune responses (109,110). DCs responds to two types of "signals": 1) direct recognition of foreign antigen, pathogens (called "danger signal") through specific receptors (named pattern recognition receptors) and 2) indirectly sensed inflammatory mediators such as TNF-alpha, IL-1beta, PGE-2 of infection. Both of these two pathways induce a well integrated program called "maturation process" which transforms peripheral DCs into the most efficient APCs and T lymphocyte activators (111-113). In a detailed review the research group of Guermonprez (108) functionally characterized five types of surface receptors triggering the maturation process in DCs: 1) toll-like receptors - TLR2 to TLR4 (114,115); 2) cytokine receptors; 3) TNF receptor family molecules; 4) receptors for immunoglobulins (most of FcR) by immune complexes or specific antibodies (116-118); and 5) sensors for cell death. T cells through CD40-dependent and independent signaling and endothelial cells *via* cell to cell contacts and secretion of cytokines help in the maturation of DCs (119,120).

The complex process of antigen uptake, internalization by receptor-mediated endocytosis, degradation and specific loading on MHC class I and II molecules was named "antigen presentation". DCs present complexes between peptides derived from exogenous antigens and MHC antigens expressed on their surfaces to resting T cells, thereby initiating several immune responses such as the sensitization of MHC-restricted T lymphocytes, the rejection of organ transplants and the formation of T-dependent antibodies (121-126).

The T lymphocytes are capable of recognizing a number of self and non-self antigens, but they also need an immunostimulatory microenvironment to become activated and initiate an immune response (127-130). $CD4^+$ and $CD8^+$ T lymphocytes can initiate a systemic immune response when the TAAs are presented to them in the form of short peptides connected to the surface of MHC class I and II molecules together with costimulatory B7 or other molecules (127,131,132). MHC class II-restricted antigen presentation to $CD4^+$ T lymphocytes is achieved by an essentially common, multimolecular pathway, the so-called "immunological synapse" (133-136). This multimolecular interaction is subject to variation with regard to the location and extent of degradation of protein antigens and the site of peptide binding to MHC class II molecules (110). To provoke a $CD4^+$ lymphocyte activation, antigens connected to MHC class II molecules (cytoskeletally accumulated) should be presented together with costimulatory molecules (B7.1, B7.2), in case of APC interaction with CD28 in lymphocytes and adhesion molecules (usually ICAM-1), if the APC lymphocyte interaction is fulfilled *via* lymphocyte function associated antigens 1 and 3 (LFA-1 and LFA-3) (137). At the same time, activated $CD4^+$ lymphocytes are able "to help" or activate maturing (licensing) APCs, a functionally ready stage, directly to initiate $CD8^+$ lymphocytes. This activation step requires the presence of CD40 receptor on the APCs and the CD40-ligand (CD40-L or CD154), expressed on the surface of $CD4^+$ lymphocytes (133,134). The same results can be achieved employing a similar interaction between TRANCE, present on the surface of activated $CD4^+$ T lymphocytes and the TNF superfamily receptor RANK, expressed on APCs (138,139). There are a number of "molecular couples" that influence DC and T lymphocyte interaction during antigen presentation: CD11/CD18 integrins, CD80/B7-1, CD86/B7-2 and heat-stable antigen (139). Antigens presented by MHC class I molecules on previously activated APCs to $CD8^+$ lymphocytes is recognized by the T lymphocyte receptor (TCR). After the TCR-antigen interaction, cytotoxic lymphocytes (CTLs) recirculate through lymphatic organs and peripheral tissues to initiate the death of cells expressing the same antigen (139-141).

DEC-205 (gp200MR6) is a type I protein, the multilectin receptor for adsorptive endocytosis mostly expressed on DCs (including IDCs and LCs) in T lymphocyte areas of lymphatic tissues, but also, at low levels, on macrophages and T lymphocytes and on the cortical reticulo-epithelial (RE) cells of the thymic cellular microenvironment (142-145). The results indicate that thymic RE cells participate in clearance of apoptotic thymocytes through the DEC-205 protein. DEC-205 belongs to a family of C-type multilectins that also include the macrophage mannose receptor (MMR) and the phospholipase A_2 receptor (PLA_2R) (146). The antigen-presentation function is associated with the high-level expression of DEC-205, an integral membrane protein homologous to the MMR, and related receptors that are able to bind carbohydrates and mediate endocytosis. DEC-205 and MMR can serve as potential antigen-uptake receptors. After binding to DEC-205, antigens (proteins) are internalized, processed and presented in a complex with MHC class II molecules to $CD4^+$ lymphocytes (147). A number of receptors for endocytosis enter into and out of cells through early endosomes. New research defined that the DEC-205 receptor targets late endosomes or lysosomes rich in MHC II products, whereas the homologous macrophage mannose receptor (MMR), as expected, is found in more peripheral endosomes.

It is well known that not all C-type (type II) lectins on DCs serve as antigen receptors recognizing pathogens through carbohydrate structures. The ICAM-3-grabbing, non-integrin, type II lectin DC-SIGN is unique in that it regulates adhesion processes of interstitial DCs, such as DC trafficking and T-cell synapse formation, as well as antigen capture (148-150). DC-SIGN not only captures HIV-1 but instead protects it in early endosomes, allowing HIV-1 transport by DCs to lymphoid tissues, where it enhances transinfection of T lymphocytes. This recent article discusses the carbohydrate/protein recognition profile and other features of DC-SIGN that contribute to the potency of DCs to control immunity. Another type II lectin, the langerin, induces the formation of a unique endocytic compartment of LCs, the so-called Birbeck granules (148).

Proteasomes are multisubunit enzyme complexes that reside in the cytoplasm (mostly in the endoplasmic reticulum) and nucleus of eukaryotic cells. Employing selective protein degradation, proteasomes regulate a number of cytoplasmic biochemical functions including MHC class I antigen processing (151). Three constitutively expressed catalytic subunits are responsible for proteasome-regulated proteolysis. These subunits are exchanged for three homologous subunits, the immunosubunits, in IFNγ-exposed cells and in cells with "professional" and specialized antigen presenting function. Both constitutive and immunoproteasomes degrade the endogenous proteins into small peptide fragments that are capable of binding *via* specialized transporters TAP1 and TAP2 to MHC class I molecules for presentation on the cell surface to cytotoxic T lymphocytes. However, immunoproteasomes seem to fulfil this function more efficiently. IFNγ further induces the expression of a proteasome activator, PA28, which can also enhance antigenic peptide production by proteasomes. The ubiquitin-proteasome system also plays an important role in antigen presentation. However, unfortunately, neoplasms employ different ways to target the proteasome system to avoid MHC class I presentation of their CAAs.

Neuroendocrine-immune interactions in antigen presentation. During mammalian ontogenesis, the thymic "pure" endodermal epithelial anlage develops and differentiates into a complex cellular and humoral microenvironment. Beginning the 7-8th week of intrauterine development, thymic reticulo-epithelial (RE) cells chemotactically regulate (induce) numerous waves of migration of stem cells into the thymus, including the $CD34^+$, yolk sac-derived, pluripotent hematopoietic stem cells (152). *In vitro* experiments have established that $CD34^+CD38^{dim}$ human thymocytes differentiate into T lymphocytes when co-cultured with mouse fetal thymic organs. Hematopoietic stem cells for myeloid and thymic DCs, IDCs and LCs are present within the minute population of $CD34^+$ progenitors in the mammalian thymus. The RE cells express the DEC-205 receptor that is so important for antigen presentation. In addition, DCs and specialized epithelial tissue structures (such as "the nursing" thymic epithelial cells - TNCs) may also be involved in direct, cryptocrine-type cell to cell interactions with the epithelial cells of the thymus. TNCs regulate the development of immature thymocytes into immunocompetent T lymphocytes by *emperipolesis*, a highly specialized form of cell-cell interaction in which immature thymocytes are engulfed by large thymic reticulo-epithelial (RE) cells. TNCs *in vitro* are capable of rescuing an early subset of $CD4^+CD8^+$ thymocytes from programmed cell death at 32˚C, the temperature at which binding and internalization were identified. This thymocyte subpopulation later matured into a characteristic IP at the double-positive stage of T lymphocyte differentiation that is indicative of positive selection. Antigen presentation by thymic RE cells to thymocytes that undergo differentiation is one of the most important events in the selection of the T lymphocyte repertoire (153).

Interactions between the neuroendocrine and immune systems have been reported in various regions of the mammalian body including the anterior pituitary (AP), the skin and the central (thymus) and peripheral lymphatic tissue (18). The network of bone marrow derived DCs is part of the reticuloendothelial system (RES), with DCs representing the cellular mediators of these regulatory endocrine-immune interactions. Folliculo-stellate cells (FSCs) are non-hormone secreting cells that communicate directly with hormone producing cells, which is a form of neuro-endocrine-immune regulation. As a result, an attenuation of secretory responses follows stimulation of these cells. FSCs are also the cells in the AP producing interleukin-6 (IL-6) and have been identified as the interferon-γ responsive elements. FSCs express lymphatic DC markers, such as DC specific aminopeptidase, leucyl-β-naphthylaminidase, non-specific esterase, MHC class I and II molecules and various other lymphatic immunological determinants [platelet derived growth factor-α chain (PDGF-α chain), CD13, CD14 and L25 antigen]. There is strong evidence that such DCs in the AP, and similar ones in the developing thymus and peripheral lymphatic tissue, are the components of a powerful "professional" antigen-presenting DC network. These APCs contain a specialized late endocytic compartment, MIIC (MHC class II-enriched compartment), that harbors newly synthesized MHC class II antigens *en route* to the cell membrane. The limiting membrane of MIIC can fuse directly with the cell membrane, resulting in the release of newly secreted intracellular MHC class II antigen-containing vesicles (exosomes). DCs possess the ability to present foreign peptides complexed with the MHC molecules expressed on their surfaces to naive and resting T cells. There are a number of "molecular couples" that influence DC and T lymphocyte interaction during antigen presentation: CD11/CD18 integrins, intercellular adhesion molecules (ICAMs), lymphocyte function associated antigen 3 (LFA-3), CD40, CD80/B7-1, CD86/B7-2 and heat-stable antigen. The "molecular couples" are involved in adhesive or costimulatory regulations, mediating an effective binding of DCs to T lymphocytes and the stimulation of specific intercellular communications. DCs also provide all of the known co-stimulatory signals required for activation of unprimed T lymphocytes. It has been defined that DCs initiate several immune responses, such as the sensitization of MHC-restricted T lymphocytes, resistance to infections and neoplasms, rejection of organ transplants and the formation of T-dependent antibodies.

Neuroimmunologic aspects of skin inflammation are also regulated by several interacting systems (154). The modulating influence of autonomic and sensory nerves has been known for a long time. Neurokinines derived from these nerves have recently been shown to interact with antigen presentation in dermal LCs and other key functions of allergic skin disease. While some connections between the afferent function and local reflex are known, the nature of efferent regulatory effects (from brain to periphery) remains to be discovered. New research topics include the involvement of the brain-derived neurotrophic factor, in addition to the autonomic nervous system in mental stress response and insight in to the immunomodulation by proopiomelanocortins.

Materials and Methods

Tissue processing. The *in vitro* protocols described below were conducted using human postnatal thymic tissues obtained at the time of corrective cardiovascular surgery from 76 patients between the ages of 6 months and 10 years. The tissue samples were stored in RPMI 1640 (Gibco Labs., Grand Island, NY, USA) medium under sterile conditions. Thymic tissues were turned into cell suspensions for *ex vivo* IP studies.

Preparation of thymic peripheral blood and bone marrow cell suspensions. To minimize cellular autolytic changes, the fresh thymic tissue from the RPMI 1640 medium was placed in phosphate, buffered saline (PBS, Gibco) within 10-30 minutes of being received

and the experimental protocol was carried out. The thymus from each patient was pooled and dissociated after removal of the capsular and pericapsular connective tissue, fat and debris. The cleaned tissue pieces were minced with cross blades until no gross pieces remained. The PBS diluted cell suspension contained 2-5x10^9 cells/ml of undifferentiated cortical thymocytes, individual or clusters of RE cells, TNCs and other thymic cell complexes, Hassall's bodies (HBs), non-RE thymic stromal cells (DCs such as LCs and IDCs), and other accessory cells, such as macrophages and fibroblasts.

Isolation of cortical thymocytes. The thymic cell suspensions contained a large number of cells that are easily dissociable from the cortex by mechanical means. These immature cortical thymocytes were in various stages of T lymphocyte differentiation. To use the conventional Ficoll-Hypaque (Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, NJ, USA) density gradient isolation, the original thymocyte suspension was diluted at least 10 times. After isolation, a portion of the thymocytes, peripheral blood or bone marrow cells (2-5x10^5 cells/ml) was suspended in RPMI 1640 medium and treated with Tris-buffered ammonium chloride (Sigma, St. Louis, MI, USA) to lyse the contaminating erythrocytes. Following this procedure, they were cultured using 24-well, flat-bottomed tissue culture plates (Falcon Labware, Division of Becton-Dickinson, Oxnard, CA, USA) and RPMI 1640 medium, enriched with the desired combinations of growth factors, mitogens, colony stimulating factors and the appropriate cytokines, depending on the experimental aims. After 3 days in culture, the thymocytes were transferred into T25 culture flasks (Falcon). When one μl rIL-2 (generously provided by Cetus Corporation, Emeryville, CA, USA) was added to the culture, the proliferation rate remained high for the first 2-3 *in vitro* weeks. After this period the thymocytes were restimulated every second week, using the combined action of a mitogen (one μg/ml PHA) (Wellcome Diagnostics, Dartford, England) and a cellular microenvironment rich in cell secreted growth factors, and antigens provided by irradiated (3.6-4 Gy) peripheral mononuclear cells (PMNC) as "feeder cells", obtained from blood donor's "buffy coats" (target:feeder cell ratio 1:5). The thymocyte cultures were examined daily, the cell numbers counted and optimal *in vitro* conditions were maintained. The presence and number of proliferating thymoblasts (lymphoblasts) was carefully recorded. Prior to culture enzymatic thymic tissue digestion, we were unable to induce thymocyte proliferation by using any combination of mitogens, RE cell supernatant and/or rIL-2.

The biological activity of rIL-2 was measured prior to the *in vitro* experiments using a 24-hour bioassay on IL-2-dependent human splenic lymphocytes and one unit was defined as the amount inducing 50% of maximal proliferation. In thymocyte cultures and proliferation assays IL-2, IL-4 and IL-13 were used at concentrations of 5, 2, 1 and 0.5 units per 2x10^5 cells.

Isolation of thymic nurse cells (TNCs)

I. Gravity sedimentation. Since TNCs, as lymphoepithelial cell complexes, are by many times larger than any other kind of thymic cells, by employing a simple four-step unit gravity sedimentation procedure *via* fetal calf or adult human serum (both fom Gibco Labs., Chagrin Falls, OH, USA) the TNC were easily isolated from the thymic cell suspension.

II. Enzymatic tissue digestion. The thymic and another tissue homogenate were transferred (2-3-mm^2 tissue pieces) into a trypsinization bottle, containing 50 ml cold (4˚C) PBS and agitated for 10 minutes. The supernatant contained a large number of cortical thymocytes and was discarded or used for thymocyte cultures. The sedimented fraction was resuspended in 50 ml prewarmed solution of 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) (Gibco), 0.5 mg/ml dispase/collagenase (Boehringer/Mannheim Biochemical, Indianapolis, IN, USA) or 0.5 mg/ml collagenase IV (Millipore Corporation, Bedford, MA, USA) and 2 ml DNAase (Boehringer/Mannheim). The enzymatic digestion was carried out at 37˚C in an incubator for 20 minutes. This procedure was repeated 3-4 times until complete disintegration of the typical thymic tissue structure was accomplished. After each time, collection of the dissociated cells was possible. This cell suspension, containing RE cells, TNCs and HBs, was pooled in 3 ml cold (4˚C) D-MEM, supplemented with 5% heat-inactivated human serum (AB blood group, Rh negative), 1% l-glutamine (Boehringer/Mannheim), 1% MEM (Gibco), 1% sodium pyruvate (Flow Labs., Mclean, VI, USA), 1% hepes buffer (Biofluids Inc., Rockville, VA, USA) and 0.05 mg/ml gentamycin sulfate (Sigma). The suspension was gently layered over 10 ml undiluted, heat-inactivated human serum in a 40-ml conical, glass centrifuge tube for the TNC enriching procedure by sequential sedimentation at unit gravity for 15 minutes. The collected sediment was resuspended in 0.7 l complete D-MEM and overlayed over 5 ml human serum for second sedimentation in 12 ml-plastic tube (Falcon) for an additional 10 minutes. This last step was repeated and resulted in an enriched to 80% presence of TNCs. The enclosed TNC-Thy were released by mild sonication or simple overnight incubation.

Thymic stromal cell (RE & DC) cultures.

1. Whole organ cultures: 2-3-mm^2 pieces of thymic tissue were put *in toto* in organ culture, without previous enzymatic tissue digestion, using enriched D-MEM medium. The thymocytes disappeared from the culture within 3 weeks if the medium was not supplemented with rIL-2. The medium was replenished every 2-3 days depending on cell count and pH. The tissue cultures were grown in an incubator at 37˚C with a gas mixture of 5% carbon dioxide, 5% oxygen and 90% nitrogen.

2. The loose thymic tissue fragments remaining after enzymatic digestion were also placed in tissue cultures using enriched D-MEM medium and the same *in vitro* conditions as mentioned above.

3. The isolated TNCs, following morphological examination and release of the enveloped TNC-Thy, were also cultured.

4. During enzymatic tissue digestion, other thymic cellular elements were also isolated. HBs, DCs and stromal accessory cells were cultured following microscopic evaluation. These cultures were used as controls to detect optimal *in vitro* conditions to obtain pure RE cell monolayers. The thymic RE cell cultures were highly susceptible to overgrowth by always present thymic fibroblasts. Repeated treatment with 0.02% EDTA (Pharmacia) diluted in PBS resulted in pure RE monolayers, expressing cytoskeletal proteins of an epithelial nature.

Culture supernatants were collected daily for one week and used to culture autologous thymocytes immediately or stored at -70˚C prior to use. The cultures were observed daily, with a tissue culture microscope (Leitz, FRG) and representative fields were photographed.

Proliferation assay (PA) for thymocytes and peripheral blood hematopoietic cells. As was mentioned above, 2-5 x 10^5/ml cortical thymocytes or enriched peripheral blood $CD34^+$ were cultured in RPMI 1640 medium, supplemented with various combinations of

growth factors (including T lymphocyte growth factor), mitogens, a variety of colony stimulation factors and cytokines. In some experiments RE cell culture supernatant was added. For PA 96-well (Falcon), flat-bottomed culture plates were used. 0.2 ml thymocyte triplicates from every experimental condition were pulsed with 1μCi/well of 3H-TdR (New England Nuclear, Boston, MA, USA). The assay required use of a tissue culture incubator at 37˚C in humidified air, containing 5% CO_2, for 4 hours. Cell harvesting onto glass fiber filters was performed using MASH harvesting equipment. The amount of 3H-TdR incorporation within the thymocyte DNA was quantitated using by a beta scintillation counter.

Transmission electronmicroscopy (TEM) of cultured thymic medullary cells (RE, DC, including LC, & IDC) and macrophages. The cultured DCs, RE cells and macrophages after a short time *in vitro* stay were attached to the culture dish surface, forming a monolayer. They were removed using gentle digestion with collagenase IV (Millipore Corp., Bedford, MA, USA) for 5 minutes at 37˚C, followed by pelleting at 600-800 rpm for an additional 5 minutes. The cell pellets were fixed in 3% glutaraldehyde (Fischer Scientific), diluted in 0.1 M Sorenson's buffer at pH 7.2 for one hour at room temperature (20-23˚C), followed by three washes in phosphate buffer. Postfixation was performed with 1% solution of osmium tetroxide for 30 minutes, followed by washing in 0.9% NaCl for another 30 minutes. Staining "*en bloc*" with 0.5% uranyl magnesium acetate, diluted in 0.9% NaCl for one hour in dark, at 4˚C, followed by washing in saline for an additional 30 minutes. After dehydration in ascending concentrations of ethanol, the tissue microcultures were embedded in Epox (E.F. Fullam, Schenectady, NY, USA). Ultrathin sections were stained secondarily with uranyl acetate and lead citrate and were examined and photographed under a Siemens Elmiskop IA, transmission electronmicroscope at 80 kV. The cell types were identified by their TEM nuclear and cytoplasmic characteristics.

Scanning electronmicroscopic (SEM) procedure for tissue samples. Small portions (1-2 mm^2) of thymic tissue were fixed for 2-4 hours in 2.5-4% glutaraldehyde diluted in 0.1M phosphate buffer at room temperature, and at 4˚C in a regular refrigerator. Postfixation was carried out employing 1% OsO_4 diluted in phosphate buffer (kept in dark). After three washes in phosphate buffer, dehydration was carried out in ascending dilutions of ethanol. After processing in the critical point dryer (CPD), sputter coating was performed. Scope and photography of the thymic tissues was carried out with a JEOL-JSM-35C Scanning Electronmicroscope (SEM) at 380 to 60,000 times magnifications.

Immunocytochemistry. Antibodies. A well-chosen library of monoclonal antibodies was employed to observe the maturation level and differentiation pathway of thymocytes and T lymphocytes and to identify the IP of stromal RE, DCs and accessory cells of the thymic microenvironment. In this way we were able to characterize the professional, specialized for antigen presentation, intrathymic cell types and the IP changes during maturation of thymocytes into immunocompetent T lymphocytes.

The following antibody library was employed in our immunocytochemical observations on human and other vertebrate thymuses:

1) anti-endocrine and anti-epithelial: A_2B5, Thy-1 and anti-epithelial membrane antigen (EMA);

2) hematopoietic: CLA (anti-HLe-1 directed against a 200-220 kD cell surface receptor), Leu-2/a (against $CD8^+$, cytotoxic/suppressor T-lymphocytes), Leu-3/a,3/b (for identification of $CD4^+$, helper lymphocytes), anti-CD34, Leu-7, Leu-11/b, Leu-14, Leu-19, Interleukin-2 receptor (IL-2R) and Leu-M5 (for monocytes/macrophages);

3) MHC molecules: HLA-A,B,C, HLA-DR and HLA-DP;

4) anti-thymic stromal cells: TE-3, TE-4, TE-7, TE-8, TE 15, TE 16 and TE 19 (developed in the laboratory of Dr. Haynes and provided to us);

5) intermediate filaments: vimentin, S-100 protein, MAP-1 (microtubules), MAP-2, MAP-5, cytokeratins 13 and 18, desmosomal cytokeratin, AE1, AE2, AE3, AE5, and AE8;

6) tumor markers: B18.7.7 and D14 (anti-carcinoembryonic antigen (CEA));

7) oncogenes and related protein products: anti-c-erbB-2, anti-c-erbB-3, anti-c-myc and anti-c-ras;

8) cell cycle related cyclins, proliferation markers and DNA repair related proteins: anti-p53, Ki-67, anti-$p34^{cdc2}$, anti-cyclin A and anti-cyclin D; and

9) growth factors: anti-TGF-β type II receptor (TGF-βIIR).

I. Immunoalkaline phosphatase antigen detection technique. We used the following immunoalkaline phosphatase cytochemical method, modified by us for antigen detection in formalin-fixed, paraffin-wax-embedded thymus tissues. The technique has been determined to be a highly sensitive, indirect, four-to six-step immunocytochemical method, which combines the biotin-streptavidin based ABC-method with enzyme-linked (alkaline phosphatase) immunohistochemistry. Briefly, following deparaffinization in three changes of xylene substitute (Shandon-Lipshaw, Pittsburgh, PA, USA) for 20 to 30 minutes, rehydration was carried out employing descending dilutions of alcohol (100% to 50%) to TBS. An initial blocking step using 1% glacial acetic acid mixed with the working buffer for 10 minutes was necessary to eliminate the endogenous AP activity from the tissues. Use of levamisole solution is also described in our earlier observations. As we explained earlier in our papers, GAA inhibition was preferred because of the possible presence of levamisole-resistant AP iso-enzyme. The second blocking step was conducted with a purified mixture of proteins (Shandon-Lipshaw) from various species for 5-10 minutes to block cross-reactive antigenic epitopes. Excess serum was removed from the area surrounding the sections. The tissue sections were then incubated for 90-120 minutes with the particular primary antibody. Next, incubation with the secondary antibody, which was either a biotinylated, whole goat anti-rabbit or goat anti-mouse IgG molecule (IgG molecule diluted by ICN Biomedicals, Inc., Aurora, OH, USA), was carried out for 20 minutes. Streptavidin conjugation was accomplished by incubation with AP conjugated streptavidin for 20 minutes. Color visualization of the primary antigen-antibody (Ag-Ab) reaction was accomplished with an alkaline phosphatase (AP) kit I (Vector Laboratories, Burlingame, CA, USA) which contains AS-TR with Tris-HCl buffer at pH 8.2, added for 28-40 minutes to allow formation of a stable red precipitate. Sections were counterstained with a diluted solution of Gill's hematoxylin (Richard-Allan, Kalamazoo, MI, USA). The tissue slides were then dehydrated in ascending concentrations of alcohol (60% to 100%) to xylene substitute (Shandon-Lipshaw), in which they were kept overnight to ensure complete morphological clearing. The stained tissue sections were mounted using a solution specially designed for use following morphological clearing in xylene substitute (Shandon-Lipshaw).

II. Immunoperoxidase antigen detection technique. The following ABC method was employed for the immunocytochemical detection of antigens in thymus specimens. Briefly, following deparaffinization in three fresh changes of xylene substitute (Shandon-Lipshaw), a tissue rehydration employing descending grades of alcohols (100% to 40%) to PBS was performed. The tissue sections were never allowed to dry-out before being moved to the next solution. After 30 minutes incubation with 0.6% H_2O_2 in methanol to block the endogenous H_2O_2, a good rinse with running tap water and PBS for 2-8 minutes was performed. The second blocking step required 5 minutes incubation in non-specific protein mixture solution (Shandon-Lipshaw). The incubation time with the primary MoABs, as with any primary antibody, depended on the developers instructions, but it was usually between 60 to 120 minutes at room temperature. Next, we applied the secondary antibody for 20 minutes (on paraffin-wax sections, the whole anti-rabbit or anti-mouse IgG antibody was used as secondary antibody directed against the IgG of the original species of the primary antibody), followed by incubation for 30-40 minutes in 8 to 10 drops of the streptavidin/biotinylated horseradish peroxidase H complex (ABC). The binding of the biotinylated antibody to streptavidin/peroxidase complexes occurs with an extremely high affinity (10^{-19}M) (BioWhitaker, Inc., Walkersville, MD, USA). Color visualization of the primary Ag-Ab reaction was accomplished using diamino-benzidine (DAB) or amino-ethyl-carbazol (AEC). The brown color was enhanced with copper sulfate and diluted 1:100 Gill's hematoxylin (Richard-Allan) was used for gentle nuclear counterstaining. After the employment of DAB solution and the appropriate counterstain, the tissue slides were dehydrated, cleared and mounted in a manner identical to that described above.

Controls in immunocytochemistry. To accurately assess the specificity of the T lymphocyte differentiation antigens and MHC class I and II antigens observed in this study, we investigated the immunoreactivity of several normal human tissues: adult and fetal thymic, tonsil, spleen, thyroid, lung, liver, kidney, heart, pancreas, ovary, prostate, small intestine, large intestine and brain tissue sections, all included in one multitissue block (DAKO Corporation, Lot: 5935B). Additional controls for all employed MoABs included:
1) omission of the primary MoAB;
2) using only the enzymatic developer solution to detect the presence of endogenous peroxidase or alkaline phosphatase activity; and
3) use of MOPC 21 mouse myeloma IgG_1 (ICN Biomedicals, Inc.) as a replacement for the primary MoAB to determine non-specific myeloma protein binding to the antigen epitopes of the screened tissues.

Evaluation of the immunoreactivity. Qualitative and quantitative evaluation of the percent of antigen-positive cells and the intensity of immunostaining were conducted using a light microscope (Olympus America, Inc., Melville, NY, USA) counting 100-200 cells from each of five to eight distinct areas in non-necrotic thymic and positive and negative control tissues. Artifacts were avoided, while, on the other hand, morphologically characteristic areas were sought out.

Quantitative evaluation: (++++) over 90% of the total cell number are positive; (+++) 50% to 90% of the total cell number are positive; (++) 10% to 50% of the total cell number are positive; (+) 1% to 10% of the total cell number are positive; (±) under 1% of the total cell number are positive; (-) negative.

Qualitative evaluation: (A) very intense red/brown staining; (B) strong red/brown staining; (C) light red/brown staining; (D) negative staining.

The significance of DCs in anti-neoplastic immunotherapy. The growth and metastatic spread of neoplasms, to a large extent, depends on their capacity to evade host immune surveillance and overcome host defenses. All tumors express antigens that are recognized, to a variable extent, by the immune system, but in many cases an inadequate immune response is elicited because of partial antigen masking or ineffective activation of effector cells (155,156). Tumor Associated Antigens (TAAs) presented in the context of MHC class I complexes on either the neoplastic cell itself or on antigen-presenting cells are only inducing immunological tolerance but not the production of CAA-specific cytotoxic T lymphocytes (127). The presence of co-stimulatory molecules, such as B7-1 and B7-2, on antigen-presenting cells and the secretion of IL-2 promote the differentiation of recruited $CD8^+$ lymphocytes into cytotoxic T lymphocytes (157).

Dynamic and permanent changes in the IP of cells following their neoplastic transformation have been explored in numerous immunocytochemical studies (158-164). Expression of novel antigens not usually expressed in the surrounding normal cells and re-expression of developmental antigens has also been well established. Many further alterations in the physiology of cells following neoplastic transformation are contained within the cytoplasm and nucleus of the cell itself, thus making molecules associated with such changes less than ideal targets for immunotherapy. Furthermore, these regulatory molecules are of quite low immunogenicity due to the fact that their presence usually represents more of a quantitative dysregulation than a qualitative one as such antigens are components of normal cells. The down-regulation of major histocompatibility complex (MHC) molecule expression and antigen processing in such cells has also been described. The establishment and maintenance of a humoral milieu unfavorable for *in situ* immune activation and neoplastic cell lysis poses yet another difficulty. Neoplastic cell escape from the host's immune effectors is most often caused by weak immunogenicity of TAAs, antigen masking, or overall immunosuppression, a characteristic of advanced stage neoplastic disease. Failure of antigen processing or binding to MHC molecules, inadequate or low-affinity binding of MHC complexes to T lymphocyte receptors, or inadequate expression of co-stimulatory adhesion molecules in conjunction with the antigen-presenting MHC complex may all lead to poor immunogenicity of neoplasm-associated peptides and impaired antitumor response (109,165,166). Neoplasm induced defects are known to occur in all major branches of the immune system (167). Antigen presentation to T lymphocytes appears to be one of the steps that is highly deficient in a number of human neoplasms, thereby making it that much more difficult to generate specific cellular mediators of the host anti-neoplastic immune response (168).

DCs are crucial orchestrators of the adaptive immune response (169). Antigen presentation is a very important regulatory element for the induction of cellular immune responses, which is why one of the main goals of tumor immunotherapy is to control and enhance tumor antigen presentation (170). Thus, the basic immunobiology

of DCs (171) is still being widely investigated to allow the development of effective DC-based immunotherapy protocols for the treatment of human malignancies (172,173).

Efficient MHC class I and II presentation is developed when antigens are synthesized endogenously by the DCs. This is fully achieved in patients with acute or chronic myelogenous leukemia which, upon cytokine stimulation, undergo cellular differentiation into DCs that contain the full repertoire of neoplastic antigens (174-176). In other neoplasms, desired immunotherapy antigens must be delivered to the DCs *ex vivo*. The identification of a large number of CAAs has suggested new possibilities for more effective, individualized antineoplastic immunotherapy. However, multiple mechanisms may contribute to the ability of tumors to escape antitumor immune responses. Tumor antigen heterogeneity, modulation of HLA expression and immune suppressive mechanisms may occur at any time during tumor cell progression and can affect the outcome of therapeutic immune intervention. In particular, the appearance of altered HLA class I phenotypes during the progression of neoplasms may have important biological and medical implications due to the role of these molecules in T and NK cell functions. Exhaustive tumor tissue studies are necessary before deciding whether a particular patient is suitable for inclusion in T cell-based immunotherapy protocols. However, immune suppressive mechanisms may occur at any time during neoplastic cell progression and can affect the outcome of immunotherapeutic intervention (177). To overcome this deficit, it is possible to employ "professional" antigen-presenting DCs. DCs can be purified from the spleen, bone marrow and peripheral or cord blood. This *in vitro* propagation of tumor-specific CTLs is hampered by the necessity for large amounts of professional APCs used for periodical cycles of restimulation (178). The principle of the treatment is to prime the purified DCs with CAAs and to reinject them into the neoplasm-bearing patient. The sensitization to the CAAs could be obtained employing the crude extract of neoplastic cells or purified antigen, which will lead to MHC class II restricted antigen presentation to $CD4^+$ T lymphocytes (155). Unfortunately these peptides have rapid turnover on the surface of DCs and their efficacy is subsequently limited (179,180). Recently, several computer-assisted approaches have been developed to predict CTL epitopes within larger protein sequences based on proteasome cleavage specificity. The availability of such programs, as well as a general insight into the proteasome-mediated steps in MHC class I antigen processing, provides us with a rational basis for the design of new anti-neoplastic T lymphocyte vaccines (151).

Survivin is present during embryonic and fetal ontogenesis, but it is down-regulated in normal adult cell and tissues, and has dual effects: antiapoptotic and as a regulator of the cell cycle. However, it becomes re-expressed during oncogenesis. Almost all types of neoplastic cells contain survivin, a TAA and member of the inhibitor of the apoptosis protein (IAPs) family, considered to play a pivotal role in early oncogenesis (overexpressed during G(2)/M-phase in wide variety of neoplastically transformed cells). The expression (detection) of survivin (almost exclusively in proliferating neoplastic cells) is considered an important prognostic factor of many human neoplasms. Therefore, survivin is an attractive target for the development of broadly applicable tumor vaccines (181). Interestingly, the authors revealed that up-regulation of survivin by DCs was carried out upon stimulation with TNF-alpha.

Employment of neopastic cell-derived exosomes (cell secretory compartment), a defined source of tumor rejection antigens released in membrane vesicles, for vaccination could be the most efficient approach (182). Exosomes transfer a number of neoplastic cell-specific antigens to DCs and induce peptide-specific, MHC class I restricted presentation to T lymphocytes clones and induce neoplastic antigens specific CTL responses in cancer patients.

Dendritic cells with a MHC class $II^+/CD1a^+/CD3^-/CD14^-/CD20^-$ membrane IP (typical for DCs) can be isolated from peripheral blood mononuclear cells (PBMC) *in vitro* by the addition of IL-4 and GM-CSF (183-185). Interleukin-13 (IL-13) is as effective as IL-4 and, combined with GM-CSF, influences the differentiation pathway of DCs in a comparable manner. Human mature DCs have also been generated in large numbers *in vitro* by culturing $CD34^+$ hematopoietic progenitors in GM-CSF and tumor necrosis factor-α (TNF-α) enriched medium for 12 days (166,186). On days 5 to 7, the DC progenitors differentiated into two subsets ($CD1a^+$ and $CD14^+$) in tissue culture, both of which matured between days 12 and 14 into cells with a typical DC morphology and IP: CD80, CD83, CD86, CD58 and high HLA class II expression. $CD1a^+$ progenitors give rise to LCs characterized by the presence of typical Birbeck granules and the expression of Lag antigen and E-cadherin. In contrast, $CD14^+$ progenitors mature into DCs lacking Birbeck granules and the other two LC markers, but expressing CD2, CD9, CD68 and the coagulation factor XIIIa, described in dermal DCs (187). Both mature DCs have been shown to be equally efficient in stimulating allogeneic $CD45RA^+$ naive T lymphocytes. $CD14^+$ progenitors are bipotent in nature, as demonstrated by their ability to differentiate into macrophage-like cells, lacking accessory function to T lymphocytes in response to M-CSF. Strobl and co-investigators (188) have recently reported that the factors necessary for DC growth *in vitro* are poorly characterized and that the cytokine combination of GM-CSF and TNF-α, and stem cell factor (SCF) in the absence of serum supplementation, is inefficient in inducing DC maturation (differentiation). The authors further demonstrated that transforming growth factor-β1 (TGF-β1) supplementation is required for substantial DC development in the absence of serum. Thus, $CD34^+$ (best known as an endothelial cell marker (189)) stem cells in serum-free conditions required the following cytokine combination for differentiation into DCs: GM-CSF + TNF-α + SCF + TGF-β. A significant number (21% ± 7%) of DCs grown in TGF-β1 supplemented, but not plasma supplemented, medium showed presence of Birbeck granules and their marker, the Lag molecule. The presence of TGF-β1 in the culture medium also diminished the number of cells with monocytic features. Human DCs, with an 80-85% purity, were also isolated from peripheral blood mononuclear cells (PBMCs) by negative selection for T lymphocytes, B lymphocytes, NK cells, monocytes and granulocytes (190,191).

Passive immunotherapeutic interventions involve efforts to augment the neoplastic cells' antigenicity through vaccination with immunogenic peptides, administration of tumor infiltrating immune effector cells *in vitro* expanded and activated, *in vivo* effector cell expansion with cytokine therapies, or genetic modification of either immune effectors or neoplastic cells with cytokine genes or genes encoding costimulatory molecules to effectively activate the immune response (192-196). A variety of adoptive cellular strategies, aimed at boosting the patient's immune system, have been tested in the management of human neoplastic diseases. Despite the drawbacks associated with *ex vivo* cell manipulation and upscaling, several such approaches have been assessed in the clinic (197,198).

The disclosure of the human genome sequence and rapid advances in genomic expression profiling have revolutionized our knowledge about molecular changes in neoplastic diseases (199). Rapidly growing gene expression databases and improvements in bioinformatics tools set the stage for new approaches using large-scale molecular information to develop specific therapeutics in cancer (200). On the one hand, the ability to detect clusters of genes differentially expressed in normal and malignant tissue may lead to widely applicable targeting of defined molecular structures. On the other hand, analyzing the "molecular fingerprint" of an individual neoplasm raises the possibility of developing customized immunotherapeutical regiments. One approach to using the emerging new datasets for the development of novel therapeutics is to identify genes that are specifically expressed in neoplasms as targets for immune intervention employing the method of "reverse immunology" for the screening of potential candidate genes by bioinformatics in order to identify the immunogenicity of candidate CAAs (199). Gene transfer can also be employed in the case of a cloned antigen (like the co-stimulatory molecule B7.1) and would lead to the MHC class I restricted priming of $CD8^+$ CTLs (178). Unfortunately, following gene transfer, the efficacy of transduction is still very low, but with an increase in our understanding, this difficulty has the possibility of being overcome. Reinjection of DCs primed with neoplastic cell lysate leads to the protection of mice against a tumor challenge. The mode and place of administration, the nature of employed DCs and the technology of sensitization may all depend on the malignancy and metastatic potential.

What kind of whole tumor cells, apoptotic or necrotic, are more efficient for tumor vaccines? A group of scientists in Hannover, Germany, compared the effect of apoptosis *versus* necrosis on the effective antigen presentation by DCs, employing various tumor models (201). Their experimental data determined that only apoptotic whole tumor cell vaccines were capable of inducing a DC-mediated, potent antineoplastic immune response. In contrast, necrotic cell vaccines produced a strong, localized macrophage response.

Gene-engineered DCs are currently being tested in antineoplastic immunotherapy throughout the world. Genetic immunotherapy with DCs can also be engineered to express tumor antigens, having the potential advantages of endogenous epitope presentation by both MHC class I and II molecules. Another advantage of using DCs is that DCs can be gene-modified to express immunostimulatory molecules that further enhance their antigen-presenting function (132).

Numerous routes to employing DCs for antineoplastic immunotherapy are being tested, ranging from direct *in situ* expansion and activation of DCs to adoptive transfer of *ex vivo*-generated DCs. Numerous techniques have also been designed to optimize DC maturation and their migratory abilities, for effective tumor antigen delivery to DCs, and induction of tumor-specific as well as, helper immune responses, *in vivo*. However, the results of recent preclinical studies and the diversity of the clinical phase I trials that are currently underway indicate that still little is known about the exact mechanisms by which DCs modulate tumor immunity. This lack of knowledge brings with it the concern that premature clinical trials might not yield the desired results and might even be harmful to, rather than promote, the concept of DC-based tumor immunotherapy.

DCs have also been used to help improve the efficacy of tumor vaccines (202). Certainly we are still far from the "ideal vaccination" which should be cell free, employing immunorelevant, neoplasm-specific antigens, regardless of patients' HLA haplotype and the production should allow large volume manufacturing. Recently clinical investigators have developed genetically-modified tumor cell vaccines (203,204). Irradiated tumor cells transduced with and expressing various cytokines, such as IL-2, IL-6, IL-12, lymphotactin, IFN-γ, or GM-CSF, or co-stimulatory molecules, such as B7-1, were capable of eliciting their trafficking into lymph nodes and their interaction with T lymphocytes (205-207). These gene-modified DC vaccines also produced a direct regression of pre-existing neoplasms, thereby curing the experimental animals. Induction of strong immune responses in neoplasm-bearing animals against non-immunogenic or weakly immunogenic malignancies supports the research view that active immunization of cancer patients deserves further consideration.

Another possibility to ensure endogenous antigen synthesis has been to fuse DCs to neoplastically transformed cells. Gong and co-workers (208) demonstrated that murine DCs fused to colon cancer cells enhanced the formation of neoplasm-specific CTL both in *in vitro* and *in vivo*. In a recent experimental study, a transgenic murine model expressing polyomavirus middle T oncogene and mucin 1 TAA was used to determine the preventive effect of a vaccine containing DCs fused to breast carcinoma cells (209). The animals developed mammary carcinoma between the ages of 65 to 108 days with 100 percent penetrance. No spontaneous CTLs were identified. The prophylactic vaccination of these genetically modified mice induced polyclonal CTL activity and rendered 60 percent of the animals free of neoplastic disease by the end of the 180 days experiment. These results indicate that prophylactic vaccination with dendritic/tumor fusion cells is capable of inducing sufficient anti-neoplastic immunity to counter the process of oncogenesis of powerful oncogenic products. A similar type of tumor vaccine was successful in the prevention of human cervical cancer (210). The fusion cell vaccine experimental approach has been applied in several other antineoplastic immunotherapy protocols, including the immunoprevention of colorectal cancer, confirming the immunogenicity of such tumor vaccines (211,212).

In vivo DCs are found in a number of tissues and reside in direct proximity to extracellular matrix proteins (213-215). Since extracellular matrix proteins affect differentiation and location of cells in tissues, a number of research observations have investigated potential effects of extracellular matrix proteins on differentiation and maturation of dendritic cells. DCs were isolated and enriched from $CD34^+$ human cord blood stem cells in the presence of GM-CSF and tumor necrosis factor (TNF)-α for 6-days and subsequently cultured for an additional 6-days period on tissue culture plates coated with various extracellular matrix proteins. Among the extracellular matrix proteins tested, exposure to fibronectin stimulated DC/LC cell differentiation as indicated by the 50% increase in the number of cells expressing the Birbeck granule-associated marker Lag and displaying numerous Birbeck granules. Adhesion on fibronectin was shown to be specifically mediated by the integrin α5β. Because laminin and collagen were unable to cause similar changes in LC development, these results suggest that fibronectin may cause changes affecting cellular differentiation of progenitors. Hematopoietic progenitors may exhibit maturational regulated differences in response to both matrix molecules and cytokines.

Human cord blood, $CD34^+$ progenitors cultured in the presence of GM-CSF and TNF-α generate a heterogeneous population of DCs including Langerhans-like DCs (LLDCs) and monocytes

(216). The authors noticed that IL-4 exerts different effects in cultures according to the cells considered. Thus, IL-4 favors DC components at the expense of monocytic development and permits long-time persistence of DCs, that can be maintained up to one month in culture. These results show an IL-4-dependent inhibition of proliferation and emergence of $CD14^+$ cells. Notably, however, IL-4 also acts on the DC precursors. Thus, IL-4 enhances survival and delays maturation of LLDCs from $CD1a^+$ $CD14^-$ precursors. In addition, IL-4 also favors orientation of $CD14^+CD1a^-$ DC/monocyte precursors towards dermal-type $CD1a^+$ DC. DCs recovered from IL-4-treated cultures displayed reduced allostimulatory capacity, but this function was restored upon IL-4 weaning. A significant discovery suggested that a short (48hour) IL-4 pulse is sufficient to favor DC development. These *in vitro* experiments demonstrate that IL-4 positively regulates DC development at several levels on distinct precursor cells.

DC-based tumor vaccines in clinical trials. DC-based immunotherapy was employed in clinical trials in melanoma, lymphoma, myeloma, renal and prostate cancer patients (217-221). Numerous clinical trials testing DC-based vaccines against neoplasms are in progress and partial clinical efficacy has been already proved. In some cases, bone marrow-derived DCs have been employed to treat established experimental neoplasms by unleashing a cellular immune response against TAAs (222). An *ex vivo* gene transfer technique with viral and non-viral vectors provided such antigens into DCs' antigen-presenting molecules. This gene transfer technique is often used to obtain expression of TAAs and, hence, used thereby to formulate the antineoplastic immunotherapeutic vaccines. The efficacy of the approaches is greatly enhanced if DCs are transfected with more than one gene encoding immunostimulating factors. In some cases, such as with IL-12, IL-7 and CD40L genes, injection inside experimental malignancies of thus transfected DCs induces complete tumor regression in experimental animal models. In this case, TAAs are captured by DCs by still unclear mechanisms and transported to lymphatic organs where productive antigen presentation to T lymphocytes takes place. Transfection of genes will further strengthen the immunogenicity of CAAs and the antigen presentation efficacy of DCs. New immunotherapeutical strategies will soon join the clinical research being conducted.

Because of cases mostly resistant to chemotherapy, renal cell carcinoma (RCC) has been a testing ground for immunotherapy for decades (223,224). The approval of IL-2 for immuno- treatment of RCC was a landmark "proof of principle" showing that agents working solely *via* the immune system can cause long lasting neoplasm remission. *In vitro* strategies to expand and load DCs with antigens have now led to human vaccine trials in RCC (at the University of California at Los Angeles, a phase 1 clinical trial) and a number of other malignancies.

Malignant lymphomas (MLs) are clonal neoplasms of lymphatic origin. By definition, all cells of the malignant clone have undergone the same rearrangement of antigen receptor genes and express identical antigen receptor molecules (immunoglobulin for B lymphocytes, T cell receptor for T lymphocyte MLs). The hypervariable that stretches within the variable regions of these receptors are considered true tumor-specific antigens ('idiotypes') (225). In several animal models, protective humoral or cellular immunity was induced against the ML by vaccination with the neoplasm-derived idiotype. Successful experimental immunization strategies in animals include idiotype protein vaccines combined with various adjuvants, genetically-or immunologically-modified ML cells, idiotype-presenting DCs, idiotype-encoding viral vectors and DNA immunization. Firm evidence for the induction of lymphoma-specific immunity has also been obtained from human idiotype vaccination trials. Furthermore, some trials have provided strong but hitherto formally unproven evidence for the clinical benefit of idiotype-vaccinated patients. Alternative vaccination approaches are based on immunologically-modified neoplastic cells. Future research efforts should involve efforts to identify the most efficacious vaccination route, on definitive proof of clinical efficacy, and on the development of new protocols to produce individually designed idiotype vaccines.

Over the last decade, the incidence of malignant melanomas (MMs) has been continuously increasing worldwide (226). Surgery is the treatment of choice in the early stages of primary lesions. Advanced MM, however, is resistant to chemotherapy and radiotherapy. Therefore, there is an essential need for new, possibly more effective treatments. In the last few years, biotherapy such as immunotherapy has been receiving quite a bit of attention. Unfortunately, systemic administration of immunostimulatory factors is very often associated with severe side-effects. Thus, concepts of specific immunotherapies, such as immunogene therapy, have been developed. Currently, various gene therapy strategies of MM are being evaluated in multiple clinical trials carried out all over the world (217,227). As was mentioned before, the new tendencies include gene-modified tumor vaccines (GMTV), modified with genes encoding cytokines or co-stimulatory molecules and DCs modified with genes encoding CAA or immunostimulatory factors. Since January 1996, in the Department of Cancer Immunology USOMS, (at Great Poland Cancer Center in Poznan, Poland), a GMTV has been tested in MM patients. More than 220 patients were enrolled in this study of GMTV consisting of melanoma cells modified with genes encoding IL-6 and its agonistic soluble receptor (sIL-6R). More than 25% of the patients were observed to have objective clinical responses and significant life extensions. The encouraging results formed the basis for design of a phase III prospective, randomized clinical study.

Conclusion

Dendritic cells are the most effective, "professional" antigen-presenting cells that capture antigens in the periphery, migrate centrally and present the processed antigens in the context of MHC and appropriate co-stimulatory molecules to T lymphocytes for the initiation of an immune response.

Dendritic cells and probably the thymic reticulo-epithelial (RE) cells are capable of performing important immunoregulatory functions by presenting antigens in the form of peptides bound to cell-surface MHC molecules to T lymphocytes. It is a fact that the intimate events of intrathymic T lymphocyte maturation regulated by DCs and other professional APCs support the action of the various putative thymic hormones, driving the immature thymocytes (prethymocytes) to a stage of maturity.

Antigen presentation is a critical regulatory element for the induction of cellular immune responses. Therefore, one of the principal current goals of anti-neoplastic immunotherapy is to control and enhance tumor antigen

presentation. In this respect, DCs are now being widely investigated as immunotherapeutic agents for the treatment of disseminated malignancies.

During the progression of neoplastic disease and the constant IP changes of tumor cells, the appearance of altered HLA class I phenotypes may have important immunobiological and immunotherapeutical implications due to the role of these molecules in T and NK cell functions.

The fundamental immunobiology of DCs is still being widely investigated to allow the development of effective DC-based immunotherapy protocols for the treatment of human malignancies. In the last decade, experimental protocols for *in vitro* growth and maturation of large quantities of DCs from their $CD34^+$ bone marrow-derived hematopoietic stem cells have been carried out employing several lineage-producing cytokine cocktails. This strategy enables the generation of functionally mature DCs, even from advanced neoplasm patients whose antigen presentation is suppressed by neoplasm derived molecules. The ability to culture autologous DCs *ex vivo* has influenced the development of protocols for genetically engineering them. Thus, the era of antigen directed, active, specific immunotherapy is born.

There is scientific evidence showing that DCs and thymic RE cells share surface receptors that play a crucial role in antigen presentation. There are also tight regulation connections between the neuroendocrine and the immune system that should be investigated in the process of antigen presentation. Without neuroendocrine regulation, there is no proper immune function. Neuroendocrine regulation is currently not encompassed in the tumor vaccine design. It is suggested that future research into the neuroendocrine question could improve the quality of DC-based tumor vaccines.

In vitro strategies to expand and load DCs with TAA antigens have now led to human vaccine trials in renal cell carcinoma and a number of other malignancies such as malignant melanomas, breast carcinomas and lymphomas. We are sure that new immunotherapeutical strategies and protocols will soon join the clinical research being conducted worldwide.

References

1 Wu L, Scollay R, Egerton M *et al*: CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. Nature *349*: 71-74, 1991.

2 Wu L, Li CL and Shortman K: Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. J Exp Med *184*: 903-911, 1996.

3 Shortman K and Caux C: Dendritic cells development: multiple pathways to nature's adjuvants. Stem Cells *15*: 409-419, 1997.

4 Shortman K and Wu L: Parentage and heritage of dendritic cells. Blood *97*: 3325, 2001.

5 Wu L, D'Amico A, Hochrein H *et al*: Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. Blood *98*: 3376-3382, 2001.

6 Shortman K and Liu YJ: Mouse and human dendritic cell subtypes. Nat Rev Immunol *2*: 151-161, 2002.

7 O'Keeffe M, Hochrein H, Vremac D *et al*: Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmocytoid pre-DC2 and CD11+ DC1 precursors. Blood *101*: 1453-1459, 2003.

8 Tan PS, Gavin AL, Barnes N *et al*: Unique monoclonal antibodies define expression of FcgammaRI on macrophages and mast cell lines and demonstrate heterogeneity among subcutaneous and other dendritic cells. J Immunol *170*: 2549-2556, 2003.

9 Kim YJ and Broxmeyer HE: 4-IBB ligand stimulation enhances myeloid dendritic cell maturation from human umbilical cord blood CD34(+) progenitor cells. J Hematother Stem Cell Res *11*: 895-903, 2002.

10 Nestle FO, Banchereau J and Hart D: Dendritic cells: on the move from bench to bedside. Nat Med *7*: 761-765, 2001.

11 Anjuere F, Martinez del Hoyo G, Martin P and Ardavin C: Langerhans cells develop from a lymphoid-committed precursor. Blood *96*: 1633-1637, 2000.

12 Bednar B: Dendritic resident cells and their immunohistologic determination. Ceskoslov Patologie *31*: 9-16, 1995.

13 Paglia P, Girolomoni G, Robbiati F, Granucci F and Ricciardi-Castagnoli P: Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses *in vivo*. J Exp Med *178*: 1893-1901, 1993.

14 Lenz A, Heine M, Schuler G and Romani N: Human and murine dermis contain dendritic cells, isolation by means of a novel method and phenotypical and functional characterization. J Clin Invest *92*: 2587-2596, 1993.

15 Steinman RM and Cohn ZA: Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med *137*: 1142-1162, 1973.

16 Markgraf R, von Gaudecker B and Müller-Hermelink HK: The development of the human lymph node. Cell Tissue Res *225*: 387-413, 1982.

17 Witmer MD and Steinman RM: The anatomy of peripheral lymphoid organs with emphasis on accessory cells: light-microscopic immunocytochemical studies of mouse spleen, lymph node and Peyer's patch. Am J Anat *170*: 4655-4681, 1984.

18 Bodey B, Bodey B Jr and Kaiser HE: Dendritic type, accessory cells within the mammalian thymic microenvironment. Antigen presentation in the dendritic neuro-endocrine-immune cellular network. In Vivo *11*: 351-370, 1997.

19 von Gaudecker B and Müller-Hermelink HK: Ontogeny and organization of the stationary non-lymphoid cells in the human thymus. Cell Tissue Res *207*: 287-306, 1980.

20 Kaiserling E, Stein H and Muller-Hermelink HK: Interdigitating reticulum cells in the human thymus. Cell Tiss Res *155*: 47-55, 1974.

21 Crivellato E, Mallardi F, Basa M and Zweyer M: Osmium-zinc iodide reacts with interdigitating cells in the mouse lymph nodes and spleen. Z mikroskop-anat Forsch *104*: 476-484, 1990.

22 Crivellato E, Baldini G, Basa M and Fusaroli P: The three-dimensional structure of interdigitating cells. Italian J Anat Embryol *98*: 243-258, 1993.

23 Kelly RH, Balfour BM, Armstrong JA and Griffiths S: Functional anatomy of lymph nodes. II. Peripheral lymph-borne mononuclear cells. Anat Rec *190*: 5-22, 1978.

24 Kamperdijk EWA, de Leeuw JHS and Hoefsmit ECM: Lymph node macrophages and reticulum cells in the immune response; the secondary response to paratyphoid vaccine. Cell Tissue Res *227*: 277-290, 1982.

25 Fossum S and Vaalard JL: The architecture of rat lymph nodes. I. Combined light and electronmicroscopy of lymph node cell types. Anat Embryol *167*: 229-246, 1983.

26 Hart DNJ and Fabre JW: Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissue of rat heart and other tissues, but not brain. J Exp Med *154*: 347-361, 1981.

27 Hart DNJ and McKenzie JL: Interstitial dendritic cells. Int Rev Immunol *6*: 128-149, 1990.

28 Klug H and Mager B: Ultrastructure and function of interdigitating cells in the guinea pig thymus. Acta Morph Acad Sci Hung *27*: 11-19, 1979.

29 Klug H: Elektronenmikroskopische Untersuchungen zur Phagocytose strahlengeschädigter Lymphozyten im Thymus von Ratten. Z Zellforsch *68*: 43-56, 1965.

30 Duijvestijn AM and Kamperdijk EW: Birbeck granules in interdigitating cells of thymus and lymph node. Cell Biol Int Rep *6*: 655, 1982.

31 Duijvestijn AM, Sminia T, Kohler YG, Janse EM and Hoefsmit EC: Rat thymus micro-environment: an ultrastructural and functional characterization. Adv Exp Med Biol *149*: 441-446, 1982.

32 Duijvestijn AM, Kohler YG and Hoefsmit EC: Interdigitating cells and macrophages in the acute involuting rat thymus. An electron-microscopic study on phagocytic activity and population development. Cell Tiss Res *224*: 291-301, 1982.

33 Miyazawa T, Sato C and Kojima K: Thymic phagocytosis and reduction in the negative surface charge of thymocytes after X irradiation. Radiat Res *79*: 622-629, 1979.

34 Higley HR and O'Morchoe CC: Morphometric analysis of thymic medullary non-lymphoid cell changes during postnatal development. Dev Comp Immunol *8*: 711-719, 1984.

35 van Ewijk W, Verzijden JH, van der Kwast TH and Luijcx-Meijer SW: Reconstitution of the thymus dependent area in the spleen of lethally irradiated mice. A light and electron microscopical study of the T-cell microenvironment. Cell Tiss Res *149*: 43-60, 1974.

36 Heusermann U, Stutte HJ and Müller-Hermelink HK: Interdigitating cells in the white pulp of the human spleen. Cell Tiss Res *153*: 415-417, 1974.

37 Ferreira-Marques J: Systema sensitivum intra-epidermicum. Die Langerhansschen Zellen als Rezeptoren des hellen Schmerzes: Doloriceptores. Arch Dermatol Syph *193*: 191-250, 1951.

38 Niebauer G: Über die interstitiellen Zellen der Haut. Hautarzt *7*: 123-126, 1956.

39 Richter R: Studien zur Neurohistologie der nervösen vegetativen Peripherie der Haut bei verschiedenen chronischen infektiösen Granulomen mit besonderer Berücksichtigung der Langerhansschen Zellen; Tuberkolosen der Haut. Arch Klin Exp Dermatol *202*: 466-495, 1956.

40 Richter R: Studien zur Neurohistologie der nervösen vegetativen Peripherie der Haut bei verschiedenen chronischen infektiösen Granulomen mit besonderer Berücksichtigung der Langerhansschen Zellen; tertiäre Syphilide der Haut. Arch Klin Exp Dermatol *202*: 496-508, 1956.

41 Richter R: Studien zur Neurohistologie der nervösen vegetativen Peripherie der Haut bei verschiedenen chronischen infektiösen Granulomen mit besonderer Berücksichtigung der Langerhansschen Zellen; Leishmaniosis cutis. Arch Klin Exp Dermatol *202*: 509-517, 1956.

42 Richter R: Studien zur Neurohistologie der nervösen vegetativen Peripherie der Haut bei verschiedenen chronischen infektiösen Granulomen mit besonderer Berücksichtigung der Langerhansschen Zellen; Lepra. Arch Klin Exp Dermatol *202*: 518-555, 1956.

43 Niebauer G: Über die Dendritenzellen bei Vitiligo. Dermatologica *130*: 317-324, 1965.

44 Niebauer G und Sekido N: Über die Dendritenzellen der Epidermis. Eine Studie über die Langerhans-Zellen in der normalen und ekzematösen Haut des Meerschweinchens. Arch Klin Exp Dermatol *222*: 23-42, 1965.

45 Masson P: My conception of cellular nevi. Cancer *4*: 9-38, 1951.

46 Billingham RE and Medawar PB: "Desensitization" to skin homografts by injections of donor skin extracts. Ann Surg *137*: 444-449, 1953.

47 Fan J and Hunter R: Langerhans cells and the modified technic of gold impregnation by Ferreira-Marques. J Invest Dermatol *31*: 115-121, 1958.

48 Fan J, Schoenfeld RJ and Hunter R: A study of the epidermal clear cells with special references to their relationship to the cells of Langerhans. J Invest Dermatol *32*: 445-450, 1959.

49 Billingham RE and Silvers WK: Re-investigation of the possible occurrence of maternally induced tolerance in guinea pigs. J Exp Zool *160*: 221-224, 1965.

50 Billingham RE and Silvers WK: Some biological differences between thymocytes and lymphoid cells. Wistar Inst Sympos Monogr *2*: 41-51, 1964.

51 Schuler G and Steinman RM: Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. J Exp Med *161*: 526-546, 1985.

52 Witmer-Pack MD, Olivier W, Valinsky J, Schuler G and Steinman RM: Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. J Exp Med *166*: 1484-1498, 1987.

53 Strunk D, Rappersberger K, Egger C *et al*: Generation of human dendritic cells/Langerhans cells from circulating $CD34^+$ hematopoietic progenitor cells. Blood *87*: 1292-1302, 1996.

54 Ardavin C, Wu L, Li CL and Shortman K: Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. Nature *362*: 761-763, 1993.

55 Galy A, Travis M, Cen D and Chen B: T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity *3*: 459-473, 1995.

56 Marquez C, Trigueros C, Fernandez E and Toribio ML: The development of T and non-T cell lineages from $CD34^+$ human thymic precursors can be traced by the differential expression of CD44. J Exp Med *181*: 475-483, 1995.

57 Res P, Martinez-Caceres E, Jaleco AC *et al*: $CD34^+CD38^{dim}$ cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. Blood *87*: 5196-5206, 1996.

58 Langerhans P: Über die Nerven der menschlichen Haut. Virchow's Arch A (Pathol Anat) *44*: 325-338, 1868.

59 Silberberg I: Apposition of mononuclear cells to Langerhans cells in contact allergic reactions: an ultrastructural study. Acta Dermatol Venereol *53*: 1-12, 1973.

60 Birbeck MS, Breathnach AS and Everall JD: An electron microscope study of basal melanocytes and high-level clear cells (Langerhans cells) in vitiligo. J Invest Dermatol *37*: 51-64, 1961.

61 Silberberg I, Baer RL and Rosenthal SA: Circulating Langerhans cells in a dermal vessel. Acta Dermato-Venereol *54*: 81-85, 1974.

62 Silberberg-Sinakin I, Fedorko ME, Baer RL *et al*: Langerhans cells: target cells in immune complex reactions. Cell Immunol *32*: 400-416, 1977.

63 Silberberg-Sinakin I, Gigli I, Baer RL and Thorbecke GJ: Langerhans cells: role in contact hypersensitivity and relationship to lymphoid dendritic cells and to macrophages. Immunol Rev *53*: 203-232, 1980.

64 Bucana CD, Munn CG, Song MJ, Dunner K Jr and Kripke ML: Internalization of Ia molecules into Birbeck granule-like structures in murine dendritic cells. J Invest Dermatol *99*: 365-373, 1992.

65 Henkes W, Syha J and Reske K: Nucleotide sequence of rat invariant gamma chain cDNA clone pLRgamma34.3. Nucleic Acids Res *16*: 11822, 1988.

66 Bakke O and Dobberstein B: MHC class II associated invariant chain contains a sorting signal for endosomal compartments. Cell *63*: 707-716, 1990.

67 Lotteau V, Teyton L, Peleraux A *et al*: Intracellular transport of class II MHC molecules directed by invariant chain. Nature *348*: 600-605, 1990.

68 Naujokas MF, Morin M, Anderson MS, Peterson M and Miller J: The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. Cell *74*: 257-268, 1993.

69 Hashimoto K and Tarnowski WM: Some new aspects of the Langerhans cell. Arch Dermatol *97*: 450-464, 1968.

70 Hashimoto K: Langerhans' cell granule. An endocytotic organelle. Arch Dermatol *104*: 148-160, 1971.

71 Olah I, Dunay C, Rohlich P and Toro I: A special type of cells in the medulla of the rat thymus. Acta Biol Acad Sci Hung *19*: 97-113, 1968.

72 van Haelst U: Light and electron microscopic study of the normal and pathological thymus of the rat. I. The normal thymus. Zeitschr Zellforsch Mikroskop Anat *77*: 534-553, 1967.

73 van Haelst U: Light and electron microscopic study of the normal and pathological thymus of the rat. II. The acute thymic involution. Zeitschr Zellforsch Mikroskop Anat *80*: 153-182, 1967.

74 Warchol JB, Brelinska R and Jaroszewski J: Granules of Langerhans cells in the thymus contain gold. Experientia *40*: 75-76, 1984.

75 Zelickson AS: The Langerhans cell. J Invest Dermatol *44*: 201-212, 1965.

76 Breathnach AS and Wyllie LM: Electron microscopy of melanocytes and Langerhans cells in human fetal epidermis at fourteen weeks. J Invest Dermatol *44*: 51-60, 1965.

77 Wolff K: The fine structure of the Langerhans cell granule. J Cell Biol *35*: 468-473, 1967.

78 Wolff K: The Langerhans cell. Curr Prob Dermatol *4*: 79-145, 1971.

79 Plzak J, Holikova Z, Dvorankova B *et al*: Analysis of binding of mannosides in relation to langerin (CD207) in Langerhans cells of normal and transformed epithelia. Histochem J *34*: 247-253, 2002.

80 Rowden G: Immuno-electron microscopic studies of surface receptors and antigens of human Langerhans cells. Br J Dermatol *97*: 593-608, 1977.

81 Rowden G, Lewis MG and Sullivan AK: Ia antigen expression on human epidermal Langerhans cells. Nature *268*: 247-248, 1977.

82 Rowden G: Expression of Ia antigens on Langerhans cells in mice, guinea pigs, and man. J Invest Dermatol *75*: 22-31, 1980.

83 Veerman AJ: On the interdigitating cells in the thymus-dependent area of the rat spleen: a relation between the mononuclear phagocyte system and T-lymphocytes. Cell Tiss Res *148*: 247-257, 1974.

84 Nossal GJ, Abbot A, Mitchell J and Lummus Z: Antigens in immunity. XV. Ultrastructural features of antigen capture in primary and secondary lymphoid follicles. J Exp Med 127:277-290, 1968.

85 Steinman RM and Witmer MD: Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc Natl Acad Sci USA *75*: 5132-5136, 1978.

86 Berman B and Gigli I: Complement receptors on guinea pig epidermal Langerhans cells. J Immunol *124*: 685-690, 1980.

87 Hammerling GJ and McDevitt HO: Antigen-binding structures on the surface of T lymphocytes. Israel J Med Sci *11*: 1331-1341, 1975.

88 Klein J and Hauptfeld V: Ia antigens: their serology, molecular relationships, and their role in allograft reactions. Transplant Rev *30*: 83-100, 1976.

89 Shreffler DC and David CS: The H-2 major histocompatibility complex and the I immune response region: genetic variation, function, and organization. Adv Immunol *20*: 125-195, 1975.

90 Nagao S, Inaba S and Iijima S: Langerhans cells at the sites of vaccinia virus inoculation. Arch Dermatol Res/Archiv fur Dermatol Forsch *256*: 23-31, 1976.

91 Seite S, Zucchi H, Moyal D *et al*: Alterations in human epidermal Langerhans cells by ultraviolet radiation: quantitative and morphological study. Br J Dermatol *148*: 291-299, 2003.

92 Katz SI, Tamaki K and Sachs DH: Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature *282*: 324-326, 1979.

93 Frelinger JG, Hood L, Hill S and Frelinger JA: Mouse epidermal Ia molecules have a bone marrow origin. Nature *282*: 321-323, 1979.

94 Rausch E, Kaiserling E and Goos M: Langerhans cells and interdigitating reticulum cells in the thymus-dependent region in human dermatopathic lymphadenitis. Virchows Archiv - B Cell Pathol *25*: 327-343, 1977.

95 Hoffman-Fezer G, Rodt H and Thierfelder S: Immunohistochemical identification of T- and B-lymphocytes delineated by the unlabeled antibody enzyme method. II. Anatomical distribution of T- and B-cells in lymphoid organs of nude mice. Beitr Pathol *161*: 17-26, 1977.

96 Hoffman-Fezer G, Rodt H, Götze D and Thierfelder S: Anatomical distribution of T and B lymphocytes identified by immunohistochemistry in the chicken spleen. Int Arch Allergy Appl Immunol *55*: 86-95, 1977.

97 Cocchia D and Miani N: Immunocytochemical localization of the brain-specific S-100 protein in the pituitary gland of adult rat. J Neurocytol *9*: 771-782, 1980.
98 Baes M, Allaerts W and Denef C: Evidence for functional communication between folliculo-stellate cells and hormone-secreting cells in perfused anterior pituitary cell aggregates. Endocrinology *120*: 685-691, 1987.
99 Vankelecom H, Carmeliet P, van Damme J, Billiau A and Denef C: Production of interleukin-6 by folliculo-stellate cells of the anterior pituitary gland in a histiotypic cell aggregate culture system. Neuroendocrinology *49*: 102-106, 1989.
100 Nakajima T, Yamaguchi H and Takahashi K: S-100 protein in folliculo-stellate cells of the rat of the pituitary anterior lobe. Brain Res *191*: 523-531, 1980.
101 Allaerts W and Denef C: Regulatory activity and topological distribution of folliculo-stellate cells in rat anterior pituitary cell aggregates. Neuroendocrinology *49*: 409-418, 1989.
102 Allaerts W, Jeucken PHM, Hofland LJ and Drexhage HA: Morphological, immunohistochemical and functional homologies between pituitary folliculo-stellate cells and lymphoid dendritic cells. Acta Endocrinol *125*: 92-97, 1991.
103 Carmeliet P, Vankelecom H, van Damme J, Billiau A and Denef C: Release of interleukin-6 from anterior pituitary cell aggregates: developmental pattern and modulation by glucocorticoids and forskolin. Neuroendocrinology *53*: 29-34, 1991.
104 Allaerts W, Jeucken PHM, Bosman FT and Drexhage HA: Relationship between dendritic cells and folliculo-stellate cells in the pituitary: immunohistochemical comparison between mouse, rat and human pituitaries. *In*: Dendritic Cells in Fundamental and Clinical Immunology (Kamperdijk *et al.*, eds), Plenum Press, New York, 1993, pp 637-642.
105 Takahashi K, Yamaguchi H, Ishizeki J, Nakajima T and Nakazato Y: Immunohistochemical and immunoelectron microscopic localization of S-100 protein in the interdigitating reticulum cells of the human lymph node. Virchows Arch (Cell Pathol) *37*: 125-135, 1981.
106 Allaerts W, Jeucken PHM, Bosman FT and Drexhage HA: Relationship between dendritic cells and folliculo-stellate cells in the pituitary: immunohistochemical comparison between mouse, rat and human pituitaries. Adv Exp Med Biol *329*: 637-642, 1993.
107 Jones TH and Kennedy RL: Cytokines and hypothalamic-pituitary function. Cytokine *5*: 531-538, 1993.
108 Guermonprez P, Valladeau J, Zitvogel L *et al*: Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol *20*: 621-667, 2002.
109 Steinman RM: Dendritic cells and immune-based therapies. Exp Hematol *24*: 859-862, 1996.
110 Robinson JH and Delvig AA: Diversity in MHC class II antigen presentation. Immunology *105*: 252-262, 2002.
111 Kalinski P, Hilkens CM, Wierenga EA and Kapsenberg ML: T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today *20*: 561-567, 1999.
112 Banchereau J, Briere F, Caux C *et al*: Immunobiology of dendritic cells. Annu Rev Immunol *18*: 767-811, 2000.
113 Lutz MB, Assmann CU, Girolomoni G and Ricciardi-Castagnoli P: Different cytokines regulate antigen uptake and presentation of a precursor dendritic cell line. Eur J Immunol *26*: 586-594, 1996.
114 Aderem A and Ulevitch RJ: Toll-like receptors in the induction of the innate immune response. Nature *406*: 782-787, 2000.
115 Visintin A, Mazzoni A, Spitzer JH *et al*: Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol *166*: 249-255, 2001.
116 Jurgens M, Wollenberg A, Hanau D *et al*: Activation of human epidermal Langerhans cells by engagement of the high affinity receptor for IgE, Fc epsilon RI. J Immunol *155*: 5184-5189, 1995.
117 Regnault A, Lankar D, Lacabanne V *et al*: Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med *189*: 371-380, 1999.
118 Geissmann F, Launay P, Pasquier B *et al*: A subset of human dendritic cells expresses IgA Fc receptor (CD89), which mediates internalization and activation upon cross-linking by IgA complexes. J Immunol *166*: 346-352, 2001.
119 Thery C and Amigorena S: The cell biology of antigen presentation in dendritic cells. Curr Opin Immunol *13*: 45-51, 2001.
120 Galluci S and Matzinger P: Danger signals: SOS to the immune system. Curr Opin Immunol *13*: 114-119, 2001.
121 Steinman RM and Nussenzweig MC: Dendritic cells: features and functions. Immunol Rev *53*: 127-148, 1980.
122 Steinman RM: The dendritic cell system and its role in immunogenicity. Annual Rev Immunol *9*: 271-296, 1991.
123 Monaco JJ: Structure and function of genes in the MHC class II region. Curr Opin Immunol *5*: 17-20, 1993.
124 Neefjes JJ and Momburg F: Cell biology of antigen presentation. Curr Opin Immunol *5*: 27-34, 1993.
125 Germain RN: MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell *76*: 287-299, 1994.
126 Ossevoort MA, Kleijmeer MJ, Nijman HW, Geuze HJ, Kast WM and Melief CJM: Functional and ultrastructural aspects of antigen processing by dendritic cells. Adv Exp Med Biol *378*: 227-231, 1995.
127 Matzinger P: Tolerance, danger, and the extended family. Annu Rev Immunol *12*: 991-1045, 1994.
128 Nanda NK and Sercarz E: A truncated T cell receptor repertoire reveals underlying immunogenicity of an antigenic determinant. J Exp Med *184*: 1037-1043, 1996.
129 Lee PP, Yee C, Savage PA *et al*: Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med *5*: 677-685, 1999.
130 Pardoll DM: Inducing autoimmune disease to treat cancer. Proc Natl Acad Sci USA *96*: 5340-5342, 1999.
131 Bodey B, Bodey B Jr, Siegel SE and Kaiser HE: Failure of cancer vaccines: the significant limitations of this approach to immunotherapy. Anticancer Res *20*: 2665-2676, 2000.
132 Ribas A, Butterfield LH, Glaspy JA and Economou JS: Cancer immunotherapy using gene-modified dendritic cells. Curr Gene Ther *2*: 57-78, 2002.
133 Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF and Heath WR: Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*: 478-480, 1998.
134 Ridge JP, Di Rosa F and Matzinger P: A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. Nature *393*: 474-478, 1998.

135 Grakoui A, Bromley SK, Sumen C *et al*: The immunological synapse: a molecular machine controlling T cell activation. Science *285*: 221-227, 1999.

136 Malissen B: Dancing the immunological two-step. Science *285*: 207-208, 1999.

137 Wulfing C and Davis MM: A receptor/cytoskeletal movement triggered by costimulation during T cell activation. Science *282*: 2266-2269, 1998.

138 Bachmann MF, Wong BR, Josien R, Steinman RM, Oxenius A and Choi Y: TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. J Exp Med *189*: 1025-1031, 1999.

139 Lu Z, Yuan L, Zhou X, Sotomayor E, Levitsky HI and Pardoll DM: CD40-independent pathways of T cell help for priming of CD8(+) cytotoxic T lymphocytes. J Exp Med *191*: 541-550, 2000.

140 Chinnaiyan AM, Hanna WI, Orth K *et al*: Cytotoxic T-cell-derived granzyme B activates the apoptotic protease ICE-LAP3. Curr Biol *6*: 897-899, 1996.

141 Froelich CJ, Dixit VM and Yang X: Lymphocyte granule-mediated apoptosis: matters of viral mimicry and deadly proteases. Immunol Today *19*: 30-36, 1998.

142 Inaba K, Swiggard WJ, Inaba M *et al*: Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. I. Expression on dendritic cells and other subsets of mouse leukocytes. Cell Immunol *163*: 148-156, 1995.

143 Guo M, Gong S, Maric S *et al*: A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. Hum Immunol *61*: 729-738, 2000.

144 Kato M, Neil TK, Fearnley DB, McLellan AD, Vuckovic S and Hart DN: Expression of multilectin receptors and comparative FITC-dextran uptake by human dendritic cells. Int Immunol *12*: 1511-1519, 2000.

145 Small M and Kraal G: *In vitro* evidence for participation of DEC-205 expressed by thymic cortical epithelial cells in clearance of apoptotic thymocytes. Int Immunol *15*: 197-203, 2003.

146 Jiang W, Swiggard WJ, Heufler C *et al*: The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature *375*: 151-155, 1995.

147 Mahnke K, Guo M, Lee S *et al*: The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation *via* major histocompatibility complex class II-positive lysosomal compartments. J Cell Biol *151*: 673-684, 2000.

148 Valladeau J, Ravel O, Dezutter-Dambuyant C *et al*: Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. Immunity *12*: 71-81, 2000.

149 Geijtenbeek TB, Krooshoop DJ, Bleijs DA *et al*: DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. Nat Immunol *1*: 353-357, 2000.

150 Geijtenbeek TB, Engering A and Van Kooyk Y: DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology. J Leukoc Biol *71*: 921-931, 2002.

151 Sijts A, Zaiss D and Kloetzel PM: The role of the ubiquitin-proteasome pathway in MHC class I antigen processing: implications for vaccine design. Curr Mol Med *1*: 665-676, 2001.

152 Bodey B: Neuroendocrine influence on thymic haematopoiesis *via* the reticulo-epithelial cellular network. Exp Opin Therap Targets *6*: 57-72, 2002.

153 Kasai M, Hirokawa K, Kajino K *et al*: Difference in antigen presentation pathways between cortical and medullary thymic epithelial cells. Eur J Immunol *26*: 2101-2107, 1996.

154 Darsow U and Ring J: Neuroimmune interactions in the skin. Curr Opinion Allergy Clin Immunol *1*: 435-439, 2001.

155 Timmerman JM and Levy R: Dendritic cell vaccines for cancer immunotherapy. Annu Rev Med *50*: 507-529, 1999.

156 Foss FM: Immunologic mechanisms of antitumor activity. Semin Oncol *29(3 Suppl 7)*: 5-11, 2002.

157 Porgador A and Gilboa E: Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. J Exp Med *182*: 255-260, 1995.

158 Bodey B, Bodey B Jr and Kaiser HE: Apoptosis in the mammalian thymus during its normal histogenesis and under various *in vitro* and *in vivo* experimental conditions. In Vivo *12*: 123-134, 1998.

159 Bodey B, Bodey B Jr, Siegel SE and Kaiser HE: Over-expression of endoglin (CD105): A marker of breast carcinoma-induced neo-vascularization. Anticancer Res *18*: 3621-3628, 1998.

160 Bodey B, Bodey B Jr, Siegel SE and Kaiser HE: Immunophenotypical (IP) analysis and immunobiology of childhood primary brain tumors. Anticancer Res *19*: 2973-2992, 1999.

161 Bodey B, Bodey B Jr, Siegel SE and Kaiser HE: Immunocytochemical detection of MMP-3 and -10 expression in hepatocellular carcinomas. Anticancer Res *20*: 4585-4590, 2000.

162 Bodey B, Bodey B Jr, Gröger AM, Siegel SE and Kaiser HE: Immunocytochemical detection of Homeobox B3, B4, and C6 gene product expression in lung carcinomas. Anticancer Res *20*: 2711-2716, 2000.

163 Bodey B, Bodey B Jr, Siegel SE and Kaiser HE: Matrix metalloproteinase expression in malignant melanomas: tumor-extracellular matrix interactions in invasion and metastasis. In Vivo *15*: 57-64, 2001.

164 Bodey B, Siegel SE and Kaiser HE: MAGE-1, a cancer-testis antigen, expression in childhood astrocytomas as an indicator of tumor progression. In Vivo *16*: 583-588, 2002.

165 Nair SK, Snyder D, Rouse BT and Gilboa E: Regression of tumors in mice vaccinated with professional antigen-presenting cells pulsed with tumor extracts. Int J Cancer *70*: 706-715, 1997.

166 Paquette RI, Hsu NC, Kiertscher SM *et al*: Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potent antigen-presenting cells. J Leukocyte Biol *64*: 358-367, 1998.

167 Carbone JE and Ohm DP: Immune dysfunction in cancer patients. Oncology (Huntingt) *16*: 11-18, 2002.

168 Pioche C, Salomon B and Klatzmann D: Cellules dendritiques et therapie cellulaire antitumorale. Pathologie Biologie *43*: 904-909, 1995.

169 Turnbull E and Macpherson G. Immunobiology of dendritic cells in the rat. Immunol Rev *184*: 58-68, 2001.

170 Gunzer M and Grabbe S: Dendritic cells in cancer immunotherapy. Crit Rev Immunol *21*: 133-145, 2001.

171 Gallucci S, Lolkema M and Matzinger P: Natural adjuvants: endogenous activators of dendritic cells. Nat Med *5*: 1249-1255, 1999.

172 Gabrilovich DI, Ciernik IF and Carbone DP: Dendritic cells in antitumor immune responses. I. Defective antigen presentation in tumor-bearing hosts. Cell Immunol *170*: 101-110, 1996.

173 Gabrilovich DI, Corak J, Ciernik IF, Kavanaugh D and Carbone DP: Decreased antigen presentation by dendritic cells in patients with breast cancer. Clin Cancer Res *3*: 483-490, 1997.
174 Eibl B, Ebner S, Duba C *et al*: Dendritic cells generated from blood precursors of chronic myelogenous leukemia patients carry the Philadelphia translocation and can induce a CML-specific primary cytotoxic T-cell response. Genes Chromosomes Cancer *20*: 215-223, 1997.
175 Choudhury BA, Liang JC, Thomas EK *et al*: Dendritic cells derived *in vitro* from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. Blood *93*: 780-786, 1999.
176 Charbonnier A, Gaugler B, Sainty D, Lafage-Pochitaloff M and Olive D: Human acute myeloblastic leukemia cells differentiate *in vitro* into mature dendritic cells and induce the differentiation of cytotoxic T cells against autologous leukemias. Eur J Immunol *29*: 2567-2578, 1999.
177 Ruiz-Cabello F, Cabrera T, Lopez-Nevot MA and Garrido F: Impaired surface antigen presentation in tumors: implications for T cell-based immunotherapy. Semin Cancer Biol *12*: 15-24, 2002.
178 Iezzi G, Protti MP, Rugarli C and Bellone M: B7.1 expression on tumor cells circumvents the need of professional antigen presentation for *in vitro* propagation of cytotoxic T cell lines. Cancer Res *56*: 11-15, 1996.
179 Amoscato AA, Prenovitz DA and Lotze MT: Rapid extracellular degradation of synthetic class I peptides by human dendritic cells. J Immunol *161*: 4023-4032, 1998.
180 Ludewig B, McCoy K, Pericin M *et al*: Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self-reactive CTL by dendritic cells. J Immunol *166*: 3678-3687, 2001.
181 Altieri DC: Validating survivin as a cancer therapeutic target. Nat Rev Cancer *3*: 46-54, 2003.
182 Wolfers J, Lozier A, Raposo G *et al*: Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med *7*: 297-303, 2001.
183 Piemonti L, Bernasconi S, Luini W *et al*: IL-13 supports differentiation of dendritic cells from circulating precursors in concert with GM-CSF. Eur Cytokine Network *6*: 245-252, 1995.
184 Barratt-Boyes SM, Henderson RA and Finn OJ: Chimpanzee dendritic cells with potent immunostimulatory function can be propagated from peripheral blood. Immunology *87*: 528-534, 1996.
185 Hanada K, Tsunoda R and Hamada H: GM-CSF-induced *in vivo* expansion of splenic dendritic cells and their strong costimulation activity. J Leukocyte Biol *60*: 181-190, 1996.
186 Caux C, Vanbervliet B, Massacrier C *et al*: $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF and TNF alpha. J Exp Med *184*: 695-706, 1996.
187 Hochrein H, Jahrling F, Kreyschh HG and Sutter A: Immunophenotypical and functional characterization of bone marrow derived dendritic cells. Adv Exp Med Biol *378*: 61-63, 1995.
188 Strobl H, Riedl E, Scheinecker C *et al*: TGF-beta 1 promotes *in vitro* development of dendritic cells from $CD34^+$ hemopoietic progenitors. J Immunol *157*: 1499-1507, 1996.
189 Yamazaki K and Eyden BP: Ultrastructural and immunohistochemical observations on intralobular fibroblasts of human breast, with observations on the CD34 antigen. J Submicr Cytol Pathhol *27*: 309-323, 1995.
190 Nijman HW, Kleijmeer MJ, Ossevoort MA *et al*: Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells. J Exp Med *182*: 163-174, 1995.
191 Ossevoort MA, Kleijmeer MJ, Nijman HW, Geuze HJ, Kast WM and Melief CJM: Functional and ultrastructural aspects of antigen processing by dendritic cells. Adv Exp Med Biol *378*: 227-231, 1995.
192 Kaplan JM, Yu Q, Piraino ST *et al*: Induction of antitumor immunity with dendritic cells transduced with adenovirus vectorencoding endogenous tumor-associated antigens. J Immunol *163*: 699-707, 1999.
193 Kirk CJ and Mule JJ: Gene-modified dendritic cells for use in tumor vaccines. Hum Gene Ther 11:797-806, 2000.
194 Furumoto K, Arii S, Yamasaki S *et al*: Spleen-derived dendritic cells engineered to enhance interleukin-12 production elicit therapeutic antitumor immune responses. Int J Cancer *87*: 665-672, 2000.
195 Hirschowitz EA, Weaver JD, Hidalgo GE and Doherty DE: Murine dendritic cells infected with adenovirus vectors show signs of activation. Gene Ther *7*: 1112-1120, 2000.
196 Jenne L, Schuler G and Steinkasserer A: Viral vectors for dendritic cell-based immunotherapy. Trends Immunol *22*: 102-107, 2001.
197 Paul S, Calmels B and Acres RB: Improvement of adoptive cellular immunotherapy of human cancer using *ex-vivo* gene transfer. Curr Gene Ther *2*: 91-100, 2002.
198 Bodey B: Spontaneous regression of neoplasms: new possibilities for immunotherapy. Exp Opin Biolog Therapy *2*: 459-476, 2002.
199 Maecker B, Von Bergwelt-Baidon, Anderson KS, Vonderheide RH and Schultze JL: Linking genomics to immunotherapy by reverse immunology-- 'immunomics' in the new millennium. Curr Mol Med *1*: 609-619, 2001.
200 Onaitis M, Kalady MF, Pruitt S and Tyler DS: Dendritic cell gene therapy. Surg Oncol Clin N Am *11*: 645-660, 2002.
201 Scheffer SR, Nave H, Korangy F *et al*: Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response *in vivo*. Int J Cancer *103*: 205-211, 2003.
202 Steinman RM and Pope M: Exploiting dendritic cells to improve vaccine efficacy. J Clin Invest *109*: 1519-1526, 2002.
203 Gilboa E: Immunotherapy of cancer with genetically modified tumor vaccines. Semin Oncol *23*: 101-107, 1996.
204 Topf N and Schmiegel WH: Immuntherapie mit genetisch modifizierten Tumorzellen. Internist *37*: 374-381, 1996.
205 Zhang W, He L, Yuan Z *et al*: Enhanced therapeutic efficacy of tumor RNA-pulsed dendritic cells after genetic modification with lymphotactin. Hum Gene Ther *10*: 1151-1161, 1999.
206 Klein C, Bueler H and Mulligan RC: Comparative analysis of genetically modified dendritic cells and tumor cells as therapeutic cancer vaccines. J Exp Med *191*: 1699-1708, 2000.
207 Armstrong TD and Jaffee EM: Cytokine modified tumor vaccines. Surg Oncol Clin N Am *11*: 681-696, 2002.
208 Gong J, Avigan D, Chen D *et al*: Activation of antitumor cytotoxic T lymphocytes by fusions of human dendritic cells and breast carcinoma cells. Proc Natl Acad Sci USA *97*: 2715-2718, 2000.
209 Xia J, Tanaka Y, Koido S *et al*: Prevention of spontaneous breast carcinoma by prophylactic vaccination with dendritic/tumor fusion cells. J Immunol *170*: 1980-1986, 2003.

210 Schultz J: Success of vaccine offers promise of cervical cancer prevention. J Natl Cancer Inst *95*: 102-104, 2003.
211 Shu S and Cohen P: Tumor-dendritic cell fusion technology and immunotherapy strategies. J Immunother *24*: 99-100, 2001.
212 Lollini PL, De Giovanni C, Nicoletti G *et al*: Immunoprevention of colorectal cancer: a future possibility? Gastroenterol Clin N Am 31:1001-1014, 2002.
213 Bell D, Young JW and Banchereau J: Dendritic cells. Adv Immunol *72*: 255-324, 1999.
214 Banchereau J, Briere F, Caux C *et al*: Immunobiology of dendritic cells. Annu Rev Immunol *18*: 767-811, 2000.
215 Staquet MJ, Jacquet C, Dezutter-Dambuyant C and Schmitt D: Fibronectin upregulates *in vitro* generation of dendritic Langerhans cells from human cord blood $CD34^+$ progenitors. J Invest Dermatol *109*: 738-743, 1997.
216 Rougier N, Schmitt D and Vincent C: IL-4 addition during differentiation of CD34 progenitors delays maturation of dendritic cells while promoting their survival. Eur J Cell Biol *75*: 287-293, 1998.
217 Nestle FO, Alijagic S, Gilliet M *et al*: Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med *4*: 328-332, 1998.
218 Zitvogel L, Angevin E and Tursz T: Dendritic cell-based immunotherapy of cancer. Ann Oncol *11 (suppl.) 3*: 199-205, 2000.
219 Panelli MC, Wunderlich J, Jeffries J *et al*: Phase I study in patients with metastatic melanoma of immunization with dendritic cells presenting epitopes derived from the melanoma-associated antigens MART-1 and gp100. J Immunother *23*: 487-498, 2000.
220 Small EJ, Fratesi P, Reese DM *et al*: Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. J Clin Oncol *18*: 3894-3903, 2000.
221 Cohen L, De Moor C, Parker PA and Amato RJ: Quality of life in patients with metastatic renal cell carcinoma participating in a phase I trial of an autologous tumor-derived vaccine. Urol Oncol *7*: 119-124, 2002.
222 Tirapu I, Rodriguez-Calvillo M, Qian C *et al*: Cytokine gene transfer into dendritic cells for cancer treatment. Curr Gene Ther *2*: 79-89, 2002.
223 Kugler A, Stuhler G, Walden P *et al*: Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med *6*: 332-336, 2000.
224 Gitlitz BJ, Figlin RA, Pantuck AJ and Belldegrun AS: Dendritic cell-based immunotherapy of renal cell carcinoma. Curr Urol Rep *2*: 46-52, 2001.
225 Veelken H and Osterroth F: Vaccination strategies in the treatment of lymphomas. Oncology *62*: 187-200, 2002.
226 Perales MA and Wolchok JD: Melanoma vaccines. Cancer Invest *20*: 1012-1026, 2002.
227 Wysocki PJ, Karczewska A and Mackiewicz A: Gene modified tumor vaccines in therapy of malignant melanoma. Otolaryngol Pol *56*: 147-153, 2002.

Received July 15, 2003
Revised November 4, 2003
Accepted December 18, 2003