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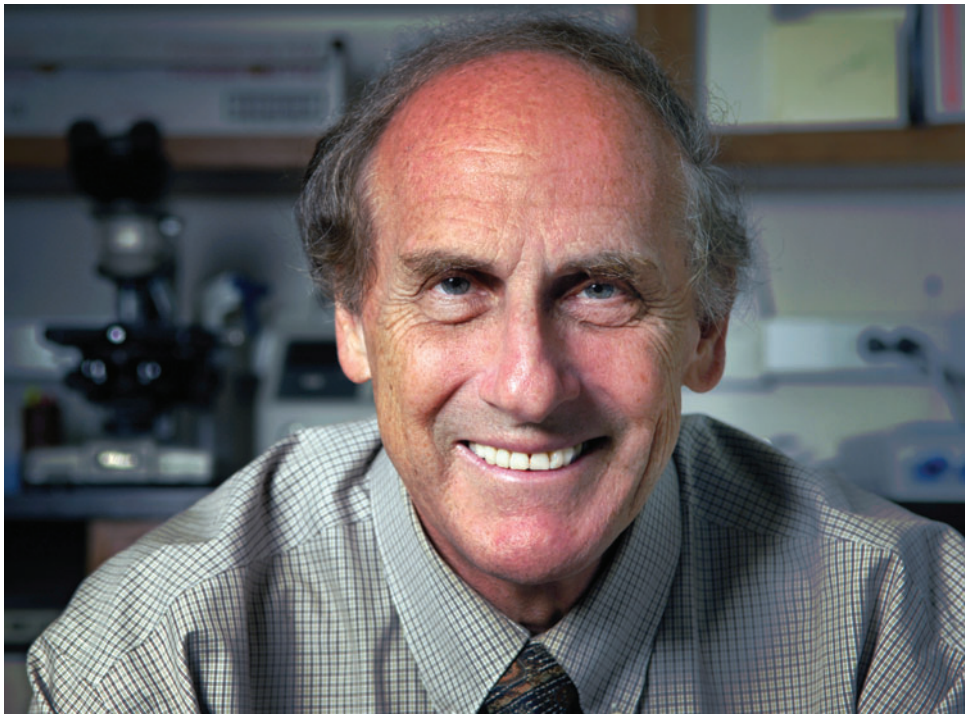

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Decisions About Dendritic Cells: Past, Present, and Future

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Abstract

A properly functioning adaptive immune system signifies the best features of life. It is diverse beyond compare, tolerant without fail, and capable of behaving appropriately with a myriad of infections and other challenges. Dendritic cells are required to explain how this remarkable system is energized and directed. I frame this article in terms of the major decisions that my colleagues and I have made in dendritic cell science and some of the guiding themes at the time the decisions were made. As a result of progress worldwide, there is now evidence of a central role for dendritic cells in initiating antigen-specific immunity and tolerance. The in vivo distribution and development of a previously unrecognized white cell lineage is better understood, as is the importance of dendritic cell maturation to link innate and adaptive immunity in response to many stimuli. Our current focus is on antigen uptake receptors on dendritic cells. These receptors enable experiments involving selective targeting of antigens in situ and new approaches to vaccine design in preclinical and clinical systems.

DECIDING TO STUDY IMMUNOLOGY

As explained elsewhere (1, 2), I had the good fortune to grow up in Sherbrooke, Quebec, attend McGill University in Montreal, and then study medicine at Harvard Medical School and Massachusetts General Hospital in Boston. All along, my teachers made it fun to learn, perhaps so much so that my decision to focus on immunology did not emerge until the end of my education in medicine. Then I became fascinated with clonal selection theory by reading *Clonal Selection Theory of Acquired Immunity* (1959) by MacFarlane Burnet (3). The theory tried to explain one of the hallmarks of the immune system: its unique diversity and ability to recognize determinants or antigens from a spectrum of infections, tumors, transplants, self tissues, and allergens. Burnet envisaged an elegant repertoire of clones, each with an antibody receptor specific for one antigen; immunization required an initial selection step by the antigen binding to its receptor. One of the amazing triumphs of immunology during my subsequent career was to see Burnet's repertoire unraveled through discoveries of how adaptive T and B cells are formed, each expressing a single receptor and together an unparalleled diverse library of specificities.

During medical training in the late 1960s, I attended a late afternoon set of seminars in modern immunology organized by Kurt Bloch at Massachusetts General Hospital. One of the lectures described that macrophages were accessories needed to initiate immunity. We were taught that when a macrophage takes up an antigen, an immunogenic RNA-antigen complex is formed, and this instructed immune cells to start making a specific antibody (4–6). This was my first exposure to the idea that clonal selection is not straightforward to initiate; somehow antigen has to interact with RNA from a macrophage. The scenario seemed hard to believe given what was already known about subcellular compartments and their membrane barriers. Nevertheless, the role of accessory cells in immunity seemed to be a critical mystery to unravel.

During this same period of training in medicine, a curious episode of fate involved a so-called throwaway journal that medical students received gratis in their hospital mailboxes. An issue arrived that caught my attention because it described the new field of cell-mediated immunity and how important it could be for medicine. The throwaway article on cell-mediated immunity kept citing a series of early reviews for the new field in the *British Medical Bulletin* of 1965.

When I turned to that issue, Peter Medawar, the father of transplantation, wrote, "We are still generally ignorant of how a homograft reaction starts" (7, p. 98). And James Gowans, who discovered that lymphocytes are the mediators of immunity, wrote, "Very little is known about the way in which antigens from vascularized grafts reach the lymphoid cells of the host" (8, p. 107). How could there be uncertainty about the initiation of the most powerful immune response in the body, when all that seemed necessary was for the foreign antigens on transplants to select clones with receptors specific to these determinants?

Deciding how to approach this problem was something I struggled with for two years before beginning my postdoctoral experiments. In contrast, it was not a struggle to decide that I needed to work with Zanvil Cohn and James Hirsch at The Rockefeller University. They were leaders in the modern cell biology of phagocytes, and these were the cells deemed to be critical accessories to initiate immunity. Fortunately, I gained a position in their Laboratory of Cellular Physiology and Immunology. When I began my postdoctoral research, I did not have a hypothesis that a new cell type must exist to understand how immunity begins. Rather, I had a commitment to what I thought was a major problem: How does the body decide to make an immune response, especially a cell-mediated one, when antigen enters the body? Or to put it another way, how is Burnet's selection of T cell clones initiated?

The decision to emphasize problems that appear central to medicine is something I will never abandon, and, of course, I am not alone in this. The relevance of immunology to so many disease states (**Figure 1**) is not something one just mentions in a search for grant funds. Instead, it is a thrilling driving force for choosing which experiments and experimental systems to pursue. I dislike the much-used distinction between basic and clinical immunology. Research on diseases and in patients are both basic. For example, the simultaneous discovery of TNF by Anthony Cerami at Rockefeller and Lloyd Old at Memorial Sloan Kettering Cancer Center was fundamental, but was it not equally basic for Marc Feldmann and Ravinder Maini in London to make the shocking discovery that anti-TNF antibodies were able to block severe inflammatory diseases in people with rheumatoid arthritis? The word "translational" can be helpful in one sense, by indicating to our community of supporters that we are studying disease and often patients. But too often the term implies that medical progress comes from a simple translation or implementation of basic studies and that research with patients and pathogens is not part of the discovery equation. This is untrue. Research attempts to uncover the unknown, whether it is clinical, cellular, or molecular (**Figure 1**).

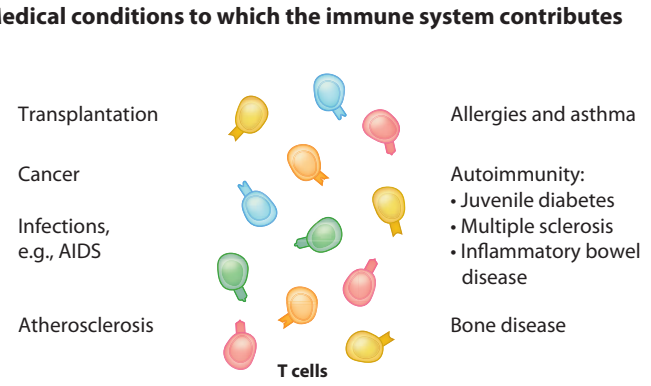

Figure 1

The immune system contributes to various medical conditions, either to protect against disease, including with vaccination and immune therapies, or to contribute to pathology and symptoms. At the bottom of the figure are areas being studied more recently for their immune involvements: atherosclerosis and bone disease. All these conditions either are becoming more frequent or, in the case of a disease such as cancer, are decreasing very little. Also, new infections always evolve, most notably AIDS, which was not known when I began my career.

DISCOVERING A NEW CELL TYPE

The route to identifying dendritic cells was not direct (1, 2). The key decision was to examine the spleen and move away from the peritoneal cavity, which remains a focus of macrophage research. Others had shown that spleen cell suspensions are special because they can be used to study the initiation of antibody responses in culture (9). But why? Once we looked at the spleen cells, we quickly observed novel cells, dendritic cells as we called them, and began to try to understand them. They did not look like macrophages, and this was soon reinforced with more functional distinctions (10, 11).

It was invaluable that Zanvil Cohn and all the scientists in the lab had a rich experience with macrophage biology. This provided a huge boost to work out that dendritic cells are entirely different from macrophages, even if others at the time did not agree. Also critical was that The Rockefeller University was the birthplace of many discoveries in modern cell biology and subcellular structure. David Sabatini's glutaraldehyde fixation method used for electron microscopy and cytochemistry preserved dendritic cells in their distinctive form, which was similar to what we observed in the living state by phase contrast microscopy. Other major breakthroughs in cell biology were the identification of lysosomes by Christian de Duve (12) and the elegant mechanisms from Cohn, Hirsch, and colleagues (13) on uptake and delivery of particles to digestive lysosomes in macrophages. It was quickly shown that dendritic cells have few standard lysosomes by morphology or acid phosphatase staining, and phagocytosis was tough to demonstrate. These cells were unusual.

By 1978, eight years after beginning research at Rockefeller, I still had the important problem of elucidating how immunity is initiated. I was aware of a cell type that did not look like or behave like any monocyte or macrophage that had been encountered. I enjoyed the unshakable patience and wisdom of Zanvil Cohn. I could identify the distinct dendritic cell by its unusual cell shape and organelles so that I could eventually purify it. Then I observed its high expression of major histocompatibility complex (MHC) molecules with effective antibodies that had just become available, and I began to assay function (14).

I wonder whether I could succeed today in obtaining my first grant, AI13013, now in its 36th year, to pursue these dendritic cells. Many funding sources provide research support almost exclusively on the basis of the specifics and feasibility of what one plans to do. Research funding should instead prioritize individuals who have done special groundwork and want to use their discoveries to pursue an important problem. In other words, funding should be determined by what the investigator brings to the table from his or her past work and the importance of the problem he or she chooses to study. It is simply illogical to award funds for a feasible and detailed future approach, in which case the biological unknown is likely doomed to be incremental.

In writing my first grant, even after spotting unusual cells, I could not have hypothesized that dendritic cells would prove to be unique initiators of immunity. In the early 1970s, there were several possible roles for "accessory cells," one being the retention of intact antigen, particularly immune complexes on the cell surface. This was observed in vivo on "dendritic macrophages" (now called follicular dendritic cells, FDCs) (15). I did manage to show with Lei Chen that persistence in vivo is truly on the cell surface of the FDC (16, 17). This meant that FDCs are very different from macrophages, on which I had failed previously to show retention of intact antigen and immune complexes in spite of large amounts of endocytosis (18). But I was also unable to show binding of intact antigen or antibody complexes to the newly recognized dendritic cells.

In fact, it took almost five years of effort, largely on cultures that allowed the massive expansion of antibody-forming cells (19), to decide to study the in vitro counterpart of Medawar's transplant rejection, the mixed leukocyte reaction (MLR) (20). The dendritic cells proved to be the principal and surprisingly potent stimulator cells, whereas MHC-bearing macrophages and B cells were weak. It took another five years for Robert Lechler and Richard Batchelor in London to report that dendritic cells are unique inducers of transplant rejection in vivo (21). During these slow early years, I benefited from essential support from the Leukemia Society of America, the American Heart Association, and the Irma T. Hirschl Fund, and again, I had the unique encouragement of Zanvil Cohn. Then in the late 1970s, after the initial years of struggle, the pace changed markedly when I was joined by PhD students Michel Nussenzweig and Wes van Voorhis, and by Kayo Inaba, fresh from her PhD in Kyoto.

DISSECTING THE AFFERENT AND EFFERENT LIMBS OF CELL-MEDIATED IMMUNITY

As mentioned, the first functional assays we used to identify the immune-initiating function of dendritic cells did not involve the addition of antigens that needed to be processed. Instead, we used responses in the MLR, a reaction in which T cells largely recognize endogenous peptides complexed to the foreign MHC. A similar situation took place in the laboratories of William Bowers and Jon Austyn, who observed the potent accessory function of dendritic cells relative to other cell types using a polyclonal mitogenesis assay in which T cells were treated with sodium periodate (22, 23). But then we began to move forward with immunity to specific added antigens (still the focus of the lab), for which the reactive T cell clones are rare. However, we were not initially thinking of antigen processing to produce

peptide MHC products. This was unraveled only later from the work of many other investigators, particularly Emil Unanue, Alain Townsend, Jack Strominger, Don Wiley, and Pam Bjorkman.

Nonetheless, dendritic cells were quickly shown to effectively present complex antigens to T cells. Nussenzweig demonstrated that dendritic cells present exogenous antigen to T cells in an MHC-restricted fashion and that they induce specific cytolytic T cell responses (24). He co-cultured the dendritic cells with T cells, irradiated trinitrophenyl (TNP)-modified thymocytes, and found that the T cells developed MHC-restricted cytolytic activity. Although not appreciated at the time, this was also the first demonstration of "cross-priming" by dendritic cells. Wes van Voorhis showed that human blood contains dendritic cells similar to the ones we had found in mice (25), and he studied presentation of *Candida* to proliferating T cells. Inaba, as she had begun to do during her PhD (26), analyzed a system employing sheep red blood cells, a classical antigen at the time to study helper T cell function in antibody formation (27). In all these systems, small numbers of dendritic cells elicited a T cell response, while much larger numbers of other cell types were inactive. Early reviews emphasized the features of this newly uncovered lineage of white cells (28, 29).

These findings made us want to understand what the MHC is doing when expressed on other cell types. Inaba and I decided to study distinct cell clusters—5–10 cells in width—which we routinely observed in our cultures when dendritic cells were initiating immunity. The clusters contained most of the dendritic cells in the culture, and these were bound to lymphocytes. The clusters proved to be the sites for the onset of lymphocyte proliferation or "blastogenesis," but then the responding "blasts" moved away from the cluster. When purified, the primed T cells showed responses to other cell types presenting antigen. For example, Inaba found that B cells fail to initiate T cell immunity to a soluble protein but could interact vigorously with the antigen-specific and MHC-restricted T blasts that are first induced by dendritic cells (30, 31). James Young, Sumi Koide, and Jon Austyn extended this two-step mechanism to other assays for successful T cell responses (23, 32, 33).

The experiments led by Inaba were long lasting in two respects, as summarized in later reviews on the importance of dendritic cells in immunogenicity (34, 35). First, dendritic cells are not simply antigen-presenting cells but, in addition, are specialized accessories for initiating immunity. All cells that express MHC molecules can use these to present antigen—but primarily to activated T cells. Second, we proposed an in vivo counterpart for the findings with cell clusters, based on several prior sets of observations: that immune responses begin in lymphoid organs; that T blasts pour into the thoracic duct lymph from lymphoid tissues several days after the onset of an immune response; and that the main place that dendritic cells can be found is in T cell zones (36). Therefore, we envisaged that dendritic cells would initiate the "afferent limb" of immunity by presenting antigen in the T cell areas of lymphoid tissues in vivo, and later the activated T cells would leave via the lymph, enter the thoracic duct and then the blood, and finally reach the inflamed tissues to bring about the efferent or effector limb of immunity.

These early assays, as well as the antigens that dominated research in immunology at the time, may seem remote to younger readers, but the underlying themes live on. In particular, the availability of dendritic cells makes it possible to initiate immunity with intact specific antigens. It was not necessary to focus on preprocessed peptides and various mitogens. Without knowing about antigen processing, we were finding that dendritic cells were carrying out the two series of events needed for T cells to start their protective and pathogenic functions, i.e., dendritic cells allowed T cells to recognize antigen (later peptide MHC complexes) and to respond to it (later accessory or costimulatory functions).

Table 1 International symposia on dendritic cells in fundamental and clinical immunology

	City	Date	Organizers
I	Yamagata City, Japan	June 1990	Y. Imai
II	Amsterdam, Holland	June 1992	E.C.M. Hoefsmit, P. Nieuwenhuis, E.W.A. Kamperdijk, A.C. Dijkstra
III	Annecy, France	October 1994	J. Banchereau, D. Schmitt, L. Valette
IV	Venice, Italy	October 1996	P. Riccardi-Castagnoli, G. Girolomoni, A. Lanzavecchia
V	Pittsburgh, USA	September 1998	M. Lotze, J. Banchereau, R. Steinman
VI	Port Douglas, Australia	May 2000	K. Shortman, D. Hart, P. Holt, P. Wood
VII	Bamberg, Germany	September 2002	G. Schuler, A. Steinkasserer, G. Stingl
VIII	Bruges, Belgium	October 2004	M. Moser, K. Thielemans, T. Boon
IX	Edinburgh, Scotland	September 2006	G. MacPherson, J. Liversidge, J. Austyn
X	Kobe, Japan	October 2008	M. Furue, K. Inaba, S. Koyasu, K. Matsushima
XI	Lugano, Switzerland	September 2010	A. Lanzavecchia, M.G. Manz, F. Sallusto
XII	Daegu, South Korea	October 2012	H-Y. Kim, Y-S. Bae, C-K. Lee

A relatively small but international community was actively contributing to dendritic cell research in fundamental and clinical immunology in the 1980s. Sizeable international biennial meetings dedicated to this theme began in 1990 (**Table 1**) and in alternating years at Keystone Symposia in the United States (**Table 2**). The organizers of these conferences include many of the leaders in dendritic cell biology over the years. Nevertheless, before the early 1990s, dendritic cells were not really on the main stage of immunology. This was largely because immunology was profitably absorbed with the crucial understanding of MHC restriction, antigen processing and presentation, and the T cell receptor. These events could be studied, at least initially, with already immunized T cells and T cell lines, clones, and hybridomas. Isolating dendritic cells for this kind of antigen presentation research was not critical initially. But today, direct attention to dendritic cells is valuable for many mechanistic studies, e.g., antigen uptake and presentation; the links between innate and adaptive immunity; T cell differentiation; dynamics of the immune system in situ; stimulation of other lymphocytes, especially NK cells; and clinical immunology.

TRACING THE DEVELOPMENT OF DENDRITIC CELLS: THEIR MATURATION AND DERIVATION FROM BONE MARROW PROGENITORS

Understanding development is essential to defining a cell lineage. Our first experiments in this sphere came through a decision by Gerold Schuler from Innsbruck to join our lab to determine how epidermal Langerhans cells relate to spleen dendritic cells. He discovered what we termed dendritic cell maturation (37). We prefer the term "maturation" to "activation" because the latter typically refers to an on-off event or restricted series of events, whereas what we were observing was the large-scale differentiation of a cell lineage, which is called maturation when, for example, myelocytes become neutrophils or normoblasts become red cells. Dendritic cell maturation is the critical link between innate and adaptive T cell–dependent immunity.

The concept is that dendritic cells respond quickly to environmental changes and differentiate extensively to become mature or immunogenic accessory cells. Microbes are not the only sources of these stimuli. There are many other sources, including the two most

Table 2 Keystone Symposia on dendritic cells

Year and place	Symposium title	Organizers
1995 Taos, NM	Dendritic Cells: Antigen Presenting Cells of T and B Lymphocytes	Jacques Banchereau and Ralph Steinman
1998 Santa Fe, NM	Cellular and Molecular Biology of Dendritic Cells	Ralph Steinman, Michel Nussenzweig, and Jacques Banchereau
2001 Taos, NM	Dendritic Cells: Interfaces with Immunobiology and Medicine	Ralph M. Steinman, Anne O'Garra, and Jacques Banchereau
2003 Keystone, CO Joint with	Dendritic Cells: Interfaces with Immunobiology and Medicine Cell Biology of the Immune Response	Ralph M. Steinman, Anne O'Garra, and Jacques Banchereau Ira Mellman, Richard Flavell, and Ralph M. Steinman
2005 Vancouver, BC	Dendritic Cells at the Center of Innate and Adaptive Immunity: Eradication of Pathogens and Cancer and Control of Immunopathology	Anne O'Garra, Jacques Banchereau, and Alan Sher
2007 Keystone, CO Joint with	Intracellular and Intercellular Signaling in Dendritic Cell Function Imaging Immune Responses	Muriel Moser, Caetano Reis e Sousa, and Yong-Jun Liu Ronald N. Germain and Ellen A. Robey
2009 Banff, Alberta Joint with	Dendritic Cells Pattern Recognition Molecules and Immune Sensors of Pathogens	Giorgio Trinchieri, Gwendalyn J. Randolph, and Sebastian Amigorena Jenny P. Ting, Richard A. Flavell, and Luke A.J. O'Neill
2011 Santa Fe, NM Joint with	Dendritic Cells and the Initiation of Adaptive Immunity Cancer Control by Tumor Suppressors and Immune Effectors (Tumor Immunology)	Ira Mellman, Michel C. Nussenzweig, Virginia Pascual, and Federica Sallusto Laurence Zitvogel, Anna Karolina Palucka, and Mark J. Smyth
2013 Venue to be determined	Understanding Dendritic Cell Biology to Improve Human Disease	Miriam Merad and Bart Lambrecht

powerful settings for cell-mediated immunity, graft rejection and contact hypersensitivity, which take place in ostensibly nonmicrobial settings. Maturation occurs whenever epidermal dendritic cells, and spleen dendritic cells in later experiments, are placed in culture. Maggi Pack found that one critical factor for the maturation of Langerhans cells was GM-CSF (38).

The surprise behind all this was that we had previously assumed that dendritic cells in vivo are fully ready to initiate immunity because of their high levels of MHC class II (MHCII) molecules in spleen and in skin. But when Ira Mellman and his colleagues at Yale decided to bring expertise in cell biology to dendritic cells, they found that the MHCII products are largely sequestered within the endocytic system (39). There, the MHCII molecules wait for a maturation stimulus to trigger endosomal acidification (40) and thereby the catabolism of protein antigens and the MHCII-associated invariant chain. These two key steps are required to allow peptide-MHCII complexes to form inside the endocytic system, followed by their subsequent display at the cell surface (41, 42). More research is needed, but the endocytic system—its regulation and composition—is turning out to be one of the hallmark differences between dendritic cells and macrophages.

Niki Romani likewise made surprising findings with dendritic cells from the skin. The immature cells capture antigens while the mature ones are ineffective; in contrast, the mature cells are very strong immune stimulators for T cells specific for antigens captured earlier (43). This finding was one of the early indications in

immunology that the initiation of immunity requires two large components: (*a*) antigen capture and presentation and (*b*) the expression of many accessory functions. I still prefer the term "accessory" because it encompasses the many specializations of dendritic cells for initiating immunity. These go beyond formal costimulation of the T cell receptor.

Many investigators use the word maturation when only "phenotypic maturation" has been documented. Typically, this means increased expression of CD40/80/86 and more recently PD-L1/CD274 and PD-L2/CD273. Phenotypic maturation is not identical to functional maturation or immunogenicity. Many changes that comprise the maturation phenotype are actually secondary to inflammatory cytokines, which Shin-ichiro Fujii and Kanako Shimizu found when they studied functional maturation mediated by natural killer T (NKT) cells in vivo (44, 45). A big gap currently is the incomplete molecular understanding of functional maturation, i.e., what events take place directly when a dendritic cell encounters a microbial product, an alarmin, an innate NK cell, or a CD40 ligand on a mast cell and platelet. Cytokine production by dendritic cells is a critical initial step in maturation, but many cells make cytokines. One needs in the future to identify the constellation of changes in dendritic cells, not only cytokines, that leads to the initiation of the appropriate immune response, and sometimes to inappropriate ones. These include allergy as reviewed by Bart Lambrecht and Hamida Hammad from Ghent (46) and systemic lupus erythematosus as reviewed by Lars Ronnblom and Virginia Pascual from Uppsala and Dallas (47).

The other area of dendritic cell development that we decided to study in the early days of the field was the identification of progenitors. With Gerold Schuler and Kayo Inaba, we began this demanding project in mice (48–50), while Jacques Banchereau, Christophe Caux, and colleagues in Dardilly were doing similar experiments with human $CD34^+$ progenitors (51). The first concept was that dendritic cells develop from a common myeloid progenitor that gives rise to granulocytes, macrophages, and dendritic cells. In our cultures, the different myeloid progeny were separated on the basis of plastic adherence and clustering properties, allowing us to show the typical morphology and phenotypic features of dendritic cells, including the later finding that the immature forms are capable of modest but clear phagocytic activity (52). Soon thereafter, using monocytes rather than marrow progenitors, Federica Sallusto and Antonio Lanzavecchia (53) and Romani, Schuler, and colleagues (54) determined that a combination of GM-CSF and IL-4 (or IL-13) can induce the initial differentiation of human monocytes to acquire phenotypic features associated with dendritic cells and that development is completed following application of a maturation stimulus (55). Consistent with the idea that maturation is the essential link between innate and adaptive immunity, Sallusto and Lanzavecchia studied lipopolysaccharide (LPS) as a maturation stimulus (55), before the identification of responsible Toll-like receptors (TLRs).

These methods to develop large numbers of monocyte-derived dendritic cells in vitro changed the field because investigators could more easily study their immunizing properties. This included Inaba's use of antigen-loaded dendritic cells to immunize healthy mice (52, 56) and later Madhav Dhodapkar's and Nina Bhardwaj's research showing that antigen-loaded dendritic cells could immunize humans (57, 58). But still, something important was missing. Despite the use of GM-CSF in these systems for dendritic cell development, Ken Shortman in Melbourne showed that mice deficient in GM-CSF and GM-CSF receptor can have quite normal numbers of dendritic cells in the steady state (59).

Several investigators have uncovered the missing link in vivo, another hematopoietin, flt-3L. Eugene Maraskovsky, with the former Immunex Corporation in Seattle, found that repeated injection of flt-3L into mice (60) and humans (61) leads to a dramatic 10- to 15-fold expansion of dendritic cells. The laboratories of Li Wu in Melbourne (62) and Markus Manz in Bellinzona (63) established

that the dendritic cell progenitor in marrow is responsive to flt-3L. Waskow and Liu, working in the Nussenzweig laboratory at Rockefeller, defined the progenitors of dendritic cells in the bone marrow (64–66). They discovered intermediates in the myeloid differentiation pathway that define the split between dendritic cells and monocytes during development. In addition, they showed that under steady-state conditions, dendritic cell–committed precursors emigrate from the bone marrow and seed lymphoid and nonlymphoid tissues, where they divide under the control of flt-3L to fill the dendritic cell compartment. Thus, in the steady state, a critical part of the definition of the dendritic cell lineage is its dependence on flt-3L. An exception is the epidermal Langerhans cell, which as reviewed by Miriam Merad and colleagues has a separate origin (67).

Frederic Geissmann, now in London, defined a common bone marrow progenitor that gives rise to both monocytes and dendritic cells. The split between the two pathways was defined by Liu and Nussenzweig (64): Monocytes remain dependent upon M-CSF, and the dendritic cells remain dependent on flt-3L. The dendritic cell progenitor moves into lymphoid and nonlymphoid organs (64), the latest example being Kang Liu's research with the meninges of the brain (68). Niroshana Anandasabapathy is leading a new clinical study with Celldex Therapeutics to pursue flt-3L in people. We want to confirm that this hematopoietin can expand many different types of dendritic cells roughly 10-fold in people. After following U.S. Food and Drug Administration guidelines to reevaluate in detail new lots of flt-3L clinical product for safety and efficacy, the product can then be used, for example, to test whether dendritic cell expansion can enhance immune control in vaccination and in autoimmunity.

But there may be a way for monocytes to become dendritic cells in vivo in parallel with the much-used human monocyte tissue culture system also mentioned above. Cheolho Cheong, Ines Matos, and Chae Gyu Park found a surprising pathway for this differentiation, surprising because it had been overlooked for so long. The monocyte–to–dendritic cell conversion occurs when mice are given a high dose of LPS or gram-negative bacteria (69). Rapidly, within 6 h, blood monocytes move into peripheral lymph nodes and differentiate via TLR4, Trif, and CD14 into typical functional dendritic cells. Importantly, the addition of LPS to mouse or human monocytes does not directly convert monocytes to dendritic cells; rather, additional, still unknown events must occur in mice that allow this transition to take place. Also uncertain are the functions of monocyte-derived, flt-3L-independent cells in many tissues, particularly lung and intestinal lamina propria. These are often called dendritic cells, but more study of their antigen-presenting functions is required, as Saurabh Mehandru is now undertaking. Curiously, the first nonlymphoid tissue in which functional flt-3L-dependent and M-CSF-dependent dendritic cells have been studied side by side is probably the most demanding one, the mouse aorta, as shown recently by Jaehoon Choi and Cheolho Cheong (70).

Monocyte-derived dendritic cells in vivo share a property with their cultured counterparts, which is the capacity to present nonreplicating proteins on MHC class I (69). This is termed "cross-presentation" and is a hallmark of one subset of dendritic cells in lymphoid tissues, the $CD8^+$ subset. This allows monocyte-derived dendritic cells to cross-present antigens to $CD8^+$ T cells from immune complexes, as shown by Sebastian Amigorena in Paris (71), and from dying infected cells, as shown by Matthew Albert and Nina Bhardwaj in New York (72).

The advances in knowledge of dendritic cell development that are coming from many laboratories now make it possible to better understand and work with the lineage. The future will yield even more clarity when the driving forces for the gene-expression programs of dendritic cells are unraveled at the levels of transcription factors and microRNAs. Several relevant transcription factors have been identified for dendritic cell development, e.g.,

the E2-2 zinc finger protein is selective for a particular pathway of dendritic cell development, in this case plasmacytoid dendritic cells (73). Likewise, the driving forces for dendritic cell function must be defined after development, i.e., to account for dendritic cell properties in the steady state and during maturation.

USING DEC-205 TO DIRECT DENDRITIC CELLS IN SITU, INCLUDING THE CONTROL OF IMMUNE TOLERANCE

By the 1990s, the dendritic cell field was still quite limited in terms of molecular tools. Early on, Nussenzweig had used a panel of available monoclonal antibodies to study the cell surface of dendritic cells; the main positive finding was the high expression of MHC products (74). The expression of high levels of the CD11c integrin was found some years later and shown to be useful to enrich dendritic cells (75, 76). However, CD11c is not cell-type specific, as is the case for the other leukocyte integrins, CD11a and CD11b. The latter integrins were initially thought to be cell specific and were termed lymphocyte function–associated antigen and Mac-1, respectively. Others have commented on the usefulness of high CD11c expression to identify many dendritic cells, as well as clear CD11c expression on other cell types (77). Expression of this integrin by itself should not be used to define the dendritic cell lineage, especially when function and development are not brought to bear on the analysis.

To gain more discrimination, we decided to isolate the molecule recognized by a monoclonal antibody, NLDC-145, that Georg Kraal in Amsterdam had found (78). The target for the NLDC-145 antibody was intriguing because it was mainly expressed on dendritic cells in the T cell areas and on cortical thymic epithelium and other epithelia (79). As shown by William Swiggard during his PhD studies, the NLDC-145 antigen turned out to be a 205-kD protein (80). Swiggard obtained some distinctive peptide sequences, and Wanping Jiang, a postdoctoral fellow in the Nussenzweig laboratory, cloned the cDNA (81). The sequence predicted 10 external lectin domains, hence the new name DEC-205, given that it was a decalectin and was expressed on both dendritic cells and epithelial cells.

We could not have foreseen what emerged from the cloning. The molecule was a cousin of the macrophage mannose receptor that Siamon Gordon in Oxford had studied; this receptor was later shown to be expressed on macrophages and sinus lining endothelium in vivo, but not in the T cell areas (82). Phil Stahl in St. Louis had studied the mannose receptor as an early example of receptor-mediated adsorptive endocytosis. As predicted from the sequence homology, DEC-205 mediates adsorptive uptake via coated pits into the endocytic system and greatly enhances the efficiency of antigen presentation (81, 83).

A human DEC-205 counterpart was quickly identified (84). Since that time, a plethora of uptake receptors, especially type II transmembrane proteins with a single external C-type lectin domain, have been found on different types of dendritic cells in situ. Curiously, DEC-205 is the only one at this point that has been visualized on many dendritic cells in the T cell areas of human and mouse lymphoid tissues (85). Gaelle Breton and Maggi Pack are now searching for good reagents, and, together with Paul and Klara Racz in Hamburg, we are trying to overcome the lack of information on the expression of various dendritic cell lectins in humans and nonhuman primates in situ.

The natural ligands for DEC-205 are still unknown, but we decided early on to use antibodies as surrogate ligands. Nussenzweig realized that the way to move forward was to engineer the heavy chain of the anti-DEC monoclonal antibody to express different antigens. In this way, the consequences of targeting antigens to dendritic cells could be studied in vivo without having to isolate the dendritic cells or generate them from precursors.

The first antigen that was engineered into anti-DEC-205 was a peptide from hen egg

lysozyme (HEL), which dominates the $CD4^+$ T cell response of H-2k mice. The injected antibody targeted to the dendritic cells in the T cell area, as expected from the natural expression of the corresponding antigen, but the consequence of antigen presentation provided a major surprise, which was recorded with HEL-specific transgenic T cells (86). Daniel Hawiger and Nussenzweig showed that the T cells first underwent clonal expansion, but then the T cells disappeared and the animal became specifically tolerant to HEL. Laura Bonifaz made similar findings with ovalbumin-specific $CD8^+$ T cells (87), while Kristen Tarbell, Xunrong Luo, and Sayuri Yamazaki used dendritic cells to expand and induce regulatory T cells (Tregs) specific for a beta cell autoantigen in culture (88, 89). Kang Liu targeted dying cells to dendritic cells in vivo, and again the consequence was deletion and tolerance or unresponsiveness (90). Before these discoveries, the thinking in the field had been that dendritic cells in lymphoid tissues were already in a mature immunogenic state, yet the new results led to the opposite interpretation. Dendritic cells in the steady state function in peripheral tolerance.

Antigen targeting is enabling researchers to probe the induction of antigen-specific regulatory or suppressor T cells in the intact animal. Harald von Boehmer in Boston (91), Karsten Mahnke in Heidelberg (92), and Sayuri Yamazaki in our lab (93) showed that targeting of a foreign protein to dendritic cells in vivo in the steady state allowed some $Foxp3^-$ $CD4^+$ T cells to become $Foxp3^+$. To date, however, most model systems in vivo have not involved true self antigens or large-scale induction of $Foxp3^+$ Tregs. Juliana Idoyaga is addressing these issues in ongoing experiments with an autoantigen from myelin that drives multiple sclerosis in mice. The concept that dendritic cells can bring about peripheral tolerance in vivo should lead to methods for the antigen-specific silencing of immunity, as would be desirable in allergy, autoimmunity, transplantation, and perhaps atherosclerosis.

DISTINGUISHING DENDRITIC CELL SUBSETS

Many different laboratories decided to look for additional molecular markers to identify and understand dendritic cells. A surprising result was that in the steady state, the markers were expressed by some but not all dendritic cells. For example, Ken Shortman in Melbourne found CD8 on a subset of classical dendritic cells in mouse lymphoid organs (94); Yong-Jun Liu in Dardilly with Banchereau found CD11c and CD4 on myeloid and plasmacytoid subsets, respectively, of human blood dendritic cells (95); and Joern Schmitz at the Miltenyi Corporation in Germany described blood dendritic cell antigens, BDCA-1, -2, and -3, which identify two types of myeloid dendritic cells (BDCA-1 and -3) and plasmacytoid dendritic cells (BDCA-2) (96). Curiously, these molecules have not yet been adequately represented in functional studies.

The opposite is the case for a plethora of molecules that enhance antigen uptake and presentation, which also frequently mark subsets of dendritic cells (**Figure 2**). This began with DEC-205, discussed above, which is expressed at high levels on one subset of dendritic cells in mice. Diana Dudziak with Nussenzweig found that another dendritic cell marker, identified by the first 33D1 monoclonal to dendritic cells (97), is also a lectin expressed by one type of dendritic cell (98). Juliana Idoyaga pursued langerin as a dendritic cell subset marker that can mediate uptake and presentation of antigens (98, 99). Actually, receptors for innate immunity, for both uptake (usually lectins) and signaling (usually TLR), are often expressed more on one dendritic cell subset than on another (**Figure 2**).

One complex example uncovered by Inaba in Kyoto is the preferential uptake of dying cells by the $CD8^+$ subset of dendritic cells in vivo. This uptake is followed by efficient cross-presentation to $CD8^+$ T cells. Not only are the dying infected cells processed onto MHC class I, as in the initial discovery (72), but the endogenous antigens in transformed

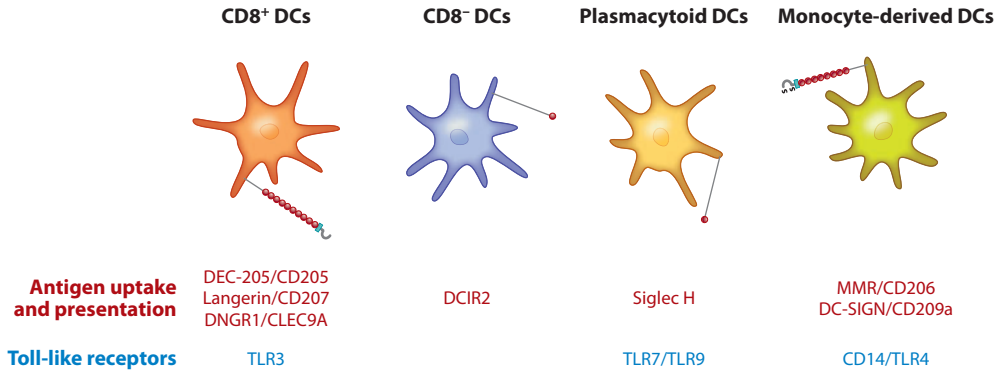

Figure 2

Different types of dendritic cells in mice and markers used to identify them. These markers are often lectins for antigen uptake, although treml4 identified by Hiroaki Hemmi (134) is an Ig superfamily member discovered in our efforts to find receptors that bind necrotic cells. In addition to innate receptors for antigen uptake and presentation (*red*), dendritic cell subsets can prioritize different innate receptors for signaling, especially Toll-like receptors (*blue*). Langerhans cells (*not shown*) are likely to be a distinct additional subset. Comparable groups of dendritic cells are found in humans, but many of the actively used markers are different from the mouse.

cells are also processed, as shown by Marion Subklewe and Christian Munz for Epstein Barr virus latency gene products (100) and by Palucka, Banchereau, and their team in Dallas studying several melanoma antigens (101). For me, a particular unknown is the handling of self and environmental antigens within dying cells to maintain peripheral tolerance. I am struck by the evidence from Gordon MacPherson in Oxford that dendritic cells are always carrying intestinal epithelial cell contents via lymphatics and on to the dendritic cells in the T cell area of the mesenteric lymph node (102). This seems like an efficient way to display the "harmless" to the immune system and bring about peripheral tolerance. Special subsets of dendritic cells may be involved.

Because of distinctions in innate receptors (**Figure 2**), one could surmise that each subset is designed to bring about rapid innate responses to the wide range of self and nonself components with which the immune system must deal. This was proposed early on by Yong-Jun Liu, who discovered that plasmacytoid dendritic cells were unique in being able to make large amounts of type I interferon in response to nonreplicating viruses (103). The raison d'être for the different dendritic cell subsets is receiving considerable attention because a more complete picture is needed, as summarized by two of the leaders in the field, William Heath and Frank Carbone in Melbourne (104). I suspect that the in vivo targeting of antigens within monoclonal antibodies to dendritic cell lectins will help decipher the function of dendritic cell subsets in intact animals and humans. Better genetic tools are being developed to deplete dendritic cells and their subsets (as reviewed in 105), but targeting allows one to assess and direct the immune system in vivo, as is needed to approach disease.

DEVELOPING DENDRITIC CELL–BASED VACCINES

Many diseases that involve the immune system often interfere with dendritic cell function, as occurs with microbial pathogens and tumors. Alternatively, the disease exploits dendritic cells, as occurs in allergy, autoimmunity, and transplantation (reviewed in 106). Paul Cameron and Melissa Pope, now Robbiani, studied HIV-1 in tissue culture and found that dendritic cells serve as a conduit to ferry virus to its major site for replication, T cells (107, 108). But the reciprocal to pathogenesis is also true: Dendritic cell science provides the means

to prevent and combat disease. This is especially the case for vaccination.

Vaccination is the route to so many medical success stories, and it depends upon the induction of antigen-specific, protective immune memory. Current vaccines largely prevent infection but not the other types of disease depicted in **Figure 1**, and they work primarily by inducing protective antibodies. Medicine now needs to discover T cell–based vaccines that enhance resistance to cancer and to infectious diseases such as AIDS and tuberculosis. Conceivably, antigen-specific Tregs can also be induced to suppress unwanted immune reactions, something we are intrigued by because of Uri Sela's findings. He showed not only that dendritic cells are special inducers of Foxp3$^+$ Tregs, but also that the latter can persist for months in vivo during the suppression of inflammatory graft-versus-host disease (109). A challenge now is to learn how to expand disease-suppressive Tregs in vivo.

We decided to enter the vast realm of vaccines twice. The first time in the 1990s stemmed from discoveries showing that antigen-loaded dendritic cells could immunize mice and, later, healthy volunteers. Initial research in cancer patients was led by Gerold and Beatrice Schuler in Erlangen (110) and Palucka, Faye, and Banchereau in Dallas (111). Other investigators, beginning with Ron Levy and Ed Engleman at Stanford with lymphoma (112), also reported that ex vivo antigen-loaded dendritic cells could immunize patients with cancer antigens (reviewed in 113, 114). However, the measured immune responses seem weak and have yet to be linked with prolonged survival. The newly licensed Dendreon Corporation's Provenge vaccine for advanced prostate cancer might be based on dendritic cell immunization to a nonmutated prostate self antigen (115), but this remains unclear.

The big obstacle to research with ex vivo–derived dendritic cells is organizational: How do we optimize two-arm studies of the many variables that lead to the induction of durable and broad anticancer immunity, and how do we gain financial support for immunotherapy? It is often written that ex vivo dendritic cell therapy is complicated from a procedural point of view. I completely disagree. The technology has advanced to the stage where machines handle the monocyte-enriched fraction derived from a leukapheresis and differentiate the monocytes into dendritic cells in very large numbers. If a single leukapheresis could lead to the preparation of several dozen effective, nontoxic vaccinations with a broad spectrum of tumor antigens, it seems unwarranted to stifle the field as being too complicated. Granted, the scientific obstacles are substantial, as reviewed by Kees Melief and Carl Figdor from Leiden and Nijmegen (116, 117), but research needs to take place with proper support and organization. This is true for the entire field of cancer immunology in people. It remains a major mystery why immune approaches to cancer are so underemphasized relative to other modalities, which immune therapies should also be able to complement, as reviewed by Laurence Zitvogel and Guido Kroemer from Paris (118).

The second time we decided to enter the vaccine realm, and where we remain active, stemmed from the above discoveries of antigen-specific uptake receptors as means to allow dendritic cells, or subsets of them, to capture vaccine antigens efficiently. Prior to the identification of DEC-205, beginning with Mary Crowley's work, relatively large doses of antigen, 100 μg or more, were being injected in vivo (119). But once foreign proteins were introduced into anti-DEC-205 monoclonal antibodies, Daniel Hawiger, Laura Bonifaz, and Christine Trumpfheller found that the antigens became highly immunogenic in low doses (86, 120–122). Enhanced immunization specifically required DEC-205. Binding of the fusion monoclonal antibody to Fc receptors was minimized by mutations introduced by longstanding colleague Jeffrey Ravetch, and the observed increase in immunity using anti-DEC fusion monoclonal antibody was abolished in DEC knockout mice. Interestingly, the introduction of proteins into a monoclonal antibody is in many cases an excellent way to manufacture defined antigens for vaccines. More

importantly, one has an opportunity to direct and harness dendritic cell science with the targeting antibody.

So far, approximately 1 μg of antibody fusion protein leads to sizeable $CD4^+$ T cell responses in mice, and cross-priming of $CD8^+$ T cells is also evident. Leonia Bozzacco made an important finding on cross-presentation, i.e., it is possible to achieve cross-presentation to $CD8^+$ T cells with DEC-205-targeted HIV gag protein in many MHC haplotypes (123); this breadth of cross-presentation will be essential for vaccination. Likewise, for cancer proteins, where we focus on nonmutated but hyperexpressed cancer antigens such as HER2 and mesothelin, Bei Wang finds that DEC-205 targeting makes it feasible for small amounts of proteins to elicit immunity, including $CD8^+$ T cell immunity or cross-priming (124; B. Wang, N. Zaidi, L.Z. He, K. Zhang, J.M.Y. Kuroiwa, T. Keler, R.M. Steinman, submitted manuscript). Our emphasis is on "one-for-all" vaccines for broad groups of cancer patients, with the goal to start the immune response effectively. Then the patient's dendritic cells, if maturing during the local killing of tumor cells, will have a chance to take over, present dying tumor cells, and spread the immune response to the plethora of mutant proteins in solid tumors.

The biggest decision currently is selecting the stimulus that needs to be delivered to bring about appropriate dendritic maturation for strong helper and killer T cell immunity. In the case of vaccines to resist cancer and infection, the dendritic cells need to be steered away from their steady-state tolerogenic functions, e.g., by a stimulus that can mimic the innate signaling that takes place during an infection. This research was given a huge boost with the definition of a new spectrum of clinically feasible innate stimuli, i.e., synthetic agonists for families of microbial recognition receptors, first by Shizuo Akira and colleagues in Osaka (125). This synthetic microbial agonist field means that one chemical compound should in principle mimic the action of a whole class of microbes, e.g., RNA viruses and gram-negative bacteria.

As a result of experiments with a consortium of investigators in Germany, including Klaus Uberla, Paul and Klara Racz, Christiane Stahl Hennig, and Ralf Ignatius, synthetic double-stranded RNA (poly IC) appeared to be promising adjuvant (126). Considerable research in mice by Paula Longhi came to the same conclusion and established that poly IC gained its potency as an adjuvant by being a strong inducer of innate interferon production (127). Marina Caskey and Sarah Schlesinger took these findings into the clinic to address some important questions together with Rafick Sékaly and colleagues in Port St. Lucie. They have found that these synthetic compounds can reliably stimulate a broad innate immune response in people and that the compounds really are microbial mimics, reproducing to a considerable extent the innate response of people receiving the successful live attenuated yellow fever vaccine (128).

Research with a new synthetic TLR4 agonist, glucosyl pyranosyl lipid A (GLA), is also underway by Longhi together with Steve Reed from Seattle (129). Different classes of adjuvants might be needed to tailor the immune response to the particular pathogen and to optimize protective immunity. A good model for protection induced by dendritic cell–targeted protein vaccines is the ongoing PhD thesis work of Ines Matos. She is using a protozoan parasite protein to show the value of dendritic cell targeting for inducing protection against a human pathogen, *Leishmania major*, that infects mice, like humans, through the skin.

The need for new vaccines based on T cell immunity is driving dendritic cell biology in an exciting way because it allows scientists to focus on directing the antigen-specific immune response in intact animals and people, including patients with cancer (117, 130). The goal is to select and guide those rare clones in Burnet's repertoire so that the clones provide the appropriate response. Transgenic T cells can provide excellent tools in this research, but we encounter instances in which the responses of transgenic T cells do not represent what one observes with the polyclonal repertoire. Here

are four examples of recent findings focused on understanding dendritic cells in the real-life context of vaccination with defined proteins and adjuvants:

1. Developing a vaccine from basic principles requires that one understand how innate immunity and adaptive immunity are linked in vivo. Most cells make responses to innate stimuli, but dendritic cells need to be engaged to gain control of adaptive immunity. Impressively, within only 4 h of administering an adjuvant like poly IC or GLA, the antigen-capturing dendritic cells have become immunogenic, able to directly immunize the T cells of a naive animal (127, 129).
2. Dendritic cells have many different receptors capable of bringing about antigen presentation. To date, Juliana Idoyaga and Christine Trumpfheller find that different receptors on the same CD8$\alpha\alpha$ dendritic cell subset in mice mediate similar T cell priming when targeted with antigen (131).
3. In a collaboration to study antigen targeting in nonhuman primates with Robert Seder at NIH, we found surprisingly that priming with a dendritic cell–targeted protein vaccine allowed the animals to make an unusually large $CD8^+$ T cell response to a boost with a replication-defective recombinant NYVAC vector (132). The NYVAC vector from Gepi Pantaleo and Mariano Esteban was itself not detectably immunogenic, even with two doses. Yet the $CD8^+$ T cell response to NYVAC was vigorous when the primate immune system had been primed with a clinical grade protein vaccine that targeted HIV gag to human DEC-205.
4. Scott Barbuto, a current PhD student in the laboratory, decided to learn to target both the antigen and innate stimulus selectively to dendritic cells. He is proving that dendritic cells alone are sufficient for initiating immunity.

Our vaccine research is being extended by physician scientists Marina Caskey, Bryan Yipp, and Niroshana Anandasabapathy to studies in healthy volunteers. The program is being directed by our clinical director, Sarah Schlesinger, with enormous help from Sarah Pollak and Lauren Sinnenberg. Our first proof-of-concept study uses HIV gag p24 as the antigen targeted within human anti-human DEC-205 monoclonal antibody; the latter is manufactured through an active collaboration with Tibor Keler at Celldex Therapeutics (133). The first adjuvant being tested is poly IC. In addition to adjuvant choice, our HIV protein vaccine research has to address some additional key gaps, such as the inclusion of HIV envelope to elicit antibody responses and the induction of immunity at mucosal surfaces.

While it is exciting to be able to pursue this research, our experience is dramatizing the powerful obstacles to obtaining financial support to learn to direct the immune system in people. Fortunately, I have had a tremendous boost from The Rockefeller University and its research hospital, the first center for research on human subjects in the United States, and also from New York City, where bright minds abound and the community takes on considerable responsibility in its support for research.

DECIDING TO STAY ON THE PATH OF VACCINE SCIENCE IN PRECLINICAL MODELS AND IN HUMAN SUBJECTS

It has been an amazing privilege to watch the progress of immunology from the early days of clonal selection and cell-mediated immunity to the present. This progress may seem overwhelming for investigators who are now beginning in the field, when they encounter hundreds of molecules with their CD numbers (in contrast there were only two markers when I began, thy-1 or theta for T cells and surface Ig for B cells), and dozens of cytokines, chemokines, cell types, signal transducers, and transcription factors. On the other hand, young scientists, the key to our future, can easily understand that

all the progress makes it easier to address the search for better prevention and treatment of conditions influenced by the immune system (**Figure 1**). The existence of these conditions says loudly and clearly that there are huge discoveries yet to be made to move forward. Our laboratory is committed to vaccines as a driving force for future discoveries.

The complexity of adaptive immunity is humbling, but at the same time it is stimulating to be part of a profession that is making great progress, particularly with the introduction of several new pharmaceuticals, mainly antibodies. My initial decision was to learn how antigen-specific immunity is initiated. It is exciting to see how dendritic cells are providing routes to the control of antigen-specific T cell immunity in its different helper, killer, and regulatory forms. In humans, this will form the basis for a myriad of future medical advances to deal with the conditions listed in **Figure 1**. It was exciting when dendritic cells appeared first as novel cells, and it remains exciting that these cells represent a novel force in medicine.

EDITOR'S NOTE

During the last four and one-half years of his life, Ralph Steinman lived with pancreatic cancer. Tragically, he succumbed to the disease just two and one-half days before receiving the Nobel Prize for Physiology or Medicine and just three weeks after submitting this manuscript.

DISCLOSURE STATEMENT

The author was on the scientific advisory boards of Argos Therapeutics and Celldex Therapeutics and has fiduciary interests in Celldex. Both companies design dendritic cell–based vaccines.

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