HOST DEFENSE, DENDRITIC CELLS AND THE HUMAN LUNG

(Het afweersysteem, dendritische cellen en de long)

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

van Haarst, Jan Maarten Willem

Host defense, dendritic cells and the human lung/
Jan Maarten Willem van Haarst; [ill.: Tar van Os]. - Rotterdam:
Erasmus Universiteit, Afdeling Immunologie. - Ill.
Thesis Rotterdam. - With ref. - With summary in Dutch.
ISBN 90-73436-22-2
NUGI 743
Subject headings: dendritic cells / airway infections / lungs

HOST DEFENSE, DENDRITIC CELLS AND THE HUMAN LUNG

HET AFWEERSYSTEEM, DENDRITISCHE CELLEN EN DE LONG

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. P.W.C. Akkermans M. A.
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 17 mei 1995 om 15.45 uur.

door

Jan Maarten Willem van Haarst

geboren te Spijkenisse

PROMOTIE-COMMISSIE:

Promotor: prof. dr. H.A. Drexhage

Co-promotor: dr. H.C. Hoogsteden

Overige leden: Prof. dr. R. Benner
Prof. dr. E.C.M. Hoefsmit
Prof. dr. H.M. Jansen

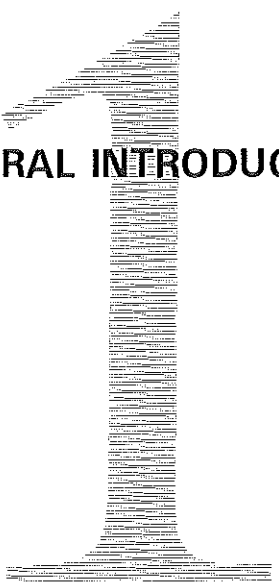
This thesis is based on collaborative studies of the departments of Immunology and Pulmonary Diseases of the Erasmus University and University Hospital Rotterdam-Dijkzigt.

The work in this thesis was financially supported by the Dutch Asthma Foundation.

The printing of this thesis was financially supported by the Dutch Asthma Foundation, the Department of Immunology of the Erasmus University, Astra Pharmaceutics B.V., Boehringer Ingelheim B.V., Bayer B.V., UCB Pharma B.V., Zambon Nederland B.V. en Ciba Geigy B.V.

Cover : Sofieke van den Hout
Illustrations : Tar van Os.
Printing : Ridderprint, Ridderkerk.

CONTENTS



GENERAL INTRODUCTION

GENERAL INTRODUCTION

Objectives

This chapter provides a short overview of host defense mechanisms in general, an introduction on the role of granulocytes, monocytes and macrophages herein, and a review on dendritic cells. Especially, the role of these cells in the lung and in pulmonary disease is emphasized. The last part of this chapter explains the aims of the investigations described in this thesis.

The immune response in a nut shell.

Host defense mechanisms protect the body against microorganisms and other foreign structures. These mechanisms can be divided in nonspecific, or innate, and specific, or acquired, immunity[1]. In both branches of immunity the several types of leukocytes (white blood cells) play a dominant role. Nonspecific defense comprises the natural barriers that protect against invading microorganisms, the complement system, and various types of white blood cells including granulocytes, monocytes, macrophages and natural killer cells that are able to neutralize microorganisms and foreign material by phagocytosis and/or killing. The natural barriers consist of the epithelial surfaces of the body, such as the skin and the bronchial epithelium. In the lung, junctional complexes between bronchial epithelial cells physically prevent the invasion of microorganisms. In addition, microorganisms and other particles are removed from the respiratory tract by the "tapis roulant": the mucus film propelled towards the oropharyngeal cavity by the cilia of the epithelial cells[2]. The mucus not only serves as vehicle for particle transport, but also contains antibacterial and antiviral proteins[3].

Specific immunity involves the recognition of foreign structures (antigens), the discrimination between self and non-self, and the generation of immunologic memory. A crucial step in the initiation of an immune response is the presentation of antigens to and the stimulation of T cells. Cells capable of antigen-presentation and the stimulation of T cells are called antigen-presenting cells (APC) and comprise monocytes, macrophages, B cells and in particular dendritic cells (DC). Recognition of the presented antigen by the T cell and the generation of costimulatory signals by the APC result in proliferation, cytokine production and a change in marker expression of the T cell. The T cells thereby regulate the activation of several effector cells such as B cells (that produce antibodies), and macrophages. These effector

mechanisms are directed to eliminate the antigen[1].

Granulocytes

Three types of mature granulocytes can be distinguished: basophilic, eosinophilic and neutrophilic granulocytes. Although basophils and eosinophils play a major role in asthma[4,5], we will here focus on the latter type of granulocytes: the neutrophil. Since neutrophilic granulocytes comprise by far the largest class of leucocytes in the blood (approximately 60%), these cells will be further referred to as granulocytes.

Like all leucocytes, neutrophils are derived from the bone marrow, where precursors proliferate and differentiate under the influence of colony-stimulating factors, most importantly granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). After release from the bone marrow, neutrophils circulate for about 10 h. in the blood, and then migrate to the tissues where they live only 1-2 days.

Neutrophil accumulation is one of the first events in an inflammatory response. Cells migrate towards the inflammatory site in response to chemotaxins, which include factors derived from bacteria (e.g. formyl-methionyl peptides), complement factors (C5a) and chemotaxins elaborated by immune cells or tissue cells (e.g. leukotriene B4, interleukin[IL]-8)[6]. Intracellular signal transduction may differ between various chemotaxin receptors triggered, but the stimulation of phospholipase C leading to the production of polyphosphoinositols and diacyl glycerol is a common pathway of chemotaxin receptor binding[7]. The activation of second messengers results in a rapid and transient polymerization of actin filaments responsible for neutrophil motility and migration towards the site of inflammation. Migration through the endothelial wall is preceded by rolling along and adherence to endothelial cells. Adherence of neutrophils to the endothelium is also enhanced by chemotactic factors via the upregulation of adhesion molecules on both cells.

Neutrophils are able to eliminate microorganisms or other particles by phagocytosis. Phagocytosis is strongly facilitated if these particles are coated with complement factors or immunoglobulins for which receptors are present on the neutrophil plasma membrane. Killing of the ingested microorganisms in the phagosome is preceded by fusion with azurophilic and specific granules that contain bactericidal proteins, by the production of reactive oxygen species and by lowering the pH in the phagosome. The

respiratory burst includes the activation of NADPH-oxidase resulting in the production of superoxide(O_2^-), from which other bactericidal oxygen radicals can be generated, especially H_2O_2, HO• and hypohalite ions via myeloperoxidase[8,9]. Extracellular degranulation also occurs, especially of specific granules and in the case of reversed endocytosis (e.g. in response to immune complexes on a solid surface)[10,11].

A normal neutrophil accumulation and function are critical for pulmonary host defense. Both severe granulocytopenia (e.g. after chemotherapy) and a deficient function (e.g. absent oxidative responses in chronic granulomatous disease) give rise to potentially fatal infections[12,13]. An increased susceptibility for pulmonary infections is also encountered in conditions with an impaired chemotactic ability of neutrophils, such as burn injuries, viral infections and in rare diseases such as the Kartagener syndrome, the Chediak Higashi syndrome and the hyperimmunoglobulin E syndrome[14,15].

G-CSF has been used successfully to restore the numbers of neutrophils and to prevent infections after chemotherapy[16]. In addition, several animal studies have shown that G-CSF might also be effective in the restoration of neutrophil function and host resistance to infections[17,18,19,20].

Although neutrophils constitute an important first line of defense against invading microorganisms, accumulation and activation of neutrophils may also cause severe tissue damage by the elaboration of proteolytic enzymes such as elastase and free oxygen radicals as for example in the adult respiratory distress syndrome and pulmonary emphysema[21,22,23,24].

Monocytes and macrophages

Monocytes are rounded cells with a horseshoe-shaped nucleus. Their cytoplasm contains myeloperoxidase, acid phosphatase and nonspecific esterase. Characteristic immunocytologic markers of monocytes are CD14, CD64 and CD68[25]. Macrophages display a marked phenotypical and functional heterogeneity. These cells are usually larger than monocytes, and they are strongly positive for acid phosphatase and nonspecific esterase. Macrophages typically express CD68, RFD7 and RFD9[26].

Monocyte production in the bone marrow is enhanced by GM-CSF and monocyte colony-stimulating factor. After migration to the tissues these cells are able to mature into macrophages and possibly into dendritic cells (see later).

Like granulocytes, monocytes and macrophages also respond to chemotactic stimuli and are equipped with the machinery for phagocytosis and killing (including the production of oxygen radicals). Beside these phagocytic functions, monocytes and macrophages are able to produce a large array of cytokines and other mediators, and these cells are able to present antigens to T cells (see below).

Two major macrophage populations can be discriminated in the lung: alveolar macrophages which reside in the alveoli and in the conducting airways and interstitial macrophages residing in the various connective tissue compartments of the lung[27,28]. Although alveolar macrophages are able to phagocytose large numbers of microorganisms, these cells have a limited bactericidal capacity and a reduced capacity to generate oxygen radicals[29,30]. This decreased potential to generate oxygen radicals is probably due to their close contact with surfactant[31]. The chemotactic ability of alveolar macrophages is also limited[32]. Although macrophages in general are capable to present antigen to and stimulate T cells, alveolar macrophages have been described to be poor T cell stimulators, and these cells are even able to suppress T cell proliferation[33].

In lung cancer as well as in other malignancies, monocyte chemotaxis is decreased[34,35,36,37,38]. This decreased monocyte chemotactic ability has been associated with the presence of p15E-like immunosuppressive factors in the serum and in malignant effusions[39]. In patients with chronic purulent infections of the upper airways a decreased monocyte chemotactic ability together with p15E-like serum factors has been described as well[40].

Dendritic cells nomenclature and definition

DC are specialized antigen-presenting cells that are able to cluster with and to stimulate (naive) T cells, in contrast to mononuclear phagocytes, DC are poorly phagocytic. DC were named after their remarkable "dendritic" morphology: an irregular outline with broad lamellipodia or veils[41]. Additional key features of DC are the expression of MHC class II molecules, and the absence or a juxtanuclear spot of acid phosphatase[42].

DC are also called leucocyte dendritic cells to distinguish them from follicular dendritic cells (FDC). Here, leucocyte refers to the bone marrow origin and the leucocyte common antigen (CD45) expression of DC. The (leucocyte) dendritic cell family comprises the Langerhans cell in the epidermis, the veiled cell in the lymph, the interdigitating cell in the paracortical areas of the

lymph node, and dendritic cells in the thymus, the spleen, the lung and in almost all organs of the body[43]. DC preferentially interact with T cells, and DC home to T cell areas in the lymphoid organs. In contrast to the leucocyte DC, FDC are $CD45^-$ and have probably a local tissue origin. FDC are exclusively present in the germinal centers of the lymph node in close contact with B cells, where these cells catch and retain immune complexes for transfer to B cells[44,45]

Morphology and marker pattern of dendritic cells

Besides the dendritic morphology described in the previous paragraph, DC characteristically show an eccentric bean-shaped or lobulated nucleus, with a nucleo-cytoplasmic ratio varying from 1:2 to 1:4. Electron microscopy reveals an electrolucent cytoplasm. The nucleus is mainly euchromatic. The cytoplasm contains smooth and rough endoplasmic reticulum, polyribosomes, a Golgi apparatus and mitochondria, and sometimes vacuoles. DC do not possess an abundant amount of (phago)lysosomes characteristic for monocytes and macrophages, but do have an endocytic apparatus including endosomes and lysosomes, which is often concentrated in a juxtanuclear position[46,47].

Langerhans cells, a subpopulation of dendritic cells present in the epidermis, are characterized by the presence of Birbeck granules[48]. These cells express CD1a. Similar cells have been described in nasal[49] and bronchial epithelium[50]. In addition, Birbeck granules have been described in veiled cells from skin-draining lymph[51] and in interdigitating cells after immune stimulation[52].

The marker pattern of DC is characterized by a strong expression of MHC class I and II molecules and adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), leucocyte function antigen 3 (LFA3), B7, CD11/CD18[53,54]. Although these markers are essential for DC function, they are not specific for DC. In addition, these cells may express RFD1, L25, CD1, S100, CD83, which are somewhat more specific for DC[55,56,57]. CD1a is a particularly interesting marker, since, outside the thymus, it is normally only expressed by epidermal LC and their homologues in the airways[58,59]. DC differ from monocytes in that CD14 expression is low or absent. Fc receptors for IgG are absent on most DC, but are expressed by Langerhans cells[60,61]. There are contradictory reports on the expression of other myeloid markers, such as CD13 and CD33. This will be discussed below.

The distinction of DC from monocytes, however, may be sometimes very difficult, because monocytes, especially after activation, may exhibit a

dendritic morphology as well[62], and dendritic cells in situ (especially in non-lymphoid organs) may express CD14, Fcγ receptors and other "macrophage" markers[63,64,65,66].

Antigen presentation

The primary function of DC is the uptake and the degradation of exogenous antigen into antigenic peptides, and subsequently the transfer of antigenic peptides bound to MHC class II molecules to the cell membrane for presentation to T cells. All nucleated cells present fragments of endogenously produced proteins via MHC class I molecules, whereas the presentation of exogenous antigens only occurs in class II positive APC.

Class I and class II MHC molecules are produced in the rough endoplasmic reticulum. Peptides derived from endogenously produced proteins are transported from the cytosol into the rough endoplasmic reticulum via the TAP1 and TAP2 transporter molecules. These small peptides (8-10 amino acids) subsequently bind to the groove of MHC class I molecules, which are then directed to the cell membrane[67]. In the rough endoplasmic reticulum, binding of endogenous peptides to MHC class II molecules is blocked by the invariant chain, which also targets the MHC class II molecule to the endocytic compartments (late endosomes and lysosomes). These compartments contain peptides derived from exogenous proteins, that have been internalized via coated pits or in a receptor-mediated fashion. After the degradation of the invariant chain, exogenous peptides bind to the groove of the MHC class II molecules, which are then transferred to the cytoplasmic membrane to present these antigenic peptides to T cells[68,69]. T cells coexpressing the CD4 molecule next to the T cell receptor interact with MHC class II - peptide complexes, whereas CD8 positive T cells interact with class I molecules. Although exogenous proteins are primarily presented by MHC class II molecules, it has been demonstrated that presentation on class I molecules may occur as well[70], and that the endosomal and the nonendosomal processing pathways are accessible for both endogenous and exogenous antigens[71].

Interactions between antigen-presenting cells and T-cells

As stated above, APC present antigenic peptides to T cells. To allow this process to take place, a close proximity of the cell membranes of the T cell and the APC is required. To establish such a contact, the adhesion molecules LFA3 and ICAM-1 on the cell membrane of the APC interact with LFA1 and CD2 respectively on the T cell[72,73]. Other adhesion molecules may play an additional role[74]. Recognition of the peptide MHC molecule complex by the

T-cell receptor is a crucial step in T-cell stimulation, but other, costimulatory signals are required as well. These signals are generated by mediators, especially cytokines such as IL-1 and IL-6, and costimulatory molecules including the adhesion molecules mentioned above, CD4 and CD8 molecules that bind to sites on MHC class II and class I molecules respectively[75], and the interaction of molecules of the B7 family on the APC with CD28 and CTLA-4 on the T cell membrane[76]. Costimulation by B7/CD28 interactions is required for resting T cells, but not for memory T cells[77]. DC are very well equipped with costimulatory molecules, but, in general, DC produce only low amounts, if any, of various cytokines[78].

T-cell stimulation results in proliferation and cytokine production. Based on the profile of cytokine production, two major T helper (Th) cell subsets can be distinguished: Th1 and Th2. Th1 cells produce predominantly IL-2, IFN-γ and TNF-β, and are the principal effectors of cell-mediated immunity against intracellular microbes and delayed-type hypersensitivity reactions. Th2 cells produce IL-4, IL-5 and IL-10, thereby promoting the production of IgE (and IgG4) by B cells and stimulating eosinophil activation[79]. In the absence of strongly polarizing signals, however, most $CD4^+$ Th cells produce a less pronounced cytokine profile, and are designated Th0. The direction of T cell differentiation may be a crucial step in the pathogenesis of many diseases. In the pathogenesis of allergy and asthma for instance, the development of Th2 responses is thought to play a central role[80]. Many factors have been suggested to influence the direction of Th differentiation into either Th1 or Th2[81]. These factors include the nature of the antigen[82], the amount of antigen, the type of APC[83,84], the presence of cytokines and other microenvironmental factors. IFN-α, TGF-β, IL-12 and IL-1RA induce differentiation towards the Th1 subset. Conversely, IL-4 and prostaglandin E2[85,86] direct T cells towards a Th2 cytokine profile. The APC might promote Th1 or Th2 differentiation by the production of cytokines or by the expression of certain molecules on the cytoplasmic membrane. Except for the type of APC (DC, macrophage or B cell), the source of the APC may be important as well. Splenic DC, for instance, have been shown to regulate Th1 versus Th2 responses differently from DC isolated from Peyer's patches[87]. The exact role of the APC and especially the DC in this process, however, remains to be elucidated.

Although there is ample of evidence that interaction between the APC and a T cell may result in T cell activation, only few data are available on the consequences of this interaction for the APC. Studies on B cells have revealed that cross-linking of MHC class II molecules, for instance during the interaction with T cells, provides a signal inducing B7 expression[88,89], and an identical mechanism of activation has been suggested for DC[76,78]. For

monocytes it has been shown that crosslinking of adhesion molecules, for instance LFA3, induces the production of the costimulatory cytokines IL-1 and TNF-α[90]. Conversely, APC can be stimulated by cytokines produced by activated T cells.

Dendritic cells versus other APC

B cells, monocytes, macrophages and dendritic cells are the classical APC. Under normal conditions most other cells lack MHC class II molecules and are unable to present exogenous antigen to T cells. However, upon activation some of these "non-classical APC" express MHC class II molecules and develop the capacity to present antigens as well. Unstimulated bronchial epithelial cells, for instance, are unable to present antigen, but acquire a low antigen-presenting capability after induction of MHC class II expression by IFN-γ[91,92]. Conversely, all regular APC constitutively express MHC class II antigens and are able to present antigen, but their capacity to stimulate T cells differs. DC have the most potent T cell stimulating (accessory) capability. Moreover, DC are able to stimulate naive, unprimed T cells, whereas other APC can only stimulate previously primed, memory T cells[93,94,95,96].

Additional functions of dendritic cells

To exert their function as specialized APCs, DC generate multiple intercellular contacts with T cells. Hereto DC are equipped with an extremely motile cytoplasmic membrane, which contains adhesion molecules for T cells[97,98]. These close contacts with T cells result in the formation of typical clusters of a DC with several T cells. Cluster formation is an essential feature of DC, and is required for the stimulation of T cells[99].

As discussed above, DC primarily present antigens to MHC class II-restricted $CD4^+$ T cells. However, DC are also potent inducers of class I restricted $CD8^+$ cytotoxic T lymphocyte responses, as has been shown for viruses and viral antigens[100,101], whereas macrophages were unable to stimulate such cytotoxic T lymphocyte responses. With respect to interactions with non T cells, DC probably only indirectly (via T cells) interact with B cells. However, for in vitro primary T dependent B cell responses, clustering of DC with T cells and B cells is required for the development of antibody-producing cells[102,103].

DC are known to be non- or poorly phagocytic, but precursors of mature lymphoid DC, such as LC, are able to phagocytize. Fresh LC are good at antigen uptake and processing, but are relatively poor in antigen

presentation. After 2 days of culture, however, phagocytic and processing capacities have been downregulated, whereas the cells have become excellent in antigen presentation and T cell stimulation[104,105]. This is in keeping with the concept of peripheral antigen uptake and processing and antigen presentation and T cell stimulation after migration into the lymph node.

Origin and kinetics of dendritic cells

The general concept on DC ontogeny and kinetics is that 1.) these cells are derived from the bone marrow; 2.) after the release from the bone marrow, DC, or their direct precursors, travel through the peripheral blood and enter the peripheral (non-lymphoid) tissues, where DC may internalize and process antigens; subsequently 3.) these cells travel through the peripheral lymph to the paracortical areas of the lymph node, where they present antigens to T cells. The exact origin of DC, however, is still controversial. Some authors propose a completely separate DC lineage in the bone marrow, whereas others suggest a myeloid origin or propose the peripheral blood monocyte as the precursor of the DC.

The first reports on the isolation of DC from human blood involved density gradients, adherence steps and overnight culture[60,46]. Because DC obtained by these procedures lacked most myelo-monocytic markers, including CD13, CD14, CD33 and Fcγ receptors, and because DC did not respond to M-CSF or G-CSF, it was suggested that DC represent a separate lineage[54]. However, overnight culture during these isolation procedures may have affected marker expression. For instance, CD14 expression is downregulated during culture[106].
In a recent paper, in which fresh blood DC were obtained by negative selection for CD3, CD19, CD14 and CD16, it was shown that these DC expressed the myeloid markers CD13 and CD33, and were dimly positive for CD14, suggesting a myeloid origin of DC[107]. The observation that precursors of DC proliferate under the influence of the myeloid growth factor GM-CSF further supports this concept[108].

In other studies, including those of our own laboratory, cells with a phenotype of DC and LC and an enhanced accessory potential compared to blood monocytes were cultured from peripheral blood monocytes, supporting the concept that the DC is a monocyte-derived cell[109,110,111]. Upon culture, monocyte CD14 expression is downregulated[102 102]. However, it has not been completely clarified whether these cells are equally potent APC as lymphoid DC isolated by others.

Several groups have tried to unravel the nature of the DC lineage by culturing DC from proliferating $CD34^+$ progenitors. CD34 is expressed by stem cells and early precursors of the myeloid and lymphoid lineages[112]. $CD34^+$ stem cells from neonatal cord blood were shown to contain DC progenitors, which proliferated in colony-forming unit-like clusters and matured to DC and monocytes under influence of a combination of GM-CSF and TNF-α, suggesting a common DC-monocyte precursor[113]. Caux and colleagues showed that $CD34^+$ progenitors from the bone marrow differentiate under the influence of GM-CSF and TNF-α into two subsets of DC. The first subset is positive for CD14 and CD1a, but does not contain Birbeck granules, whereas the second subset is CD14 negative, but expresses CD1a and contains Birbeck granules[114]. In addition to GM-CSF and TNF-α, stem cell factor (c-kit ligand) has been shown to enhance the GM-CSF and TNF-α induced differentiation of $CD34^+$ precursors into DC[115].

As discussed above, DC in the peripheral tissues, such as LC, are less distinct from monocytes than DC after isolation in vitro, since these DC often express CD14 and Fcγ receptors, commonly associated with cells from the monocyte lineage. Therefore, it has been suggested that LC are derived from monocytes[116]. In support of this hypothesis, the LC marker CD1a can be induced on PBM by GM-CSF[117], and in the blood of burn patients $CD1a^+$ cells have been described that coexpress the monocyte marker CD14[118].

In conclusion, DC are myeloid cells, closely related to cells of the monocyte-macrophage lineage and possibly part of it. However, the exact relationship between DC and the monocyte lineage remains to be established.

Dendritic cells in the lung

In the mouse and the human lung, dendritic cells were first described by Sertl[119]. Holt described the intraepithelial DC in the human bronchus as a tightly meshed network, and hypothesized an important function as an intraepithelial trap for inhaled antigens[120].

Purification of DC from rat and human lung showed that these pulmonary DC are potent antigen-presenting cells, whereas AM are able to suppress T cell stimulation[33,121,122]. Nicod later described an improved isolation method, based on autofluorescence, for the purification of DC from minced and digested human lung tissue[123]. These cells were shown to produce only low quantities of IL-1α, IL-1ß and TNFα compared to PBM and AM, and these DC did not require these cytokines for the stimulation of T cells[124]. These pulmonary DC expressed high levels of adhesions molecules, which were shown to be crucial for their capability to stimulate T cells[125].

Pulmonary DC, however, are not a homogenous population of cells. Several animal studies reported a marked heterogeneity in marker expression as well as in function[126,127,128]. Moreover, differences were reported associated to their anatomical location: intraepithelial DC were shown to share many features with epidermal Langerhans cells, whereas parenchymal DC showed the characteristics of more mature DC[129].

Only few data are available on the ontogeny of the DC system in the lung. One study in the newborn rat reported a virtual absence of MHC class II-expression on airway DC, and it was suggested that this immaturity of airway DC might contribute to the increased susceptibility to infections and elevated risk of the induction of allergic disease in infancy[130]. In contrast, two other studies showed the appearance of MHC class II-positive DC at day 16 of gestation that slowly increased and reached adult levels 1-3 weeks after birth[131,132].

The density, distribution and phenotype of airway DC populations in the adult rat was shown to be reflected by the level of stimulation provided by inhalation of irritant stimuli[133]. In humans, cigarette smoking also increased the numbers of DC/LC in the bronchoalveolar lavage fluid and in lung tissue[134,135]. Inhalation of LPS or moraxella catarrhalis induced a rapid recruitment of MHC class II-positive DC precursors into the airway epithelium. Peak levels of MHC class II positive cells were attained at 2 h. after inhalation. After 24 h. these cells had developed into morphologically typical DC. Within 2 to 3 days most of the DC then migrate into the draining lymph node[136,137]. Rat splenic DC when instilled intratracheally, also have been shown to migrate into the paracortical areas of the draining lymph node to elicit primary T-cell responses[138,139]. The mechanisms regulating DC influx have not been fully elucidated, but may include the production of chemotactic factors by bronchial epithelial cells and the release of chemotactic neuropeptides by autonomic nerve fibers. In the rat lung, a close proximity was observed between substance-P containing nerve fibers and DC. Moreover substance-P was shown to be chemotactic for DC[140]. Human bronchial epithelial cells produce chemotactic factors in diseases such as asthma and in response to stimuli like cigarette smoke, endotoxin and histamine. These factors include chemotaxins (e.g. LTB4, IL-8, MCP-1, GM-CSF) for neutrophils, eosinophils, monocytes and probably also for DC[141,142,143,144,145]. Of these factors especially GM-CSF has been proposed as an important local factor for the recruitment and differentiation of DC in the lung[146]. Besides epithelial cells, macrophages may also suppress or augment DC function[147,148]. It is not known how migration from the bronchus to the lymph nodes is induced. In the cutaneous immune response, however, TNF-α was suggested to provide a stimulus for LC

migration to the lymph node[149].

DC in lung disease

Langerhans cell histiocytosis comprises a group of diseases characterized by a local or generalized hyperplasia of cells with the features of TLC. This group of disorders encompasses histiocytosis X, Hand-Schüller-Christian syndrome, Letterer-Siwe disease, eosinophilic granuloma of the bone and self-healing reticulo-histiocytosis[150]. The nature of this group of diseases has long been elusive, but a recent study suggests that all forms of LC histiocytosis are based on a clonal proliferation of LC, and that this group of diseases should be considered as a neoplastic disease with variable biologic behavior[151].

Considering the role of DC/LC in allergic diseases, the numbers of LC in the skin in atopic dermatitis and in the nasal mucosa of patients with allergic rhinitis have been found to be increased, and these LC displayed more surface IgE and Fc receptors for IgE[152,153,154,155,156]. The numbers of LC were shown to be higher during the grass-pollen season, and after allergen provocation there was a rapid threefold increase in epithelial LC and later (at 24 h.) also in the lamina propria[157,158]. Such studies have not been performed in asthma, however, increased numbers of RFD1$^+$ cells (a marker for DC) in the bronchial mucosa have been reported, which decreased during treatment with inhaled corticosteroids[159]. In allergic rhinitis, corticosteroid spray also reduced the numbers of LC[160]. Similar studies in the rat show that glucocorticoid administration inhibits the influx of DC in the airway wall[161]. Studies on monocytes and on epidermal LC suggest that glucocorticoids downregulate the T cell stimulatory potential of these cells[162,163,164].

The functional role of DC/LC in allergic inflammation has not been completely established. The resistance to eosinophilic airway inflammation in MHC class II-deficient mice, however, indicates the requirement of APC - T cell interactions in the pathogenesis of this condition[165]. In some experimental conditions DC and LC have been shown to promote preferentially T helper 2 responses[166,167,168], and might thereby a factor in the development of allergy and asthma. IgE on DC/LC may facilitate antigen presentation and trigger ongoing IgE synthesis[169,170]. In atopic dermatitis, IgE$^+$ LC have been demonstrated to promote strongly T cell responses to house dust allergen, whereas IgE$^-$ LC from either patients or controls did not[171].

Besides a role in allergic diseases, DC have been implicated in the

pathogenesis of bronchiolitis obliterans associated with chronic rejection of lung allografts. The infiltration of recipient DC together with $CD4^+$ T cells in the graft is an early event in the pathogenesis of this condition, possibly associated with the stimulation of alloreactive T cells by DC[172,173,174]. An increased infiltration of DC (together with macrophages and T cells) has also been described in bronchiectasis[175,176].

In several types of human cancer, the infiltration of DC/LC in the primary tumor correlated with an improved survival and suppressed metastatic spread[177]. Such a favorable correlation with survival rates has also been described for the infiltration of DC in lung carcinoma[178]. DC are thought to play a role in the immunosurveillance against tumor cells. In animal studies, the ability of DC has been demonstrated to present tumor-associated antigens and to stimulate tumor-specific T cells[179,180]. In contrast to DC/LC, the extent of macrophage infiltration in malignant tumor is not associated with a prolonged survival. Although macrophages may display anti-tumor cytotoxicity and stimulate lymphocytes, the production of growth factors by these cells may enhance tumor growth[181,182].

Aims of the investigations described in this thesis

PART ONE

Since DC are equipped with unique properties to present antigens to and to stimulate (naive) T cells, these cells are thought to play an essential role in the initiation (and in the propagation) of immune responses and thereby in the pathogenesis of many lung diseases. To extend the knowledge and our understanding of the role of DC in the normal immune response in the lung, we have tried to define the immunophenotype, distribution, cytokine production, accessory cell function and sensitivity for glucocorticoids of populations of pulmonary DC, and we compared these cells with alveolar macrophages from human lung (chapters 2 to 5).

In chapter 2 we investigated the distribution and immunophenotype of mononuclear phagocytes and DC in "normal" lung tissue from patients undergoing lobectomy or pneumectomy.

In chapter 3 a separation method for cells from the bronchoalveolar lavage (BAL) based on autofluorescence is described. With this method, we were able to obtain two distinct populations of cells: the high autofluorescent (HAF) cells were typical AM, and the low autofluorescent (LAF) cells

displayed features of DC, but also showed some monocyte characteristics.

In chapter 4 we examined the effects of glucocorticoids on immunophenotype and function of dendritic LAF cells isolated from the human BAL.

In chapter 5 we addressed the question whether LAF cells isolated from the BAL are able to produce IL-1, IL-6 and TNF-α and to what extent these cytokines contribute to their function as APC. Moreover, we investigated whether subpopulations of LAF cells differed in the ability to produce these cytokines.

PART TWO

Patients with chronic recurrent purulent infections of the lower airways constitute a significant clinical problem, since 1. the infections do not resolve or quickly relapse despite appropriate antibiotic therapy, 2. such patients regularly present at all clinics, and 3. the cause of the susceptibility for these infections is often elusive, and hence treatment can be only symptomatic. In chapter 6 and 7 we studied the function of peripheral blood monocytes and granulocytes (chemotactic responsiveness and superoxide production) in these patients.

REFERENCES

1. Kuby, J. 1992. Overview of the immune system. *In* Immunology. W.H. Freeman and company, New York. 1-19.
2. Rennard, S.I., J.D. Beckmann and R.A. Robbins. 1991. Biology of airway epithelial cells. *In* The Lung: scientific foundations. Crystal, R.G., J.B. West et al. eds. Raven Press Ltd. New York. 157-167.
3. Rogers, D.F. 1994. Airway goblet cells: responsive and adaptable frontline defenders. *Eur. Respir. J.* 7:1690-1706
4. Bousquet, J., P. Chanez, J.Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Goddard and F-B. Michel. 1990. Eosinophilic inflammation in asthma. *N. Engl. J. Med.* 323:1033-1039.
5. MacGlashan, D.W. and L.M. Lichtenstein. 1991. Basophils. *In* The Lung: scientific foundations. Crystal, R.G., J.B. West et al. eds. Raven Press Ltd. New York. 591-600.
6. Senior, R.M. and C.C. Daughaday. 1991. Chemotaxis. *In* The Lung: scientific foundations. Crystal, R.G., West, J.B. et al. eds. Raven Press Ltd. New York. 131-139.
7. Snyderman, R. and R.J. Uhing. 1988. Phagocytic cells: stimulus response coupling mechanisms. *In* Gallin, J.I., I.M. Goldstein and R. Snyderman eds. Inflammation: basic principles and clinical correlates. Raven Press, New York. 309-324.
8. Babior, B.M. 1978. Oxygen-dependent microbial killing by phagocytes. *N. Engl. J. Med.* 298:659-668.

9. Babior, B.M. 1978. Oxygen-dependent microbial killing by phagocytes. *N. Engl. J. Med.* 298:721-725.
10. Abramson, S.L., H.L. Malech and J.I. Gallin. 1991. Neutrophils. *In* The Lung: scientific foundations. Crystal, R.G., J.B. West et al. eds. Raven Press Ltd. New York. 553-563.
11. Goldstein, I.M. 1987. Granulocytes. *In* Basic and Clinical Immunology. Sites, D.P., J.D. Stobo and J.V. Wells eds. Appleton & Lange, East Norwalk, CT. 106-113.
12. Holroyd, K.J. and R.G. Crystal. 1991. Deficiency in host defense. *In* The Lung: scientific foundations. Crystal, R.G., J.B. West et al. eds. Raven Press Ltd. New York. 1913-1924.
13. Infections and immunity. 1993. G. Virella. *In* Introduction to medical immunology. G. Virella, editor. Marcel Dekker, Inc. New York. 233-250.
14. Gallin, J.I. 1980. Disorders of phagocyte chemotaxis. *Ann. Intern. Med.* 92:520-538.
15. Abramson, J.S. and E.L. Mills. 1988. Depression of neutrophil function induced by viruses and its role in secondary microbial infections. *Rev. Infect. Dis.* 10:326-341.
16. Crawford, J., H. Ozer, R. Stoller, D. Johnson, G. Lyman, I. Tabbara, M. Kris, J. Grous, V. Picozzi, G. Rausch et al. 1992. Reduction by granulocyte colony-stimulating factor of fever and neutropenia induced by chemotherapy in patients with small-cell lung cancer. *N Engl. J. Med.* 325:164-170.
17. Sartorelli, K., G.M. Silver and R.L. Gamelli. 1991. The effect of granulocyte colony-stimulating factor (G-CSF) upon burn-induced defective neutrophil chemotaxis. *J. Trauma* 31:523-530.
18. Nelson, S., C. Mason, G. Bagby and W. Summer. 1994. The contrasting effects of anti-tumor necrosis factor antibody and granulocyte colony-stimulating factor. *Am. J. Respir. Crit. Care Med.* 149:A872.
19. Abramson, J.S. and H.R. Hudnor. 1994. Effect of priming polymorphonuclear leukocytes with cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF] and G-CSF) on host resistance to Streptococcus pneumoniae in chinchillas infected with influenza A virus. *Blood* 83:1929-1934.
20. Hebert, J.C., M. O'Reilly and R.L. Gamelli. 1990. Protective effect of recombinant human granulocyte colony-stimulating factor against pneumococcal infections in splenectomized mice. *Arch. Surg.* 125:1075-1078.
21. Weiland J.E., W.B. Davis, J.F. Holter, J.R. Mohammed, P.M. Dorinsky and J.E. Gadek. 1986. Lung neutrophils in the adult respiratory distress syndrome: clinical and pathophysiological significance. *Am. Rev. Respir. Dis.* 133:218-225.
22. McGowan, S.E. and G.W. Hunnighake. 1989. Neutrophils and emphysema. *N. Engl. J. Med.* 321:968-970.
23. Janoff, A. 1983. Biochemical links between cigarette smoking and pulmonary emphysema. *J. Appl. Physiol.* 55:285-293.
24. Hogg, J.C. 1991. Neutrophil traffic. *In* The Lung: scientific foundations. Crystal, R.G., J.B. West et al. eds. Raven Press Ltd. New York. 565-579.
25. Beelen, R.H.J., H.J. Bos, E.W.A. Kamperdijk and E.C.M. Hoefsmit. 1989. Ultrastructure of monocytes and macrophges. *In* Human Monocytes. Zembala, M. and G.L. Asherson eds. Academic Press Ltd. 7-16.
26. Stachura, J. 1989. Cytochemistry of monocytes and macrophages. *In* Human Monocytes. Zembala, M. and G.L. Asherson eds. Academic Press Ltd. 28-36.
27. Lohmann-Matthes, M-L., C. Steinmüller and G. Franke-Ullmann. 1994. Pulmonary macrophages. *Eur. Respir. J.* 7:1678-1689.
28. Herscowitz, H.B. 1985. In defense of the lung: paradoxical role of the pulmonary alveolar macrophage. *Ann. Allergy* 55:634-648.
29. Hoidal, J.R., D. Schmeling and P.K. Peterson. 1981. Phagocytosis, bacterial killing and metabolism by purified human lung phagocytes. *J. Infect. Dis.* 144:61-71.
30. Fals, A.O.S. and Z.A. Cohn. 1986. The alveolar macrophage. *J. Appl. Physiol.* 60:353-

369.

31. Geerstma, M.F., H.R. Broos, M.T. van Barselaar, P.H. Nibbering and R. van Furth. 1993. Lung surfactant suppresses oxygen-dependent bactericidal functions of human blood monocytes by inhibiting the assembly of the NADPH oxidase. *J. Immunol.* 150:2391-2400.
32. Beaty, C.D., T.R. Martin and C.B. Wilson. 1991. Signal transduction in human alveolar macrophages: diminished chemotactic response to FMLP correlates with a diminished density of G_i proteins and FMLP receptors. *Am. J. Respir. Cell Mol. Biol.* 5:87-92.
33. Holt, P.G., A. Degebrodt, C. O'Leary, K. Krska and T. Plozza. 1985. T-cell activation by antigen-presenting cells from lung tissue digests: suppression by endogenous macrophages. *Clin. Exp. Immunol.* 62:586-593.
34. Nielsen, H., J. Bennedsen and P. Dombernowsky. 1982. Normalization of defective monocyte chemotaxis during chemotherapy in patients with small cell anaplastic carcinoma of the lung. *Cancer Immunol. Immunother.* 14:13-15.
35. Nielsen, H., J. Bennedsen, S.O. Larsen, P. Dombernowsky and K. Viskum. 1982. A quantitative and qualitative study on blood monocytes in patients with bronchogenic carcinoma. *Cancer Immunol. Immunother.* 13:93-97.
36. Snyderman, R., L. Meadows, W. Holder and S. Wells. 1978. Abnormal monocyte chemotaxis in patients with breast cancer: evidence of a tumor-mediated effect. *J. Natl. Cancer Inst.* 60:737-740.
37. Kjeldsberg, C.R. and G.D. Pay. 1978. A qualitative and quantitative study of monocytes in patients with malignant solid tumors. *Cancer* 41:2236-2241.
38. Kay, A.B. and J.G. McVie. 1977. Monocyte chemotaxis in bronchial carcinoma and cigarette smokers. *Br. J. Cancer* 36:461-466.
39. Cianciolo, G., J. Hunter, J. Silva, J.S. Haskill and R. Snyderman. 1981. Inhibitors of monocyte responses to chemotaxins are present in human cancerous effusions and react with monoclonal antibodies to the p15(E) structural protein of retroviruses. *J. Clin. Invest.* 68:831-844.
40. Plassche-Boers, E.M. van de, M.Tas, M. de Haan-Meulman, M. Kleingeld and H.A. Drexhage. 1988. Abnormal monocyte chemotaxis in patients with chronic purulent rhinosinusitis: an effect of retroviral p15E-related factors in serum. *Clin. Exp. Immunol.* 73:348-354.
41. Steinman, R.M. and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. *J. Exp. Med.* 137:1142-1162.
42. Steinman, R.M. and M.C. Nussenzweig. 1980. Dendritic cells: features and functions. *Immunological Rev.* 53:127-147.
43. Hoefsmit, E.C.M., J.M.S. Arkema, M.G.H. Betjes, C.E.G. Havenith, E. van Vught, R.H.J. Beelen and E.W.A. Kamperdijk. 1993. Heterogeneity of dendritic cells and nomenclature. *Adv. Exp. Med. Biol.* 329:481-486.
44. Tew, J.G. 1993. Follicular dendritic cells and dendritic cell nomenclature. *Adv. Exp. Med. Biol.* 329:467-468.
45. Knight, S.C. and A.J. Stagg. 1993. Antigen-presenting cell types. *Curr. Opin. Immunol.* 5:374-382.
46. Voorhis, W.C. van, L.S. Hair, R.M. Steinman and G. Kaplan. 1982. Human dendritic cells. Enrichment and characterization from peripheral blood. *J. Exp. Med.* 155:1172-1187.
47. Kamperdijk, E.W.A., E. van Vught, C.D. Richters and R.H.J. Beelen. 1994. Morphology of dendritic cells. *In* Immunopharmacology of macrophages and other antigen-presenting cells, Academic Press Ltd. pp.45-61.
48. Birbeck, M.S., A.S. Breathnach and J.D. Everall. 1961. An electron microscopic study of basal melanocytes and high level clear cells (Langerhans cells) in vitiligo. *J. Invest. Dermatol.* 37:51-64.

49. Fokkens, W.J., D.M. Broekhuis-Fluitsma, E. Rijntjes, T.M. Vroom and E.C.M. Hoefsmit. 1991. Langerhans cells in nasal mucosa of patients with grass-pollen allergy. *Immunobiol.* 182:135-142.
50. Richard, S., S. Barbey, A. Pfister, P. Scheinmann, F. Jaubert and C. Nezelof. 1987. Mise en evidence de cellules de Langerhans dans l'epithelium bronchique humain. *CR. Acad. Sci. Paris* T305 serie III:35-39.
51. Knight, S.C. 1984. Veiled cells - <<dendritic cells>> of the peripheral lymph. *Immunobiol.* 168:349-361.
52. Kamperdijk, E.W.A., M.L. Kapsenberg, M. van den Berg and E.C.M. Hoefsmit. 1985. Characterization of dendritic cells, isolated from normal and stimulated lymph nodes of the rat. *Cell Tissue Res.* 242:469-474.
53. King, P.D. and D.R. Katz. 1990. Mechanisms of dendritic cell function. *Immunol. Today* 11:206-211.
54. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* 9:271-296.
55. Poulter, L.W., D.A. Campbell, C. Munro and G. Janossy. 1986. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scan. J. Immunol.* 24:351-357.
56. Kabel, P.J., M. de Haan, R.D. van der Gaag and H.A. Drexhage. 1987. Intrathyroidal dendritic cells. *J. Clin. Endocrin. Metab.* 66:199-207.
57. Barclay, A.N., M.L. Birkeland, M.H. Brown, A.D. Beyers, C. Somoza and A.F. Williams. 1993. CD1. *In* The leukocyte antigen facts book. Academic Press, London. 102-103.
58. Fithian, E., P.Kung, G. Goldstein, M. Rubenfeld, C. Fenoglio and R. Edelson. 1981. Reactivity of Langerhans cells with hybridoma antibody. *Proc. Natl. Acad. Sci. USA* 78:2541-2544.
59. Murphy, G.F., A.K. Bhan, S. Sato, T.J. Harrist and M.C. Mihm. 1981. Characterization of Langerhans cells by the use of monoclonal antibodies. *Lab. Invest.* 45:465-468.
60. Kamperdijk, E.W.A., E. van Vught, C.D. Richters and R.H.J. Beelen. 1994. Morphology of dendritic cells. *In* Immunopharmacology of macrophages and other antigen-presenting cells, Academic Press Ltd. pp.45-61.
61. Metlay, J.P. and R.M. Steinman. 1989. Control of the immune response at the level of antigen-presenting cells: comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* 47:45-116.
62. Knight, S.C., J. Farrant, A. Bryant, A.J. Edwards, S. Burman, A. Lever, J. Clarke and A.D.B. Webster. 1986. Non-adherent, low-density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. *Immunol.* 57:595-603.
63. Knight, S.C. and A.J. Stagg. 1993. Antigen-presenting cell types. *Curr. Opinions Immunol.* 5:374-382.
64. Spry, C.J., A.J. Pflug, G. Janossy and J.H. Humphrey. 1980. Large mononuclear (veiled) cells with <<Ia-like>> membrane antigens in human afferent lymph. *Clin. Exp. Immunol.* 39:750-755.
65. Stingl, G., E.C. Wolff-Schreiner, W.J. Pichler, G.F. Gschnait, W. Knapp and K. Wolff. 1977. Epidermal Langerhans cells bear Fc and C3 receptors. *Nature* 268:245-246.
66. Franklin, W.A., D.Y. Mason, K. Pulford, B. Falini, E. Bliss, K.C. Gatter, H. Stein, L.C. Clarke and J.O'D. McGee. 1986. Immunohistological analysis of human mononuclear phagocytes and dendritic cells by using monoclonal antibodies. *Lab. Invest.* 54:322-335.
67. Monaco, J.J. 1992. A molecular model of MHC class-I-restricted antigen processing. *Immunol. Today* 13:173-178.
68. Neefjes, J.J. and H.L. Ploegh. 1992. Intracellular transport of MHC class II molecules. *Immunol. Today* 13:179-183.

69. Harding, C.V. 1993. Cellular and molecular aspects of antigen processing and the function of class II MHC molecules. *Am. J. Respir. Cell Mol. Biol.* 8:461-467.
70. Rock, K.L., L. Rothstein, S. Gamble and C. Fleischacker. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J. Immunol.* 150:438-446.
71. Jin, Y., J.W.K. Shih and I. Berkower. 1988. Human T cell response to the surface antigen of hepatitis B virus (HBsAg). *J. Exp. Med.* 168:293-306.
72. Böhmig, G.A., J. Kovarik, W. Holter, E. Pohanka and G.J. Zlabinger. 1994. Specific downregulation of proliferative T cell alloresponsiveness by interference with CD2/LFA-3 and LFA-1/ICAM-1 in vitro. *J. Immunol.* 152:3720-3728.
73. Teunissen, M.B.M., H.A.H. Rongen and J.D. Bos. 1994. Function of adhesion molecules lymphocyte function-associated antigen-3 and intercellular adhesion molecule-1 on human epidermal Langerhans cells in antigen-specific T cell activation. *J. Immunol.* 152:3400-3409.
74. Dustin, M. and T.A. Springer. 1991. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu. Rev. Immunol.* 9:27-66.
75. Collins, T.L., P.D. Kassner, B.E. Bierer and S.J. Burakoff. 1994. Adhesion receptors in lymphocyte activation. *Current Opinion Immunol.* 6:385-393.
76. Allison, J.P. 1994. CD28-B7 interactions in T cell activation. *Current Opinion Immunol.* 6:414-419.
77. Azuma, M., M. Cayabyab, D. Buck, J.H. Philips and L.L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. *J. Exp. Med.* 175:353-360.
78. Hart, D.N.J. and V.L. Calder. 1994. Human dendritic cells function and cytokine production. *In* Immunopharmacology of macrophages and other antigen-presenting cells, eds. C.A.F.M. Bruijnzeel-Koomen and E.C.M. Hoefsmit, Academic Press Ltd. London pp 63-91.
79. Mosmann, T.R. and R.L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* 7:145-173.
80. Anderson, G.P. and A.J. Coyle. 1994. T_H2 and 'T_H2-like' cells in allergy and asthma: pharmacological perspectives. *TiPS* 15:324-332.
81. Romagnani, S. 1994. Human Th1 and Th2 subsets: "eppur si muove!" *Eur. Cytokine Netw.* 5:7-12.
82. Del Prete, G.F., M. De Carli, C. Mastromauro, D. Macchia, R. Biagiotti, M. Ricci and S. Romagnani. 1991. Purified protein derivate of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J. Clin. Invest.* 88:346-350.
83. De Becker, G., T. Sornasse, N. Nabavi, H. Bazin, F. Thielemans, J. Urbain, O. Leo and M. Moser. 1994. Immunoglobulin isotype regulation by antigen-presenting cells in vivo. *Eur. J. Immunol.* 24:1523-1528.
84. Hsieh, C-S., A.B. Heimberger, J.S. Gold, A. O'Garra and K.M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an *αβ* T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci. USA* 89:6065-6069.
85. Snijdewint, F.G.M., P. Kalinski, E.A. Wierenga, J.D. Bos and M.L. Kapsenberg. 1993. Prostaglandin E_2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J. Immunol.* 150:5321-5329.
86. Betz, M. and B.S. Fox. 1991. Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J. Immunol.* 146:108-113.
87. Everson, M.P., W.J. Koopman, J.R. McGhee and K.W. Beagly. 1994. Dendritic cells regulate development of alloantigenic and mitogenic Th1 versus Th2 responses. *In*

Dendritic cells in fundamental and clinical Immunology, in press.

88. Nabavi, N., G.J. Freeman, A. Gault, D. Godfrey, L.M. Nadler and L.H. Glimcher. 1992. Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. *Nature* 360:266-268.

89. Cambier, J.C., M.K. Newell, L.B. Justement, J.C. McGuire, K.L. Leach and Z.Z. Chen. 1987. Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. *Nature* 327:629-632.

90. Webb, D.S.A., Y. Shimizu, G.A. van Seventer, S. Shaw and T.L. Gerrard. 1990. LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. *Science* 249:1295-1297.

91. Kalb, T.H., M.T. Chuang, Z. Marom and L. Mayer. 1991. Evidence for accessory cell function by class II MHC antigen-expressing airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 4:320-329.

92. Mezzetti, M., M. Soloperto, A. Fasoli and S. Mattoli. 1991. Human bronchial epithelial cells modulate CD3 and mitogen-induced DNA synthesis but function poorly as antigen-presenting cells compared to pulmonary macrophages. *J. Allergy Clin. Immunol.* 87:930-938.

93. Fuchs, E.J. and P. Matzinger. 1992. B cells turn off virgin but not memory T cells. *Science* 258:1156-1159.

94. Croft, M., L.M. Bradley and S.L. Swain. 1994. Naive versus memory CD4 T cell response to antigen. *J. Immunol.* 152:2675-2685.

95. Inaba, K. and R.M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717-1735.

96. Ronchese, F. and B. Hausmann. 1993. B lymphocytes in vivo fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. *J. Exp. Med.* 177:679-690.

97. Drexhage, H.A., J.W. Lens, J. Cvetanov, E.W.A. Kamperdijk, R. Mullink and B.M. Balfour. 1980. Veiled cells resembling Langerhans cells. *In* Mononuclear Phagocytes, R. van Furth, ed. Martinus Nijhoff. The Hague, the Netherlands. pp. 235-272.

98. Freudenthal, P.S. and R.M. Steinman. 1990. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc. Natl. Acad. Sci. USA* 87:7698-7702.

99. Austyn, J.M., D.E. Weinstein and R.M. Steinman. 1988. Clustering with dendritic cells precedes and is essential for T-cell proliferation in a mitogenesis model. *Immunology* 63:691-696.

100. Nair, S., J.S. Babu, R.G. Dunham, P. Kanda, R.L. Burke and B.T. Rouse. 1993. Induction of primary antiviral cytotoxic, and proliferative responses with antigens administered via dendritic cells. *J. Virol.* 67:4062-4069.

101. Macatonia, S.E., P.M. Taylor, S.C. Knight and B.A. Askonas. 1989. Primary stimulation by dendritic cells induces antiviral proliferation and cytotoxic T cell responses in vitro. *J. Exp. Med.* 169:1255-1264.

102. Inaba, K., M.D. Witmer and R.M. Steinman. 1984. Clustering of dendritic cells, helper T cells and histocompatible B cells during primary antibody responses in vitro. *J. Exp. Med.* 160:858-876.

103. Inaba, K., A. Granelli-Piperno and R.M. Steinman. 1983. Dendritic cells induce T lymphocytes to release B cell-stimulating factors by an interleukin 2-dependent mechanism. *J. Exp. Med.* 158:2040-2057.

104. Romani, N. and G. Schuler. 1989 Structural and functional relationships between epidermal Langerhans cells and dendritic cells. *Res. Immunol.* 140:895-898.

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

106. Hal, P.Th.W. van, H.C. Hoogsteden, A.A. te Velde, J.M. Wijkhuis, C.G. Figdor and C.

Hilvering. 1990. Induction of a macrophage-like phenotype on peripheral blood monocytes by interleukin 4 is inhibited by glucocorticoids. *In* Heterogeneity of mononuclear phagocytes in interstitial lung disease. H.C. Hoogsteden, thesis. Rotterdam.

107. Thomas, R., L.S. Davis and P.E. Lipsky. 1993. Isolation and characterization of human peripheral blood dendritic cells. *J. Immunol.* 150:821-834.

108. Inaba, K., R.M. Steinman, M. Witmer-Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* 175:1157-1167.

109. Kabel, P.J., M. de Haan-Meulman, H.A.M. Voorbij, M. Kleingeld, E.F. Knol and H.A. Drexhage. 1989. Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator-purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation. *Immunobiol.* 179:395-411.

110. Peters, J.H., J. Ruppert, R.K.H. Gieseler, H.M. Najar and H. Xu. 1990. Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage? *Pathobiology* 59:122-126.

111. Rossi, G. 1992. Development of a Langerhans cell phenotype from peripheral blood monocytes. *Immunol. Lett.* 31:189-198.

112. Andrews, R.G., J.W. Singer and I.D. Bernstein. 1986. Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. *Blood* 67:842-845.

113. Santiago-Schwarz, F., E. Belilos, B. Diamond and S.E. Carsons. 1992. TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J. Leukoc. Biol.* 52:274-281.

114. Caux, C., C. Massacrier, B. Vandervliet, B. Dubois, C. Dezutter-Dambuyant, D. Schmitt and J. Banchereau. 1994. Characterization of human $CD34^+$ derived dendritic / Langerhans cells (DLC). *Dendritic cells in fundamental and clinical immunology*, in press.

115. Young, J.W., P. Szabolcs, E.D. Feller and M.A.S. Moore. 1994. Large numbers of immunostimulatory dendritic cells can be generated from $CD34^+$ bone marrow progenitors under the aegis of c-kit ligand, GM-CSF and TNF-*a*. *In* Dendritic cells in fundamental and clinical immunology, in press.

116. Hoefsmit, E.C.M., A.M. Duijvestijn and E.W.A. Kamperdijk. 1982. Relationship between Langerhans cells, veiled cells and interdigitating cells. *Immunobiol.* 161:255-265.

117. Kasinrerk, W., T. Baumruker, O. Majdic, W. Knapp and H. Stockinger. 1993. CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 150:579-584.

118. Gothelf, Y., D. Hanau, H. Tsur, N. Sharon, E. Sahar, J-P. Cazenave and E. Gazit. 1988. T6 positive cells in the peripheral blood of burn patients: are they the Langerhans cell precursors? *J. Invest. Dermatol.* 90:142-148.

119. Sertl, K., T. Takemura, E. Tschachler, V.J. Ferrans, M.A. Kaliner, and E.M. Shevach. 1986. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma and visceral pleura. *J. Exp. Med.* 163:436-451.

120. Holt, P.G., M.A. Schon-Hegrad, M.J. Philips and P.G. McMenamin. 1989. Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* 19:597-601.

121. Rochester, C.L., E.M. Goodell, J.K. Stoltenberg and W.E. Bowers. 1988. Dendritic cells from rat lung are potent accessory cells. *Am. Rev. Respir. Dis.* 138:121-128.

122. Nicod, L.P., M.F. Lipscomb, J.C. Weissler, C.R. Lyons, J. Albertson and G.B. Toews. 1987. Mononuclear cells in human lung parenchyma. Characterization of a potent accessory cell not obtained by bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 136:818-823.

123. Nicod, L.P., M.F. Lipscomb, G.B. Toews and J.C. Weissler. 1989. Separation of potent and poorly functional human lung accessory cells based on autofluorescence. *J. Leukoc. Biol.* 45:458-465.

124. Nicod, L.P., B. Galve-de Rochemonteix and J-M. Dayer. 1990. Dissociation between allogenic T cell stimulation and interleukin-1 or tumor necrosis factor production by human dendritic cells. *Am. J. Respir. Cell Mol. Biol.* 2:512-522.

125. Nicod, L.P. and F. El Habre. 1992. Adhesion molecules on human lung dendritic cells and their role in T-cell activation. *Am. J. Respir. Cell Mol. Biol.* 7:207-213.

126. Xia, W., E.E. Schneeberger, K. McCarthy and R.L. Kradin. 1991. Accessory cells of the lung. II. Ia^+ pulmonary dendritic cells display cell surface antigen heterogeneity. *Am. J. Respir. Cell Mol. Biol.* 5:276-283.

127. Pollard, A.M. and M.F. Lipscomb. 1990. Characterization of murine lung dendritic cells: similarities to Langerhans cells and thymic dendritic cells. *J. Exp. Med.* 172:159-167.

128. Kradin, R.L., W. Xia, K. McCarthy and E.E. Schneeberger. 1993. $FcR^{\pm}$ subsets of Ia^+ pulmonary dendritic cells in the rat display differences in their abilities to provide accessory co-stimulation for naive ($Ox\text{-}22^+$) and sensitized ($Ox\text{-}22^-$) T cells. *Am. J. Pathol.* 142:811-819.

129. Lin Gong, J., K.M. McCarthy, J. Telford, T. Tamatani, M. Miyasaka and E.E. Schneeberger. 1992. Intraepithelial airway dendritic cells: a distinct subset of pulmonary dendritic cells obtained by microdissection. *J. Exp. Med.* 175:797-807.

130. Nelson, D.J., C. McMenamin, A.S. McWilliam, M. Brenan and P.G. Holt. 1994. Development of the airway intraepithelial dendritic cell network in the rat from class II major histocompatibility (Ia)-negative precursors: differential regulation of Ia expression at different levels of the respiratory tract. *J. Exp. Med.* 179:203-212.

131. McCarthy, K.M., J. Lin Gong, J.R. Telford and E.E. Schneeberger. 1992. Ontogeny of Ia^+ accessory cells in fetal and newborn rat lung. *Am. J. Respir. Cell Mol. Biol.* 6:349-356.

132. van Rees, E.P., M.B. van der Ende and T. Sminia. 1991. Ontogeny of macrophage subpopulations and Ia-positive dendritic cells in pulmonary tissue of the rat. *Cell Tissue Res.* 263:367-373.

133. Schon-Hegard, M.A., J. Oliver, P.G. McMenamin and P.G. Holt. 1991. Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J. Exp. Med.* 173:1345-1356.

134. Casolaro, M.A., J-F. Bernaudin, C. Saltini, V.J. Ferrans and R.G. Crystal. 1988. Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am. Rev. Respir. Dis.* 137:406-411.

135. Soler, P., A. Morreau, F. Basset and A.J. Hance. 1989. Cigarette smoking induces changes in the number and differentiated state of pulmonary dendritic cells / Langerhans cells. *Am. Rev. Respir. Dis.* 139:1112-1117.

136. McWilliam, A.S., D. Nelson, J.A. Thomas and P.G. Holt. 1994. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* 179:1331-1336.

137. Holt, P.G., S. Haining, D.J. Nelson and J.D. Sedgwick. 1994. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* 153:256-261.

138. Havenith, C.E.G., A.J. Breedijk, M.G.H. Betjes, W. Calame, R.H.J. Beelen and E.C.M. Hoefsmit. 1993. T cell priming in situ by intratracheally instilled antigen-pulsed dendritic cells. *Am. J. Respir. Cell Mol. Biol.* 8:819-824.

139. Havenith, C.E.G., P.P.M.C. van Miert, R.H.J. Beelen and E.C.M. Hoefsmit. 1993. Migration of dendritic cells into the draining lymph nodes of the lung after intratracheal

instillation. *Am. J. Respir. Cell Mol. Biol.* 9:484-488.

140. Kradin, R., W. Xia, C. Pinto and L-H. Zhao. 1994. Substance P-containing unmyelinated C-fibers are present in the immediate proximity of airway dendritic cells and may promote their migration during inflammation. *Am. J. Respir. Crit. Care Med.* 149:A267.

141. Koyama, S., S.I. Rennard, G.D. Leikauf and R.A. Robbins. 1990. Bronchial epithelial cells release monocyte chemotactic activity in response to smoke and endotoxin. *J. Immunol.* 147:972-979.

142. Sousa, A.R., S.J. Lane, J.A. Nakhosteen, T. Yoshimura, T.H. Lee and R.N. Poston. 1994. Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am. J. Respir. Cell Mol. Biol.* 10:142-147.

143. Cromwell, O., Q. Hamid, C.J. Corrigan, J. Barkans, Q. Meng, P.D. Collins and A.B. Kay. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1β and tumour necrosis factor-α. *Immunology* 77:330-337.

144. Bellini, A., H. Yoshimura, E. Vittori, M. Marini and S. Mattoli. 1993. Bronchial epithelial cells of patients with asthma release chemoattractant factors for T lymphocytes. *J. Allergy Clin. Immunol.* 92:412-424.

145. Proud, D. 1993. The epithelial cell as a target and effector cell in airway inflammation. *In* Asthma: physiology, immunopharmacology and treatment. Eds. Academic Press Ltd. London. 199-209.

146. Tazi, A., F. Bouchonnet, M. Grandsaigne, L. Boumsell, A.J. Hance and P. Soler. 1993. Evidence that granulocyte-macrophage colony-stimulating factor regulates the distribution and differentiated state of dendritic cells / Langerhans cells in human lung and lung cancers. *J. Clin. Invest.* 91:566-576.

147. Holt, P.G., M.A. Shon-Hegard and J. Oliver. 1988. MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen-presenting activity by endogenous macrophage populations. *J. Exp. Med.* 167:262-274.

148. Gong, J.L., K.M. McCarthy, R.A. Rogers and E.E. Schneeberger. 1994. Interstitial lung macrophages interact with dendritic cells to present antigenic peptides derived from particulate antigens to T cells. *Immunology* 81:343-351.

149. Cumberbatch, M. and I. Kimber. 1992. Dermal tumour necrosis factor-α induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans' cell migration. *Immunol.* 75:257-263.

150. Favara, B.E. 1991. Langerhans' cell histiocytosis: pathobiology and pathogenesis. *Semin. Oncol.* 18:3-7.

151. Willman, C.L., L. Busque, B.B. Griffith, B.E. Favara, K.L. McClain, M.H. Duncan and G. Gilliland. 1994. Langerhans'-cell histiocytosis (histiocytosis X) - a clonal proliferative disease. *N. Engl. J. Med.* 331:154-160.

152. Bieber, T. and J. Ring. 1991. Atopic eczema, Langerhans cells and allergy. *Int. Arch. Allergy Appl. Immunol.* 99:204-207.

153. Bieber, T. 1994. FcεRI on human Langerhans cells: a receptor in search of new functions. *Immunol. Today* 15:52-53.

154. Buckley, C.C., C. Ivison, L.W. Poulter and M.H.A. Rustin. 1992. FcεRII/CD23 receptor distribution in patch test reactions to aeroallergens in atopic dermatitis. *J. Invest. Dermatol.* 99:184-188.

155. Fokkens, W.J., T.M. Vroom, E. Rijntjes and P.G.H. Mulder. 1989. CD1a (T6), HLA-DR-expressing cells, presumably Langerhans cells, in nasal mucosa. *Allergy* 44:167-172.

156. Fokkens, W.J., A.F. Holm, E. Rijntjes, P.G.H. Mulder and T.M. Vroom. 1990. Characterization and quantification of cellular infiltrates in nasal mucosa of patients with grass-pollen allergy, non-allergic patients with nasal polyps and controls. *Int. Arch. Allergy Appl. Immunol.* 93:66-72.

157. Fokkens, W.J., T.M. Vroom, E. Rijntjes and P.G.H. Mulder. 1989. Fluctuation of the number of CD1a (T6)-positive dendritic cells, presumably Langerhans cells, in the nasal mucosa of patients with an isolated grass-pollen allergy before, during and after the grass-pollen season. *J. Allergy Clin. Immunol.* 84:39-43.

158. Fokkens, W.J., T. Godthelp, A.F. Holm, P.G.H. Mulder and T.M. Vroom. 1991. Dynamics of nasal Langerhans cells in patients with allergic rhinitis and non-allergic controls during allergen provocation, a biopsy study. *In* The pathogenesis of allergic rhinitis. Fokkens, W.J., thesis. Rotterdam. 83-92.

159. Burke, C., C.K. Power, A. Norris, A. Condez, B. Schmekel and L.W. Poulter. 1992. Lung function and immunopathological changes after inhaled corticosteroid therapy in asthma. *Eur. Respir. J.* 5:73-79.

160. Fokkens, W.J., T. Godthelp, A.F. Holm, P.G.H. Mulder, T.M. Vroom and E. Rijntjes. 1991. The effect of nasal corticosteroid spray on Langerhans cells in the nasal mucosa. *In* The pathogenesis of allergic rhinitis. Fokkens, W.J., thesis. Rotterdam. 107-114.

161. Holt, P.G., S. Haining, D.J. Nelson and J.D. Sedgwick. 1994. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* 153:256-261.

162. Rhodes, J., J. Ivanyi and P. Cozens. 1986. Antigen presentation by human monocytes: effects of modifying major histocompatibility complex class II antigen expression and interleukin 1 production by using recombinant interferons and corticosteroids. *Eur. J. Immunol.* 16:370-375.

163. Hirschberg, H., T. Hirschberg, H. Nousiainen, L.R. Braathen and E. Jaffe. 1982. The effect of corticosteroids on antigen presenting properties of human monocytes and endothelial cells. *Clin. Immunol. Immunopathol.* 23:577-585.

164. Braathen, L.R. and H. Hirscherg. 1984. The effect of short-term corticosteroid incubation on the alloactivating and antigen-presenting capacity of human epidermal Langerhans cells. *Br. J. Dermatol.* 111:295-302.

165. Brusselle, C.G., J.C. Kips, J.H. Tavernier, J.G. van der Heyden, C.A. Cuvelier, R.A. Pauwels and H. Bluethmann. 1994. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin. Exp. Allergy* 24:73-80.

166. Hauser, C., C.M. Snapper, J. Ohara, W.E. Paul and S.I. Katz. 1989. T helper cells grown with hapten-modified cultured Langerhans' cells produce interleukin 4 and stimulate IgE production by B cells. *Eur. J. Immunol.* 19:245-251.

167. Simon, J.C., P.D. Cruz, P.R. Bergstresser and R.E. Tigelaar. 1990. Low dose ultraviolet B-irradiated Langerhans cells preferentially activate $CD4^+$ cells of the T helper 2 subset. *J. Immunol.* 145:2087-2091.

168. Bellini, A., E. Vittori, M. Marini, V. Ackerman and S. Mattoli. 1993. Intraepithelial dendritic cells and selective activation of Th2-like lymphocytes in patients with atopic asthma. *Chest* 103:997-1005.

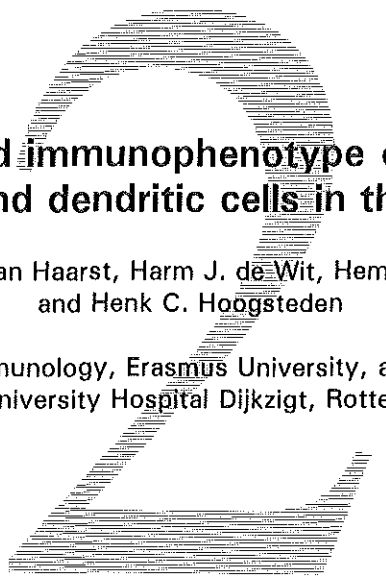
170. Mudde, G.C., T.T. Hansel, F.C. van Reijsen, B.F. Osterhof and C.A.F.M. Bruijnzeel-Koomen. 1990. IgE: an immunoglubulin specialized in antigen capture? *Immunol. Today* 11:440-442.

171. Fokkens, W.J., C.A.F.M. Bruijnzeel-Koomen, Th.M. Vroom, E. Rijntjes, E.C.M. Hoefsmit, G.C. Mudde and P.L.B. Bruijnzeel. 1990. The Langerhans cell: an underestimated cell in atopic disease. *Clin. Exp. Allergy* 20:627-638.

172. Mudde, G.C, F.C. van Reijsen, G.J. Boland, G.C. de Gast, P.L.B. Bruijnzeel and C.A.F.M. Bruijnzeel-Koomen. 1990. Allergen presentation by epidermal Langerhans' cells from patients with atopic dermatitis is mediated by IgE. *Immunol.* 69:335-341.

173. Al-Dossari, G.A., V.R. Kshettry, J. Jessurun and M. Bolman. 1994. Experimental large-animal model of obliterative bronchiolitis after lung transplantation. *Ann. Thorac. Surg.* 58:34-40.

174. Uyama, T., J.B. Winter, G. Groen, C.R.H. Wildevuur, Y. Monden and J. Prop. 1992. Late airway changes caused by chronic rejection in rat lung allografts. *Transplantation* 54:809-812.
175. Yousem, S.A., L. Ray, I.L. Paradis, J.A. Dauber and B.P. Griffith. 1990. Potential role of dendritic cells in bronchiolitis obliterans in heart-lung transplantation. *Ann. Thorac. Surg.* 49:424-428.
176. Lapa E Silva, J.R., J.A.H. Jones, P.J. Cole and L.W. Poulter. 1989. The immunological component of the cellular inflammatory infiltrate in bronchiectasis. *Thorax* 44:668-673.
177. Lapa E Silva, J.R., D. Guerreiro, B. Noble, L.W. Poulter and P.J. Cole. 1989. Immunopathology of experimental bronchiectasis. *Am. J. Respir. Cell Mol. Biol.* 1:297-304.
178. Becker, Y. 1992. Anticancer role of dendritic cells (DC) in human and experimental cancers - a review. *Anticancer Res.* 12:511-520.
179. Zeid, N.A. and H.K. Muller. 1993. S100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. *Pathology* 25:338-343.
180. Cohen, P.J., P.A. Cohen, S.A. Rosenberg, S.I. Katz and J.J. Mulé. 1994. Murine epidermal Langerhans cells and splenic dendritic cells present tumor-associated antigens to primed T cells. *Eur. J. Immunol.* 24:315-319.
181. Grabbe, S., S. Bruvers, R.L. Gallo, T.L. Knisely, R. Nazareno and R.D. Granstein. 1991. Tumor antigen presentation by murine epidermal cells. *J. Immunol.* 146:3656-3661.
182. Mantovani, A., B. Botazi, F. Colttaa, S. Sozzani and L. Ruco. 1992. The origin and function of tumor-associated macrophages. *Immunol. Today* 13:265-270.
183. Sone, S. 1986. Role of the alveolar macrophage in pulmonary neoplasias. *Biochim Biophys. Acta* 823:227-245.



Distribution and immunophenotype of mononuclear phagocytes and dendritic cells in the human lung

Jan Maarten W. van Haarst, Harm J. de Wit, Hemmo A. Drexhage, and Henk C. Hoogsteden

Department of Immunology, Erasmus University, and Department of Pulmonary Medicine, University Hospital Dijkzigt, Rotterdam, the Netherlands

Distribution and Immunophenotype of Mononuclear Phagocytes and Dendritic Cells in the Human Lung

Jan Maarten W. van Haarst, Harm J. de Wit, Hemmo A. Drexhage, and Henk C. Hoogsteden

Department of Immunology, Erasmus University, and Department of Pulmonary Medicine, University Hospital Dijkzigt, Rotterdam, the Netherlands

Mononuclear phagocytes and dendritic cells (DC) play an important role in the immune response in the lung. DC act in the afferent phase of the immune response by presenting antigen to T cells, while macrophages play a role in the efferent phase by exerting phagocytic/cytotoxic functions. We investigated the localization and the marker pattern of these cells in the human lung. Macrophages, identified as large, rounded, acid phosphatase-positive cells, were mainly detected in the alveolar spaces, in the lumen of the bronch(iol)us, and in the bronchoalveolar lavage (BAL). They were positive for major histocompatibility complex (MHC) class II antigens (DR, DQ), CD68, RFD7, RFD9, and partly positive for RFD1. Irregularly shaped cells with a marker pattern comparable to that of blood-derived DC (positive for DR, DQ, L25, RFD1, and CD68) were predominantly observed in the epithelium and subepithelial tissue of the bronch(iol)us and in the bronchus-associated lymphoid tissue. In the epithelium, approximately 30% of these cells were positive for CD1a (OKT6). In the subepithelial tissue, these DC formed characteristic small clusters with T cells. The BAL, the alveolar spaces, and the alveolar walls contained only a small number of DC. These immunohistologic data suggest that the bronch(iol)us is well equipped to initiate immune responses. The high number of macrophages in the alveolar compartment, which have been described to suppress T cell proliferation, together with low numbers of DC, makes the alveolar compartment less suited for mounting an immune response.

Monocytes and macrophages have important phagocytic and cytotoxic functions and produce an array of mediators (1). Although these cells are capable of presenting antigen to T cells and thereby stimulating proliferation, alveolar macrophages (AM) in high numbers have been described to suppress T cell proliferation (2). The most potent stimulators of proliferative T cell responses are dendritic cells (DC), and in contrast to other antigen-presenting cells DC are able to activate unprimed (resting) T cells (3). Typical immunohistologic features of DC are their bean-shaped eccentric nucleus, the prominent cytoplasmatic protrusions, the enzyme histochemistry (juxtanuclear spot or absence of acid phosphatase), and the strong major histocompatibility complex (MHC) class II expression (4). DC are considered by some authors to represent a separate, bone marrow-derived lineage (CD45 positive) (5), while others suggest a monocyte origin (6, 7). The Langerhans cell, carrying the marker CD1a, is a member of the DC family and is thought to be the precursor of the interdigitating cell in the lymph node (8, 9).

Macrophages reside mainly in the alveolar space and in the lumen of the bronchus and bronchiolus. DC have been described in both the human lung (10–12) as well as in the rat (13) and in the mouse (10) lung. A further characterization of their marker pattern and a more precise localization of these cells may improve our understanding of the local immune responses in the normal lung. We therefore examined the presence and distribution of macrophages and DC in the human lung, using an array of markers (Table 1). Moreover, we compared the marker pattern of lung DC and macrophages with those of blood monocytes and blood-derived DC.

(Received in original form June 15, 1993 and in revised form November 1, 1993)

Address correspondence to: J. M. W. van Haarst, M.D., Department of Immunology Ee838, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, the Netherlands.

Abbreviations: alveolar macrophages, AM; bronchoalveolar lavage, BAL; bronchus-associated lymphoid tissue, BALT; dendritic cells, DC; major histocompatibility complex, MHC; monoclonal antibody, mAb; phosphate-buffered saline, PBS.

Am. J. Respir. Cell Mol. Biol. Vol. 10. pp. 487–492, 1994

Materials and Methods

Cells and Tissues

Normal lung tissue was obtained from 14 patients undergoing (partial) lung resection for bronchial carcinoma. The patients were all current smokers or ex-smokers. Tissue specimens remote from the tumor were used, which contained smaller bronchi, bronchioli, alveolar tissue, and blood vessels. Lung tissue was gently inflated with optimal cutting

TABLE 1

Monoclonal antibodies used in this study

Monoclonal Antibody	Main Specificity
OKIa*	HLA-DR
Anti-HLA-DR†	HLA-DR
Anti-HLA-DR-biotin†	HLA-DR
Leu10†	HLA-DQ
RFD1¶	DC, AM, and B cells
L25§	DC and B cells
My4‡	CD14 (monocytes)
LeuM3†	CD14 (monocytes)
UCHM1/	CD14 (monocytes)
KIM6**	CD68 (macrophages)
KIM7**	CD68 (macrophages)
EBM11††	CD68 (macrophages)
RFD7¶	Macrophages
RFD9¶	Macrophages
OKT6*	CD1a (Langerhans cells)
Leu4†	CD3 (T cells)
B4†	CD19 (B cells)
5D3¶¶	Cytokeratin 8 and 18
M616/	Factor VIII

* Ortho Diagnostics, Raritan, NJ.
† Becton Dickinson, San Jose, CA.
‡ Dr. L. W. Poulter, London, UK.
§ Dr. T. Takami, Gifu, Japan.
‖ Coulter Clone, Hialeah, FL.
/ Dr. P. C. L. Beverley, London, UK.
** Behring, Marburg, Germany.
†† Dako, Glostrup, Denmark.
¶¶ Organon Teknika, Apeldoorn, the Netherlands.

TABLE 2

Comparison of marker patterns of DC of the bronchial/bronchiolar wall, blood-derived DC, peripheral blood monocytes, and AM

	Bronchial DC	Blood-derived DC	Blood Monocytes	AM
Enzyme				
Acid phosphatase	−	−	−	+
Nonspecific esterase	−	±	+	+
Monoclonal antibody				
OKIa (HLA-DR)	+	+	+	+
RFD1	+	+	−	+/−
L25	+	+	±/−	±/−
OKT6 (CD1a)	+/−	−	−	−
My4 (CD14)	+/−	+	+	−
LeuM3 (CD14)	−	−	+	−
UCHM1 (CD14)	−	−	+	−
KIM7 (CD68)	+	+	+	++
RFD7	−	−	−	+
RFD9	−	−	−	+
OKT3 (CD3)	−	−	−	−
B4 (CD19)	−	−	−	−

++ = strongly positive; + = positive; ± = weak; − = negative; +/− = partly positive.

temperature cryoembedding material (O.C.T. tissue-Tek; Miles, Elkhart, IN) 1:1 diluted in phosphate-buffered saline (PBS) and embedded in O.C.T. tissue-Tek. Specimens were frozen and stored at −80°C. Cryostat sections (6 μm) were cut and brought on poly-L-lysine-coated slides and air-dried.

Bronchoalveolar lavage (BAL) cells were obtained from healthy, volunteering individuals. This procedure was approved by the Medical Ethical Committee. Four subjects were smokers and five were nonsmokers.

Blood monocytes of healthy laboratory staff were isolated from heparin blood. Heparin blood was diluted 1:1 with PBS, and mononuclear cells were obtained by Ficoll-Paque (1.079 g/ml; Pharmacia, Uppsala, Sweden) density gradient separation (15 min, 1,000 × *g*). Cells were washed twice in PBS, and subsequently Percoll (1.063 g/ml; Pharmacia) density gradient separation was performed, which yielded a purity of 70 to 95% monocytes as determined by nonspecific esterase staining.

Blood-derived DC with a potent, accessory cell function (potently acting as stimulator cells in mixed lymphocyte reactions) and a morphology compatible with DC (Table 2) were obtained as described by Kabel and colleagues (7). In short, blood monocytes were cultured overnight (16 h, 37°C, 5% CO_2) under nonadherent conditions (polypropylene tubes; Falcon, Lincoln Park, NJ), yielding 15 to 35% DC. Cytocentrifuge preparations were prepared from the various cell suspensions.

Immunohistology

Monoclonal leukocyte-specific antibodies were used as listed in Table 1. L25 is a monoclonal antibody (mAb) directed against B cells and DC (14, 15) and was a generous gift of Dr. T. Takami (Gifu, Japan). RFD1 recognizes a class II-associated epitope present on activated B cells, DC, and AM (16). As controls we used nonspecific murine IgG1, IgG2, and IgM. Staining with these negative controls was invariably negative.

The cytocentrifuge preparations and the histologic sections were fixed in acetone (Merck, Stuttgart, Germany) for 10 min and thereafter incubated with normal rabbit serum (Dako, Glostrup, Denmark) 10% in PBS for 10 min before incubating for 1 h with the appropriate mAb. After washing 3 times with PBS, the slides were incubated for 30 min with rabbit anti-mouse antiserum conjugated with horseradish peroxidase (RaMHRP; Dako) diluted in PBS with 10% normal human serum. The slides were rinsed 3 times in PBS and stained with diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO). Selected slides were stained for either acid phosphatase or nonspecific esterase (α-naphthyl esterase). Counterstaining was performed with hematoxylin (Merck, Darmstadt, Germany). Finally, the slides were dehydrated in ethanol (Merck) and embedded in Depex mounting medium (BDH, Poole, UK).

For double immunostaining, fixation and subsequent incubation with first-step mAb followed by RaMHRP was performed as described above. After blocking with normal mouse serum (1:10), the slides were incubated with a directly biotinylated second mAb. Slides were rinsed 3 times in PBS and incubated with streptavidine-biotin complex conjugated with alkaline phosphatase (Dako). Slides were sequentially stained with 3-amino-9-ethylcarbazole (AEC; Sigma) and

Fast Blue BB Base (Sigma). Finally, the slides were rinsed in distilled water and mounted in glycerin gelatin (Chroma, Stuttgart, Germany).

Identification and Enumeration of Marker-positive Cells

The slides were evaluated independently by two investigators (J.M.H. and H.J.W.). For each tissue compartment (alveolar wall, alveolar space, bronchial epithelium, and subepithelial tissue), criteria were (*1*) the relative number of positively stained cells (in comparison with other compartments and other mAbs), (*2*) the shape of the stained cells, (*3*) whether the stained cells were positive for acid phosphatase, and (*4*) whether in double-stained slides there was a complete, a partial, or no overlap in the staining pattern of the two mAbs used.

Enumeration of marker-positive cells/mm² of tissue section was performed on a VidasRT (Kontron Elektronik GmbH, Neufarn, Germany) image analysis computer. The areas of the bronchial epithelium and the subepithelial tissue were outlined and measured interactively. Subepithelial tissue was determined to be the area between the epithelial basement membrane and underlying structures such as smooth muscle, cartilage, blood vessel, or alveolar space. The area of the alveolar tissue mass (alveolar walls + alveolar macrophages) was identified as contrasted to the alveolar space and measured on the basis of its grey values. The number of marker-positive cells in the mentioned areas was counted interactively using the VidasRT system.

Results

Bronchial and Bronchiolar Epithelium

Irregularly shaped cells with dendritic processes extending between the epithelial cells were found in the epithelium of the bronchi and bronchioli in all patients (Figure 1). These cells stained for MHC class II (245/mm² epithelium; Table 3), L25 (174/mm²), and RFD1 (154/mm²) (Figure 2A), and a part of these cells (78/mm², approximately 30%) reacted strongly with CD1a, while no reactivity was observed with RFD7, RFD9, acid phosphatase, and nonspecific esterase. These irregularly shaped cells were scattered along the epithelial barrier and were predominantly situated close to the basement membrane. Large, oval, acid phosphatase-positive cells reacting with RFD7 and RFD9 were only sporadically observed in the epithelium (1 ± 2/mm²).

TABLE 3

Numbers of acid phosphatase (APh)-negative cells staining for respectively OKIa, L25, RFD1, and OKT6 and numbers of APh-positive cells per mm² of epithelium, subepithelial tissue, and alveolar tissue

Compartment	Epithelium	Subepithelial	Alveolar
APh-negative			
OKIa	245 ± 61	320 ± 186	NC*
L25	174 ± 73	224 ± 75	26 ± 10
RFD1	154 ± 58	216 ± 94	31 ± 15
OKT6	78 ± 44	Nil	Nil
APh-positive	1 ± 2	22 ± 19	249 ± 161

* NC = not counted; see text for explanation. Data represent the mean ± SD.

Subepithelial Tissue

A heterogeneous infiltrate of mononuclear cells was found in the subepithelial tissue of the bronchus and bronchiolus of all patients, consisting mainly of round, CD3-positive T cells and irregularly shaped, MHC class II–positive cells, with long cytoplasmic extensions. Interestingly, these cells were not equally distributed around the lumen, but they were mainly located in small clusters of dendritic-shaped cells together with T cells under the bronchial epithelium (Figures 1B and 2B).

Besides HLA-DR (320/mm²; Table 3), most of these irregularly shaped cells stained for HLA-DQ, L25 (224/mm²), RFD1 (216/mm²), and CD68 (KIM7). In these cells, no reactivity with acid phosphatase, nonspecific esterase, RFD7, RFD9, Leu4, and B4 was detected. Furthermore, these cells did not contain any inclusion bodies. There was a difference

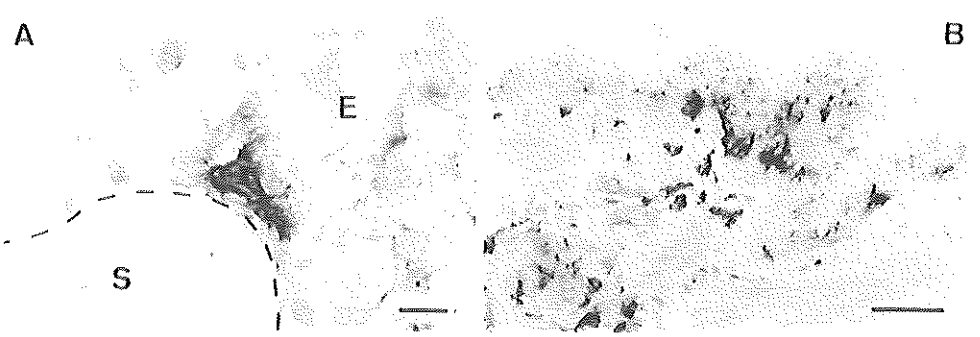


Figure 1. A. CD1a (OKT6)-positive cell in the bronchial epithelium; note the bean-shaped nucleus and the cytoplasmic extensions; E = epithelium; S = subepithelial tissue. Bar = 10 μm. B. Clusters of irregularly shaped cells stained with L25; one cluster directly beneath and in the bronchial epithelium, with intraepithelial dendritic-shaped cells showing long cytoplasmic protrusions towards the bronchial lumen; a second cluster is located in a deeper layer of the subepithelial tissue. Bar = 100 μm.

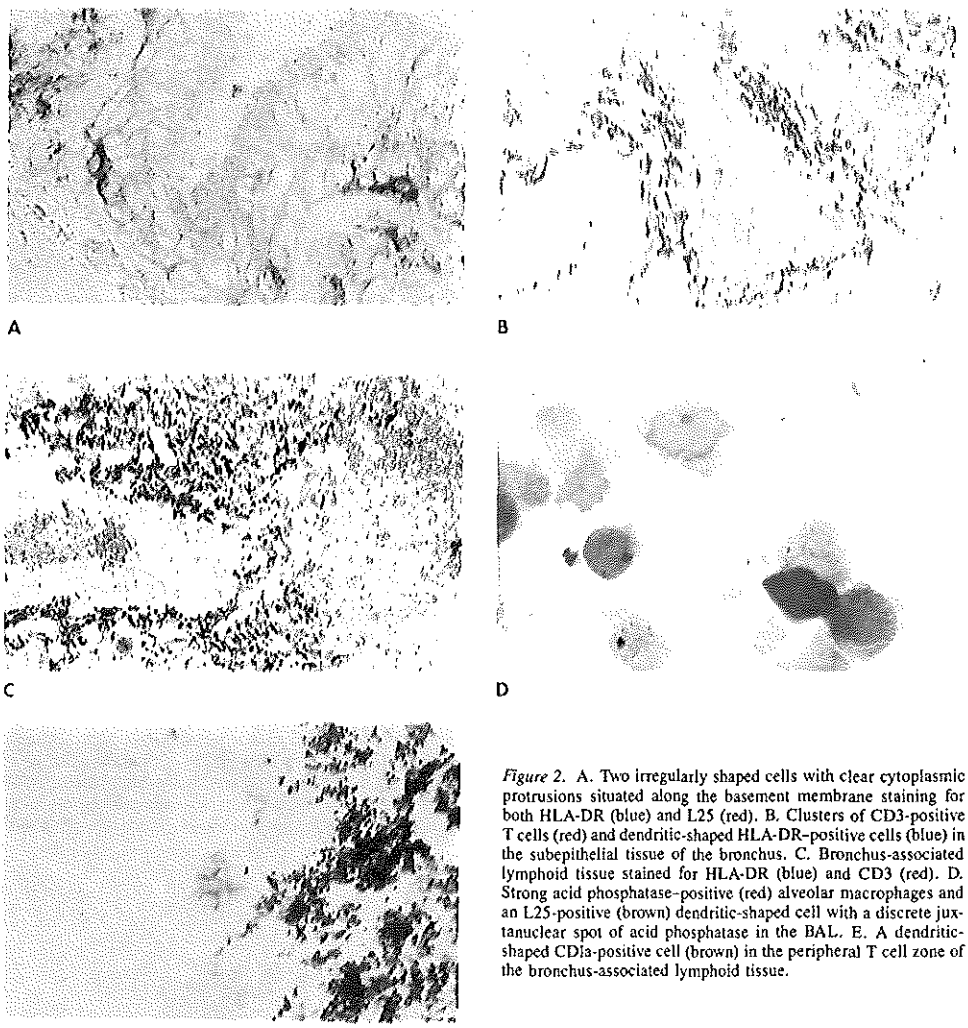


Figure 2. A. Two irregularly shaped cells with clear cytoplasmic protrusions situated along the basement membrane staining for both HLA-DR (blue) and L25 (red). B. Clusters of CD3-positive T cells (red) and dendritic-shaped HLA-DR-positive cells (blue) in the subepithelial tissue of the bronchus. C. Bronchus-associated lymphoid tissue stained for HLA-DR (blue) and CD3 (red). D. Strong acid phosphatase-positive (red) alveolar macrophages and an L25-positive (brown) dendritic-shaped cell with a discrete juxtanuclear spot of acid phosphatase in the BAL. E. A dendritic-shaped CDla-positive cell (brown) in the peripheral T cell zone of the bronchus-associated lymphoid tissue.

in the reactivity of mAbs specific for CD14: virtually no staining was found with LeuM3 and UCHM1, while My4 did react with the irregularly shaped cells. Double staining with anti-class II and My4 revealed a partial overlap. Compared with T cells and the irregularly shaped cells, only a few large, rounded cells staining for RFD7, RFD9, KIM7, class II, acid phosphatase (22/mm^2; Table 3), and nonspecific esterase were observed. CDla-positive cells and B cells (CD19) were only sporadically encountered in the subepithelial tissue.

Bronchus-associated Lymphoid Tissue (BALT)

BALT was determined as nonencapsulated aggregations of lymphocytes, with distinct B cell and T cell areas, in the subepithelial compartment of the bronchus (Figure 2C). BALT was encountered in two of the 12 patients. The cells in the inner zone of the BALT reacted with the B cell marker CD19 (B4). The small, outer zone of the BALT could be identified as the T cell zone on the basis of positivity for CD3

(Leu4). Dispersed through the BALT, some rounded, acid phosphatase-positive cells were observed. In the T cell zone, characteristic dendritic-shaped, acid phosphatase-negative cells could be detected that reacted with OKIa, L25, RFD1, and CD1a (Figure 2E).

Alveolar Walls

The recognition of separate HLA-DR-positive cells in the alveolar wall was impossible because of MHC class II expression by alveolar epithelial cells as well as endothelial cells. Double labeling of anti-HLA-DR with anti-cytokeratin and anti-factor VIII indicated that there were few dendritic-shaped cells present in the alveolar wall. These cells were mainly located in connective tissue in thickened alveolar walls and at bifurcations of alveolar septa. Other markers like L25, RFD1, and Leu10 (HLA-DQ) did not stain endothelium or epithelium. With these markers also occasionally positive cells (negative for acid phosphatase) were encountered in the alveolar wall (26/mm² with L25, 31/mm² with RFD1; Table 3). Reactivity with CD1a (OKT6) could not be observed in the alveolar septa.

Bronchoalveolar Lavage

The BALs obtained from healthy controls ($n = 9$) contained more than 90% large, round, strongly acid phosphatase-positive macrophages reacting with nonspecific esterase, class II, KIM7, RFD7, RFD9, and RFD1. AM were either negative or weakly positive for L25.

A very small percentage of the BAL cells ($0.36 \pm 0.27\%$) showed prominent dendritic protrusions, were strongly positive for MHC class II, and were negative for or had a spot of acid phosphatase (Figure 2D). Furthermore, these cells reacted with L25 and RFD1. Although there was a tendency for higher numbers of these cells in smokers than in nonsmokers (0.45% versus 0.28%), this difference was not statistically significant. Interestingly, OKT6 stained $2.13 \pm 1.66\%$ of the BAL cells, which were acid phosphatase negative, some with a dendritic shape, but the majority showed a more monocyte-like, rounded morphology.

Comparison of Marker Patterns

In Table 2, the marker pattern of the dendritic-shaped cells in the bronchial/bronchiolar epithelium and subepithelial tissue and large, rounded, acid phosphatase-positive macrophages from the BAL and the alveolar spaces is compared with that of blood monocytes and blood-derived DC. The marker pattern of the dendritic-shaped cells in the bronchial wall is comparable to that of blood-derived DC and is clearly different from that of blood monocytes and AM.

Discussion

In this report, the distribution and marker pattern of macrophages and dendritic-shaped cells in the human lung are described. There was a predominance of macrophages in the alveolar compartment and the bronchial/bronchiolar lumen, whereas the dendritic-shaped cells were situated primarily in the epithelium and subepithelial tissue of the bronchus and bronchiolus and in the BALT. Because of the strong resemblance to the marker pattern of blood-derived DC and the lack of various monocyte-macrophage markers (Table 2), we presume these cells belong to the DC family. Of course, functional studies on isolated cells need to confirm this presumption. The positivity for My4 and CD68 (KIM7) on the other hand suggests a relationship of these DC to the monocyte-macrophage lineage.

The material used in this study was entirely from patients with bronchial carcinoma. Our tissue specimens were taken from a site remote from the tumor and showed a histomorphologic normal appearance, except for the sequelae of cigarette smoking and urban life. Since all patients were current smokers or ex-smokers living in an urban surrounding, our data might be influenced by these environmental factors. The numbers of AM and monocyte-like cells (17) have been described as being elevated in the BAL of smokers. Increased numbers of total inflammatory cells and polymorphonuclear leukocytes have also been described in lung tissue of current smokers and ex-smokers (18–20). Nonsmokers might therefore differ in the numbers of macrophages and DC in their lungs. Unfortunately, we were unable to obtain lung tissue from nonsmokers, and further studies are needed to clarify this item.

In the epithelium of the bronchial/bronchiolar wall, a subpopulation of the DC carried the Langerhans cell-marker CD1a (approximately 30%), which is in agreement with the description of Holt and associates (12). Others have detected Birbeck granulae in these cells (11), suggesting a close relationship with the Langerhans cell of the skin. The tightly meshed network of DC as described by Holt and associates was locally present (12), but in general we observed a more scattered pattern of DC in the epithelium, even on tangentially cut sections. The DC in the subepithelial tissue of the bronchial/bronchiolar wall only occasionally showed CD1a expression, and there was a stronger overlap with markers of the monocyte-macrophage lineage (CD14 and CD68). It has been described, however, that My4 (CD14) not only reacts with monocytes, but reacts with interdigitating cells, interstitial DC, unfixed Langerhans cells, and endothelium as well (21–23). Other CD14 mAbs, like UCHM1 and LeuM3, have a smaller specificity and did not (or only sporadically) stain cells in the bronchial wall. The same differences in reactivity with anti-CD14 mAbs were found with our blood-derived DC (Table 2). Different mAbs of the CD68 cluster (KIM6, KIM7, and EBM11) showed comparable staining patterns in our slides. CD68, which is often used as a macrophage marker, also reacts with Langerhans cells in the skin, spleen DC, and interdigitating cells (24, 25). In addition, CD68 is present on freshly isolated blood DC and is rearranged to a juxtanuclear spot upon culture (26).

DC were also observed in the T cell zone of the BALT. This is in line with previous observations in the BALT of mouse lung (27). Only in two of the 14 patients was BALT encountered. Although BALT has been described in the human lung, it is argued that it is normally not present in the lungs of healthy, nonsmoking individuals (28) (see discussion above).

We found a considerably lower number of DC in the alveolar compartment as compared with the numbers in the bronchial wall. Although DC have previously been described in the human lung (10, 11), this difference in compartmentalization has not been noted. Interestingly, a comparable distribution has been described for T cells in the human lung (29).

Our data are in contrast to those of Soler and co-workers (11), who described the presence of a large number of CDla (OKT6)-positive cells in the alveolar wall. Sertl and colleagues (10) reported a lack of positivity of pulmonary DC for CDla (OKT6). In our sections, which showed no background staining, strongly positive CDla-positive cells could be detected in the bronchial epithelium and the BALT, whereas the alveolar compartment was completely negative for CDla.

In support of our immunohistologic data, clearly showing DC in the human lung, are studies in which DC were isolated from the human lung. These cells were strong stimulators of allogenic T cell responses *in vitro* (30). The DC in the bronchus observed by us are likely to have such T cell–stimulatory properties, since they were often seen in close contact with T cells and since their morphology and marker pattern is virtually the same as that of interdigitating cells and blood-derived DC, which are strong T cell stimulators as well (7). It is unclear, however, whether the clusters of DC and T cells found by us represent the same active process of clustering of these cells *in vitro*, which is essential for T cell stimulation (31).

Macrophages were abundantly present in the alveolar compartment. AM have been described as inhibiting the stimulation of proliferative responses by antigen-presenting cells (2, 32). Our data therefore suggest this compartment to be less well equipped to initiate immune responses. This view is strengthened by the scarce presence of DC in the alveolar wall and the BAL. The bronchus and bronchiolus, showing the clear presence of DC, are better suited for mounting an immune response than the alveolar compartment. The concept that the bronchial compartment of the lung is the primary site for the initiation of immune responses in the lung is supported by the development of lymphoid tissue (BALT) in the bronchus/bronchiolus in a few patients and the presence of high endothelial venules in the bronchus in healthy subjects (33).

Acknowledgments: This study was supported by a grant of the Netherlands Asthma Foundation. We gratefully acknowledge Glaxo b.v. (Zeist, the Netherlands) for paying the costs of the color prints. We wish to thank Dr. T. Takami for the supply of the L25 monoclonal antibody, Tar van Os for the preparation of the photomicrographs, and Martha Canning for correction of the English language.

References

1. Crystal, R. G. 1991. Alveolar macrophages. *In* The Lung: Scientific Foundations. R. G. Crystal, editor. Raven Press, New York. 527–538.
2. Holt, P. G., A. Degebrodt, C. O'Leary, K. Krska, and T. Plozza. 1985. T-cell activation by antigen-presenting cells from lung tissue digests: suppression by endogenous macrophages. *Clin. Exp. Immunol.* 62:586–593.
3. Inaba, K., and R. M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717–1735.
4. Van Voorhis, W. C., L. S. Hair, R. M. Steinman, and G. Kaplan. 1982. Human dendritic cells, enrichment and purification from peripheral blood. *J. Exp. Med.* 155:1172–1187.
5. Austyn, J. M. 1987. Lymphoid dendritic cells. *Immunology* 62:161–170.
6. Peters, J. H., J. Ruppert, R. K. H. Gieseler, H. M. Najar, and R. Xu. 1991. Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage? *Pathobiology* 59:122–126.
7. Kabel, P. J., M. de Haan-Meulman, H. A. M. Voorbij, M. Kleingeld, E. F. Knol, and H. A. Drexhage. 1989. Accessory cells with a morphology and a marker pattern of dendritic cells can be obtained from elutriator purified blood monocyte fractions, an enhancing effect of metrizamide in this differentiation. *Immunobiology* 179:395–411.
8. Hoefsmit, E. C. M., A. M. Duijvestijn, and E. W. A. Kamperdijk. 1982. Relation between Langerhans cells, veiled cells and interdigitating cells. *Immunobiology* 161:255–265.
9. Romani, N., and G. Schuler. 1989. Structural and functional relationships between epidermal Langerhans cells and dendritic cells. *Res. Immunol.* 140:895–898.
10. Sertl, K., T. Takemura, E. Tschachler, V. J. Ferrans, M. A. Kaliner, and E. M. Shevach. 1986. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma and visceral pleura. *J. Exp. Med.* 163:436–451.
11. Soler, P., A. Moreau, F. Basset, and A. J. Hance. 1989. Cigarette smoking-induced changes in the number and differentiated state of pulmonary dendritic cells/Langerhans cells. *Am. Rev. Respir. Dis.* 139:1112–1117.
12. Holt, P. G., M. A. Schon-Hegrad, M. J. Philips, and P. G. McMenamin. 1989. Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* 19:597–601.
13. Holt, P. G., M. A. Schon-Hegrad, J. Oliver, B. J. Holt, and P. G. McMenamin. 1990. A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *Int. Arch. Allergy Appl. Immunol.* 91:155–159.
14. Ishii, Y., T. Takami, Y. Kokai, H. Yuasa, J. Fujimoto, and T. Takei. 1985. A novel human B cell antigen shared with lymphoid dendritic cells: characterization by a monoclonal antibody. *Clin. Exp. Immunol.* 61:624–632.
15. Kabel, P. J., M. de Haan, R. D. van der Gaag, and H. A. Drexhage. 1987. Intrathyroidal dendritic cells. *J. Clin. Endocrin. Metab.* 66:199–207.
16. Poulter, L. W., D. A. Campbell, C. Munro, and G. Janossy. 1986. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scand. J. Immunol.* 24:351–357.
17. Hoogsteden, H. C., P. Th. W. van Hal, J. M. Wijkhuis, W. Hop, A. P. K. Verkaik, and C. Hilvering. 1991. Expression of the CD11/CD18 cell surface adhesion glycoprotein family on alveolar macrophages in smokers and nonsmokers. *Chest* 100:1567–1571.
18. Bosken, C. H., J. Hards, K. Gatter, and J. C. Hogg. 1992. Characterization of the inflammatory reaction in the peripheral airways of cigarette smokers using immunocytochemistry. *Am. Rev. Respir. Dis.* 145:911–917.
19. Niewoehner, D. E., J. Kleinerman, and D. B. Rice. 1974. Pathologic changes in the peripheral airways of young cigarette smokers. *N. Engl. J. Med.* 291:755–758.
20. Wright, J. L., J. E. Hobson, P. D. Pare, and J. C. Hogg. 1988. Airway inflammation and peribronchial attachments in the lungs of nonsmokers, current and ex-smokers. *Lung* 166:277–286.
21. Hogg, N., and M. A. Horton. 1987. Myeloid antigens: new and previously defined clusters. *In* Leucocyte Typing III. A. J. McMichael, editor. Oxford University Press, Oxford. 576–602.
22. McKenzie, J. L., G. F. Reid, M. E. J. Beard, and D. N. J. Hart. 1987. Reactivity of myeloid antibodies with human dendritic cells. *In* Leucocyte Typing III. A. J. McMichael, editor. Oxford University Press, Oxford. 668–671.
23. Gadd, S. 1989. Cluster report: CD14. *In* Leucocyte Typing IV. W. Knapp, editor. Oxford University Press, Oxford. 787–794.
24. Pulford, K. A. F., A. Sipos, J. L. Cordell, W. P. Stross, and D. Y. Mason. 1990. Distribution of the CD68 macrophage/myeloid associated antigen. *Int. Immunol.* 2:973–980.
25. Kelly, P. M. A., E. Bliss, J. A. Morton, J. Burns, and J. O. D. McGee. 1988. Monoclonal antibody EBM/11: high cellular specificity for human macrophages. *J. Clin. Pathol.* 41:510–515.
26. Beljes, M. G. H., M. C. Haks, C. W. Tuk, and R. H. J. Beelen. 1991. Monoclonal antibody EBM11 (anti-CD68) discriminates between dendritic cells and macrophages after short term culture. *Immunobiology* 183:79–87.
27. Breel, M., M. van der Ende, T. Sminia, and G. Kraal. 1988. Subpopulations of lymphoid and non-lymphoid cells in the bronchus associated lymphoid tissue (BALT) of the mouse. *Immunology* 63:657–662.
28. Pabst, R., and I. Gehrke. 1990. Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans? *Am. J. Respir. Cell Mol. Biol.* 3:131–135.
29. Kaltreider, H. B. 1991. Normal immune response. *In* The Lung: Scientific Foundations. R. G. Crystal, editor. Raven Press, New York. 499–510.
30. Nicod, L. P., M. F. Lipscomb, J. C. Weissler, C. R. Lyons, J. Albertson, and G. B. Toews. 1987. Mononuclear cells in the human lung parenchyma: characterization of a potent accessory cell not obtained by bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 136:818–823.
31. Austyn, J. M., D. E. Weinstein, and R. M. Steinman. 1988. Clustering with dendritic cells precedes and is essential for T-cell proliferation in a mitogenesis model. *Immunology* 63:691–696.
32. Schauble, T. L., W. R. Boom, C. K. Finegan, and E. A. Rich. 1993. Characterization of suppressor function of human alveolar macrophages for T lymphocyte responses to phytohemagglutinin: cellular selectivity, reversibility, and early events in T cell activation. *Am. J. Respir. Cell Mol. Biol.* 8:89–97.
33. Poston, R. N., A. Duijvestijn, and J. Bousquet. 1992. HEV in normal human bronchi. *Immunol. Today* 13:329–330.

Dendritic cells and their precursors isolated from human bronchoalveolar lavage: immunocytologic and functional properties

Jan Maarten W. van Haarst, Henk C. Hoogsteden, Harm J. de Wit, Gert T. Verhoeven, Carin E.G. Havenith, and Hemmo A. Drexhage

Department of Immunology and Pulmonary Medicine, Erasmus University and Universital Hospital Dijkzigt, Rotterdam, the Netherlands, and Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee

Dendritic Cells and their Precursors Isolated from Human Bronchoalveolar Lavage: Immunocytologic and Functional Properties

Jan Maarten W. van Haarst, Henk C. Hoogsteden, Harm J. de Wit, Gert T. Verhoeven, Carin E. G. Havenith, and Hemmo A. Drexhage

Departments of Immunology and Pulmonary Medicine, Erasmus University and University Hospital Dijkzigt, Rotterdam, the Netherlands, and Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee

Human bronchoalveolar lavage (BAL) has been described to contain, besides a large number of alveolar macrophages (AM) (~95%), small numbers of monocyte-like cells (~2%) and dendritic cells (DC) (~0.4%). To separate AM (high autofluorescence) from DC, we used a fluorescence activated cell sorter (FACS) to separate BAL cells into a low autofluorescent (LAF) fraction and a high autofluorescent (HAF) fraction. Immunocytologic and functional properties of these fractions were investigated. The LAF fraction was composed of acid phosphatase (APh)- and RFD9-negative cells, which were strongly positive for HLA-DR, L25, RFD1, and CD68. A portion of these cells expressed CD1a (22%) and My4 (60%). The marker pattern of these cells is reminiscent to that of intraepithelial bronchial DC and to that of blood DC. The majority of the LAF cells had a monocyte-like morphology, but after overnight culture the percentage of LAF cells with long cytoplasmic extensions (DC morphology) was strongly augmented (from 18 to 51%). The HAF fraction contained 100% AM, strongly positive for APh, HLA-DR, CD68, RFD7, and RFD9. In culture, the LAF cells formed clusters with T cells and vigorously stimulated the proliferation of allogeneic T cells and naive (CD45RO-negative) T cells. BAL and LAF cells produced higher responses in nonsmokers than in smokers. In contrast, HAF cells did not form clusters with T cells and did not stimulate allogeneic T cell proliferation. HAF cells even suppressed mitogen-driven T cell proliferation. We conclude that human BAL cells can be separated with a FACS into (*1*) high autofluorescent AM with T cell–suppressive properties and (2) low autofluorescent accessory cells with a very strong T cell–stimulatory potential. These accessory cells show many features of dendritic cells, but do have some monocyte characteristics as well.

Dendritic cells (DC) and alveolar macrophages (AM) play an important role in the immune response of the human lung.

DC are professional antigen-presenting cells and are able to initiate immune responses. In contrast to macrophages and other antigen-presenting cells, DC are able to activate naive (unprimed) T cells (1). Immunocytologic features of dendritic cells are their irregular outline, their bean-shaped eccentric nucleus, an absence of acid phosphatase (APh) (or the enzyme concentrated in a juxtanuclear spot), and a strong surface expression of major histocompatibility complex (MHC) class II antigens (2–4).

AM have important phagocytic and cytotoxic functions and produce an array of mediators. Although macrophages are in general regarded as antigen-presenting cells, AM are poor stimulators or even suppressors of T cell proliferation (5, 6). Immunocytologic features of AM are a rounded morphology and strong APh positivity (7).

Bronchoalveolar lavage (BAL) of healthy subjects yields a cell population containing greater than 90% AM. In addition to AM, small percentages of lymphocytes (4%), granulocytes (2%), monocyte-like cells (2%), and DC (0.4%) can be observed in the human BAL (8, 9).

The abundance of AM in BAL has allowed elaborate studies on the functional properties of these cells. The number of studies on DC in BAL and lung is limited, however, due to the difficult purification procedures required and the low number of these cells present. Nicod and colleagues recently described the isolation and purification of DC from minced and digested human lung tissue, based on the

(Received in original form December 20, 1993 and in revised form February 15, 1994)

Address correspondence to: J. M. W. van Haarst, M.D., Department of Immunology, Ee838, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, the Netherlands.

Abbreviations: acid phosphatase, APh; alveolar macrophages, AM; bronchoalveolar lavage, BAL; dendritic cells, DC; fluorescence activated cell sorter, FACS; high autofluorescent (fraction), HAF; low autofluorescent (fraction), LAF; major histocompatibility complex, MHC; mixed lymphocyte reactions, MLR; monoclonal antibody, mAb; peripheral blood monocytes, PBM; phytohemagglutinin, PHA; phosphate-buffered saline, PBS; sideward scatter, SSC.

Am. J. Respir. Cell Mol. Biol. Vol. 11. pp. 344-350, 1994

autofluorescence of AM (10). Havenith and co-workers have described a similar technique to isolate monocyte-like cells and DC from the rat BAL fluid after BCG instillation (11).

Here we describe the isolation and purification of DC and their precursors from human BAL fluid using a similar separation technique (fluorescence activated cell sorting [FACS]), based on the difference in autofluorescence between DC and AM (see also references 10 and 11). In addition, we tested the immunocytologic and functional properties of the DC isolated from human BAL.

Materials and Methods

Bronchoalveolar Lavage

After informed consent, BAL was performed on individuals during anesthesia for routine elective surgery. Subjects with a history of pulmonary or systemic disease were excluded. Seven smokers (more than five cigarettes a day) and five lifelong nonsmokers were included in the study (mean age 36 yr, range 28 to 57 yr). BAL was performed with a flexible bronchoscope placed in wedge position in the right middle lobe. Four aliquots of 50 ml saline were subsequently instilled and aspirated. BAL fluid (total volume ranged from 110 to 150 ml) was collected in siliconized bottles. The procedure has been approved by the Medical Ethical Committee of the Erasmus University and University Hospital Dijkzigt.

Isolation and Purification of DC and AM

BAL cells were kept at 4°C, washed twice in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 0.45% glucose, and subsequently filtered through a 55 μm and a 30 μm gauze. BAL cells were sorted on a FACS-Vantage (Becton Dickinson, Erembodegem, Belgium) with a 488 nm laser. Sort windows were generated on autofluorescence (FL1, 530 nm) to create a population with a low autofluorescence (LAF cells) and a population with a high autofluorescence (HAF cells), and on forward scatter (FSC) to exclude small cells (lymphocytes) and debris. For the LAF fraction, an additional sort criterion was set on sideward scatter (SSC) to exclude cells with a high SSC (granulocytes and macrophages). In selected experiments, cells were incubated with OKT6 conjugated with a fluorochrome to subdivide the LAF fraction. Peripheral blood monocytes (PBM) and blood DC (used as a control population) were purified according to techniques described in detail elsewhere (12, 13). In short, monocytes were isolated from heparin blood or buffy coats by subsequent Ficoll-Paque (1,079 g/ml; Pharmacia, Uppsala, Sweden) and Percoll (1.063 g/ml; Pharmacia) density gradient separation. After exposure to triiodothyronine (T3, 30 min, 37°C), monocytes were cultured under nonadherent conditions for 16 h, yielding a population containing 30 to 45% DC.

Monoclonal Antibodies

The following monoclonal antibodies (mAbs) were used: OKIa (HLA-DR) and OKT6 (CD1a) (Ortho Diagnostics Systems, Beerse, Belgium); Leu4 (CD3), B4 (CD19), and LeuM3 (CD14) (Becton Dickinson, San Jose, CA); My4 (CD14) (Coulter Immunology, Hialeah, FL); UCHL1 (CD45RO) (Serotec, Oxford, UK); 32.2 (CD64) (Medarec Tuc., West Lebanon, NH); and KIM7 (CD68) (Behring, Marburg, Germany). L25 is an mAb directed against B cells and DC (14, 15) and was a generous gift of Dr. T. Takami (Gifu, Japan); RFD7 and RFD9 are macrophage markers, RFD1 recognizes a class II-associated epitope present on activated B cells, DC, and AM (16). RFD7, RFD9, and RFD1 were kindly provided by Dr. L. W. Poulter (London, UK).

Immunocytology

Cytocentrifuge preparations were prepared of original BAL cells, of the FACS-sorted fractions, and after DC-lymphocyte culture (see below). Of the FACS-sorted fractions, cytospins were prepared immediately after sorting as well as after overnight culture under nonadherent conditions (16 h, 37°C, 5% CO_2 in polypropylene tubes; Falcon, Lincoln Park, NJ).

Cytocentrifuge preparations were fixed in acetone (Merck, Darmstadt, Germany) for 10 min and thereafter incubated with normal rabbit serum 10% (Dako, Gostrup, Denmark) in PBS for 10 min before incubating for 1 h with the appropriate mAb. Subsequently, the slides were either incubated with rabbit anti-mouse antiserum conjugated with horseradish peroxidase (RaMHRP; Dako) for diaminobenzidine (Sigma Chemicals, Axel, the Netherlands) staining, or with alkaline phosphatase anti-alkaline phosphatase for Fast Blue BB Base (Sigma) staining. Most slides were stained for APh, and selected slides were stained for nonspecific esterase. Counterstaining was performed with hematoxylin.

Mixed Lymphocyte Cultures

Responder T cells were isolated from buffy coats of healthy blood donors. Buffy coats were diluted 1:1 with PBS, and mononuclear cells were obtained by Ficoll-Paque (1.079 g/ml; Pharmacia) density gradient separation (15 min, 1,000 *g*). Monocytes and lymphocytes were separated by Percoll (1.063 g/ml; Pharmacia) density gradient centrifugation. Lymphocytes were depleted from B cells and residual monocytes by adherence in a nylon wool column (Polyscience, Warrington, PA) (1 h, 37°C, 5% CO_2). For some experiments, naive CD45RO-negative T cells were obtained by depleting T cells from CD45RO-positive memory T cells. T cells were incubated with UCHL1 (CD45RO) and subsequently with goat anti-mouse IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD45RO-positive cells were depleted with a magnetic cell separator (MACS; Miltenyi Biotec). The various purified T cell populations were stored in liquid nitrogen until use.

For mixed lymphocyte reactions (MLR), various stimulator cell populations were irradiated with 2,000 rad. Various numbers of stimulator cells were cultured with 150,000 allogeneic T cells in RPMI 1640 (GIBCO, Breda, the Netherlands) to which 10% human A^+ serum, penicillin, and streptomycin were added, to a total volume of 200 μl. After 5 days, 0.5 μCi [^{3}H]thymidine was added and the cells were harvested 16 h later. Scintillation was counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland). Cultures were always performed in triplicate. Negative controls ([^{3}H]thymidine incorporation in T cells alone and in the various stimulator cell fractions without T cells) were always less than 500 counts per minute (cpm).

In some phytohemagglutinin (PHA)- or LAF cell-induced T cell proliferation assays, the effect of co-culture with HAF cells was tested. The percentage of inhibition caused by co-culture with HAF cells was calculated from th

proliferative responses as: 100 − [(STIM + HAF)/STIM × 100]; STIM = PHA or LAF cells.

To visualize the interaction between stimulator and responder cells, 15,000 stimulator cells were cultured with 150,000 T cells using 200 μl culture medium in 96-well, flat-bottom plates. At 4 and 24 h, the number of clusters (a group of at least four cells closely attached to each other) was counted with an inverted microscope, the cells (clustered and nonclustered) were harvested, and cytocentrifuge slides were prepared.

Statistics

The numbers of BAL and LAF cells in smokers and nonsmokers were compared with a *t* test. Normal distributions of the proliferative responses were obtained after logarithmic transformation (log(cpm)). Dose-response curves of BAL cells, LAF cells, and HAF cells in the MLR were compared with a *t* test using the log(cpm). *P* values < 0.05 (corrected for multiple analysis) were deemed significant. Using the same method, the effect of co-culture of HAF cells in PHA-driven T cell proliferation was evaluated (17).

Results

Cell Recovery and Viability

The BAL fluids of the smokers contained 46 ± 17 × 10^6 cells (mean ± SD). Cell sorting with the FACS-Vantage yielded 33 ± 12 × 10^4 cells (0.72%) in the LAF fraction. The BAL fluids of the nonsmokers contained only 8.9 ± 6.5 × 10^6 cells, from which 3.0 ± 0.6 × 10^4 cells (0.34%) were obtained in the LAF fraction. Differences between smokers and nonsmokers were significant ($P < 0.01$) both for absolute numbers and percentages.

The viability of BAL cells was 81 ± 8%. The LAF fraction was always more than 95% viable, whereas the HAF cells had a viability of 73%. Viability figures were comparable between smokers and nonsmokers.

Morphology and Immunocytology

In Table 1, the (immuno-)phenotypic characteristics of the original BAL cells, the LAF and HAF fractions, PBM, and blood-derived DC are shown. Due to the limited availability of material, only four to 10 slides per marker could be investigated. There were no gross differences in the percentage of positive cells for any marker between smokers and nonsmokers; therefore, we made no distinction between these two groups in the data on BAL, LAF, and HAF cells in Table 1.

The LAF fraction predominantly consisted (85%) of rounded large monocytic cells that were negative for APh or had only a juxtanuclear spot of this enzyme activity. These LAF cells were negative or weakly positive for nonspecific esterase. There was a low level of contamination with APh-, RFD7-, and RFD9-positive larger macrophages (9 to 11%) and lymphocytes (6%). In some cases, low numbers (< 5%) of granulocytes and epithelial cells were present. The APh-negative LAF cells had a bean-shaped eccentric nucleus. Whereas most of these cells showed a more or less rounded morphology (82 ± 9%) (Figure 1a), only a minor part (18%) showed an irregular outline with long cytoplasmic extensions. After overnight culture under nonadherent conditions (37°C, polypropylene tubes), there was a marked increase in the LAF fraction of cells showing such cytoplasmic extensions or veils (Figure 1c), and values of up to 51 ± 6% dendritic/veiled cells were obtained. These morphologic changes could not be observed after a short incubation period of 1 h at 37°C. Immunocytologically, the APh-negative (or APh-spot) LAF cells showed a high expression of HLA class II molecules and the DC-markers RFD1 and L25. Twenty-two percent of the cells expressed the Langerhans cell marker OKT6 (CD1a); My4 (CD14) was expressed by 60% of the cells. Most LAF cells were negative for LeuM3 (CD14), 32.2 (CD64), RFD7, and RFD9. After overnight culture, there was a marked decrease in the percentage of cells positive for LeuM3 and My4 (Table 1). For the other markers, such changes were not detected (data not shown). Note that the immunocytologic profile of the monocytic LAF

TABLE 1

*Percentages of cells exhibiting cytoplasmic extensions (DC shape) and percentages of marker-positive cells of total BAL cells and of LAF and HAF cell fractions**

	LAF	HAF	BAL	Blood DC	PBM	Bronchial DC
DC shape (F)	18 ± 9	0	0	+	−	+
DC shape (C)	51 ± 6	0	0			
APh	12 ± 7	100 ± 0	93 ± 3	−	−	−
OKIa	90 ± 7	94 ± 9	89 ± 5	+	+	+
RFD1	57 ± 17	48 ± 35	46 ± 31	+	−	+
L25	84 ± 6	75 ± 26	77 ± 19	+	−	+
OKT6	22 ± 10	0 ± 0	2 ± 2	−	−	+/−
My4 (F)	60 ± 3	6 ± 6	ND	+	+	+/−
My4 (C)	26 ± 9	ND	ND			
LeuM3 (F)	15 ± 6	0 ± 0	ND	−	+	−
LeuM3 (C)	6 ± 4	ND	ND			
32.2	14 ± 15	72 ± 9	ND	ND	ND	ND
KIM7	78 ± 16	100 ± 0	ND	+	+	+
RFD7	5 ± 6	60 ± 17	ND	−	−	−
RFD9	12 ± 7	75 ± 15	ND	−	−	−
Leu4	6 ± 4	0 ± 0	2 ± 2	−	−	−

* For the DC shape and My4 and LeuM3 expression, data of freshly isolated cells (F) and of overnight cultured cells (C) are shown. The data represent the mean ± SD of four to 10 slides of smokers and nonsmokers together. For comparison, the reactivity with these markers of blood-derived DC, PBM, and bronchial epithelial DC (as was described in an earlier study [7]) is shown; + = positive, − = negative, +/− = partially positive, ND = not done.

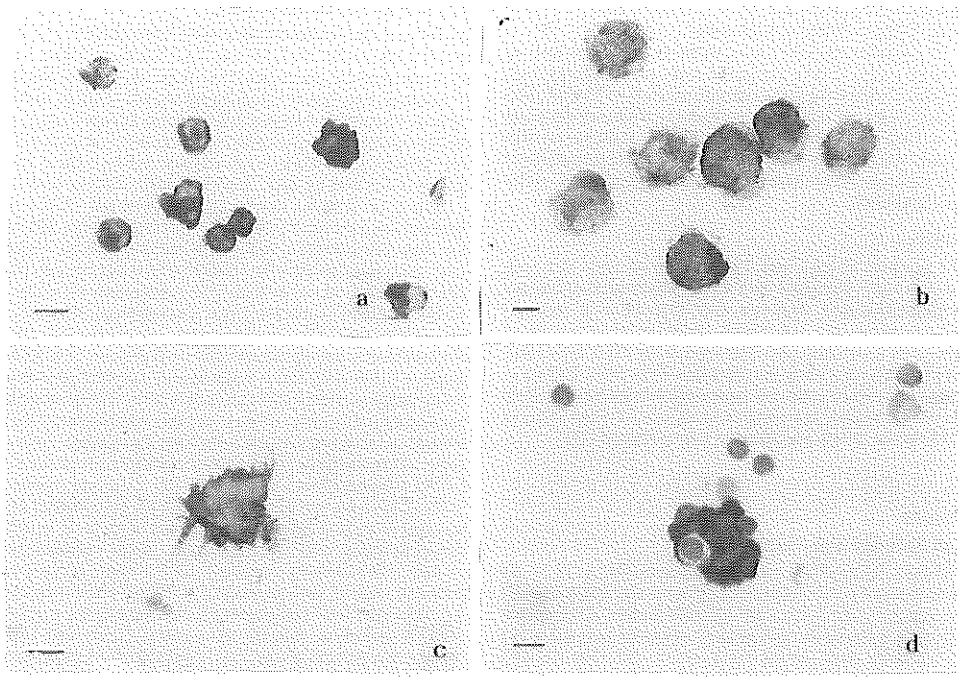


Figure 1. LAF cells are monocyte-like cells that are strongly positive for L25 and negative for APh and exhibit (freshly isolated) a rounded morphology (a). HAF cells are large rounded cells, strongly positive for APh and weakly positive for L25 (b). During overnight culture, LAF cells develop long cytoplasmic extensions (c). An intimate cluster of a LAF cell (stained with OKIa) with several T cells caught in its long extensions (d). Bar = 10 μm.

cells, though mostly rounded, parallels that of blood DC but not that of PBM (Table 1).

HAF cells were all rounded cells with a round or oval nucleus and all were strongly positive for APh and nonspecific esterase (characteristic AM; Figure 1b). HAF cells were also strongly positive for CD68, RFD9, and MHC class II molecules, but only a portion of these cells was positive for RFD1 and RFD7. L25, though positive, was usually expressed more weakly on these cells than on LAF cells. The HAF fraction was completely devoid of lymphocytes, DC, and the monocyte-like cells described in the LAF fraction. Overnight culture did not change the morphologic appearance of the HAF cells (they did not obtain a dendritic/veiled appearance).

Mixed Lymphocyte Cultures

As is shown in Figure 2, LAF cells vigorously stimulated allogeneic T cell proliferation. Significant proliferation was already obtained at stimulator-responder ratios of 1:40. LAF cell–induced T cell responses were comparable to those of blood DC, which we described earlier (9). BAL cells were poor stimulators of allogeneic T cell proliferation; HAF cells were almost nonstimulatory and only had some effect at a stimulator-responder ratio of 1:1. In all ratios, proliferative responses produced by LAF cells, BAL cells, and HAF cells were significantly different. BAL and LAF cells of nonsmokers produced higher T cell responses in comparison with those of smokers. These differences were statistically significant ($P < 0.05$) for the stimulator-responder ratios of 1:40 to 1:5 of the BAL cells. There were no differences in the responses induced by HAF cells between smokers and nonsmokers.

Since LAF cells showed a marked heterogeneity in the expression of several markers (e.g., CD1a), we wondered whether or not this phenotypical heterogeneity was associated with a functional heterogeneity within the LAF cell population. In two experiments, CD1a-positive cells were isolated from the LAF fractions via FACS sorting. These CD1a-positive LAF cells induced even higher responses than total LAF cells (74,937 cpm versus 43,821 cpm).

PBM were poor stimulators of total CD3-positive T cells when compared to LAF cells (Figure 3). In contrast to PBM, LAF cells were capable of stimulating CD45RO-negative, naive T cells (Figure 3). In these experiments, PHA responses of total T cells and naive T cells were comparable.

During mixed lymphocyte culture, LAF cells formed clusters with allogeneic T cells and allogeneic naive T cells. As early as 4 h after the beginning of the culture, some small

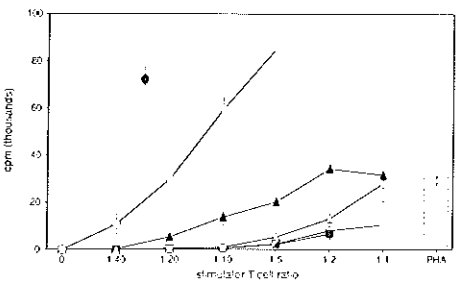


Figure 2. MLR of BAL cells (*triangles*), LAF cells (*circles*), and HAF cells (*squares*) of five nonsmokers (*closed symbols*) and five smokers (*open symbols*) in different stimulator-to-T cell ratios. LAF cells of nonsmokers could only be tested in a ratio of 1:30; 10 μg/ml PHA was used as a positive control. Data represent the mean ± SEM.

clusters of just a few cells could be observed. After 24 h, many more clusters had been formed (Table 2), which grew slowly in size over the following days. In cytospin preparations, clusters always contained one or more dendritic LAF cells and many T cells. The T cells were often located in the veils or cytoplasmic extensions of the LAF cells (Figure 1d). The LAF cells in the clusters strongly reacted with the DC markers OKIa, L25, RFD1, and CD1a. Surprisingly, however, there were also LAF cells showing classic veils or long cytoplasmic extensions and strong positivity for DC markers, but which did not cluster with T cells. PBM formed only a few clusters with T cells after 24 h. HAF cells, on the contrary, did not form clusters with T cells in culture, but some superficial HAF cell–T cell interactions could be observed in the cytospin preparations.

Since in all experiments (see Figures 2 and 3) HAF cells were extremely poor stimulators in the MLR, and since other investigators reported immunosuppressive properties of AM, we tested the possible inhibitory effect of co-culture of these cells on PHA-driven T cell proliferation. HAF cells, when co-cultured at a ratio of 1:5 to T cells, showed a variable but significant inhibition of PHA-driven T cell proliferation of 43 ± 22% (range 0 to 94%, $n = 8$, $P < 0.05$; Figure 4). With a HAF cell–T cell ratio of 1:1, a comparable percentage of 54 ± 44% inhibition was observed (range 0 to 97%, $n = 5$, NS). There were no differences between smokers and nonsmokers regarding the immunosuppressive properties of the HAF cells. The HAF cell–induced inhibition of PHA-driven mitogenesis could not be reduced by the addition of indomethacin or by the nitric oxide synthesis inhibitor *N*-monomethylarginine (NMMA). In these experiments ($n = 4$), HAF cells (1:5) inhibited PHA-induced responses for 75% (48 to 94%) versus 79% (58 to 95%) with indomethacin (1 μg/ml) added, 73% (44 to 93%) with NMMA (0.5 mM) added, and 74% (51 to 94%) with both substances added. Other concentrations of these substances tested did not have any effect either (not shown). In contrast to the immunosuppressive activity in PHA-driven T cell proliferation, HAF cells in a ratio of 1:5 to T cells enhanced T cell stimulation by LAF cells by 25 ± 8% ($n = 3$). However, in a higher HAF cell–T cell ratio (1:1), LAF cell–stimulated T cell proliferation was reduced by 26% compared to LAF cells cultured with T cells alone.

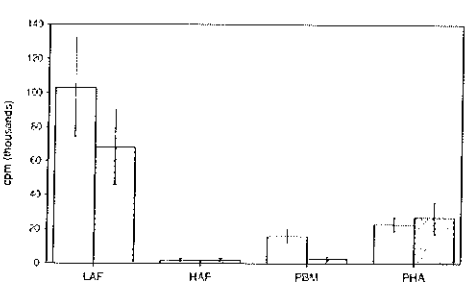


Figure 3. Comparison of MLR with allogeneic T cells (*open bars*) and with allogeneic naive CD45RO-negative T cells (*hatched bars*). LAF cells (1:30), HAF cells (1:5), PBM (1:5), and PHA (10 μg/ml) were used as stimulators. LAF cells and HAF cells used for these experiments, were obtained from three nonsmokers and from one smoker. Data represent the mean ± SD.

TABLE 2

*Numbers of clusters in six microscopic fields (200×) after 4 and 24 h of culture of 15,000 LAF cells, HAF cells, or PBM, together with 150,000 T cells**

	4 h	24 h
LAF	16 ± 9	55 ± 16
HAF	0	0
PBM	0	16 ± 5

* Data represent the mean ± SD of four experiments.

Discussion

This report shows that we were able to separate human BAL cells on the basis of autofluorescence into two immunocytologically and functionally different fractions. The LAF cells were potent accessory cells and stimulated naive T cell proliferation in an allogeneic MLR, whereas the HAF cells were very poor stimulators in an MLR and even suppressed T cell proliferation in PHA-driven cultures.

Freshly isolated LAF cells predominantly showed a rounded, monocyte-like morphology, but their marker pat-

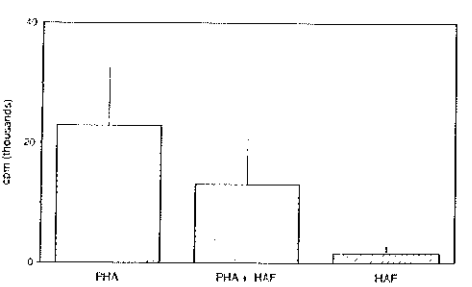


Figure 4. T cell proliferation induced by PHA, by PHA + HAF cells (1:5), and by HAF cells (1:5) alone. Data represent the mean ± SD.

tern was characteristic of DC. Monocyte-like cells in the BAL were until now regarded as precursors of mature AM (18). However, upon overnight culture under nonadherent conditions, half of the LAF cells developed cytoplasmic extensions, whereas the other half kept their monocyte-like appearance. This suggests that the rounded LAF cells rather differentiate into DC than into AM. Moreover, the marker pattern of the rounded LAF cells was comparable to that of blood-derived DC and to that of DC in the bronchial wall (9). In contrast to PBM, LAF cells expressed L25, RFD1, CDla, and high amounts of MHC class II antigens. On the other hand, LAF cells lacked typical macrophage markers (RFD7, RFD9, and APh). KIM7 (CD68), which is generally known as a pan-macrophage marker, was detected on LAF cells but has also been described on DC by us and others (9, 19–21). CD14 expression was only detected with one of the two mAbs used on fresh LAF cells and was down-regulated during overnight culture. Although CD14 is generally regarded as a monocyte marker, we and others have described the reactivity of DC with some CD14 mAbs in (lung) tissue (4, 7). We conclude that the LAF cell fraction of the human BAL contains a population of monocytic cells that are the precursors of dendritic/veiled cells.

In addition to these strong morphologic and immunocytologic similarities of LAF cells with DC, LAF cells formed large clusters with T cells and vigorously stimulated allogeneic T cell proliferation. Clustering with T cells is an important feature of DC and is essential for the induction of T cell proliferation (22). Since the LAF cells could potently stimulate (and cluster with) naive CD45RO-negative T cells and since stimulation of naive (unprimed) T cells is a hallmark of DC (4), these data also clearly show that the functioning of LAF cells from BAL fluid compares with the functional capabilities of DC. The notion that CDla-positive LAF cells were even more potent stimulators of allogeneic (naive) T cell responses further supports this view, but also shows the immunohistochemical and functional heterogeneity within the LAF cell fraction. This heterogeneity was noted with other markers as well (e.g., My4 and RFD1) and with the cluster behavior of the cells (some LAF cells formed large clusters with allogeneic T cells, whereas others did not). The heterogeneity in the LAF cell fraction might reflect the coexistence of different cell lineages or differences in maturation and activation states of one lineage.

With regard to our separation method, autofluorescence has been correlated to the number of lysosomes (23), which is very low in DC and high in macrophages (24). The separation of low autofluorescent DC (and monocyte-like cells) from high autofluorescent macrophages has been previously described for human lung tissue (10) and for rat BAL cells (11). Twenty to thirty percent of the bronchial intraepithelial DC are positive for the Langerhans cell marker CDla, while DC elsewhere in the lung are negative for this marker (9, 25). Nicod and colleagues (10) did not observe CDla expression in DC isolated from minced and digested lung tissue. This suggests that DC isolated from digested lung tissue are probably derived from interstitial DC, whereas our LAF cells—that are partially positive for CDla—are at least in part derived from bronchial epithelial DC.

Although our experiments were not designed to assess differences between smokers and nonsmokers, we found a higher percentage of LAF cells recovered from the BAL of smokers than from nonsmokers. This is in accordance with the higher percentage of monocyte-like cells (resembling our LAF cells) in the BAL of smokers described before (8). This increased influx of monocyte-like cells (and DC) in the lungs can be explained by the earlier described observation that bronchial epithelial cells release monocyte chemotactic factors (leukotriene B_4) in response to cigarette smoke (26). On the other hand, it is likely that AM also contribute to the influx of monocytes, since (*1*) numerous effects of cigarette smoke on their function have been described (27), (*2*) AM are able to produce several monocyte chemotactic factors (e.g., MCP-1 [28]), and (*3*) AM from smokers have been shown to spontaneously produce chemotactic factors for neutrophils (29). In contrast to the higher percentages of LAF cells in smokers, BAL cells and LAF cells of smokers produced lower T cell responses in the MLR than those of nonsmokers. These functional differences between BAL cells of smokers and nonsmokers are comparable with the data of Laughter and associates (30). The mechanism by which smoking lowers the functional capabilities of these LAF cells remains to be elucidated. In AM, it has been shown that cigarette smoke (among other effects) lowers the expression of the adhesion molecules CD11/CD18 (8) and diminishes interleukin-1 production (31), both of which might affect the interactions of AM with T cells. Whether smoking affects the function of LAF cells in a similar way remains speculative. Except for direct or indirect functional impairment of LAF cells, smoking might induce differentiation towards phagocytic nonstimulatory AM. However, further research is needed to test these hypotheses.

The HAF cells consisted of 100% strongly APh-positive AM with a high expression of CD68, RFD7, RFD9, and MHC class II antigens. Despite the MHC class II positivity, the AM had a poor T cell–stimulatory capacity in the MLR and they did not form clusters with T cells. Only in very high stimulator-to-responder ratios was a low stimulation observed. This poor stimulatory capability of the AM could not be attributed to poor cell viability, since all adherent AM were still viable after 6 days of culture. Although macrophages in general are regarded as antigen-presenting cells, there is a large body of evidence that AM are able to exert immunosuppressive functions as well (5, 6, 32–34). Our data concerning the ability of HAF cells to inhibit PHA-driven T cell proliferation are consistent with these earlier studies. Several mediators (nitric oxide [35], prostaglandins [36], interleukin-1 receptor agonist [37], and transforming growth factor-β [38]) and cell-cell contact (39) have been proposed to be involved in AM-mediated suppression of T cell proliferation. In our hands, this inhibition was not reduced by prostaglandin or nitric oxide synthesis inhibition. Concerning the strength of inhibition, however, there was a large interindividual variability, which suggests a variable activity and/or a variable number of suppressor macrophages in the various BALs. This variability was not related to smoking habits, which is in accordance with the data of Weissman and colleagues (40). Several surface markers (RFD1, RFD7) on the AM also showed a marked heterogeneity, which has been attributed to differences in stimulator and suppressor populations of AM (41). When HAF cells co-cultured with LAF cells were compared with LAF cells alone, inhibition of T cell proliferation was only detected with a very high HAF cell–T cell ratio (1:1), whereas in lower ratios (1:5), T

cell proliferation was enhanced. This also suggests a critical balance between stimulator and suppressor activity in AM. On the other hand, this observation shows that in the same HAF cell-T cell ratio, proliferative responses can be either enhanced (LAF cell responses) or suppressed (PHA responses) depending on the stimulatory signals for the T cells involved.

From our report, it can be concluded that two functionally and morphologically completely different cell populations can be obtained from human BAL using FACS sorting on the basis of autofluorescence. The LAF cell population contains functional DC and their precursors. The HAF cell population consists of AM. This method enables the investigation of DC (precursors) and AM of human BAL separately, in various disease states of the lung.

Acknowledgments: This study has been supported by a research grant of the Nederlands Astma Fonds. We thank Marella de Bruin for introducing us to FACS sorting, Tar van Os for the preparation of photographs and figures, Wim Hop (Department of Biostatistics, Erasmus University) for his support with the statistical analysis, and Martha Canning for correction of the English language.

References

1. Inaba, K., and R. M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717–1735.
2. Van Voorhis, W. C., L. S. Hair, R. M. Steinman, and G. Kaplan. 1982. Human dendritic cells, enrichment and purification from peripheral blood. *J. Exp. Med.* 155:1172–1187.
3. Austyn, J. M. 1987. Lymphoid dendritic cells. *Immunology* 62:161–170.
4. Knight, S. C., and A. J. Stagg. 1993. Antigen-presenting cell types. *Curr. Opinion Immunol.* 5:374–382.
5. Holt, P. G. 1979. Alveolar macrophages. II. Inhibition of lymphocyte proliferation by purified macrophages from the rat lung. *Immunology* 37:429–436.
6. Herscowitz, H. B., R. E. Conrad, and K. J. Pennline. 1980. Alveolar macrophage-induced suppression of the immune response. *Adv. Exp. Med. Biol.* 121:459–484.
7. Crystal, R. G. 1991. Alveolar macrophages. *In* The Lung: Scientific Foundations. R. G. Crystal, editor. Raven Press, New York. 527–538.
8. Hoogsteden, H. C., P. T. W. van Hal, J. M. Wijkhuis, W. Hop, A. P. K. Verkaik, and C. Hilvering. 1991. Expression of the CD11/CD18 cell surface adhesion glycoprotein family on alveolar macrophages in smokers and nonsmokers. *Chest* 10:487–492.
9. van Haarst, J. M. W., H. J. de Wit, H. A. Drexhage, and H. C. Hoogsteden. 1994. Distribution and immunophenotype of mononuclear phagocytes and dendritic cells in the human lung. *Am. J. Respir. Cell Mol. Biol.* 10:487–492.
10. Nicod, L. P., M. F. Lipscomb, J. C. Weissler, C. R. Lyons, J. Albertson, and G. B. Toews. 1987. Mononuclear cells in the human lung parenchyma, characterization of a potent accessory cell not obtained by bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 136:818–823.
11. Havenith, C. E. G., A. J. Breedijk, P. P. M. C. van Miert, N. Blijleven, W. Calame, R. H. J. Beelen, and E. C. M. Hoefsmit. 1993. Separation of alveolar macrophages and dendritic cells via autofluorescence: phenotypical and functional characterization. *J. Leukoc. Biol.* 53:504–510.
12. Kabel, P. J., M. de Haan-Meulman, H. A. M. Voorbij, M. Kleingeld, E. F. Knol, and H. A. Drexhage. 1989. Accessory cells with a morphology and a marker pattern of dendritic cells can be obtained from elutriator purified blood monocyte fractions, an enhancing effect of metrizamide in this differentiation. *Immunobiology* 179:395–411.
13. Mooij, P., P. J. Simons, M. de Haan-Meulman, H. J. de Wit, and H. A. Drexhage. The effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells. A role of GM-CSF, TNFα and IL-6. *J. Endocrinol.* (In press)
14. Ishii, Y., T. Takami, Y. Kokai, H. Yuasa, J. Fujimoto, and T. Takei. 1985. A novel human B cell antigen shared with lymphoid dendritic cells: characterization by a monoclonal antibody. *Clin. Exp. Immunol.* 61:624–632.
15. Kabel, P. J., M. de Haan, R. D. van der Gaag, and H. A. Drexhage. 1987. Intrathyroidal dendritic cells. *J. Clin. Endocrinol. Metab.* 66:199–207.
16. Poulter, L. W., D. A. Campbell, C. Munro, and G. Janossy. 1986. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scan. J. Immunol.* 24:351–357.
17. Glantz, S. A. 1987. Primer of Biostatistics. McGraw-Hill, New York, NY.
18. Hoogsteden, H. C., P. T. W. van Hal, J. M. Wijkhuijs, W. Hop, and C. Hilvering. 1990. Alveolar macrophages with immature and mature morphology and peripheral blood monocytes exhibit a distinct immunophenotype. *In* Heterogeneity of Mononuclear Phagocytes in Interstitial Lung Diseases. H. C. Hoogsteden, editor. Haveka b.v., Alblasserdam, the Netherlands.
19. Pulford, K. A. F., A. Sipos, J. L. Cordell, W. P. Stross, and D. Y. Mason. 1990. Distribution of the CD68 macrophage/myeloid associated antigen. *International Immunology* 2:973–980.
20. Kelly, P. M. A., E. Bliss, J. A. Morton, J. Burns, and J. O. D. McGee. 1988. Monoclonal antibody EBM/11: high cellular specificity for human macrophages. *J. Clin. Pathol.* 41:510–515.
21. Beljes, M. G. H., M. C. Haks, C. W. Tuk, and R. H. J. Beelen. 1991. Monoclonal antibody EBM11 (anti-CD68) discriminates between dendritic cells and macrophages after short term culture. *Immunobiology* 183:79–87.
22. Austyn, J. M., D. E. Weinstein, and R. M. Steinman. 1988. Clustering with dendritic cells precedes and is essential for T cell proliferation in a mitogenesis model. *Immunology* 63:691–696.
23. Kradin, R. L., K. M. McCarthy, F. I. Preffer, and E. E. Schneeberger. 1986. Flow-cytometric and ultrastructural analysis of alveolar macrophage maturation. *J. Leukoc. Biol.* 40:407–417.
24. Beelen, R. H. J., H. J. Bos, E. W. A. Kamperdijk, and B. C. M. Hoefsmit. 1989. *In* Human Monocytes. M. Zembala and G. L. Asherson, editors. Academic Press, London, UK. 7–26.
25. Holt, P. G., M. A. Schon-Hegrad, M. J. Philips, and P. G. McMenamin. 1989. Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* 19:597–601.
26. Koyama, S., S. I. Rennard, G. D. Leikauf, and R. A. Robbins. 1991. Bronchial epithelial cells release monocyte chemotactic activity in response to smoke and endotoxin. *J. Immunol.* 147:972–979.
27. Costabel, U., and J. Guzman. 1992. Effects of smoking on bronchoalveolar lavage constituents. *Eur. Respir. J.* 5:776–779.
28. Brieland, J. K., M. L. Jones, S. J. Clarke, J. B. Baker, J. S. Warren, and J. C. Fantone. 1992. Effect of acute inflammatory lung injury on the expression of monocyte chemoattractant protein-1 (MCP-1) in rat pulmonary alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 7:134–139.
29. Hunninghake, G. W., and R. G. Crystal. 1983. Cigarette smoke and lung destruction. *Am. Rev. Respir. Dis.* 128:833–838.
30. Laughter, A. H., R. R. Martin, and J. J. Twomey. 1977. Lymphoproliferative responses to antigens mediated by human alveolar macrophages. *J. Lab. Clin. Med.* 89:1326–1332.
31. Yamaguchi, E., N. Okazaki, A. Itoh, S. Abe, Y. Kawakami, and H. Okuyama. 1989. Interleukin-1 production by alveolar macrophages is reduced in smokers. *Am. Rev. Respir. Dis.* 140:397–402.
32. Toews, G. B., W. C. Vial, M. M. Dunn, P. Guzzetta, G. Nunez, P. Stastny, and M. F. Lipscomb. 1984. The accessory cell function of human alveolar macrophages in specific T cell proliferation. *J. Immunol.* 132:181–186.
33. Holt, P. G., A. Degebrodt, C. O'Leary, K. Krska, and T. Plozza. 1985. T cell activation by antigen-presenting cells from lung tissue digests: suppression by endogenous macrophages. *Clin. Exp. Immunol.* 62:586–593.
34. Schauble, T. L., W. H. Boom, C. K. Finegan, and E. A. Rich. 1993. Characterization of suppressor function of human alveolar macrophages for T lymphocyte responses to phytohemagglutinin: cellular selectivity, reversibility, and early events in T cell activation. *Am. J. Respir. Cell Mol. Biol.* 8:89–97.
35. Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen. 1993. Downregulation of the antigen-presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J. Exp. Med.* 177:397–407.
36. McCombs, C. C., J. P. Michalski, B. T. Westerfield, and R. W. Light. 1982. Human alveolar macrophages suppress the proliferative response of peripheral blood lymphocytes. *Chest* 82:266–271.
37. Moore, S. A., R. M. Strieter, M. W. Rolfe, T. J. Standiford, M. D. Burdick, and S. L. Kunkel. 1992. Expression and regulation of human alveolar macrophage–derived interleukin-1 receptor antagonist. *Am. J. Respir. Cell Mol. Biol.* 6:569–575.
38. Wahl, S. M., N. McCartney-Francis, and S. E. Mergenhagen. 1989. Inflammatory and immunomodulatory roles of TGF-β. *Immunol. Today* 10:258–261.
39. Rich, E. A., C. Cooper, Z. Toossi, M. L. Leonard, R. M. Stucky, R. T. Wiblin, and J. J. Ellner. 1991. Requirement for cell-to-cell contact for the immunosuppressive activity of alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 4:287–294.
40. Weissman, D. N., R. D. de Shazo, D. E. Banks, and Y. Baser. 1986. Immunomodulation by bronchoalveolar lavage cells in normal individuals and patients with bronchogenic carcinoma. *Am. J. Med. Sci.* 292:187–192.
41. Spiteri, M. A., and L. W. Poulter. 1991. Characterization of immune inducer and suppressor macrophages from the human lung. *Clin. Exp. Immunol.* 83:157–162.

GLUCOCORTICOIDS DECREASE ALLOSTIMULATORY FUNCTION AND B7 EXPRESSION OF HUMAN AIRWAY DENDRITIC CELLS IN VITRO

Jan Maarten W. van Haarst[1,2], Gert T. Verhoeven[2], Harm J. de Wit[1], Hemmo A. Drexhage[1], Gertrude M. Möller[1,2] and Henk C. Hoogsteden[2].

Dept. of Immunology[1] and Dept. of Pulmonary Medicine[2], Erasmus University and University Hospital Dijkzigt, Rotterdam, the Netherlands.

ABSTRACT

Dendritic cells (DC) are the most potent accessory cells for T cell stimulation. DC thereby play a major role in the initiation and the propagation of immune responses. Glucocorticoids (GC) exert multiple anti-inflammatory actions on immune cells. However, there are no data on the effects of GC on the function of DC. We investigated the influence of GC on the functional and immunocytologic properties of DC isolated from human bronchoalveolar lavage (BAL). Airway DC incubated with therapeutic concentrations of dexamethasone showed an impaired accessory capability in the allogenic mixed lymphocyte reaction (inhibition at dexamethasone concentrations of 10 nM: 30.7 ± 19.2%; 100 nM: 35.0 ± 23.9%; 1 μM: 50.9 ± 21.0% and 10 μM: 55.5 ± 10.5%). GC incubation significantly suppressed the expression of B7-1 (CD80). The expression of CD11b on DC was downregulated by GC incubation in 4 out of the 6 subjects tested. GC did not affect the expression of other adhesion molecules or "typical" DC markers, such as HLA-DR, RFD1, L25 and CD1a. The percentage of cells exhibiting a DC morphology was also not influenced by GC. We conclude that GC downregulate airway DC accessory capability, which may be partly explained by the repression of B7 expression. The GC induced suppression of the T cell stimulatory potential of DC might play an important role in the downregulation of bronchial inflammation in asthma.

INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells (APC) and these cells are the most potent accessory cells for the stimulation of T cells[1]. In the lung, DC are predominantly situated in and just underneath the bronchial epithelium, often in small clusters with T cells[2,3]. In asthma, increased numbers of RFD1$^+$ and CD1a$^+$ DC and (activated) T cells have been described at these locations[4,5,6]. T cells are thought to play a crucial role in the development of asthma[7], and we suggest that DC (as the major T cell stimulators) play a role herein as well. In MHC class II-deficient mice, the resistance to eosinophilic airway inflammation was attributed to the lack of mature T cells[8], but might also point to a role for class II-positive APC, such as DC.

During glucocorticoid (GC) therapy, which is considered the most effective treatment in asthma[9], the numbers of both RFD1$^+$ and CD1a$^+$ cells and (activated) T cells in the bronchial mucosa are reduced[4,5,10]. However, it is not clear, whether only the actual numbers of DC are modulated, or whether in addition, their marker expression is downregulated as well. Studies in the rat suggest that GC administration inhibits the influx of DC in the airway wall[11]. There are, however, no data on the effects of GC on marker expression or

function of DC. Studies on monocytes and on epidermal Langerhans cells suggest that GC downregulate the T cell stimulatory potential of these cells[12,13,14]. The possible effects of GC on airway DC stimulatory capacity may have consequences for the activation of T cells and the outcome of the immune response in the airways and for asthma in particular.

These considerations prompted us to study the effects of GC on the T cell stimulatory potential and the immunological marker pattern of highly purified DC isolated from the human bronchoalveolar lavage (BAL). For comparison, the effects of GC on peripheral blood monocytes (PBM) were studied as well.

MATERIAL AND METHODS

Bronchoalveolar lavage

After informed consent, BAL was performed on individuals during anaesthesia for routine elective surgery. Subjects with a history of pulmonary or systemic disease were excluded. Nine smokers (more than 5 cigarettes a day) and 7 lifelong nonsmokers were included in the study (mean age 39 yrs, range 26-49 yrs). BAL was performed with a flexible bronchoscope placed in wedge position in the right middle lobe. Four aliquots of 50 ml saline were subsequently instilled and aspirated. BAL fluid (total volume ranged from 110 to 150 ml) was collected in siliconized bottles. The procedure has been approved by the Medical Ethical Committee of the Erasmus University and University Hospital Dijkzigt.

Isolation and purification of DC

BAL cells were kept at 4°C, washed twice in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 0.45% glucose, and subsequently filtered through a 55μ and a 30μ gauze. BAL cells were sorted on a FACS-Vantage (Becton & Dickinson, Erembodegem, Belgium) with a 488 nm laser. Sort windows were generated on autofluorescence (FL1, 530 nm) to create a population with a low autofluorescence (to exclude AM), on forward scatter (FSC) to exclude small cells (lymphocytes) and debris, and on sideward scatter (SSC) to exclude cells with a high SSC (granulocytes and macrophages). As we described previously, this procedure yields a population of low autofluorescent cells, highly purified for dendritic cells and their precursors with only small contaminations of AM (±10%) and lymphocytes (±5%)[15]. This population will be further referred to as DC. Peripheral blood monocytes (PBM) (used as a control population) were purified according to techniques described in detail elsewhere[16,17]. In short, monocytes were isolated from heparin blood or buffy coats by subsequent Ficoll-Paque (1,079 g/ml; Pharmacia, Uppsala, Sweden) and Percoll (1.063 g/ml; Pharmacia) density gradient separation.

Glucocorticoid incubation

After isolation BAL DC and PBM were preincubated overnight under non-adherent conditions in RPMI containing 10% FCS (16 h., 37°C, 5% CO_2, polypropylene tubes, Falcon, Lincoln Park, NJ) with or without various concentrations of dexamethasone (Sigma, St. Louis, MO). To prevent contamination of the mixed lymphocyte reactions with dexamethasone, cells were washed 4 times after this incubation period, and between each wash cells were incubated at 37°C for 30 min. To check this procedure for residual dexamethasone contamination of DC or PBM populations, ^{3}H-dexamethasone was used. ^{3}H-dexamethasone concentration in the supernatant and the cellular ^{3}H-dexamethasone content was measured after 24 h. culture of ^{3}H-dexamethasone preincubated and washed DC or PBM. In selected experiments beclomethasone dipropionate and fluticasone propionate were used in the same procedure.

Monoclonal antibodies

The following Moab's were used: OKIa (HLA-DR) and OKM1 (CD11b) (Ortho Diagnostics Systems, Beerse, Belgium); LFA 1/2 (CD11a) and LFA 1/1 (CD18) (CLB, Amsterdam, the Netherlands); LeuM5 (CD11b) (Becton & Dickinson, San Jose, CA); My4 (CD14) and 4B4 (CD29) (Coulter Immunology, Hialeah, FL); HP2.1 (CD49d)(Immunotech, Marseille, France); BBA-3 (anti-ICAM-1, CD54)(British Biotechnology, Oxon, U.K.); OKT6 (CD1a) (American Type Culture Collection, Rockville, MD). L25 is a MoAb directed against B cells and DC[18,19] and was a generous gift of Dr.T. Takami (Gifu, Japan); RFD1 recognizes a class II-associated epitope present on activated B cells, dendritic cells and alveolar macrophages[20] and was kindly provided by Dr.L.W. Poulter, London, UK. B7-24 (anti-B7-1, CD80) was a gift from M. de Boer, Immunogenetics, Gent, Belgium. HB15a (CD83) was provided by T.F. Tedder, Boston, MA. TS2/9 (anti-LFA3, CD58) was a gift of T. Schumacher, Amsterdam, the Netherlands.

Immunocytology

Cytocentrifuge preparations were prepared of the FACS-sorted DC-fraction, immediately after sorting as well as after overnight culture in RPMI/FCS with or without 1μM dexamethasone.

Cytocentrifuge preparations were fixed in acetone (Merck, Darmstadt, Germany) for 10 min, and thereafter incubated with normal rabbit serum 10% (Dako, Gostrup, Denmark) in PBS for 10 min before incubating for 1 hr with the appropriate monoclonal antibody (MoAb). Subsequently, the slides were either incubated with rabbit-anti-mouse antiserum conjugated with horseradish peroxidase (RaMHRP; Dako) for diaminobenzidine (Sigma Chemicals, Axel, the Netherlands) staining, or with alkaline phosphatase anti-alkaline phosphatase for

Fast Blue BB Base (Sigma) staining. Most slides were stained for acid phosphatase and selected slides were stained for non-specific esterase. Counterstaining was performed with hematoxylin.

Mixed lymphocyte reactions

Responder T cells were isolated from buffy coats of healthy blood donors. Buffy coats were diluted 1:1 with PBS, and mononuclear cells were obtained by Ficoll-Paque (1.079 g/ml; Pharmacia) density gradient separation (15 min., 1000 g). Monocytes and lymphocytes were separated by Percoll (1.063 g/ml; Pharmacia) density gradient centrifugation. Lymphocytes were depleted from B cells and residual monocytes by adherence in a nylon wool column (Polyscience, Warrington, PA) (1 h., 37°C, 5% CO_2). The purified T cell populations were stored in liquid nitrogen until use.

For mixed lymphocyte reactions (MLR), the stimulator cell populations were irradiated with 2000 Rad. Stimulator cells (7,500 DC or 30,000 PBM) were cultured with 150,000 allogeneic T cells (stimulator-responder ratios 1:20 and 1:5 respectively. RPMI 1640 (Gibco, Breda, the Netherlands) to which 10% human A^+ serum, Penicillin and Streptomycin were added, was used as culture medium. Cultures were always performed in triplicate in a flat bottom 96 well plate (Falcon) with a total volume of 200 μl per well. After 5 days, 0.5 μCi ^{3}H-thymidine was added and the cells were harvested 16 h later. Scintillation was counted on a LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland). Negative controls (^{3}H-thymidine incorporation in T cells alone and in the various stimulator cell fractions without T cells) were always less than 500 counts per minute (cpm).

RESULTS

Mixed lymphocyte reactions

Figure 1 depicts the effect the contineous presence of various concentrations of dexamethasone added to MLR's of freshly isolated DC and T cells (no preincubation). Dexamethasone induced a dose-dependent inhibition of T cell proliferation as measured by ^{3}H-thymidine incorporation. To determine whether or not this effect could be (partly) explained by the influence of dexamethasone on DC, DC were preincubated overnight with or without dexamethasone, washed extensively, and added to responder T cells in the MLR. Viability of the DC after overnight preincubation was 85 ± 7% without dexamethasone and 87 ± 6% with 1μM dexamethasone. Dexamethasone contamination after the washing procedure, estimated using ^{3}H-dexamethasone, was as low as 10^{-13}M. In concentrations lower than 10^{-10}M dexamethasone did not affect PHA-stimulated T cell proliferation.

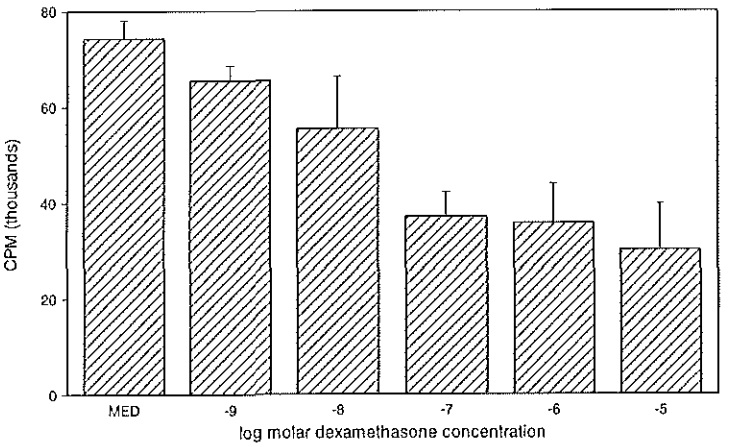


Figure 1. Effect of the continuous presence of dexamethasone (no preincubation) on the MLR of airway DC and allogenic T cells (stimulator-responder ratio 1:20). Data show mean ± SD of one representative experiment.

DC preincubated with dexamethasone produced significant lower T cell responses in the MLR compared to DC preincubated in the absence of dexamethasone (figure 2): preincubation with 10nM dexamethasone lowered DC induced T cell responses to 69.2 ± 19.2% (expressed as percentage of the control value), to 65.0 ± 23.9% with 100nM dexamethasone, to 49.1 ± 21.0% with 1μM dexamethasone, and to 44.5 ± 10.5% with 10μM dexamethasone ($n = 5\text{-}7$, $p < 0.05$ for all concentrations tested as assessed with the Wilcoxon rank sum test). Beclomethasone dipropionate and fluticasone propionate, glucocorticoids used for inhalation therapy, affected DC allostimulatory function in a similar fashion (figure 3).

With respect to smoking habits, dexamethasone induced comparable effects on DC from the BAL of smokers and those of nonsmokers: preincubation overnight with 1μM dexamethasone reduced the proliferative responses to 52.6% of control values in nonsmokers ($n = 3$) and to 46.5% in smokers ($n = 4$).

Dexamethasone had comparable effects on the accessory potential of PBM in the MLR (figure 2). Preincubation with dexamethasone reduced T cell responses to 75.7 ± 8.3% for 10 nM, 54.3 ± 17.2% for 100 nM, and to 47.7 ± 17.8% for 1 μM (percentages of control values).

Immunocytology

FACS sorting yielded a population of low autofluorescent cells with the characteristics of DC and their precursors, as has been described in detail

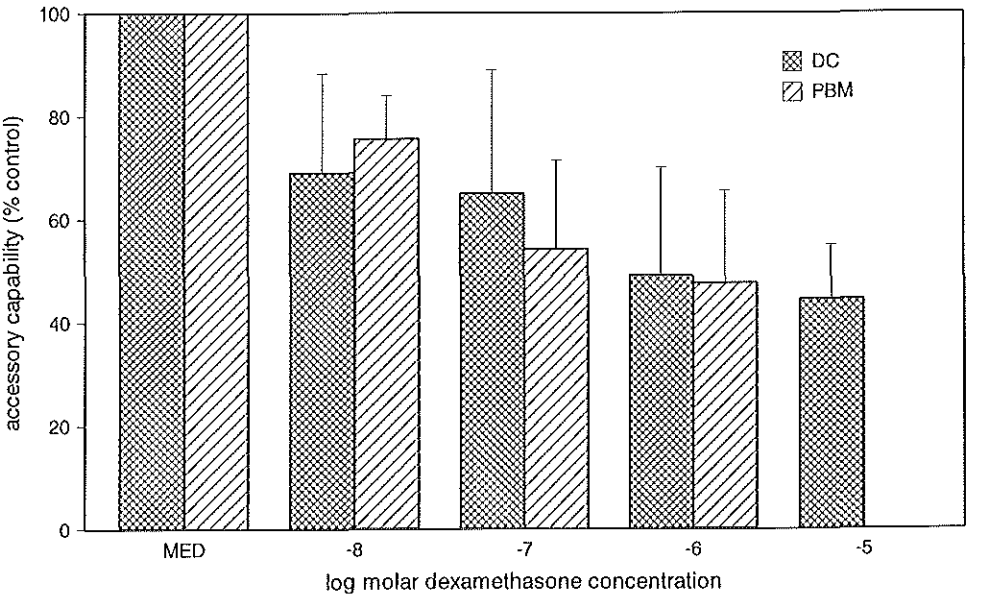


Figure 2. Effects of dexamethasone preincubation (16 h) on accessory function of airway DC and PBM in an allogenic MLR. Data represent mean ± SD of 5 to 7 experiments of ^{3}H-thymidine incorporation expressed as percentage of ^{3}H-thymidine incorporation of the controls (without dexamethasone).

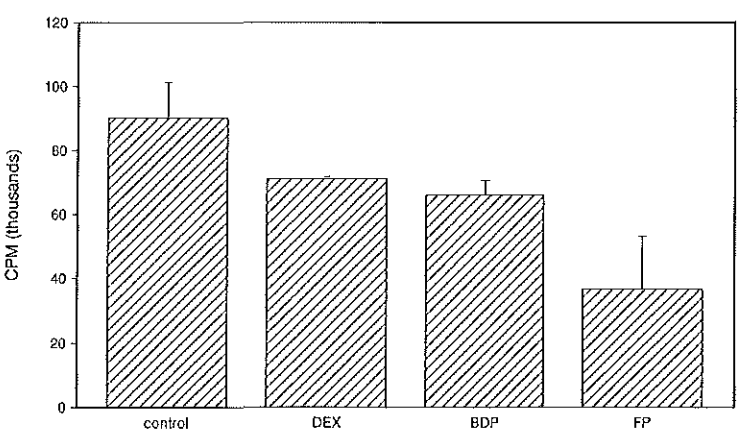


Figure 3. Comparison of accessory function in an allogenic MLR of airway DC after 16 h incubation with 100 nM dexamethasone (DEX), 100 nM beclomethasone dipropionate (BDP), 100 nM fluticasone propionate (FP), or without GC (control). Data represent mean ± SD of ^{3}H-thymidine incorporation in one of two experiments.

before[15]. These cells are negative for acid phosphatase, and bear typical DC markers. The influence of 1 μM dexamethasone (16 h) on the immunocytology of the LAF cells is shown in table 1. The percentage cells with characteristic cellular extensions (DC morphology) was comparable for cells incubated overnight with and without dexamethasone. The expression of HLA-DR, the expression of "typical" DC markers, such as RFD1, L25, CD1a and CD83, and the expression of the monocyte-lineage marker CD14 were not affected by dexamethasone (table 1). Compared to incubation with culture medium alone, dexamethasone reduced the percentage of B7-1 (CD80) positive DC from the BAL of all 6 subjects tested ($p<0.05$) (figure 4). GC strongly downregulated the percentage of CD11b positive DC in 4 out of 6 experiments. In 2 experiments, however, we did not observe any effect of GC on CD11b expression. The expression of other adhesion molecules (CD11a, CD11c, CD18, CD29, CD49d, CD54 and CD58) was not affected by GC (table 1).

	FRESH	MED	DEX
DC-morphology	11	39.0 ± 16.8	36.5 ± 15.6
MoAb's			
OKIa (HLA-DR)	86.3 ± 11.1	93.0 ± 3.7	95.0 ± 2.6
L25	(84 ± 6)	72.5 ± 15.2	74.3 ± 12.2
RFD1	(57 ± 17)	29.3 ± 16.1	25.3 ± 16.7
OKT6 (CD1a)	20.0 ± 14.5	14.8 ± 11.7	14.3 ± 10.8
HB15a (CD83)	4.5 ± 0.5	7.0 ± 0.8	5.0 ± 2.5
MY-4 (CD14)	63.0 ± 13.7	52.5 ± 30.3	52.0 ± 26.2
LFA 1/2 (CD11a)	ND	17.8 ± 11.7	16.6 ± 13.2
OKM1 (CD11b)	45.0 ± 17.1	39.3 ± 15.2	16.2 ± 10.7
LeuM5 (CD11c)	ND	57.4 ± 24.5	56.2 ± 22.2
LFA 1/1 (CD18)	ND	81.4 ± 18.3	89.4 ± 7.6
4B4 (CD29)	ND	84.2 ± 14.8	92.4 ± 4.1
HP2.1(CD49d)	ND	15.3 ± 4.1	13.7 ± 5.6
BBA3 (CD54)	60.3 ± 31.0	62.9 ± 19.1	64.3 ± 15.7
TS2/9 (CD58)	ND	95.0 ± 2.5	93.5 ± 6.0
B7-24 (CD80)	ND	12.4 ± 7.4	4.2 ± 3.9*

Table 1. Immunocytology of freshly isolated LAF cells (FRESH) and LAF cells after 16 h. incubation in culture medium alone (MED) or with dexamethasone (DEX). The data represent the mean ± SD of the percentage of cells exhibiting cellular extensions (DC-morphology) and the percentages of marker-positive cells. *, $p<0.05$ MED vs. DEX as determined with the Wilcoxon rank sum test. Data on fresh LAF cells between parenthesis are derived from a previous study[15], these stainings were not repeated in this study. Not done, ND.

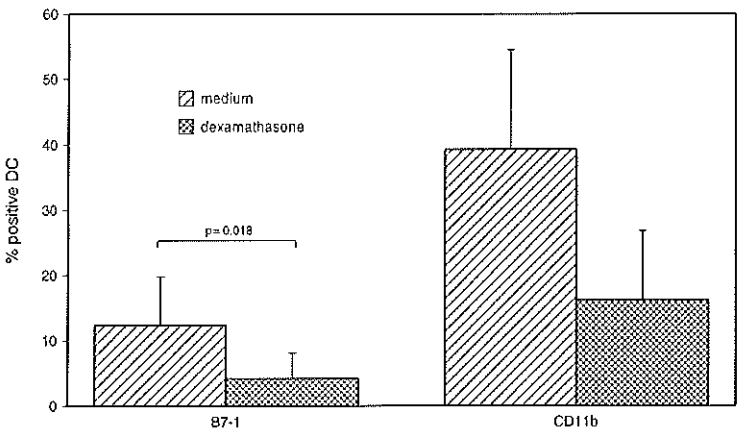


Figure 4. Dexamethasone incubation downregulates B7-1 ($p = 0.018$, $n = 7$) and CD11b ($p = 0.116$, $n = 6$) expression on airway DC. Data represent mean ± SD of the percentage positive cells.

DISCUSSION

In this report we show that therapeutic concentrations of GC significantly downregulate the expression of B7 and inhibit T cell stimulatory function of DC isolated from the BAL. The effect on the MLR could not be attributed to a reduced viability after GC incubation or to a direct effect of contaminating levels of GC after the washing procedure on T cells in the MLR. Although there are no other data on the effects of GC on the function of airway DC, our data are in agreement with studies using other APC, which demonstrated that GC suppress the accessory function of PBM[12,13] and epidermal Langerhans cells[14].

In GC therapy, the functional impairment of DC - the most potent accessory cell for the stimulation of T cells - most likely contributes to the downregulation of the activation and cytokine production of T cells. The latter is an important step in the initiation and propagation of bronchial inflammation in asthma. The effects of GC on APC may play an important role in the response to GC therapy, since corticosteroid-resistant asthma has been shown to be associated with an impaired response of APC to GC. In GC resistant asthma it has been demonstrated that GC fail to suppress monocyte complement receptor expression[21] and that GC do not inhibit the release of monocyte-derived neutrophil activating factor[22]. Moreover, mononuclear cell proliferation could not be suppressed by GC in these patients, which was attributed to a defect in their monocytic cells[23]. It must be noted, however, that the inhibitory effect of GC on T cells is also reduced in GC-resistant asthma[24].

With regard to the mechanisms of the suppressive effects, GC might inhibit cytokine production of airway DC. However, it is generally held that DC produce only very low quantities of cytokines[25]. On the other hand, epidermal Langerhans cells (sharing some properties with our BAL DC) have been described to produce a number of cytokines, including IL-1β, IL-6, MIP-1α, MIP-1β[26,27], and human pulmonary DC have been demonstrated to produce IL-1α, IL-1β and TNF-α [28]. However, these pulmonary DC produced only low amounts of cytokines compared to blood monocytes, and antibodies against IL-1 and TNF-α did not inhibit MLR's induced by these DC. In agreement with these data, we did not observe an inhibition of more than 10% with either anti-IL-1, anti-IL-6, anti-GM-CSF, anti-TNFα or anti-IFNγ in MLR's of DC from the BAL with T cells, whereas anti-IL-1 inhibited the monocyte-driven MLR for almost 60% (unpublished data). Hence: although an impairment of cytokine production might contribute to the GC effect on DC, it is unlikely that the suppressed DC function can be fully attributed to an effect of GC on cytokine production. Therefore we focussed in this study on the effects of GC on marker expression of DC.

In contrast to cytokine production, the expression of several surface markers has been shown to be essential for the accessory potential of DC[29,30,31]. MHC class II expression is required for the presentation of exogenous antigen and for the interaction with the T cell receptor and the CD4 molecule. For PBM it has been reported that GC upregulate MHC class II-expression[12,32]. In these and in other studie [33,15], the height of class II expression was not related to T cell stimulatory properties. In our study, GC did not affect MHC class II-expression on DC, which was constitutively very high. However, others have demonstrated that the glycosylation state of MHC class II molecules and other cell surface membrane molecules on DC differs from that on other APC, which might be crucial for the T cell stimulatory properties of DC[34,35,36].

Besides MHC T cell receptor contacts, adhesion molecules are required for the establishment of intimate intercellular contacts and the formation of clusters between DC and T cells[37]. For human pulmonary DC, the significance of the expression of adhesion molecules was shown by a study of Nicod[31], who demonstrated that several anti-adhesion molecule antibodies are able to block DC-induced allogenic T cell proliferation. Other studies showed that LFA-3 and ICAM-1 expression on the cell membrane of the APC and LFA-1 and CD2 expression by T cells play a major role in T cell stimulation[29]. In our experiments, GC did not repress ICAM-1 (CD54) or LFA3 (CD58) expression on DC from the BAL. In monocytic and bronchial epithelial cell lines, however, GC have been shown to suppress TNF-induced ICAM-1 expression[38,39]. In a few pilot experiments (not published), we also found that GC repressed the upregulation of ICAM-1 expression on peripheral blood monocytes during overnight culture. The constitutive ICAM-1 expression on airway DC contrasting

with a stimulator-induced upregulation of ICAM expression in other cells, might explain this difference.

B7-1 (CD80) interacts with CD28 on the T cell and thereby delivers an essential second signal for T cell activation[40]. In agreement with data from studies in mice on peripheral DC, and on human Langerhans cells[41,42], only a minor portion of the DC from human BAL expressed B7. We observed a downregulation of the B7 expression by GC incubation, which might contribute to the decrease of the functional capability of DC. Since only a small part of the airway DC was positive for B7, it is difficult to hold this phenomenon completely responsible for the lowered accessory capability of the DC. However, CTLA4-Ig (a ligand of B7) strongly inhibits allogenic T cell proliferation induced by either PBM, Langerhans cells or DC, and it was suggested that B7 expression is upregulated during the MLR[41,43]. This suggestion is supported by the observation that B7 expression is induced by cross-linking of MHC class II molecules due to interaction with T cell receptors[44]. It is tempting to hypothesize that GC also inhibit this increase in B7 expression. However, further research is needed to test this hypothesis, and other, yet unrevealed mechanisms may play a role as well.

Of the other adhesion molecules investigated, GC exposure reduced CD11b expression on DC in 4 out of 6 experiments. We speculate that this variability reflects interindividual differences in the sensitivity for GC. CD11b is the α-chain of the C3bi-receptor, but interacts with ICAM and other ligands as well. CD11b plays a role in adhesion to endothelium[45], but probably contributes little to the interaction of DC with the T cell membrane[46]. Downregulation of CD11b expression might play a role in the decreased influx of DC in the lung during GC treatment, as has been described by others[5,11].

In conclusion, our data show that GC strongly reduce the T cell stimulatory potential of human airway DC. We suggest that the inhibition of DC function contributes in the downregulation of bronchial inflammation and the concommitant improvement of bronchial hyperreactivity and clinical symptoms in GC therapy in asthma. Shifts in B7 expression, may contribute to the GC-mediated functional impairment, but the precise mechanisms, how GC compromise DC function, remain to be elucidated.

ACKNOWLEDGEMENTS

This study was supported by a grant of the Netherlands Asthma Foundation. We thank Mascha Verheggen for her support on the ^{3}H-dexamethasone estimations and Tar van Os for the preparation of the figures.

REFERENCES

1. Holt, P.G., M.A. Schon-Hegard, M.J. Philips, and P.G. McMenamin. 1989. Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* 19:597-601.
2. van Voorhis, W.C., J. Valinsky, E. Hoffman, J. Luban, L.S. Hair and R.M. Steinman. 1983. Relative efficacy of human monocytes and dendritic cells as accessory cells for T cell replication. *J.Exp. Med.* 158:174-191.
3. Rochester, C.L. and J.A. Rankin. 1991. Is asthma T cell-mediated? *Am. Rev. Respir. Dis.* 144:1005-1007.
4. Azzawi, M., B. Bradley, P.K. Jefferey et al. 1990. Activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am. Rev. Respir. Dis.* 142:1407-1413.
5. Möller, G.M., S.E. Overbeek, C.G. van Helden-Meeuwsen, J.M.W. van Haarst, E.P. Prens, D.S. Postma and H.C. Hoogsteden. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled glucocorticoids. Submitted.
6. Burke, C., C.K. Power, A. Norris, A. Condez, B. Schmekel and L.W. Poulter. 1992. Lung function and immunopathological changes after inhaled corticosteroid therapy in asthma. *Eur. Respir. J.* 5:73-79.
7. Haarst, J.M.W. van, H.J. de Wit, H.A. Drexhage and H.C. Hoogsteden. 1994. Distribution and immunophenotype of dendritic cells and mononuclear phagocytes in the human lung. *Am. J. Respir. Cell Mol. Biol.* 10:487-492.
8. Böhmig, G.A., J. Kovarik, W. Holter, E. Pohanka and G.J. Zlabinger. 1994. Specific downregulation of proliferative T cell alloresponsiveness by interference with CD2/ LFA-3 and LFA-1/ICAM-1 in vitro. *J. Immunol.* 152:3720-3728.
9. Bevilacqua, M.P. 1993. Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol.* 11:767-804.
10. Nabavi, N., G.J. Freeman, A. Gault, D. Godfrey, L.M. Nadler and L.H. Glimcher. 1992. Signalling through MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. *Nature* 360:266-268.
11. Thomas, R., L.S. Davis and P.E. Lipsky. 1993. Comparative accessory cell function of human peripheral blood dendritic cells and monocytes. *J. Immunol.* 151:6840-6852.
12. Larsen, C.P., S.C. Ritchie, R. Hendrix, P.S. Linsley, K.S. Hathrock, R.J. Hodes, R.P. Lowry and T.C. Pearson. 1994. Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. *J. Immunol.* 152:5208-5219.
13. Symington, F.W., W. Brady, and P.S. Linsley. 1993. Expression and function of B7 on epidermal Langerhans cells. *J. Immunol.* 150:1286-1295.
14. June, C.H., J.A. Bluestone, L.M. Nadler and C.B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today* 15:321-331.
15. van de Stolpe, A., E. Caldenhoven, B.G. Stade, L. Koenderman, J.A.M. Raaijmakers, J.P. Johnson and P.T. van der Saag. 1994. 12-O-tetradecanoylphorbol-13-acetate and tumor necrosis factor a-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. *J. Biol. Chem.* 269:6185-6192.
16. van de Stolpe, A., E. Caldenhoven, J.A.M. Raaijmakers, P.T. van der Saag and L. Koenderman. 1993. Glucocorticoid-mediated repression of intercellular adhesion molecule-1 expression in human monocytic and bronchial epithelial cell lines. *Am. J. Respir. Cell Mol. Biol.* 8:340-347.
17. Scheeren, R.A., G. Koopman, S. van der Baan, C.J.L.M. Meijer and S.T. Pals. 1991. Adhesion receptors involved in clustering of blood dendritic cells and T lymphocytes. *Eur. J. Immunol.* 21:1101-1105.
18. Cowing, C. and J.M. Chapdelaine. 1983. T cells discriminate between Ia antigens expressed on allogenic accessory cells and B cells: a potential function for carbohydrate side chains

on Ia molecules. *Proc. Natl. Acad. Sci. USA* 80:6000-6004.

19. Boog, C.J.P., J.J. Neefjes, J. Boes, H.L. Ploegh and C.S.M. Melief. 1989. Specific immune responses restored by alterations in carbohydrate chains on antigen-presenting cells. *Eur. J Immunol.* 19:537-542.
20. Egner, W., B.D. Hock and D.N.J. Hart. 1993. Dendritic cells have reduced cell surface membrane glycoproteins including CD43 determinants. *Adv. Exp. Med. Biol.* 329:71-73.
21. Trannoy, E., T. Manser, M.D. Cole and M.J. Daley. 1993. Monoclonal c-myc transformed macrophage cell lines; heterogeneity in ability to process and present antigen. *J. Immunol.* 151:3042-3056.
22. Gerrard, T.L., T.R. Cupps, C.H. Jurgensen and A.S. Fauci. 1984. Hydrocortisone-mediated inhibition of monocyte antigen presentation: dissociation of the inhibitory effect and expression of DR antigens. *Cell. Imunol.* 85:330-339.
23. Nicod, L.P. and F. El Habre. 1992. Adhesion molecules on human lung dendritic cells and their role for T cell activation. *Am. J. Respir. Cell Mol. Biol.* 7:207-213.
24. Teunissen, M.B.M., H.A.H. Rongen and J.D. Bos. 1994. Function of adhesion molecules lymphocyte function-associated antigen-3 and intercellular adhesion molecule-1 on human epidermal Langerhans cells in antigen-specific T cell activation. *J. Immunol.* 152:3400-3409.
25. Böhmig, G.A., J. Kovarik, W. Holter, E. Pohanka and G.J. Zlabinger. 1994. Specific downregulation of proliferative T cell alloresponsiveness by interference with CD2/ LFA-3 and LFA-1/ICAM-1 in vitro. *J. Immunol.* 152:3720-3728.
26. Nicod, L.P., B. Galve-de Rochemonteix and J-M. Dayer. 1990. Dissociation between allogenic T cell stimulation and interleukin-1 or tumor necrosis factor production by human lung dendritic cells. *Am. J. Respir. Cell Mol. Biol.* 2:515-522.
27. Schreiber, S., O. Kilgus, E. Payer, R. Kutil, A. Elbe, C. Mueller and G. Stingl. 1992. Cytokine pattern of Langerhans cells isolated from murine epidermal cell cultures. *J. Immunol.* 149:3522-3534.
28. Heufler, C., G.Topar, F. Koch, B. Trockenbacher, E. Kämpgen, N. Romani and G. Schuler. 1992. Cytokine gene expression in murine epidermal cell suspension: interleukin-1β and macrophage inflammatory protein 1α are selectively expressed by Langerhans cells but are differentially regulated in culture. *J. Exp. Med.* 176:1221-1226.
29. Hart, D.J.N. and V.L. Calder. 1994. Human dendritic cells: function and cytokine production. *In* Immunopharmacology of macrophages and other antigen-presenting cells, edited by C.A.F.M. Bruijnzeel-Koomen and E.C.M. Hoefsmit, Academic Press Ltd, London, UK, p.63-91.
30. Sher, E.R., D.Y.M. Leung, W.Surs, J.C. Kam, G. Zieg, A.K. Kamada and S.J. Szefler. 1994. Steroid asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. *J. Clin. Invest.* 93:33-39.
31. Poznansky, M.C., A.C.H. Gordon, J.G. Douglas, A.S. Krajewski, A.H. Wyllie and I.W.B. Grant. 1984. Resistance to methylprednisolone in cultures of blood mononuclear cells from glucocorticoid-resistant asthmatic patients. *Clin. Sci.* 67:639-645.
32. Wilkinson, J.R.W., A.E.G. Crea, T.J.H. Clark and T.H. Lee. 1989. Identification and characterization of a monocyte-derived neutrophil-activating factor in corticosteroid-resistant bronchial asthma. *J. Clin. Invest.* 84:1930-1941.
33. Kay, A.B., P. Diaz, J. Carmichael and I.W.B. Grant. 1981. Corticosteroid-resistant astham and monocyte complement receptors. *Clin. Exp. Immunol.* 44:576-580.
34. Poulter, L.W., D.A. Campbell, C. Munro and G. Janossy. 1986. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scan. J. Immunol.* 24: 351.
35. Kabel, P.J., M. de Haan, R.D. vd Gaag, and H.A. Drexhage. 1987. Intrathyroidal dendritic cells J *Clin. Endocrin. Metab.* 66: 199-207.
36. Ishii, Y., T.Takami, Y. Kokai, H.Yuasa, J. Fujimoto, and T. Takei. 1985. A novel human B

cell antigen shared with lymphoid dendritic cells: characterization by a monoclonal antibody. *Clin. Exp. Immunol.* 61:624-632.

37. Mooij, P., P.J. Simons, M. de Haan-Meulman, H.J. de Wit and H.A. Drexhage. The effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells. A role of GM-CSF, TNF*α* and IL-6. *J. Endocrinol.* Accepted.
38. Kabel, P.J., M. de Haan-Meulman, H.A.M. Voorbij, M. Kleingeld, E.F. Knol, and H.A. Drexhage. 1989. Accessory cells with a morphology and a marker pattern of dendritic cells can obtained from elutriator purified blood monocyte fractions, an enhancing effect of metrizamide in this differentiation. *Immunobiol.* 179:395-411.
39. van Haarst, J.M.W., H.C. Hoogsteden, H.J. de Wit, G.T. Verhoeven, C.E.G. Havenith, and H.A. Drexhage. 1994. Dendritic cells and their precursors isolated from the human bronchoalveolar lavage, immunocytological and functional properties. *Am. J. Respir. Cell Mol. Biol.* In press.
40. Braathen, L.R. and H. Hirscherg. 1984. The effect of short-term corticosteroid incubation on the alloactivating and antigen-presenting capacity of human epidermal Langerhans cells. *Br. J. Dermatol.* 111:295-302.
41. Hirschberg, H., T. Hirschberg, H. Nousiainen, L.R. Braathen and E. Jaffe. 1982. The effect of corticosteroids on antigen presenting properties of human monocytes and endothelial cells. *Clin. Immunol. Immunopathol.* 23:577-585.
42. Rhodes, J., J. Ivanyi and P. Cozens. 1986. Antigen presentation by human monocytes: effects of modifying major histocompatibility complex class II antigen expression and interleukin 1 production by using recombinant interferons and corticosteroids. *Eur. J. Immunol.* 16:370-375.
43. Holt, P.G., S. Haining, D.J. Nelson and J.D. Sedgwick. 1994. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* 153:256-261.
44. Djukanovic, R., J.W. Wilson, K.M. Britten, A.F. Walls, W.R. Roche, P.H. Howarth and S.T. Holgate. 1992. Effect of inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am. Rev. Respir. Dis.* 145:669-674.
45. Barnes, P.J. and S. Pedersen. 1993. Efficacy and safety of inhaled corticosteroids in asthma. *Am. Rev. Respir. Dis.* 148:S1-S26.
46. Brusselle, C.G., J.C. Kips, J.H. Tavernier, J.G. van der Heyden, C.A. Cuvelier, R.A. Pauwels and H. Bluethmann. 1994. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin. Exp. Allergy* 24:73-80.

CD1a$^+$ AND CD1a$^-$ ACCESSORY CELLS FROM HUMAN BRONCHOALVEOLAR LAVAGE DIFFER IN CYTOKINE PRODUCTION AND IN ALLOSTIMULATORY POTENTIAL

Jan Maarten W. van Haarst[1,2], Gert T. Verhoeven[2], Harm J. de Wit[1], Henk C. Hoogsteden[2], Reno Debets[1], and Hemmo A. Drexhage[1].

Dept. of Immunology[1] and Dept. of Pulmonary Medicine[2], Erasmus University and University Hospital Dijkzigt, Rotterdam, the Netherlands.

ABSTRACT

Recently, we described the isolation via FACS-sorting of low autofluorescent (LAF) cells from human bronchoalveolar lavage. These LAF cells showed a high potency to stimulate naive T cells and displayed an immunophenotype of dendritic cells (DC), but also showed some monocyte characteristics. We investigated the capability of LAF cells of healthy individuals to produce IL-1, IL-6 and TNF-α, and the role of these cytokines in allogenic T cell stimulation by these accessory cells. Moreover, we investigated whether subsets of LAF cells positive or negative for CD1a and CD14 differed in these capabilities.

Although LPS-stimulated LAF cells released biologically active IL-1, IL-6 and TNF, and also showed intracellular immune reactivity for IL-1, IL-6, TNF-α, neutralizing antibodies against these cytokines did not inhibit their ability to induce T cell proliferation. Marked differences were observed between CD1a$^+$ and CD1a$^-$ subsets of LAF cells: the CD1a$^+$ subset exhibited a clearly higher accessory capability than the CD1a$^-$ subset, and CD1a$^+$ cells were very poor producers of IL-1, IL-6 and TNF-α, whereas CD1a$^-$ cells were potent producers of these cytokines.

In conclusion, two different subsets of LAF cells could be identified on the basis of cytokine profile and accessory capability. CD1a$^+$ LAF cells were very potent T cell stimulators, but poor cytokine producers, and should thus be regarded as true DC. CD1a$^-$ LAF cells had a lower stimulatory capability, but were potent producers of IL-1, IL-6 and TNF-α, and displayed a morphology and marker pattern comparable with both monocytes and DC.

INTRODUCTION

In the airways, dendritic cells (DC) are located in and just underneath the bronchial epithelium, a location strategical for the uptake of inhaled antigens[1,2] . The presentation of antigens by accessory cells, such as DC, to T cells is an essential step in the initiation of immune responses. The presentation of these antigens to T cells requires interaction between the MHC-peptide complex and the T cell receptor as well as costimulatory signals. These costimulatory signals can be generated by intercellular contacts and by several cytokines, primarily IL-1[3], IL-6[4], and TNF-α[5].

Recently, we described the isolation via FACS of a population of low autofluorescent (LAF) cells from human bronchoalveolar lavage (BAL), which displayed an immunophenotype and function compatible with DC, but showed some monocytic features as well[6]. In contrast to alveolar macropha-

ges (AM), the LAF cells (strongly expressing MHC class II molecules but negative for acid phosphatase [APh]) were potent stimulators of (naive) T cells. These potent airway accessory cells probably play an important role in immune responses in the lung and in pulmonary diseases such as asthma.

Although the LAF cells are potent T cell stimulators, it is unclear to what extent cytokines contribute to this property. Besides effects on T cell stimulation, cytokines may play an important proinflammatory role as well, and may regulate the activation status of, for instance, bronchial epithelial cells or other immune cells. Therefore, we investigated 1) the production of IL-1, IL-6 and TNF-α by LAF cell populations isolated from BAL of healthy nonsmokers and smokers, and 2) the role of these cytokines in the stimulation of T cells by these LAF cells. Since we observed a heterogeneity in the expression of several surface markers in the population of LAF cells, most notably of CD1a and CD14, we also investigated cytokine production in subpopulations positive or negative for these markers.

MATERIALS AND METHODS

Bronchoalveolar lavage:
After informed consent, BAL was performed on individuals during anaesthesia for routine elective surgery. Subjects with a history of pulmonary or systemic disease were excluded. Seven smokers (more than 5 cigarettes a day) and 6 lifelong nonsmokers were included in the study (mean age 37 yrs, range 18-54 yrs). BAL was performed with a flexible bronchoscope placed in wedge position in the right middle lobe. Four aliquots of 50 ml saline were subsequently instilled and aspirated. BAL fluid (total volume ranged from 95 to 150 ml) was collected in siliconized bottles. The procedure has been approved by the Medical Ethical Committee of the Erasmus University and University Hospital Dijkzigt.

FACS-sorting of BAL cells:
BAL cells were kept at 4°C, washed twice in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 0.45% glucose, and subsequently filtered through a 100μ and a 30μ gauze. BAL cells were sorted on a FACS-Vantage (Becton & Dickinson, Erembodegem, Belgium) with a 488 nm laser. Two populations were sorted on autofluorescence (FL1, 530 nm), sideward scatter and forward scatter. As described previously, the high autofluorescent population contained 100% typical AM (APh positive), and the low autofluorescent (LAF) population contained MHC class II positive, APh negative cells with an immunophenotype of DC (including some monocyte characteristics), and a very potent T-cell stimulatory function[6]. Periphe-

ral blood monocytes (PBM) (used as a control population) were purified according to techniques described in detail elsewhere[7,8]. In short, monocytes were isolated from heparin blood or buffy coats by subsequent Ficoll-Paque (1.079 g/ml; Pharmacia, Uppsala, Sweden) and Percoll (1.063 g/ml; Pharmacia) density gradient separation.

Bioassays for cytokines:
Freshly isolated LAF cells, AM and PBM fractions at a concentration of $2x10^5$ cells/ml were incubated in RPMI 1640 (Gibco, Breda, the Netherlands) containing 10% FCS at 37°C and 5% CO_2 under nonadherent conditions (polypropylene tubes [Falcon, Lincoln Park, NJ]) with or without 2μg/ml LPS (derived from E. coli 026:B6, Difco Laboratories, Detroit, MI). After 24 h., the tubes were centrifuged, and the supernatants were collected, filtered through a 0.45 μm filter (Milipore Corp., Bedford, MA) and stored in aliquots at -80°C. The samples were tested for biologically active cytokines within 2 months. Cytospin slides were prepared from the cell pellet for immunocytochemical evaluation.

IL-1 activity was measured using a subline of the murine T cell line D10.G4.1, designated D10(N4)M as described by Hopkins and Humphreys[9]. Further improvement at our department resulted in an enhanced reproducibility of the test[10]. IL-6 content was detected in the B9 bioassay developed by Aarden and colleagues[11]. Proliferation of D10(N4)M and B9 cells was measured via ^{3}H-thymidine incorporation. The murine fibroblast cell line WEHI 164.13 was used to assay TNF bioactivity according to Espevik and Nissen-Meyer[12]. The MTT cytotoxicity test[13] served to measure WEHI cell viability. For all assays, samples were serially diluted and assayed in triplicate. Recombinant human IL-1ß (UBI, Lake Placid, NY), IL-6 (kindly provided by L.A. Aarden, Amsterdam, the Netherlands) and TNF-α (UBI) served as positive controls in the D10, B9 and WEHI assay respectively. Cytokine activities were corrected for background activity of the culture medium and calculated in U/ml with 1 U/ml corresponding to half-maximal response.

Immunocytochemistry:
For immunocytochemical stainings of cytokines, rabbit polyclonal antibodies specific for IL-1, IL-6, TNF-α and IFN-γ (Endogen, Boston, MA), and mouse monoclonal anti-GM-CSF (Genzyme, Boston, MA) were used. As controls for these antibodies, normal rabbit serum and mouse IgG (CLB, Amsterdam, the Netherlands) were used. Both specific antibodies and control sera were used at an IgG concentration of 0.1 mg/ml. For the identification of subpopulations of LAF cells, My4 (CD14) (Coulter Immunology, Hialeah, FL) and OKT6 (CD1a) (American Type Culture Collection, Rockville, MD) were used in double stainings with the anti-cytokine antibodies.

Cytocentrifuge preparations were fixed in acetone (Merck, Darmstadt, Germany) for 10 min, and thereafter incubated with 10% normal swine serum or normal rabbit serum for 10 min. The slides were incubated for 1 hr with an antibody or control serum. Subsequently, the slides were either incubated with swine-anti-rabbit antiserum or rabbit-anti-mouse antiserum both conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark). Diaminobenzidine (Sigma Chemicals, Axel, the Netherlands) was used as substrate. Counterstaining was performed with hematoxylin.

For double immunostaining, My4 or OKT6 mouse monoclonal antibodies were added together with the polyclonal antibodies, and in the second step goat-anti-mouse (Dako) was added simultaneously with the HRP-conjugated antibody. Subsequently, the slides were incubated with alkaline phosphatase anti-alkaline phosphatase (mouse) (Dako) and stained with Fast Blue BB Base (Sigma) and 3-amino-9-ethylcarbazole (Sigma) respectively.

Mixed lymphocyte reactions:
Responder T cells were isolated from buffy coats of healthy blood donors. Buffy coats were diluted 1:1 with PBS, and mononuclear cells were obtained by Ficoll-Paque (1.079 g/ml; Pharmacia) density gradient separation (15 min., 1000 g). Monocytes and lymphocytes were separated by Percoll (1.063 g/ml; Pharmacia) density gradient centrifugation. Lymphocytes were depleted from B cells and residual monocytes by adherence in a nylon wool column (Polyscience, Warrington, PA) (1 h., 37°C, 5% CO_2). The purified T cell populations were stored in liquid nitrogen until use.

For mixed lymphocyte reactions (MLR), the stimulator cell populations were irradiated with 2000 rad. Stimulator cells (7,500 LAF cells or 30,000 PBM) were cultured with 150,000 allogeneic T cells (stimulator-responder ratios 1:20 and 1:5 respectively). RPMI 1640 (Gibco, Breda, the Netherlands) to which 10% human A^+ serum, penicillin and streptomycin were added, was used as culture medium. Cultures were always performed in triplicate in a flat bottom 96 well plates (Falcon) with a total volume of 200 μl per well. After 5 days, 0.5 μCi ^{3}H-thymidine was added and the cells were harvested 16 h later. Scintillation was counted with a LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland). Negative controls (^{3}H-thymidine incorporation in T cells alone and in the various stimulator cell fractions without T cells) were always less than 500 counts per minute (cpm).

Neutralization of cytokines in the MLR:
To investigate the role of cytokines in the MLR, neutralizing polyclonal antibodies against IL-1, IL-6, GM-CSF, TNF-α or IFN-γ (Endogen) were added in the MLR at a final concentration of 10 μg/ml. As a measure of the effect

of these antibodies on the MLR, T cell proliferation in the presence of anti-cytokine antibodies, measured as ^{3}H-thymidine incorporation, was expressed as percentage of the control (T cell proliferation without anti-cytokine antibodies).

Statistics:
The Wilcoxon rank sum test was used for statistical analysis. P-values less than 0.05 were considered to be signifcant.

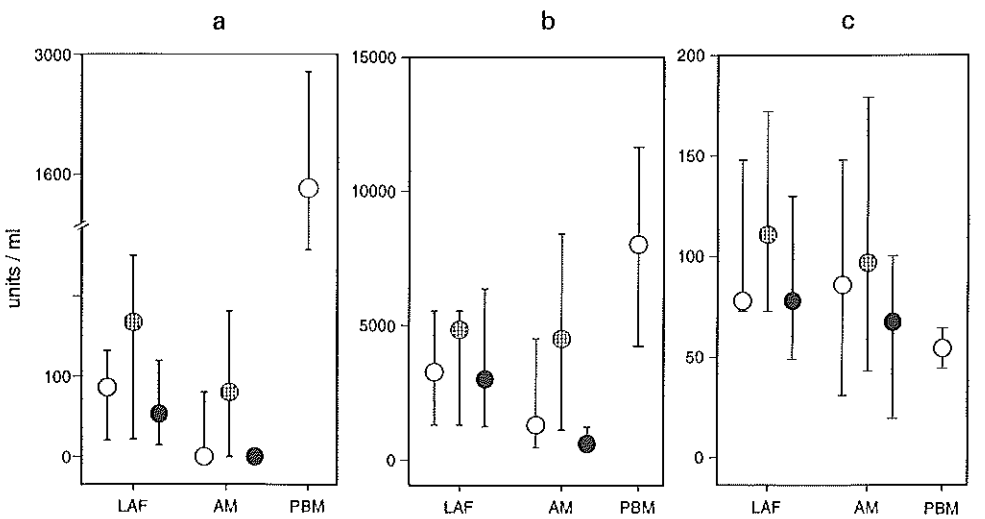


Figure 1. The production of biologically active cytokines by LPS-stimulated LAF cells, AM and PBM. Data represent medians and 25 to 75 percentile ranges of the production of IL-1 (a), IL-6 (b), and TNF (c) of nonsmokers and smokers together (open circles), and nonsmokers (dotted circles) and smokers (closed circles) separately. * indicates $p<0.05$.

RESULTS

Cytokine production

In the supernatants of unstimulated LAF cell populations, 0.5 U/ml (median, range 0-4 U/ml) of biologically active IL-1 was detected, compared to 66 U/ml (median, range 3-444 U/ml) in the LPS-stimulated fraction (figure 1a). LAF cells of smokers and nonsmokers did not differ in the production of IL-1. In response to LPS, AM produced significantly lower levels of IL-1 bioactivity than LAF cells ($p<0.05$, $n=12$). Compared to nonsmokers, AM of smokers tended to produce less IL-1, but this difference was not statistically significant ($p=0.12$, $n=6$).

LAF cells cultured in the absence of LPS produced 135 U/ml (15-482 U/ml) IL-6, which was enhanced by LPS stimulation to 2995 U/ml (796-13774 U/ml) (figure 1b). LAF cells of smokers secreted similar quantities of IL-6 as those of none smokers. IL-6 levels tended to be lower in the supernatants of LPS-stimulated AM compared to the supernatants of LAF cells ($p=0.10$), and smokers' AM tended to release lower levels of IL-6 than AM of nonsmokers ($p=0.11$).

TNF-bioactivity in the supernatants of LAF cells incubated without LPS was 14 U/ml (0-43 U/ml), and 78 U/ml (46-432 U/ml) with LPS. The levels of TNF production of LAF cells were comparable with AM and PBM (figure 1c), and there were no clear differences between smokers and nonsmokers in TNF secretion by either LAF cells or AM.

Immunocytochemistry

As described previously, the high autofluorescent cells isolated from the BAL consisted of 100% AM and the LAF fraction contained predominantly cells with a marker pattern reminiscent of DC (with some monocyte characteristics) with less than 10% contaminating cells (macrophages, lymphocytes and eosinophils). Approximately 50% of the LAF cells showed prominent cytoplasmic extensions (dendritic morphology). After 24 h. of exposure to LPS, the phenotypes of AM and LAF cells did not change significantly, apart from an increase in cytoplasmic extensions in the LAF cells.

LAF cells incubated without LPS showed low numbers (20-50%) of cells positive for intracellular IL-1, whereas LAF cells incubated with LPS most cells (50-80%) were strongly positive for IL-1. Immunoreactivity for IL-6 was only detected in LPS-stimulated LAF cells. LPS-stimulated LAF cells also stained with antibodies against TNF-α and IFN-γ (weakly) and GM-CSF. Unstimulated LAF cells, however, also stained with anti-GM-CSF and weakly with anti-TNF-α (table 1). Slides incubated with control serum and IgG were always completely negative.

Cytospin slides of AM stained with control serum were also mostly negative, but in some experiments a weak background staining was present. Therefore results with the specific antibodies were related to their appropriate controls. In AM, intracellular immunoreactivity for IL-1, IL-6 (weak), TNF-α, IFN-γ (weak) and GM-CSF was detected. Compared to LAF cells or PBM, AM exhibited a much stronger reactivity for GM-CSF. However, in AM incubated with or without LPS, differences in intracellular immunoreactivity for these cytokines could not clearly be detected (table 1).

PBM and T cells were used as controls. Roughly, the staining pattern of PBM was comparable to that of LAF cells (table 1). In contrast, stimulated T cells were negative for IL-1, but exhibited a strong IFN-γ immunoreactivity.

	LAF cells		AM		PBM	
	MEDIUM	LPS	MEDIUM	LPS	MEDIUM	LPS
IL-1	±/+	++	±	+	±/+	++
IL-6	—	+	—/±	±	—/±	+/++
TNF-α	±	±/+	±/+	±/+	—	—/±
IFN-γ	—	±	±	±	—	+/++
GM-CSF	+	+	++	++	±	+

Table 1. The presence of intracellular immunoreactive cytokines in LAF cells, AM and PBM. Negative, —; weakly positive, ±; positive, +; strongly positive, ++.

Hence, the data on intracellular immunoreactivity for cytokines roughly parallel the data on cytokine production described in the first part of the results section.

Effects of anti-cytokine antibodies on the MLR

Figure 2 shows the effects of the addition of anti-cytokine antibodies to MLRs with LAF cells or PBM as stimulator cells and allogenic T cells as responder cells. Neutralizing antibodies against IL-1 had no significant effect on LAF cells-induced T cell proliferation (96±6% [mean±SD] of control values, n=8), whereas PBM-induced T cell proliferation was decreased to 42±19% of control values (n=8, $p<0.05$). Anti-IL-6 and anti-TNF-α antibodies did not produce any significant effects on the MLRs of either LAF cells or PBM with T cells. Anti-GM-CSF had a significant, but small effect on the MLRs induced by both types of accessory cells: LAF cell-induced MLRs were reduced down to 88±15% and PBM-induced MLRs to 79±15% of control values (for both: $p<0.05$, n=8). Anti-IFN-γ had no effect on LAF cell-induced MLR's, but PBM-induced MLR's were 23% higher than control values (n=8, $p<0.05$). The addition of all 5 antibodies together to the MLR almost abrogated the responses of PBM MLRs (down to 16±21% of control values, $p<0.05$), and downregulated LAF cell-induced responses to 68±12% of control values ($p<0.05$).

Differences between CD1a$^-$ and CD1a$^+$ LAF cells:

Double immunostaining with anti-cytokine polyclonal antibodies and OKT6 (CD1a) disclosed marked contrasts between CD1a$^+$ and CD1a$^-$ LAF cells. The majority of CD1a$^-$ cells stained for IL-1, IL-6 and TNF-α, whereas the

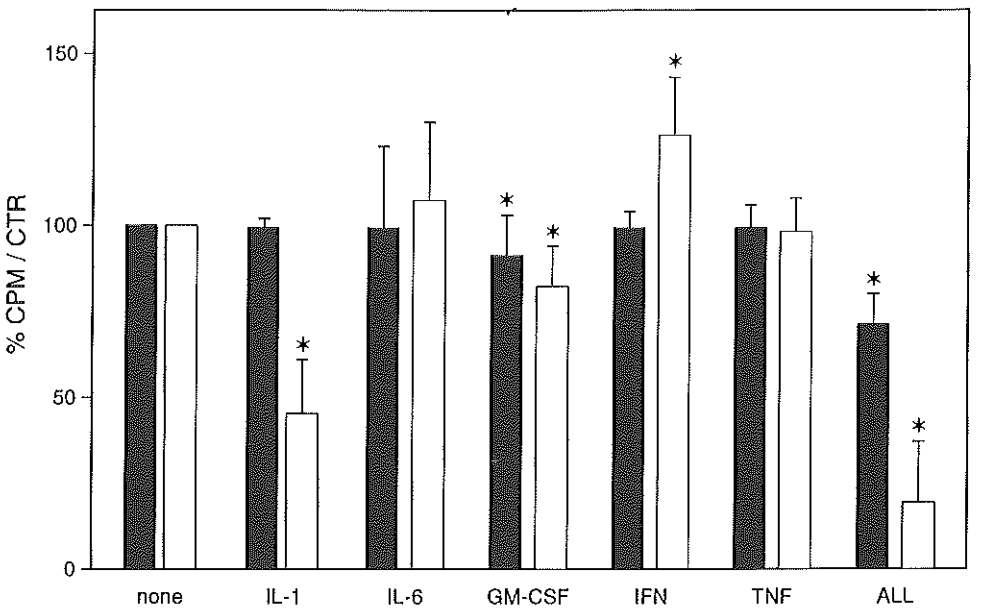


Figure 2. The effects of the addition of neutralizing anti-cytokine antibodies on the allogenic MLR with LAF cells (closed bars) or PBM (open bars) as stimulator cells. T cell proliferation in the presence of anti-cytokine antibodies is expressed as a percentage of control values (T cell proliferation without anti-cytokine antibodies). Data represent mean ± SD of 8 experiments. * indicates $p<0.05$ compared to control values.

	$CD1a^-$	$CD1a^+$	$CD14^-$	$CD14^+$
IL-1	++	−/±	++	++
IL-6	+	−	+	+
TNF-α	+	±	+	+

Table 2. Double immunostaining in cytospin preparations of LPS-stimulated LAF cells with anti-cytokine antibodies and OKT6 (CD1a) or My4 (CD14). The table depicts the intracellular immunoreactivity for cytokines of $CD1a^{-/+}$ and $CD14^{-/+}$ subpopulations. Negative, −; weakly positive, ±; positive, +; strongly positive, ++.

$CD1a^+$ cells were negative or weakly positive for IL-1, negative for IL-6, and

negative or weakly positive for TNF-α. Conversely, double staining with My4 (CD14) revealed that both My4$^-$ and My4$^+$ subsets contained cells positive for IL-1, IL-6 and TNF-α (table 2).

To validate these differences between CD1a$^-$ and CD1a$^+$ LAF cells, we labeled BAL cells with OKT6 conjugated with phycoerythrin, and separated these LAF cells fractions by FACS-sorting. The CD1a$^-$ fraction was completely depleted for CD1a$^+$ cells in two out of three experiments, but contained 1% of CD1a$^+$ cells in the third experiment. The CD1a$^+$ fraction contained 95-100% CD1a$^+$ cells. In the supernatants of LPS-stimulated CD1a$^-$ LAF cells the amounts of biologically active IL-1, IL-6 and TNF detected were comparable to unseparated LAF cells, whereas the supernatants of CD1a$^+$ LAF cells contained no detectable or low levels of IL-1, IL-6 and TNF. In contrast to their ability to produce cytokines, the CD1a$^+$ cells were more potent stimulators of allogenic T cell proliferation compared to the CD1a$^-$ LAF cells (table 3).

	Unseparated			CD1a-negative			CD1a-positive		
Exp.	1	2	3	1	2	3	1	2	3
IL-1	132	48	ND	48	59	222	0	0	4
IL-6	1236	3108	ND	872	4600	4802	0	98	257
TNF	432	500	ND	97	261	393	26	10	31
MLR	45712	21451	43043	3156	3080	25494	41509	34039	71614

Table 3. Bioactivity of IL-1, IL-6 and TNF in U/ml in the supernatants of LPS-stimulated LAF cells fractions, and stimulatory capability in the allogenic MLR (in cpm) of freshly isolated LAF cell fractions. Data show the results of three experiments. In the MLR of experiment 1 the LAF cells to T cell ratio was 1:20, and in experiment 2 and 3 a LAF cells:T ratio of 1:40 was used. ND: not done.

DISCUSSION

As we described before, LAF cells isolated from human BAL are very potent stimulators of naive T cells (an important feature of DC[14]) and show many other features of DC[6]. Our present data demonstrate that these LAF cells are able to produce IL-1, IL-6 and TNF-α. These cytokines were, however, not required for the T-cell stimulating function of the LAF cells, since neutralizing anti-cytokine antibodies did not inhibit LAF cell-driven MLRs. We further showed that the heterogeneity in CD1a expression of LAF cells is associated with functional differences: CD1a$^+$ LAF cells were more potent T cell stimulators than their CD1a$^-$ counterparts, and, in contrast to CD1a$^-$ LAF

cells, CD1a$^+$ LAF cells produced no or much lower amounts of IL-1, IL-6 and TNF-α. Such differences in cytokine production were not found in CD14$^{+/-}$ subpopulations of LAF cells.

Our observations suggest the existence of two distinct subpopulations of LAF cells in human BAL. The CD1a$^+$ LAF cells meet all the criteria of classic DC. The CD1a expression together with the presence of Birbeck granules in a subset of the CD1a$^+$ BAL cells described by Casolaro and colleagues[15], indicate that these cells resemble epidermal Langerhans cells. The limited ability of the CD1a$^+$ LAF cells to produce cytokines concurs with the general concept that DC are poor cytokine producers[16]. Several investigators reported that DC do not produce IL-1 or IL-6, whereas others detected low quantities of these cytokines in supernatants of DC[17,18,19,20,21]. In the skin, however, epidermal LC are a major source of IL-1ß[22,23,24].

The CD1a$^-$ LAF cells have a lower stimulatory capability and show a cytokine profile comparable to that of PBM. Unlike PBM, many of these cells do have dendritic processes, express RFD1 and L25, and are CD14 negative or low. These CD1a$^-$ LAF cells might contain precursors of (CD1a$^+$) DC. Several investigators have reported that PBM are able to mature into (CD1a$^+$) DC-like cells with a strong accessory capability[7,25,26]. In the rat, Holt demonstrated an influx of monocyte-like cells in the bronchial mucosa after an inflammatory stimulus that differentiated into mature DC in 1-2 days[27,28]. The alternative hypothesis is that these CD1a$^-$ cells represent precursors of mature macrophages. AM may also express RFD1 and L25[2,29], and PBM differentiating into macrophages also downregulate their CD14 expression[30]. Further research is required to assess the nature of the CD1a$^-$ LAF cells from the BAL.

The data on biologically active cytokines in the supernatants roughly correlated with the observations in immunocytochemistry. Both bioactivity and intracellular immunoreactivity for cytokines were enhanced by LPS. Unstimulated LAF cells and AM, however, usually showed immunoreactivity for IL-1 in the absence of IL-1 production. This can be explained by the cytoplasmic storage of pro-IL-1 isoforms[31]. The relatively low amounts of IL-1 bioactivity in the supernatants of AM, contrasting with the high intracellular immunoreactivity, might also be attributed to the high amounts of IL-1 receptor antagonist produced by these cells, especially in the presence of serum as in our assay[32,33].

In agreement with studies on pulmonary tissue DC and other DC[18,20,21], we showed that IL-1 is not a major costimulatory factor for T cell stimulation induced by airway DC, in which the CD1a$^+$ DC is the major stimulatory

population. Conversely, IL-1 was used by PBM as a major costimulatory factor for T cell stimulation, which is in agreement with other studies[34]. Other important cytokines (IL-6, TNF-α and IFN-γ) were also not essential for the induction of T cell proliferation by airway LAF cells either, which confirms the general concept that cytokines do not play an important role for T cell stimulation by DC[16]. Anti-GM-CSF, however, slightly reduced T cell proliferation induced by LAF cells (and PBM). This is in agreement with the concept that GM-CSF enhances T cell proliferation via augmentation of APC function[35]. We cannot explain the increased PBM-induced T cell responses in the presence of antibodies against IFN-γ.

In this study we focussed on IL-1, IL-6 and TNF-α. In addition to these cytokines, we also observed immunoreactivity for GM-CSF and IFN-γ in both LAF cells and AM. IFN-γ is usually considered to be a T cell cytokine, but, in support of our immunocytologic data, it has been described before that human AM secrete IFN-γ after stimulation[36]. AM and PBM have both been described to secrete GM-CSF[37,38]. For LAF cells and their subsets, however, further experiments are required to evaluate their ability to secrete GM-CSF and IFN-γ, and to validate our data on intracellular immunoreactivity for these cytokines.

We investigated BAL cells of both smokers and nonsmokers. There is ample of evidence that smoking affects the function of immune cells in the lung[39,40], and smokers' AM have been shown to produce less IL-1 and IL-6 compared to those of nonsmokers[41,42]. In agreement with these studies, our data did show a trend for a lower production of IL-1 and IL-6 in smokers' AM, but these differences did not reach the level of statistical significance, probably due to the small groups studied (6 smokers and 6 nonsmokers). Cytokine production of LAF cell populations did not differ between smokers and nonsmokers.

In conclusion, in LAF cells isolated from human BAL, two different subsets could be identified on the basis of CD1a expression. CD1a$^+$ LAF are classic DC with a potent T cell stimulating capacity, but a limited ability to produce cytokines. CD1a$^-$ LAF cells have a lower stimulatory capability, are potent producers of IL-1, IL-6 and TNF-α, and display a morphology and marker pattern comparable with both monocytes and DC. These cells might be the progenitors of either mature macrophages or CD1a$^+$ DC.

ACKNOWLEDGEMENTS

We thank Marie-Jose Melief for measuring TNF-bioactivity, and Tar van Os

for the preparation of the graphs. This study was supported by a grant of the Netherlands Asthma Foundation.

REFERENCES

1. Holt, P.G., M.A. Schon-Hegrad, M.J. Philips and P.G. McMenamin. 1989. Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* 19:597-601.
2. van Haarst, J.M.W., H.J. de Wit, H.A. Drexhage and H.C. Hoogsteden. 1994. Distribution and immunophenotype of mononuclear phagocytes and dendritic cells in the human lung. *Am. J. Respir. Cell Mol. Biol.* 10:487-492.
3. Oppenheim, J.J., E.J. Kovacs, K. Matsushima and S.K. Durum. 1986. There is more than one interleukin 1. *Immunol. Today* 7:45-56.
4. Lotz, M., F. Jirik, R. Kabouridis, C. Tsoukas, T. Hirano, T. Kishimoto and D. Carson. 1988. B cell stimulatory factor 2/ interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *J. Exp. Med.* 167:1253-1258.
5. Shalaby, M.R., T. Espevik, G.C. Rice, A.J. Ammann, I.S. Figari, G.E. Ranges and M.A. Paladino. 1988. The involvement of tumor necrosis factor-α and -β in the mixed lymphocyte reaction. *J. Immunol.* 141:499-503.
6. van Haarst, J.M.W., H.C. Hoogsteden, H.J. de Wit, G.T. Verhoeven, C.E.G. Havenith and H.A. Drexhage. 1994. Dendritic cells and their precursors isolated from human bronchoalveolar lavage: immunocytologic and functional properties. *Am. J. Respir. Cell Mol. Biol.* 11:344-350.
7. Kabel, P.J., M. de Haan-Meulman, H.A.M. Voorbij, M. Kleingeld, E.F. Knol and H.A. Drexhage. 1989. Accessory cells with a morphology and a marker pattern of dendritic cells can be obtained from elutriator purified blood monocyte fractions, an enhancing effect of metrizamide in this differentiation. *Immunobiol.* 179:395-411.
8. Mooij, P., P.J. Simons, M. de Haan-Meulman, H.J. de Wit and H.A. Drexhage. 1994. Effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells. A role of granulocyte-macrophage colony-stimulating factor, tumour-necrosis factor-α and interleukin-6. *J. Endocrinol.* 140:503-512.
9. Hopkins, S.J. and M. Humphreys. 1989. Simple, sensitive and specific bioassay of interleukin-1. *J. Immunol. Meth.* 120:271-276.
10. Debets, R., T. van Joost, R. Benner and E.P. Prens. 1993. Psoriatic epidermal cells release elevated levels of immunoreactive and biologically active interleukins 1 and 6: modulation of corticosteroid treatment. *In* Bernard, B.A. and B. Schroot, editors. Pharmacology of the Skin: from molecular biology to therapeutics. Karger, Basel. Vol. 5:158-166.
11. Aarden, L.A., E.R. de Groot, O.L. Schaap and P.M. Lansdorp. 1987. Production of hybridoma growth factor by human monocytes. *Eur. J. Immunol.* 17:1411-1416.
12. Espevik, T. and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor / tumor necrosis factor from human monocytes. *J. Immunol. Meth.* 95:99-105.
13. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytoxicity assays. *J. Immunol. Methods* 65:55-63.
14. Inaba, K. and R.M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717-1735.
15. Casolaro, M.A., J-F. Bernaudin, C. Saltini, V.J. Ferrans and R.G. Crystal. 1988.

Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am. Rev. Respir. Dis.* 137:406-411.

16. Hart, D.N.J. and V.L. Calder. 1993. Human dendritic cells: function and cytokine production. *In* Immunopharmacology of macrophages and other antigen-presenting cells, eds. C.A.F.M. Bruijnzeel-Koomen and E.C.M. Hoefsmit, Academic Press Ltd., London, U.K. 63-91.
17. Nicod, L.P., B. Galve-de Rochemonteix and J-M. Dayer. 1990. Dissociation between allogenic T cell stimulation and interleukin-1 or tumor necrosis factor production by human dendritic cells. *Am. J. Respir. Cell Mol. Biol.* 2:512-522.
18. Calder, V.L., T.C.R. Prickett, J.L. McKenzie and D.N.J. Hart. 1993. Analysis of cytokine and cytokine receptor production by human dendritic cells. *Adv. Exp. Med. Biol.* 329:81-85.
19. McKenzie, J.L., T.C.R. Prickett and D.N.J. Hart. 1989. Human dendritic cells stimulate allogenic T cells in the absence of IL-1. *Immunol.* 67:290-297.
20. Lafontaine, M., D. Landry, N. Blanc-Brunât, M. Pelletier and S. Montplaisir. 1991. IL-1 production by thymic dendritic cells: studies on the interrelation with DC accessory function. *Cell. Immunol.* 135:431-444.
21. Vakkila, J., M. Sihvola and M. Hurme. 1990. Human peripheral blood dendritic cells do not produce interleukin 1α, interleukin 1ß, or interleukin 6. *Scand. J. Immunol.* 31:345-352.
22. Heufler, C., G. Topar, F. Koch, B. Trockenbacher, E. Kämpgen, N. Romani and G. Schuler. 1992. Cytokine gene expression in murine epidermal cell suspensions: interleukin 1β and macrophage inflammatory protein 1α are selectively expressed in Langerhans cells but are differentially regulated in culture. *J. Exp. Med.* 176:1221-1226.
23. Enk, A.H., V.L. Angeloni, M.C. Udey and S.I. Katz. 1993. An essential role for Langerhans cell-derived IL-1ß in the initiation of primary immune responses in the skin. *J. Immunol.* 150:3698-3704.
24. Chu, C.Q., M. Field, E. Andrew, D. Haskard, M. Feldmann and R.N. Maini. 1992. Detection of cytokines at the site of tuberculin-induced delayed-type hypersensitivity in man. *Clin. Exp. Immunol.* 90:522-529.
25. Peters, J.H., J. Ruppert, R.K.H. Gieseler, H.M. Najar and H. Xu. 1990. Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage? *Pathobiology* 59:122-126.
26. Rossi, G. 1992. Development of a Langerhans cell phenotype from peripheral blood monocytes. *Immunol. Lett.* 31:189-198.
27. McWilliam, A.S., D. Nelson, J.A. Thomas and P.G. Holt. 1994. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* 179:1331-1336.
28. Holt, P.G., S. Haining, D.J. Nelson and J.D. Sedgwick. 1994. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* 153:256-261.
29. Spiteri, M.A., S.W. Clarke and L.W. Poulter. 1988. Phenotypic and functional changes in alveolar macrophages contribute to the pathogenesis of pulmonary sarcoidsis. *Clin. Exp. Immunol.* 74:359-364.
30. van Hal, P.Th.W., H.C. Hoogsteden, A.A. te Velde, J.M. Wijkhuis, C.G. Figdor and C. Hilvering. 1990. Induction of a macrophage-like phenotype on peripheral blood monocytes by interleukin 4 is inhibited by glucocorticoids. *In* Heterogeneity of mononuclear phagocytes in interstitial lung disease. H.C. Hoogsteden. Haveka b.v., Alblasserdam. 103-112.
31. Chin, J. and M.J. Kostura. 1993. Dissociation of IL-1ß synthesis and secretion in

human blood monocytes stimulated with bacterial cell wall products. *J. Immunol.* 151:5574-5585.

32. Galve-de Rochemonteix, B., L.P. Nicod, R. Chicheportiche, S. Lacraz, C. Baumberger and J-M. Dayer. 1993. Regulation of interleukin-1ra, interleukin-1α, and interleukin-1ß production by human alveolar macrophages with phorbol myristate acetate, lipopolysaccharide and interleukin-4. *Am. J. Respir. Cell Mol. Biol.* 8:160-168.
33. Kline, J.N., M.M. Monick and G.W. Hunninghake. 1992. IL-1 receptor antagonist release is regulated differently in human alveolar macrophages than in monocytes. *J. Appl. Physiol.* 73:1686-1692.
34. Dinarello, C.A. 1984. Interleukin-1. *Rev. Infect. Dis.* 6:51-95.
35. Stewart-Akers, A.M., J.S. Cairns, D.J. Tweardy and S.A. McCarthy. 1994. Granulocyte-macrophage colony-stimulating factor augmentation of T-cell receptor-dependent and T-cell receptor-independent thymocyte proliferation. *Blood* 83:713-723.
36. Nugent, K.M., J. Glazier, M.M. Monick and G.W. Hunninghake. 1985. Stimulated human alveolar macrophages secrete interferon. *Am. Rev. Respir. Dis.* 131:714-718.
37. Lee, M-T., K. Kaushanski, P. Ralph and M.B. Ladner. 1990. Differential expression of M-CSF, G-CSF and GM-CSF by human monocytes. *J. Leukoc. Biol.* 47:275-282.
38. Crystal, R.G. 1991. Alveolar macrophages. *In* The Lung: scientific foundations. Crystal, R.G., J.B. West et al. eds. Raven Press Ltd. New York. 157-167.
39. Costabel, U. and J. Guzman. 1992. Effects of smoking on brochoalveolar lavage constituents. *Eur. Respir. J.* 5:776-779.
40. Holt, P.G. 1987. Immune and inflammatory function in cigarette smokers. *Thorax* 42:241-249.
41. Yamaguchi, E., N. Okazaki, A. Itoh, S. Abe, Y. Kawakami and H. Okuyama. 1989. Interleukin 1 production by alveolar macrophages is decreased in smokers. *Am. Rev. Respir. Dis.* 140:397-402.
42. Soliman, D.M. and H.L. Twigg. 1992. Cigarette smoking decreases bioactive interleukin-6 secretion by alveolar macrophages. *Am. J. Physiol.* 263:L471-L478.

A DECREASED POLARIZATION OF GRANULOCYTES UPON CHEMOTACTIC STIMULI IN PATIENTS WITH SEVERE CHRONIC RELAPSING INFECTIONS OF THE LOWER AIRWAYS CAN BE RESTORED BY G-CSF IN VITRO

Jan Maarten W. van Haarst[1,2], Harm J. de Wit[1], Henk C. Hoogsteden[2], Joop P. van de Merwe[1], Herbert Hooijkaas[1], Wim Sluiter[3] and Hemmo A. Drexhage[1].

Dept. of Immunology[1], Pulmonology[2] and Biochemistry[3], Erasmus University and University Hospital Dijkzigt, Rotterdam, the Netherlands.

ABSTRACT

Granulocytes play a major role in host defense against bacterial infections. Severe inborn defects in granulocyte function are associated with serious infections in early childhood. More subtle disturbances in granulocyte function might contribute to an enhanced susceptibility to bacterial infections in adulthood. We investigated granulocyte functions in 18 adults with unexplained chronic recurrent infections of the lower airways (12 women, 6 men, aged 49 ± 18 y).

Granulocyte polarization (change in shape from round to polarized under the influence of chemotactic stimuli) was significantly lower in patients compared to healthy controls with both N-formyl-methionyl-leucyl-alanine (FMLP) and casein as stimulus. Ten of the 18 patients showed a polarization response to FMLP below the range of the healthy controls. Addition of granulocyte colony-stimulating factor (G-CSF) *in vitro* significantly improved polarization of patients' granulocytes, especially in those patients with a suppressed polarization response. Chemotaxis of patient granulocytes was only tested in 7 of the patients. A just significant lower chemotaxis was found as compared to healthy controls. Granulocyte superoxide production was comparable in patients and in healthy controls.

In conclusion, we observed subtle impairments in granulocyte polarization and chemotactic motility in approximately half of our selected patients with severe unexplained recurrent infections of the lower airways. The polarization defects could be restored *in vitro* with G-CSF. We hypothesize that this granulocyte hyporesponsiveness to chemotactic stimuli contributes to the recurrence and chronicity of the airway infections.

INTRODUCTION

It is well known that in many of the patients with chronic relapsing infections of the lower airways, these infections can be attributed to metabolic disturbances or anatomic abnormalities of the airways. Rare causes of chronic recurrent airway infections are well-defined impairments in host defence, such as X-linked agammaglobulinemia and common variable immunodeficiency. More subtle disturbances in host defence have been suggested as a cause of at least a part of the remaining unexplained cases with chronic recurrent airway infections. Deficiencies in immunoglobulin isotype, IgG subclasses or specific antibodies have been observed in patients (in most studies children) with chronic recurrent (airway) infections[1,2,3,4]. However, in all these studies such defects were found only in a proportion (12-58%) of these patients. Moreover, the role of these defects in the pathogenesis of chronic recurrent infections has not been completely elucidated[5].

Next to humoral immunity, granulocyte function is important in host defence against bacterial infections. Complete and inborn defects in either superoxide production or chemotaxis are associated with severe infections in early childhood. We hypothesized that more subtle disturbances in granulocyte function might be responsible for a raised susceptibility to bacterial infections leading to chronic recurrent infections of the airways (a main porte d'entree) in adulthood. We therefore investigated granulocyte polarization in response to two chemotactic stimuli in 18 patients with unexplained chronic relapsing infections of the lower airways selected in three major Rotterdam clinics in a period of one year.

Granulocyte polarization is the change of shape of the cells from round to elongated or triangular with large cytoplasmic extensions. Many important functions of human granulocytes are dependent on this ability to undergo impressive morphological changes: passage through small capillary vessels requires the rapid acquisition of elongated shape[6]; emigration from blood to inflamed tissues necessitates drastic squeezing between endothelial cells[6], ingestion of foreign particles usually involves close adaption of the phagocyte to its prey[6], and displacement along a chemotactic gradient is associated with characteristic morphological patterns[7]. In addition to the polarization, the superoxide production of the patients' granulocytes was studied as well, as was their chemotaxis using a commercially available microchemotaxis assay. The latter assay was only used in a limited number (n = 7) of the patients.

Since we observed a decreased granulocyte polarization response in some patients in preliminary experiments that was correctable by granulocyte colony stimulating factor (G-CSF) in vitro, we decided to test the effect of G-CSF addition on granulocyte polarization in all patients.

MATERIALS AND METHODS

Patient selection:
Patients with chronic relapsing bacterial infections of the lower airways and to be tested in the polarization assay were selected on clinical suspicion of immunodeficiency according to the following criteria:

- chronic production of purulent sputum (minimally 3 months a year),
- no response to antibiotic treatment or frequent recurrence despite antibiotic therapy,
- minimal duration of the disease 2 year,
- sputum culture positive,
- no use of antibiotics at the time of testing.

Excluded were patients with preexisting anatomic abnormalities (e.g. lung

sequester), patients with metabolic defects (e.g. diabetes, cystic fibrosis) and patients with well characterized immunodeficiency syndromes, such as HIV infection or severe hypo- or agammaglobulinemia (IgG < 4 g/l). Serum levels of IgG, IgA and IgM were determined by nephelometry, and IgG-subclass levels were determined with a radial immunodiffusion assay (Department of Clinical Chemistry, University Hospital Dijkzigt, Rotterdam, the Netherlands). Patients were recruited from the dept. of Pulmonology and the dept. of Immunology of the University Hospital Dijkzigt, and from pulmonary departments of the St. Franciscus Ziekenhuis and the Havenziekenhuis in Rotterdam.

On the basis of the inclusion and exclusion criteria we selected 18 patients, aged 49 ± 18 years (mean ± SD), 12 women and 6 men in the study group of severe chronic relapsing airway infections. Sputum cultures contained H. influenza, Str. pneumoniae, P. aeruginosa, M. catarrhalis and S. aureus. Controls existed of healthy laboratory staff (n = 18, ages 20-50 yrs).

Isolation of granulocytes:
Heparinized blood was obtained by venipuncture, immediately brought to our laboratory (< 2 hrs) and mixed 1:1 with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (Organon Technika, Boxtel, the Netherlands). Granulocytes and erythrocytes were separated from mononuclear cells with Ficoll-Paque (1.079 g/ml; Pharmacia, Uppsala, Sweden) density gradient centrifugation. Granulocytes were obtained by lysing the erythrocytes in icecold NH_4Cl-buffer for 20 min. Subsequently, the cells were washed twice and resuspended in RPMI 1640 (Gibco, Breda, the Netherlands).

Polarization Assay:
The granulocyte polarization assay was developed analogous to the polarization assay for monocytes described by Cianciolo[8] and by this lab[9,10]. Granulocytes ($0.2x10^6$) were incubated in a total volume of 0.25 ml in polypropylene tubes (Falcon, Oxford, CA) with or without a chemotactic stimulus at 37°C in a waterbath. After 15 min. cells were fixed by adding 0.25 ml icecold phosphate-buffered 10% (w:v) formaldehyde solution. As stimulus was used: N-formyl-Methionyl-Leucyl-Phenylalanine (FMLP) in concentrations of 0.1, 1, 10, 100 and 1000 nM, and casein (concentrations: 6.4, 3.2, 1.6, 0.8 and 0.4% w/v). To investigate the possible effect of G-CSF on granulocyte polarization, rhuG-CSF (Roche, Basel, Switzerland) was added in a concentration of 0.2 μg/ml together with 10 nM FMLP in the polarization assay. The cells were kept at 4°C until counting in a hemocytometer. Of each tube two hundred cells were counted using a light microscope. A cell was considered to be polarized if it showed multiple broadened lamellipodia and a triangular or elongated shape. The percentage granulocytes responding to the chemotactic stimulus was calculated from the percentage cells polarized in the presence of a chemotactic stimulus

minus the percentage cells polarized without a stimulus (medium control).

Microchemotaxis assay:
Granulocyte chemotaxis was tested in a commercially available microchemotaxis assay; we used the 48 well microchemotaxis chamber (Neuro Probe Inc., Cabin John, MD) as described by Falk and colleagues[11]. The chemotactic solution (25 μl 10^{-7} M FMLP in RPMI/FCS), or as control RPMI/FCS alone, was placed in the bottom wells. The upper chambers were filled with 50 μl cell suspensions containing 20,000 granulocytes. The test was always performed in quadruple. The wells were separated by a polycarbonate filter with 3 μm pores (Nuclepore, Pleasanton, CA). After 30 min. incubation at 37°C and 5% CO_2, the chemotaxis filters were removed and non-migrated cells were swept off using a windscreenwiper. Subsequently the filters were air dried and stained with Coomassie blue. Migrated cells were counted automatically using a VIDAS-RT image-analyzing computer (Kontron Elektronik GmbH, Neufarn, Germany). Granulocyte chemotaxis was calculated from the mean number of migrated cells in four wells at 1 mm^2 in the presence of FMLP minus the mean number of migrated cells without FMLP.

Cytochrome C Reduction Assay:
Superoxide production was measured as the superoxide dysmutase (SOD) inhibitable reduction of Cytochrome C as described by Pick and Mizel[12]. The assay was performed in 96 well tissue culture plates in an ELISA-reader (Molecular Devices, Menlo Park, CA) at 37°C. In each well 100,000 granulocytes with a stimulus and with or without SOD (Sigma) in a total volume of 200 μl were incubated for 15 min. As a stimulus phorbol myristate acetate (PMA, Sigma) 100 ng/ml, serum treated zymosan (STZ) 1.25 mg/ml and FMLP 1μM were used. Optical densities (OD) were measured at intervals of 18 s. at 550 nm and at 540 nm (reference wave length). From the optical densities measured, the maximum rate of superoxide production was calculated in mOD/min using Softmax (Molecular Devices) software. This value was transformed into nmol/min using the molar extinction coefficient of cytochrome C (21.100 $M^{-1}cm^{-1}$).

Statistics:
Results are shown as mean ± standard deviation. Since all data showed a normal distribution, the Student's T-test was used for statistical analysis. P-values < 0.05 were considered significant.

RESULTS

Granulocyte polarization:

For both the 18 patients with severe chronic relapsing airway infections and the healthy controls, the percentage granulocytes polarizing upon stimulation with a chemoattractant is depicted in figure 1. There was no difference in spontaneous polarization (without a stimulus) between healthy controls and patients (9.8±4.4% vs. 10.0±5.3%, n=18, p>0.5). However, with 10 nM and 100 nM FMLP as chemotactic stimulus, granulocyte polarization was

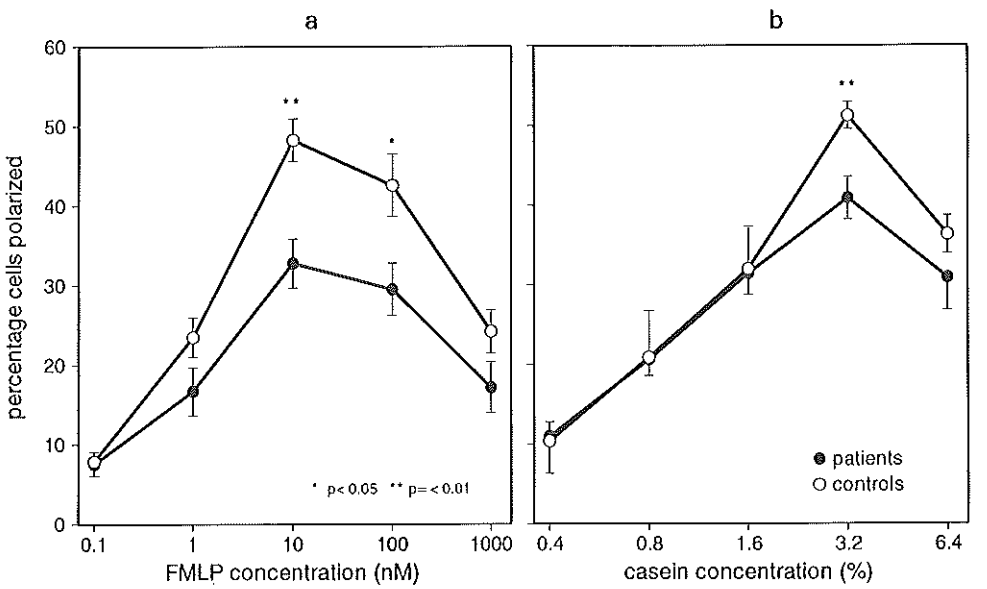


Figure 1. Granulocyte polarization (corrected for spontaneous polarization) from patients with chronic recurrent infections of the lower airways (closed circles) and healthy controls (open circles); a) dose response curves of the responses to FMLP; b) dose response curves of the responses to casein; data represent mean ± SEM; *: p<0.05; **: p<0.01.

significantly lower in our group of patients with chronic lower airway infections as compared to the healthy controls. The maximal response to the optimal concentration of FMLP was 36.1% ± 11.1% of patients compared to 51.9% ± 11.9%, (p<0.001) in the healthy controls. Ten of the 18 patients showed maximal responses below the range of the healthy controls (<40% granulocyte polarization). With 3.2% casein as a stimulus, the granulocytes of the patients also showed significantly lower responses when compared to the healthy controls, but the absolute differences between the two groups were smaller than with FMLP as a stimulus. Maximal polarization was 44.8±10.9% (patients) vs. 52.7±7.1% (controls, p=0.022), and in 4 of the 17 patients

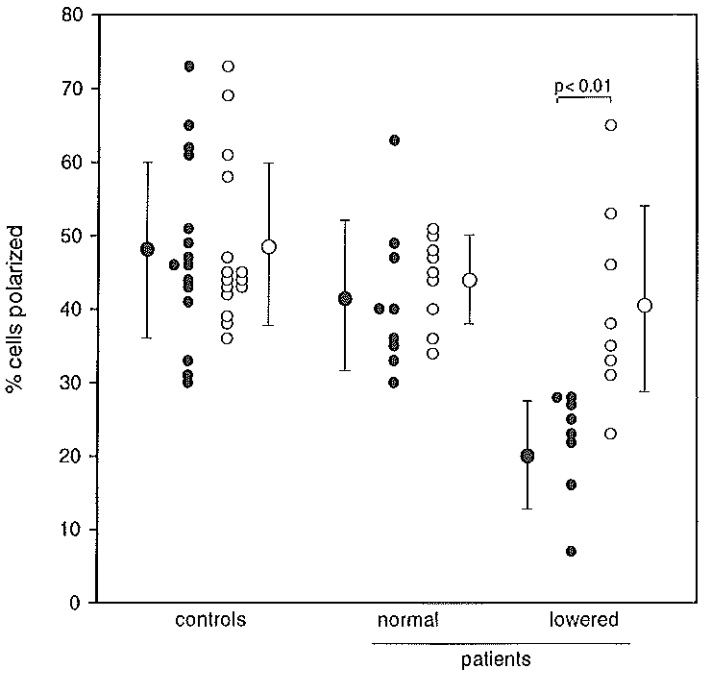


Figure 2. Granulocyte polarization in response to 10 nM FMLP in the absence (closed circles) or presence (open circles) of G-CSF. G-CSF effects on granulocyte polarization responses of healthy controls are compared to that of patients with a granulocyte polarization within (NORMAL) and below (LOWERED) the range of the healthy controls. Small circles represent individual data; Large circles represent the mean ± SD

casein-stimulated granulocyte polarization was below the range of the healthy controls (<40%).
In vitro addition of G-CSF improved FMLP-induced granulocyte polarization in the patient group (32.0±13.8% without vs. 42.7±9.7% with G-CSF, n=17, p=0.006, figure 2). In patients with a granulocyte polarization below the range of the healthy controls, G-CSF had a marked effect on the percentage of granulocytes polarized (23.7±7.7% without G-CSF vs. 41.2±11.4% with G-CSF, p<0.001, figure 2). However, in patients showing a percentage granulocyte polarization in the normal range, G-CSF had no effect on polarization (45.8±9.7% without vs. 45.2±6.1% with G-CSF). G-CSF had also no effect on the percentage granulocytes polarized of the healthy controls (48.2±12.5% vs. 48.5±11.3%, figure 2).

We tried to correlate various clinical parameters of the patients with the defects

in FMLP-induced granulocyte polarization. FMLP induced optimal granulocyte polarization was comparable in patients with normal lung function (n = 8) and patients with an obstructive lung function (n = 6, FEV1 and FEV1/FVC < 80% predicted): 37.6% vs. 36.8%, ($p > 0.5$). There was also no statistical significant difference between patients with (n = 9) and without bronchiectasis (n = 7) and those with (n = 8) or without (n = 10) concomitant chronic purulent rhinosinusitis (34.3 vs. 43.0% and 41.2% vs 34.3% respectively). Granulocyte polarization was also comparable in patients on prednisone treatment (n-5) and those without (n = 13) prednisone (39.6 vs. 37.1%, $p > 0.5$).

One patient with a defective granulocyte polarization (18% polarized granulocytes at the optimal FMLP concentration) showed a strongly elevated IgE level (3419 U/ml). Until 4 years ago, this patient had suffered from eczema, and therefore this patient met the criteria of the hyperimmunoglobulin E syndrome. Apart from this exception, total serum immunoglobulin and subclass levels were virtually within the normal range in all patients, except for slightly lowered levels of IgG and IgA in 2 patients and 1 patient respectively, not explaining their severe susceptibility for infection (in support of this notion treatment with intravenous immunoglobulin started in one had no effect).

Granulocyte chemotaxis:
Because in our experience the interassay variation in the granulocyte microchemotaxis-assay is considerable, the chemotactic activity of the granulocytes of a given patient was always determined in relation to the granulocyte chemotaxis of a paired healthy control performed in the same assay. Only 7 of our patients were tested in the microchemotaxis-assay; the results showed that this group of 7 patients indeed showed a lower chemotaxis of their granulocytes towards the chemoattractant FMLP compared to healthy control group ($p = 0.05$, figure 3). Numbers of patients were too small to carry out a valid correlation study between outcomes in the polarization assay and the chemotaxis assay (see for further details the discussion).

Superoxide production:
In earlier experiments we had established that 100 mg/ml PMA, 1,25 mg/ml STZ and 1μM FMLP were optimal stimuli in the superoxide production assay. No differences in optimal superoxide production were observed between healthy controls and patients with either PMA, STZ or FMLP as a stimulus (PMA: 518 ± 266 vs. 524 ± 256 pmol/min, STZ:168 ± 28 vs. 176 ± 36 pmol/min, FMLP: 248 ± 150 vs. 266 ± 98 pmol/min, n = 8, $p > 0.5$). When patients with a lowered granulocyte polarization (<40%, 5 of 8 patients tested) were compared to those with a normal polarization we did not observe any difference between these two groups in superoxide production either.

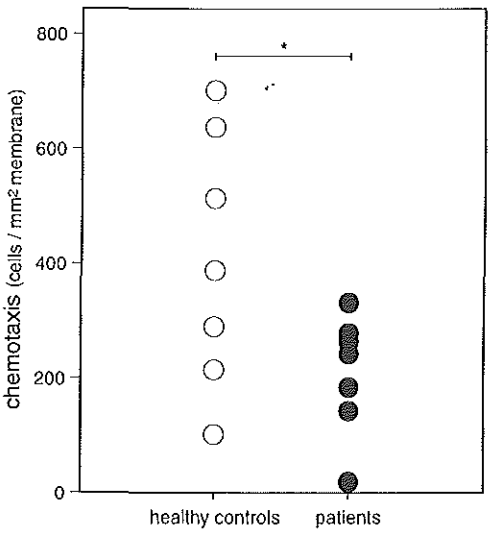


Figure 3. The chemotaxis of granulocytes of patients with chronic recurrent infections of the lower airways (closed circles) and healthy controls (open circles). *: p=0.05 (paired Student's t-test).

DISCUSSION

We investigated granulocyte function in patients with unexplained chronic recurrent infections of the lower airways. Our patient selection was primarily based on clinical symptoms; chronic production of purulent sputum, not explained by well-defined immunodeficiencies or preexisting anatomic or metabolic abnormalities, and infection proved by positive bacterial sputum culture. These patients constitute a significant clinical problem, since despite intensive and frequent therapy the infections do not resolve, and since such patients present regularly (18 patients were selected from 3 clinics in a period of 1 year).

We were able to demonstrate that these patients showed a lowered granulocyte polarization towards two tested chemotactic stimuli, and that G-CSF restored this granulocyte defect *in vitro*. In a limited series tested patients also had a marginally significant lower chemotactic ability of their granulocytes. Patient granulocytes showed normal superoxide production towards three stimuli used at optimal concentrations.

To our knowledge a defective neutrophil polarization in patients with severe infections has not been reported in the literature before. A disturbed chemotaxis however is a well recognized phenomenon in patients with burn injuries, in viral infections and in several rare diseases associated with chronic airway infections such as the Karthagener syndrome, the Chediak Higashi syndrome and the hyperimmunoglobulin E syndrome [13,14]. One of our patients met the criteria for the hyper IgE syndrome[15]. In patients with recurrent infections also involving other sites than the bronchial tree but in the absence of the above mentioned disorders, a reduced neutrophil chemotaxis has also been reported, be it in a clear minority[16,17]. In a study on 10 patients with chronically infected bronchiectasis, neutrophil chemotaxis measured in a Boyden chamber was, although lower, not significantly different from the healthy controls[18]. We also found that the granulocyte chemotactic response in our patient group tended to be lower than the healthy controls.

What is actually known about the relationship between granulocyte polarization and granulocyte chemotaxis? Displacements along a chemotactic gradient are associated with characteristic morphological patterns of polarization[7], and it has been suggested that polarization reflects an early event in the process of chemotaxis[8]. In a heterogenous group of patients (including the here described adult patients, children with recurrent infections, patients with recurrent infections at other sites, and carcinoma patients) granulocyte chemotaxis was significantly lower in patients with a relatively low polarization response ($< 75\%$ of the value of the day control) compared to patients with a polarization response of $\geq 75\%$ of the day control ($56 \pm 12\%$ vs. $78 \pm 26\%$ chemotaxis expressed as percentage of the day control, mean $\pm$ SE, $n = 15$, $p = 0.03$). It must be noted, however, that there were also clear exceptions in individual cases, and low polarization values can be found in the presence of a normal chemotaxis and vice versa. We therefore view polarization and chemotaxis as related phenomena but not as identical processes, and it is clear that the polarization assay can not fully replace the chemotaxis assay.

Considering the importance for host defence *in vivo* of the restorative effect of G-CSF on the defective granulocyte polarization, studies in animal models have provided interesting data. In mice, burn injury as well as superimposed Pseudomonas sepsis caused a depressed chemotaxis. Interestingly, in vivo administration of rhG-CSF, restored chemotaxis and resulted in an improved survival of the mice[19]. In addition, G-CSF and GM-CSF have been shown to reverse neutrophil dysfunction in influenza A virus infection in chinchillas[20]. In experimental C. albicans infection in the rat, G-CSF also enhanced pulmonary antifungal activity[21]. G-CSF has been reported to have a number of effects on neutrophils. It enhances hematopoietic neutrophil progenitor growth, neutrophil release from the bone marrow, and primes several mature neutrophil effector

functions[22]. G-CSF has also been reported to co-operate in the induction of FMLP receptors on the myeloid HL60 cell line[23], to prime FMLP-induced membrane depolarization in neutrophils[24], and to enhance FMLP receptor-mediated, but not protein kinase C-mediated, neutrophil superoxide generation[25]. The here described in vitro restoration of a defective polarization of patient granulocytes by G-CSF has not been described before.

With respect to the cellular mechanisms responsible for the impaired granulocyte polarization observed in our patients, it seems unlikely that these are due to a decreased number or avidity of FMLP receptors, since superoxide production in response to FMLP was normal. It should be noted, however, that FMLP induces different signal transduction pathways for chemotaxis and for superoxide production in human neutrophils[26]. Hence, our data might be explained by a hampered signal transduction for chemotactic responses in our patients' neutrophils. Changes in cell shape are considered to be driven by cytoplasmic cytoskeletal elements, whereas the plasma membrane behaves as a passive structure[28]. Actin filaments are likely to play a dominant role in active cell shape changes, and exposing cells to microfilament inhibitors results in major alterations of shape control [28, 29, 30]. Indeed, cytochalasin B exposure abolished the polarization response of granulocytes (data not shown). Preliminary data indicate that granulocytes of our patients defective in the polarization-assay do have disturbances in polymerization-depolymerization of actin-filaments (personal communication A.J. Verhoeven, CLB, Amsterdam, and H. Hooijkaas).

With regard to the etiology of the locomotory defects of the patient granulocytes, defects may be inborn (intrinsic) or induced by factors influencing the cell's motility[13]. An inborn defect is less likely, since in most patients the onset of the airway infections was in adulthood. For inborn defects, we would have expected a positive family history and a start of the infections in childhood. Factors influencing phagocyte function might be produced by bacteria colonizing the airways, by airway epithelial cells or by immune cells themselves. Several bacteria have been shown to produce factors that affect leucocyte function, e.g. pyocyanine of P. aeruginosa inhibits neutrophil superoxide production[31]. The production of cytokines by airway epithelial cells or various types of immune cells may also downregulate neutrophil function[32]. By the production of such factors, bacterial infection might cause an alteration of host defenses in the respiratory tract, predisposing to further infection, and the establishment of a vicious circle, as has been hypothesized before[33,34].

Five patients (28%) in our study received oral corticosteroids because of emphysema at the time of testing. Corticosteroids affect leucocyte function in various ways, but do not have any effect on chemotaxis[35]. We could also not

demonstrate any effect of corticosteroid treatment on granulocyte polarization.

The defective granulocyte polarization and its restoration in vitro by G-CSF observed by us bring up the question, whether or not patients with chronic recurrent airway infections might benefit from rhG-CSF treatment. In a pilot study, 7 patients with chronic airway infection and a decreased granulocyte polarization were treated with subcutaneous rhG-CSF[36]. In these patients infections had persisted despite multiple courses of antibiotics. Interestingly, in 3 of these patients G-CSF completely prevented further infections, and 2 patients showed a partial improvement during rhG-CSF treatment. However, to establish the possible beneficial effect of rhG-CSF administration to patients with chronic relapsing airway infections, randomized, double-blind, placebo-controlled studies are needed.

ACKNOWLEDGEMENTS

This study was supported by a grant of the Netherlands Asthma Foundation. We thank Dr.K.Y. Tan, (St. Franciscus Gasthuis, Rotterdam) and Dr. P.R.M. Hekking (Havenziekenhuis and Ikazia Ziekenhuis, Rotterdam, the Netherlands) for their contribution in the selection of patients, and Dr. Blijenberg (Dept. Clinical Chemistry, University Hospital Dijkzigt, Rotterdam) for the immunoglobulin determinations. We thank Tar van Os for the preparation of the figures.

REFERENCES

1. Gross S, Blais MS, Herrod HG.- Role of immunoglobulin subclasses and specific antibody determinations in the evaluation of recurrent infection in children. *J Pediatr* 1992;121:516-522.
2. De Baets, F, Kint J, Pauwels R, Leroy J.- IgG subclass deficiency in children with recurrent bronchitis. *Eur J Pediatr* 1992;151:274-278.
3. Popa V, Kim K, Heiner DC.- IgG deficiency in adults with recurrent respiratory infections. *Ann Allergy* 1993;70:418-424.
4. Hidalgo H, Ehlayel M, Moore C, Sorensen RU.- Assessment of anti-pneumococcal antibodies in children with recurrent infections. *Am J Respir Crit Care Med* 1994;149:A122.
5. Jefferis R, Kumararatne DS.- Selective IgG subclass deficiency: quantification and clinical relevance. *Clin Exp Immunol* 1990;81:357-367.
6. Zaffran Y, Lepedi H, Benofiel A, Capo C, Bongrand P. Role of calcium in the shape control of human granulocytes. *Blood Cells* 1993;19:115-131.
7. Wilkinson PC.- Chemotaxis and inflammation. Churchill Livingstone. Edinburgh, 1982.
8. Cianciolo GJ, Snyderman R.- Monocyte responsiveness to chemotactic stimuli is a property of a subpopulation of cells that can respond to multiple chemotactic stimuli. *J Clin Invest* 1981;67:60-68.
9. Tan IB, Drexhage HA, Scheper RJ, von Blomberg-van der Flier BME, de Haan-Meulman M,

Snow GB, Balm AJM.- Defective monocyte chemotaxis in patients with head and neck cancer. Restoration after treatment. *Arch Otolaryngol Head Neck Surg* 1986;112:942-945.

10. Plassche-Boers EM van de, Tas M, de Haan-Meulman M, Kleingeld M, Drexhage HA.- Abnormal monocyte chemotaxis in patients with chronic purulent rhinosinusitis: an effect of retroviral p15E-related factors in serum. *Clin Exp Immunol* 1988;73:348-354.
11. Falk W, Goodwin RH, Leonard EJ.- A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J Immunol Methods* 1980;33:239-247.
12. Pick E, Mizel D.- Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods* 1981;46:211-226.
13. Gallin JI.- Disorders of phagocyte chemotaxis. *Ann Intern Med* 1980;92:520-538.
14. Abramson JS, Mills EL.- Depression of neutrophil function induced by viruses and its role in secondary microbial infections. *Rev Infect Dis* 1988;10:326-341.
15. Geha RS, Leung DYM.- Hyperimmunoglobulin E syndrome. *Immunodeficiency Rev* 1989;1:155-172.
16. Brenneis H, Schmidt A, Blaas-Mautner P, Worner I, Ludwig R, Hansch GM.- Chemotaxis of polymorphonuclear neutrophils (PMN) in patients suffering from recurrent infections. *Eur J Clin Invest* 1993;23:693-698.
17. Giebink SG, Mills EL, JS Huff, Cates KL, Juhn SK, Quie PG.- Polymorphonuclear leukocyte dysfunction in children with recurrent otitis media. *J Pediatr* 1979;94:13-18.
18. Stockley RA, Shaw J, Hill SL, Burnett D.- Neutrophil chemotaxis in bronchiectasis: a study of peripheral cells and lung secretions. *Clin Sci* 1988;74:645-650.
19. Sartorelli K, Silver GM, Gamelli RL.- The effect of granulocyte colony-stimulating factor (G-CSF) upon burn-induced defective neutrophil chemotaxis. *J Trauma* 1991;31:523-530.
20. Abramson JS, Hudnor HR.- Effect of priming polymorphonuclear leukocytes with cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF] and G-CSF) on host resistance to Streptococcus pneumoniae in chinchillas infected with influenza A virus. *Blood* 1994;83:1929-1934.
21. Nelson S, Mason C, Bagby G, Summer W.- The contrasting effects of anti-tumor necrosis factor antibody and granulocyte colony-stimulating factor. *Am J Respir Crit Care Med* 1994;149:A872.
22. Cairo MS, van de Ven C, Toy C, Mauss D, Sheikh K, Kommareddy S, Modanlou H.- Lymphokines: enhancement by granulocyte-macrophage and granulocyte colony-stimulating factors of neonatal myeloid kinetics and functional activation of polymorphonuclear leukocytes. *Rev Infect Dis* 1990;12:S492-497.
23. Sakashita A, Nakamaki T, Tsuruoka N, Honma Y, Hozumi M.- Granulocyte colony-stimulating factor, and not granulocyte-macrophage colony-stimulating factor, co-operates with retinoic acid on the induction of functional N-formyl-methionyl-phenylalanine receptors in HL60 cells. *Leukemia* 1991;5:26-31.
24. Sullivan R, Griffin JD, Simons ER, Schafer AI, Meshulam T, Fredette JP, Maas AK, Gadenne A-S, Leavitt JL, Melnick DA.- Effects of recombinant human granulocyte and macrophage colony-stimulating factors on signal transduction pathways in human granulocytes. *J Immunol* 1987;139:3422-3430.
25. Tanimura M, Kobuchi H, Utsumi T, Yoshioka T, Kataoka Y, Fujita S, Utsumi K.- Neutrophil priming by granulocyte colony-stimulating factor and its modulation by protein kinase inhibitors. *Biochem Pharmacol* 1992;44:1045-1052.
26. Yasui K, Yamazaki M, Miyabayashi M, Tsuno T, Komiyama A.- Signal transduction pathways in human polymorphonuclear leukocytes for chemotaxis induced by a chemotactic factor. *J Immunol* 1994;152:5922-5929.
27. Schmid-Schönbein GW. Leukocyte biophysics. *Cell Biophys* 1990;17:107-135.
28. Hitchcock SE. Regulation of motility in non-muscle cells. *J Cell Biol* 1976;71:848-875.

29. Lichtman MA, Santillo PA, Kearney EA, Roberts GW, Weed RI. The shape and surface morphology of human leukocytes in vitro: effect of temperature, metabolic inhibitors and agents that influence membrane structure. *Blood Cells* 1976;2:507-531.
30. Tomasek JJ, Hay ED. Analysis of the role of microfilaments and microtubules in acquisition of bipolarity and elongation of fibroblasts in hydrated collagen gels. *J Cell Biol* 1984;99:536-549.
31. Muller PK, Krohn K, Muhlradt PF.- Effects of pyocyanine, a phenazine dye from Pseudomonas aeruginosa, on oxidative burst and bacterial killing in human neutrophils. *Inf Immun* 1989;57:2591-2596.
32. Steinbeck MJ, Roth JA.- Neutrophil activation by recombinant cytokines. *Rev Inf Dis* 1989;11:549-568.
33. Cole P.- Host-microbe relationship in chronic respiratory infection. *Respiration* 1989;55:(suppl.1:5-8).
34. Murphy FT, Sethi S.- Bacterial infection in chronic obstructive respiratory disease. *Am Rev Respir Dis* 1992;146:1067-1083.
35. Fukushima K, Ando M, Ito K, Suga M, Araki S.- Stimulus and cumulative dose-dependent inhibition of O_2^- production by polymorphonuclear leukocytes of patients receiving corticosteroids. *J Clin Lab Immunol* 1990;33:117-123.
36. van de Merwe JP and Hooijkaas H.- Sjögren's syndrome in patients with chronic bacterial respiratory infections and a diminished chemotactic responsiveness of monocytes and/or granulocytes: treatment with G-CSF. *In:* Homma M, Sugai S, Tojo T, Miyasaka N, Akizuki M, Eds. Sjögren's Syndrome - State of the Art, Amsterdam, Kugler Publications, 1994:373-376.

DEFECTS IN MONOCYTE POLARIZATION AND CHEMOTAXIS IN PATIENTS WITH CHRONIC RELAPSING BACTERIAL INFECTIONS OF THE LOWER AIRWAYS

Jan Maarten W. van Haarst[1,2], Harm J. de Wit[1], Meeny de Haan-Meulman[1], Wim Sluiter[3], Henk C. Hoogsteden[2] and Hemmo A. Drexhage[1].

Dept. of Immunology[1], Pulmonology[2] and Biochemistry[3], Erasmus University and University Hospital Dijkzigt, Rotterdam, the Netherlands.

ABSTRACT

Severe immunodeficiencies are a well-known cause of chronic or recurrent infections. More subtle disturbances in immune function might also contribute to an enhanced susceptibility for infections. We investigated polarization (change of shape in response to a chemotaxin), chemotaxis and superoxide production of monocytes of patients with severe chronic relapsing bacterial infections of the lower airways, that failed to respond lastingly to antibiotic treatment.
FMLP-induced monocyte polarization was significantly reduced in patients compared to healthy controls ($24.5 \pm 9.4\%$ vs. $34.3 \pm 5.9\%$, $n = 36$, $p < 0.001$) and the percentage monocytes polarized was below the range of the healthy controls in 14 (39%) of the patients.
In patients with a lowered monocyte polarization, this polarization could partially be restored in vitro by the addition of thymic peptides (TP-1 or TP5).
Monocyte chemotaxis was also lower in patients than in controls (444 vs. 698 cells/field, $p < 0.05$, $n = 11$). Superoxide production of patient monocytes did not differ from controls with either phorbol ester (PMA), serum treated zymosan (STZ) or FMLP as stimulus. The serum of the patients contained low molecular weight factors (LMWF) that were able to inhibit the polarization of healthy donor monocytes. These LMWF showed an antigenic relationship with the retroviral immunosuppressive protein p15E.
In conclusion discrete, but clear disturbances in the polarization and chemotactic ability of monocytes were observed in patients with severe chronic relapsing infections of the lower airways not responding to antibiotic treatment. These disturbances might play a role in their susceptibility for infections.

INTRODUCTION

Patients with chronic or recurrent purulent infections of the lower airways, that do not resolve despite appropriate antibiotic therapy, constitute a significant clinical problem. The high susceptibility for airway infections can sometimes be attributed to metabolic disturbances or anatomic abnormalities of the airways. Severe immunodeficiencies are rare causes of such purulent infections and mostly present with opportunistic infections as in AIDS.

More subtle disturbances in immune function might also contribute to an increased susceptibility for airway infections. With respect to B cell function, deficiencies in immunoglobulin isotype, IgG subclasses and specific antibodies have been observed in patients with chronic recurrent (airway) infections[1,2,3,4].

Recently, we described a decreased polarization (change in shape in response

to a chemotaxin) of granulocytes in patients with chronic relapsing infections of the lower airways[5]. These defects could be restored with G-CSF in vitro. Disturbances in monocyte polarization might also contribute to the increased susceptibility for infections in these patients. Several important functions of monocytes depend on their ability to undergo extensive morphological changes: passage through small capillaries[6], the emigration from blood through the vessel wall[7], and shape changes associated with phagocytosis[8] and chemotaxis[9]. Actin filaments and calcium fluxes are likely to play a dominant role in active cell shape changes[10]. To determine such a possible role of defects of shape change in monocytes, we have investigated the polarization of peripheral blood monocytes in 36 patients with chronic recurrent purulent infections of the lower airways that failed to respond lastingly to appropriate antibiotic therapy. In 10 of the 36 patients with chronic recurrent purulent airway infections the chemotaxis of the monocytes was tested as well, and compared to the outcomes of the polarization assay.

In patients with chronic purulent infections of the upper airways (rhinosinusitis), defects in monocyte polarization have been detected by us before. This decreased ability of the monocytes to polarize was related to the presence of a low molecular weight immunosuppressive factor (LMWF) in the serum of the patients[11]. This LMWF appeared to be antigenically related to the retroviral envelope protein p15E[11]. Thymic peptides were able to neutralize the immunosuppressive effects of the p15E-like serum factor and partially restored the polarization of the patients' monocytes[11]. Therefore, we also investigated the presence of such p15E-like immunosuppressive factors in the serum of patients with chronic recurrent bacterial infections of the lower airways and the in vitro effects of thymic peptides on the function of the monocytes of the patients.

PATIENTS AND METHODS

Patients:

a) Patients with therapy-refractory severe chronic relapsing bacterial infections of the lower airways were selected according to the following criteria:

- chronic production of purulent sputum (minimally 3 months a year),
- no response to antibiotic treatment or frequent recurrence despite antibiotic therapy,
- a minimal duration of the disease of 2 years,
- sputum culture positive,
- no use of antibiotics at the time of testing.

Excluded were patients with metabolic defects (e.g. diabetes, cystic fibrosis)

and patients with well characterized immunodeficiency syndromes (e.g. agammaglobulinemia, HIV infection).
Patients were recruited from the dept. of Pulmonology and the dept. of Immunology of the Dijkzigt University Hospital, and from pulmonary departments of the St. Franciscus Ziekenhuis and the Havenziekenhuis in Rotterdam.
On the basis of the inclusion and exclusion criteria 36 patients were selected from these 3 clinics in a period of 2 years. The patients aged 54 ± 17 years (mean ± SD), 24 women and 12 men. The sputum cultures contained H. influenza, Str. pneumoniae, P. aeruginosa, M. catarrhalis and S. aureus. Twenty four of the 33 evaluable patients (73%) had bronchiectasis, diagnosed on CT-scan, bronchography or as typical chest X-ray abnormalities (tramlines, infiltrates). Ten patients (28%) with chronic recurrent infections of the lower airways had concomitant chronic rhinosinusitis. Eleven patients (31%) were treated with prednisone orally 5 or 10 mg daily, because of severe obstructive pulmonary disease.
The vast majority of the patients had normal immunoglobulin and subclass levels. Two patients had lowered levels of IgG (6.83 and 4.30 g/l; normal range 8.0-18.0 g/l), IgA and IgG subclasses. A slightly lowered IgA level (0.79 g/l; normal range 0.9-4.5 g/l) was observed in another patient. We did not consider these slight abnormalities as reasons for the infections susceptibility, since intravenous γ-globulin treatment did not resolve the severe purulent airway infection. One patient was remarkable for a strongly elevated IgE level (^J^/ml), and, until 4 years ago, this patient had suffered from eczema.
b) Disease controls included patients with uninfected bronchiectasis, that did not produce sputum and did not have a history of chronic or relapsing airway infections (n = 5, ages 36-79 yrs), and patients with uninfected chronic obstructive pulmonary disease (COPD, n = 19, ages 31-81 yrs) of whom no bacteria could be cultured from the sputum in routine microbiological assays.
c) Healthy controls were laboratory staff (n = 35, ages 20-50 years).

Isolation of monocytes:
Heparin blood was obtained by venipuncture immediately transported to our laboratory (< 2 hrs), and mixed 1:1 with phosphate buffered saline (PBS). Mononuclear cells were isolated by Ficoll-Paque (1.079 g/ml; Pharmacia, Uppsala, Sweden) density gradient centrifugation. Cells were washed twice in PBS and were resuspended in RPMI 1640 (Gibco) containing 10% fetal calf serum (RPMI/FCS). Subsequently Percoll (1.063 g/ml; Pharmacia) density gradient separation was performed. The interface contained 70-95% monocytes as determined by non-specific esterase staining.

Monocyte Polarization Assay:
The monocyte polarization assay of Cianciolo and Snyderman[12] was used with

slight modifications[13]. Monocytes ($0.2x10^6$) were incubated in a total volume of 0.25 ml in polypropylene tubes (Falcon, Oxford, CA) with or without 10^{-8}M N-formyl-Methionyl-Leucyl-Phenylalanine (FMLP) at 37°C in a waterbath. After 15 min. cells were fixed by adding 0.25 ml icecold 10% (w:v) formaldehyde solution. The cells were kept at 4°C until counting in a hemocytometer. Two hundred cells were counted by two investigators independently using a light microscope. A cell was determined to be polarized if it showed broadened lamellipodia or a triangular or elongated shape. The percentage monocytes responding to the chemotactic stimulus (FMLP) was calculated from the percentage cells polarized in the presence of FMLP minus the percentage polarized in the absence of FMLP, and corrected for the purity of the monocyte suspension, as follows:

$$\% \text{ monocyte polarization} = \frac{\% \text{ polarized (FMLP-medium)}}{\% \text{ NSE-positive cells}} \times 100\%$$

With regard to the effects of thymic peptides, two commercially available preparations were used, viz a crude thymic hormone preparation (TP-1, SERONO, Madrid, Spain) and a synthetic peptide thymopentin (the 5 AA active part of thymopoietin, Ortho, Raritan, New York)[14]. Optimal concentrations of 0.1 mg and 0.01 mg respectively (see for dose-response curves[15]) were added to the monocyte polarization assay together with the chemotactic stimulus.

Monocyte Chemotaxis:
Monocyte chemotaxis was tested in a microchemotaxis assay as described by Falk[16]. We used a 48 well microchemotaxis chamber (Neuro Probe Inc., Cabin John, MD). The chemotactic solution (25 μl 10^{-8}M FMLP in RPMI/FCS), or as control RPMI/FCS alone, was placed in the bottom wells. The upper chambers were filled with 50 μl cell suspension containing 20,000 monocytes. The test was always performed in quadruple.. The wells were separated by a polycarbonate filter with 5 μm pores (Nuclepore, Pleasanton, CA). After 30 min. incubation at 37°C and 5% CO_2, the chemotaxis filters were removed and non-migrated cells were swept of using a windscreenwiper. Subsequently the filters were air dried and stained with Coomassie blue. Migrated cells were counted automatically using a VIDAS-RT image-analyzing computer (Kontron Elektronik GmbH, Neufarn, Germany). Monocyte chemotaxis was calculated from the mean number of migrated cells in four wells at 1 mm^2 in the presence of FMLP minus the mean number of migrated cells without FMLP.

Cytochrome C Reduction Assay:
Superoxide production was measured as the superoxide dysmutase (SOD) inhibitable reduction of cytochrome C as described by Pick and Mizel[17]. The assay was performed in 96 well tissue culture plates in an ELISA-reader

(Molecular Devices, Menlo Park, CA) at 37°C. In each well 100,000 monocytes with a stimulus and with or without SOD (Sigma) in a total volume of 200 μl were incubated for 15 min. As a stimulus phorbol myristate acetate (PMA, Sigma) 100 ng/ml, serum treated zymosan (STZ) 1.25 mg/ml and FMLP 1μM were used. Optical densities (OD) were measured at intervals of 18 s. at 550 nm and at 540 nm (reference wave length). From the optical densities measured, the maximum rate of superoxide production was calculated in mOD/min using Softmax (Molecular Devices) software. This value was transformed into nmol/min using the molar extinction coefficient of cytochrome C (21.100 $M^{-1}cm^{-1}$).

Effects of low molecular weight serum factors on monocyte polarization
Sera of patients and controls were diluted 1:1 with PBS, and subjected to ultrafiltration through Amicon CF25 centriflo cones (Amicon Corp., Danvers, U.S.A.) for 15 min. at 700 g (molecular weight 'cut off' point 25 kD). To assess p15E-like bioactivity, absorption experiments were performed with the low molecular weight serum fractions (LMWF). The LMWFs were incubated for 48 h. at 4°C with monoclonal antibodies (MAbs), followed by Amicon ultrafiltration. The MAbs used were a combination of 2 anti-p15E MAbs (4F5 and 19F8). In previous studies, control MAb with the same isotype did not absorb inhibitory factors from the LMWFs. Original and absorbed LMWFs were stored at -70°C until use. The ability of these LMWFs to inhibit the FMLP-induced polarization of monocytes of healthy donors was tested. LMWFs were added in the polarization assay at a final dilution of 1:60. The percentage of inhibition was calculated from the percentages monocyte polarization with FMLP alone (FMLP) and with FMLP and LMWF (LMWF), which were corrected for polarization without FMLP (medium): [1 - (LMWF - medium) / (FMLP - medium)] x 100%.

Statistics:
Results are shown as mean ± standard deviation. Data showing a normal distribution were evaluated for statistical significance using Student's t-test. Data with a non-normal distribution were analyzed with the Wilcoxon rank sum test. P-values < 0.05 were considered significant.

RESULTS

Monocyte polarization:
In healthy controls the percentage monocytes polarizing upon stimulation with FMLP was 34.3±5.9% (mean ± SD, range 23-45%, n=35). In patients with chronic airway infection this percentage was significantly lower (24.5±9.4%, n=36, $p < 0.001$, figure 1). In 14 of these 36 patients (39%) the percentage

monocytes polarized was below the range of that of the here described healthy controls and of healthy controls reported before (≤ 20%). The monocyte polarization of the disease controls was 33.4 ± 7.1% (n = 5) for the patients with uninfected bronchiectasis and 39.4 ± 13.1% (n = 19) for the COPD patients (figure 1). These values were not significantly different from those of the healthy controls. Remarkably, defects in monocyte polarization were mainly found in patients with severe relapsing bronchial infections younger than 65 years of age. Monocyte polarization defects were equally found in our group of bronchially infected patients with or without oral corticosteroid treatment, and irrespective of the presence or absence of obstructive lung disease, bronchiectasis or chronic upper airway infection.

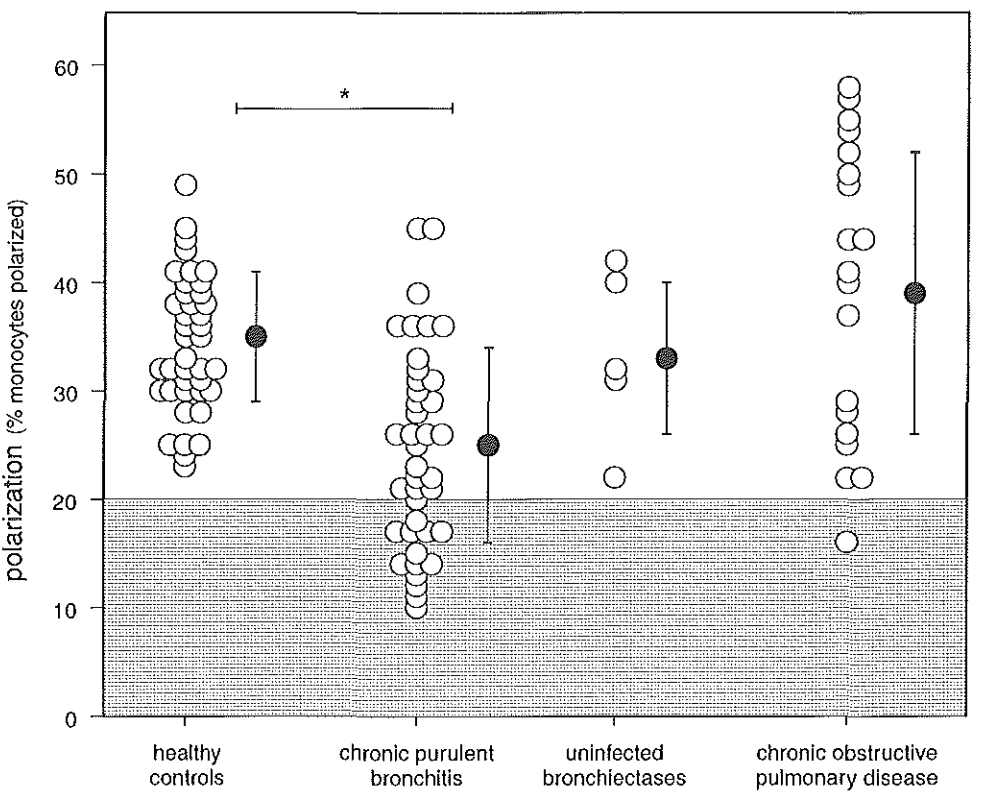


Figure 1. Monocyte polarization of healthy controls, patients with chronic relapsing infections of the lower airways, with "uninfected" bronchiectases, and chronic obstructive lung disease. Individual data and mean ± SD are shown. *: $p < 0.05$.

In a subgroup of 18 of the 36 patients described here, granulocyte function was investigated as well, as has been reported in detail elsewhere[5]. In 10 of these

18 patients (55%) we observed a decreased polarization of granulocytes in response to FMLP. Of the 18 patients in which both monocyte and granulocyte functions were investigated, combined defects in the polarization of both monocytes and granulocytes were observed in only 4 patients, 4 showed an isolated reduced monocyte polarization, 6 exhibited only a lowered polarization of granulocytes, and 4 showed a normal polarization response of both monocytes and granulocytes. Hence, correlations did not exist and defects in granulocyte and monocyte polarization must hence be considered as separate and independent abnormalities.

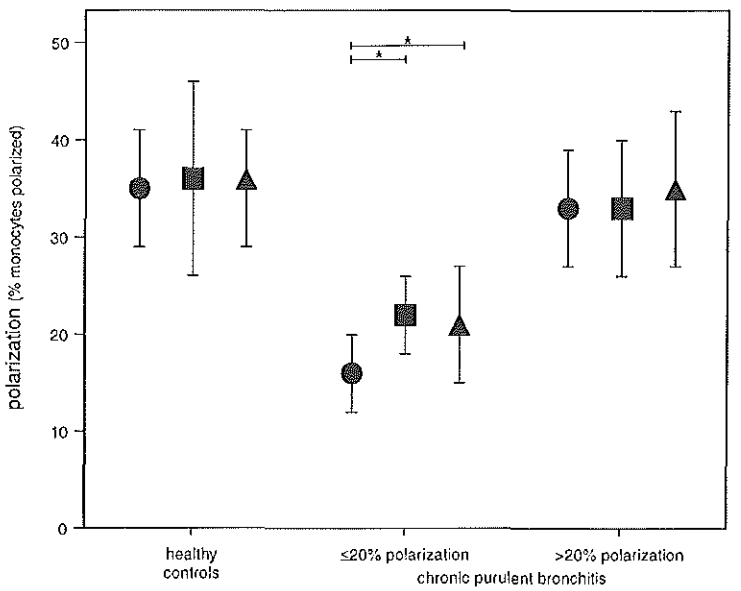


Figure 2. Effects of the addition of thymic peptides in vitro on the percentage monocyte polarization in patients and healthy controls. ●, no addition; ■, TP-1; ▲, TP5. The patients are separated in a group with a percentage FMLP-induced monocytes polarization within the range of the healthy controls (>20% polarization), and a group of patients with a percentage monocyte polarization below the range of the healthy controls (≤20% polarization). Data represent mean ± SD. *: $p<0.05$.

In patients with a percentage monocyte polarization below the range of the healthy controls, monocyte polarization partially, but significantly improved in vitro under the influence of TP-1 from 16.4±4.2% to 22.0±4.4% ($p<0.05$) and in the presence of TP5 from 16.5±3.9% to 21.5±5.7% ($p<0.01$) (both in optimal concentrations) (figure 2). In patients showing a monocyte polarization within the range of the healthy controls, the addition of TP-1 or TP5 did not alter the percentage monocytes polarized (figure 2).

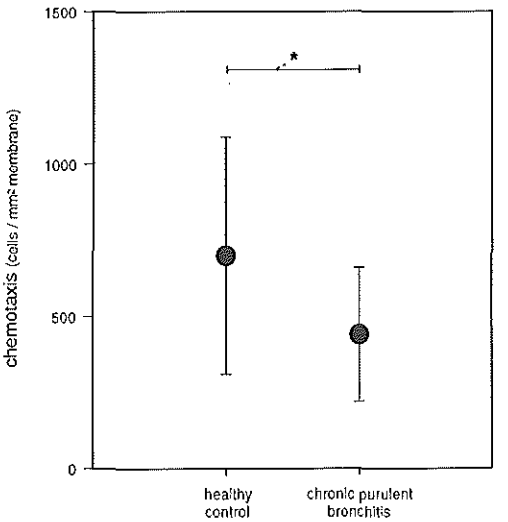


Figure 3. Numbers of monocytes migrated upon FMLP stimulation (cells/mm^2) of healthy controls and patients in the microchemotaxis assay. Data represent mean ± SD. *: $p < 0.05$

Monocyte chemotaxis:
Because the interassay variation of the microchemotaxis appeared to be high (numbers of migrated cells ranged from 68-1855 cells/mm^2); the chemotactic activity of monocytes of patients and paired healthy controls were always determined in the same assay. The results showed that the monocytes of patients with chronic relapsing bacterial infections of the lower airways exhibited a significantly lower chemotaxis (444 ± 218 vs. 698 ± 389 cells/mm^2, $n = 11$, $p < 0.05$, paired Student's t-test, figure 3). The addition of TP5 had no effect on monocyte chemotaxis of both patients and controls.

Monocyte polarization in relation to monocyte chemotaxis
Figure 4 depicts the relation between the polarization assay and the chemotaxis assay carried out on 35 monocyte suspensions of a heterogenous group of patients (including the here described patients, children with recurrent infections, patients with rhinosinusitis[11] and cancer patients[13]). In this larger panel of monocyte suspensions polarization and chemotaxis expressed as a value relative to the day control positively correlated ($r = 0.41$, $p < 0.05$, $n = 35$). Although in most cases a low polarization coincided with a low chemotaxis and a normal polarization coincided with a normal chemotaxis of monocytes, figure 4 also shows that in some cases clear discrepancies existed between the outcomes of the polarization assay and the chemotaxis assay. In the 11

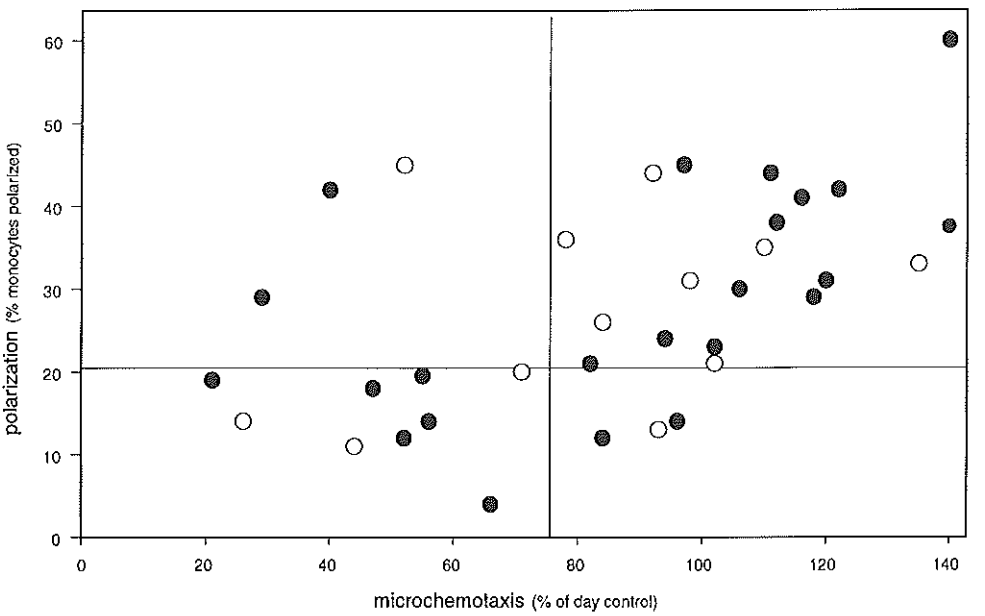


Figure 4. Correlation between data obtained in the polarization and the microchemotaxis assay in patients (n = 35) with chronic recurrent infections of the lower airways (open circles) and other patients (see text, closed circles). Correlation: $r = 0.41$, $p < 0.05$.

patients with chronic recurrent infections of the lower airways a similar trend for a positive correlation between polarization and chemotaxis could be observed, which did, however, not reach the level of statistical significance.

Superoxide production:
FMLP, PMA and STZ were used at a concentration that elicited a maximal superoxide production. In response to FMLP, monocytes of healthy controls showed a maximal rate of superoxide production of 366 ± 122 pmol/min (Mean ± SD). A similar value of 306 ± 192 pmol/min was obtained in patients with chronic lower airway infection (n = 10). With PMA as stimulus, monocytes of healthy controls and patients produced 230 ± 54 pmol/min superoxide and 196 ± 54 pmol/min respectively (n = 10). Upon addition of STZ, monocyte superoxide production amounted to 178 ± 30 pmol/min in healthy controls compared to 164 ± 38 pmol/min in patients (n = 10). Superoxide production of monocytes was not significantly different between patients and controls with either stimulus.

Low molecular weight factors in the serum of patients:
LMWFs of patients inhibited the polarization of healthy donor monocytes for

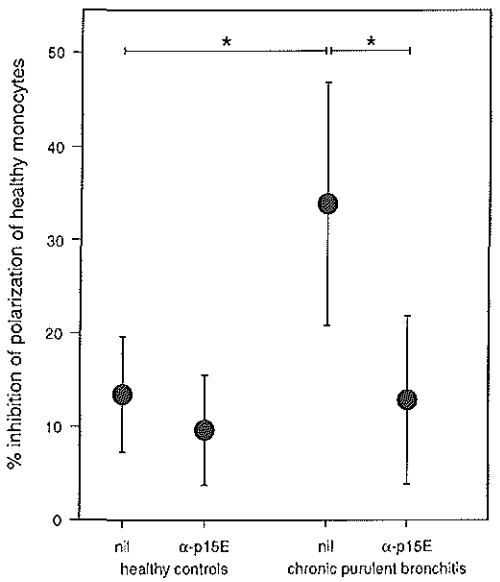


Figure 5. Percentage inhibition of unabsorbed (nil) and after absorption with anti-p15E MAbs LMWFs of the serum of patients and controls on the polarization of healthy donor monocytes. Data represent mean ± SD. *: $p < 0.05$

33,8 ± 12,2% (mean ± SD, n = 18), which was significantly higher compared to the inhibition by LMWFs of healthy controls 16,6% ± 12,2% (n = 12, figure 5). These factors that inhibit the polarization of monocytes present in the serum of our patients could be partially absorbed by monoclonal antibodies against the retroviral immunosuppressive factor p15E (figure 5). We could however not establish a correlation between the presence of LMWFs in the serum of individual patients and the performance of their monocytes in the polarization assay.

DISCUSSION

We investigated monocyte function in patients with severe, unexplained, chronic recurrent infections of the lower airways. The patients were primarily selected on the basis of clinical symptoms: chronic production of purulent sputum, infection proved by sputum culture, and quick relapse after or insufficient response to treatment with antibiotics. The regular appearance of such patients and the inability for a definitive eradication of these infections compose a significant clinical problem.

We were able to show that 14 of these 36 patients (39%) showed a monocyte polarization below the range of the healthy controls. Monocyte chemotaxis was also lower in the patient group compared to the healthy controls. Although the disturbances of the patients' monocytes in chemotaxis and polarization tended to correlate, clear discrepancies between polarization and chemotaxis could also be observed, indicating the separate nature of the two phenomena. Superoxide production was not different in monocytes from patients and controls.
Except for the presence of chronic recurrent airway infections, the lowered monocyte polarization could not be attributed to any clinical characteristic or to treatment with corticosteroids, which is in agreement with previous studies[18]. Defects in monocyte polarization, however, seemed to play only a minor role in chronic recurrent infections of the lower airways in older patients (> 65 yrs.).

Defects in the motility of monocytes are a well recognized phenomenon in patients with carcinomas, uremia, burns and certain viral infections such as AIDS[19,20,21]. An impaired monocyte responsiveness to chemotactic stimuli has also been described in patients suffering from recurrent cutaneous bacterial infections and atopic dermatitis[22]. We previously described monocyte polarization defects in patients with severe chronic infections of the upper airways[11]. In these latter patients also an impaired delayed type hypersensitivity reaction and a decreased MIF production were found in addition to the defective responses of the monocytes. Interestingly, a similar combination of immune defects (lowered chemotaxis and impaired delayed type hypersensitivity reactions) is a well recognized feature of patients with the hyperimmunoglobulin E syndrome[23]. It must be noted that one of our patients in fact met the criteria for this syndrome.

In the sera of 18 patients investigated, low molecular weight inhibitory factors were detected, which were able to suppress the polarization of healthy donor monocytes. These monocyte inhibitory factors could be partially neutralized with monoclonal antibodies against the retroviral immunosuppressive factor p15E. It is a short step to the idea that these factors directly contribute to the impairment of the cellular motility of the patients' monocytes. However, monocyte polarization defects did not correlate with the presence of low molecular weight immunosuppressive factors in serum. Hence, complex relationships must exist, and the impaired monocyte polarization of our patients cannot be entirely attributed to the presence of these factors.

Importantly, p15E-like factors have also been described in the sera of patients with chronic infections of the upper airways[11], and in serum and malignant effusions of cancer patients[24]. Besides an antigenic relation with retroviral p15E, LMWFs of cancer patients could also be absorbed by antibodies against IFNα[25]. A sequential and functional homology has been reported between a

bioactive fragment of IFNα and an immunosuppressive region of p15E, which both act as suppressors of monocyte polarization[26]. The molecular structure of the LMWFs in the here described patients with chronic recurrent infections of the lower airways thus remains to be elucidated. Tumor cells have been shown to contain antigens related to p15E and IFNα[24,28,29], and these cells are thought to be the source of the p15E/IFNα-like serum factors in cancer patients. In patients with chronic airway infections these factors might be produced by airway epithelial cells, since immunoreactivity against p15E has been described in the nasal epithelium of patients with chronic purulent rhinosinusitis and in the bronchial epithelium of patients with chronic purulent bronchitis[29].

Besides their effect on monocyte polarization, the retroviral p15E protein and synthetic peptides derived from p15E are also able to suppress other immune functions[30]. Several studies indicate that these peptides interfere with the transduction of intracellular signals (possibly by inhibiting protein kinase C)[31,32], but an inhibitory effect at the level of the FMLP receptor has also been suggested[33]. Recently, it has been found that signal transduction pathways in neutrophils for chemotaxis and superoxide production[34] are different. This might be relevant for our finding of a normal rate of superoxide production and an impaired chemotactic activity of the patients' monocytes. An inhibition at the level of the FMLP receptor can, however, not be excluded, since in the study of Yasui and colleagues[34] and in our study the FMLP concentrations used were 5 and 100 times respectively higher in the superoxide assay compared to the concentrations used in the chemotaxis and polarization assays.

The here presented data together with those reported previously[5] point to several disturbances in phagocyte locomotory function, which may contribute to the enhanced susceptibility for bacterial invasion and lead to chronic relapsing infections of the lower airways. The restoration of these defects might abrogate the increased susceptibility for infections. In the here described patient group, a lowered monocyte polarization could be partially restored in vitro by the addition of thymic peptides, whereas, as we described previously, granulocyte function can be improved by G-CSF[5]. In patients with chronic infections of the upper airways, treatment with thymic peptides indeed improved both clinical symptoms and immunological parameters[15]. Possibly, similar treatment modalities are beneficial for patients with chronic infections of the lower airways as well.

ACKNOWLEDGEMENTS

This study was supported with the Netherlands Asthma Foundation grant no. 89-27. We thank Dr. J.P. van de Merwe (Dept. of Immunology, University

Hospital Dijkzigt), Dr. K.Y. Tan (St. Franciscus Gasthuis, Rotterdam) and Dr. P.R.M. Hekking (Havenziekenhuis and Ikazia Ziekenhuis Rotterdam) for their contribution in the selection of patients. We also thank Dr. Blijenberg (Dept. of Clinical Chemistry, University Hospital Dijkzigt, Rotterdam) for the immunoglobulin determinations and Tar van Os for the preparation of the figures.

REFERENCES

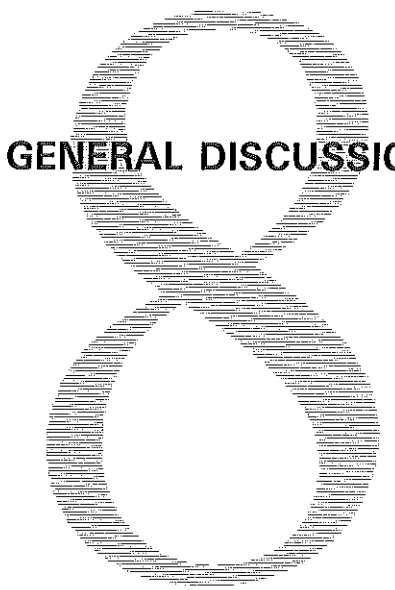
1. Gross, S., M.S. Blais and H.G. Herrod. 1992. Role of immunoglobulin subclasses and specific antibody determinations in the evaluation of recurrent infection in children. *J. Pediatr.* 121:516-522.
2. De Baets, F., J. Kint, R. Pauwels and J. Leroy. 1992. IgG subclass deficiency in children with recurrent bronchitis. *Eur. J. Pediatr.* 151:274-278.
3. Popa, V., K. Kim and D.C. Heiner. 1993. IgG deficiency in adults with recurrent respiratory infections. *Ann. Allergy* 70:418-424.
4. Hidalgo, H., M. Ehlayel, C. Moore and R.U. Sorensen. 1994. Assessment of anti-pneumococcal antibodies in children with recurrent infections. *Am. J. Respir. Crit. Care Med.* 149:A122.
5. van Haarst, J.M.W., H.C. Hoogsteden, H.J. de Wit, J.P. van der Merwe, H. Hooijkaas and H.A. Drexhage. A decreased polarization of granulocytes upon chemotactic stimuli in patients with chronic relapsing infections of the lower airways can be restored by G-CSF in vitro. Submitted.
6. Bagge, U., B.R. Johansson, J. Olofsson. 1977. Deformation of white blood cells in capillaries. *Adv. Microcirc.* 7:18-28.
7. Beesley, J.E., J.D. Pearson, A. Hutchings, J.S. Carleton, J.S. Gordon. 1979. Granulocyte migration through endothelium in culture. *J. Cell Sci.* 38:237-248.
8. Tizard, I.R., W.L. Holmes. 1974. Phagocytosis of sheep erythrocytes by macrophages: some observations under the scanning electron microscope. *J. Reticuloendothelial Soc.* 15:132-138.
9. Wilkinson, P.C. 1982. *Chemotaxis and Inflammation* Chuchill Livingstone, Edinburgh.
10. Zaffran, Y., H. Lepidi, A-M. Benoliel, C. Capo, P. Bongrand. 1993. Role of calcium in the shape control of human granulocytes. *Blood Cells* 19:115-131.
11. Plassche-Boers, E.M. van de, M. Tas, M. de Haan-Meulman, M. Kleingeld and H.A. Drexhage. 1988. Abnormal monocyte chemotaxis in patients with chronic purulent rhinosinusitis: an effect of retroviral p15E-related factors in serum. *Clin. Exp. Immunol.* 73:348-354.
12. Cianciolo, G.J. and R. Snyderman. 1981. Monocyte responsiveness to chemotactic stimuli is a property of a subpopulation of cells that can respond to multiple chemotactic stimuli. *J. Clin. Invest.* 67:60-68.
13. Tan, I.B., H.A. Drexhage, R.J. Scheper, B.M.E. von Blomberg-van der Flier, M. de Haan-Meulman,, G.B. Snow and A.J.M. Balm. 1986. Defective monocyte chemotaxis in patients with head and neck cancer. Restoration after treatment. Arch. Otolaryngol. *Head Neck Surg.* 112:942-945.
14. Goldstein, G., M. P. Scheid, E.A. Boyse, D.H. Schlesinger and J. van Wauwe. 1979. A synthetic pentapeptide with the biological activity characteristic of the thymic hormone thymopoietin. *Science* 204:1309-1310.
15. Tas, M., Leezenberg, J.A., Drexhage, H.A. 1990. Beneficial effects of the thymic hormone preparation thymostimulin in patients with defects in cell-mediated immunity and chronich

purulent rhinosinusitis. A double blind cross-over trial on improvements in monocyte polarization and clinical effects. *Clin. Exp. Immunol.* 80:304-313.

16. Falk, W., R.H. Goodwin and E.J. Leonard. 1980. A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods* 33:239-247.
17. Pick, E. and D. Mizel. 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J. Immunol. Methods* 46:211-226.
18. Rinehart, J.J., A.L. Sagone, S.P. Balcerzak, G.A. Ackerman and A.L. LoBuglio. 1975. Effects of corticosteroid therapy on human monocyte function. *N. Eng. J. Med.* 292:236-241.
19. Smith, P.D., K. Ohura, H. Masur, H. C. Lane, A.S. Fauci and S.M. Wahl. 1984. Monocyte function in the acquired immune deficiency syndrome. *J. Clin. Invest.* 74:2121-2128.
20. Gelfand, J.A. 1984. Infections in burn patients: a paradigm for cutaneous infection in the patient at risk. *Am. J. Med.* 76(5A):158-165.
21. Gallin, J.I. 1980. Disorders of phagocyte chemotaxis. *Ann. Int. Med.* 92:520-538.
22. Ternowitz, T. and T. Herlin. 1986. Defective monocyte and polymorphonuclear leukocyte chemotaxis and clinical characteristics in atopic dermatitis. *Arch. Dermatol. Res.* 278:454-459.
23. Geha, R.S. and D.Y.M. Leung. 1989. Hyperimmunoglobulin E syndrome. *Immunodeficiency Rev.* 1:155-172.
24. Cianciolo, G., J. Hunter, J. Silva, J.S. Haskill and R. Snyderman. 1981. Inhibitors of monocyte responses to chemotaxins are present in human cancerous effusions and react with monoclonal antibodies to the p15(E) structural protein of retroviruses. *J. Clin. Invest.* 68:831-844.
25. Simons, P.J., R.A.J. Oostendorp, M.P.R. Tas and H.A. Drexhage. 1994. Comparison of retroviral p15E-related factors and interferon α in head and neck cancer. *Cancer Immunol. Immunother.* 38:178-184.
26. Ruegg, C.L. and M. Strand. 1990. Identification of a decapeptide region of human interferon-alpha with antiproliferative activity and homology to an immunosuppressive sequence of the retroviral transmembrane protein p15E. *J. Interferon Res.* 10:621-626.
27. Tan, I.B., H.A. Drexhage, R.J. Scheper, B.M.E. von Blomberg-van der Flier, M. de Haan-Meulman,G.B. Snow and A.J.M. Balm. 1986. Immunohistochemical detection of retroviral p15E-related material in carcinomas of the head and neck. *Arch. Otolaryngol. Head Neck Surg.* 112:942-945.
28. Cianciolo, G.J., D. Phipps and R. Snyderman. 1984. Human malignant and mitogen-transformed cells contain retroviral p15E-related antigen. *J. Exp. Med.* 159:964-969.
29. Scheeren, R.A., R.A.J. Oostendorp, S. van der Baan, R.J. Scheper and C.J.L.M. Meijer. 1992. Distribution of retroviral p15E-related proteins in neoplastic and non-neoplastic human tissues, and their role in the regulation of the immune response. *Clin. Exp. Immunol.* 89:94-99.
30. Oostendorp, R.A.J., C.J.L.M. Meijer, and R.J. Scheper. 1993. Immunosuppression by retroviral-envelope-related proteins, and their role in non-retroviral human disease. *Crit. Rev. Oncol. Hematol.* 14:189-206.
31. Kadota, J-I, G.J. Cianciolo, R. Snyderman. 1991. A synthetic peptide homologous to retroviral transmembrane envelope proteins depresses protein kinase C mediated lymphocyte proliferation and directly inactivates protein kinase C: a possible mechanism for immunosuppression. *Microbiol. Immunol.* 35:443-459.
32. Gottlieb, R.A., E.S. Kleinerman, C.A. O'Brian, S. Tsujimoto, G.J. Cianciolo and W.J. Lennarz. 1990. Inhibition of protein kinase C by a peptide conjugate homologous to a domain of the retroviral protein p15E. *J. Immunol.* 145:2566-2570.
33. Oostendorp, R.A.J., W.M.M. Schaaper, J. Post, R.H. Meloen and R.J. Scheper. 1992.

Synthetic hexapeptides derived from the transmembrane envelope proteins of retroviruses suppress N-formylpeptide-induced monocyte polarization. *J. Leukoc. Biol.* 51:282-288.

34. Yasui, K., M. Yamazaki, M. Miyabayashi, T. Tsuno and A. Komiyama. 1994. Signal transduction pathways in human polymorphonuclear leukocytes for chemotaxis induced by a chemotactic factor. *J. Immunol.* 152:5922-5929.



GENERAL DISCUSSION

OBJECTIVES

This thesis describes populations of cells from human bronchoalveolar lavage (BAL) and lung tissue with a morphology and marker pattern similar to that of dendritic cells (DC) described by others. Two major issues will be discussed: 1. What is the nature of these cells, how do they interrelate and how do they relate to the lineages of DC and mononuclear phagocytes? 2. What is the role of these pulmonary accessory cells in airway disease?

This thesis further reports on an impaired membrane motility (polarization and chemotaxis) of monocytes and granulocytes in response to chemotactic stimuli in patients with chronic recurrent bacterial infections of the lower airways resistant to antibiotic therapy. Two important questions are: 1. How do these disturbances in leucocyte membrane motility relate to the enhanced susceptibility for infections? 2. What is the cause of these impaired responses? With regard to clinical practice, the most interesting observation is that these defects could be partially restored in vitro (and in preliminary experiments also in vivo) by thymic peptides (monocytes) or by G-CSF (granulocytes).

THE NATURE OF THE "PULMONARY ACCESSORY CELLS"

In this thesis, an isolation method for highly purified DC from human BAL using a FACS is described. In previous experiments we did not succeed to obtain such a highly purified population of DC from either minced and digested human lung tissue or BAL by conventional techniques (density gradients, adherence, panning and / or magnetic beads). We and others (Department of Cell Biology, Vrije Universiteit, Amsterdam) were unable to deplete AM efficiently from human BAL using these conventional techniques, and these procedures yielded a population containing 10-15% APh^-, MHC class II^+ cells[1]. Using FACS-sorting of BAL cells on autofluorescence, sideward scatter and forward scatter, we were able to obtain a population containing 85-90% of APh^-, MHC class II^+ low autofluorescent (LAF) cells (chapter 3). We demonstrated that these LAF cells exhibit a potent T cell-stimulatory function and a marker pattern compatible with that of DC. Previously, the purification of DC on the basis of autofluorescence using a FACS was described by Nicod[2] for lung tissue and later by Havenith for the rat BAL[3]. The method is probably based on the presence of phagocytosed material and secondary lysosomes in AM that increases both autofluorescence and sideward scatter of the cells[4]. Therefore, one may hypothesize that LAF cells have either a low phagocytic capability, such as DC, or that these cells have not yet phagocytosed.

The population of LAF cells isolated from BAL showed the same marker pattern as the dendritic-shaped cells in lung tissue, which were predominantly observed in the bronchial wall (chapters 2 and 3). These data suggest that the LAF cells are predominantly derived from the bronchial wall. Vice versa, it is likely that the dendritic-shaped cells in the bronchial wall have the same T cell stimulatory capabilities as the LAF cells. Since $CD1a^+$ DC in lung tissue were almost exclusively observed in and just beneath the bronchial epithelium and not in the alveolar compartment, the $CD1a^+$ LAF cells must certainly be derived from the bronchial wall. Because it is unlikely that cells in the deeper layers of the bronchial wall appear in the BAL, the LAF cells thus probably represent the population of bronchial intraepithelial dendritic-shaped cells with a (small) admixture of similar cells from the alveolar compartment.

Dendritic-shaped cells in lung tissue and LAF cells in BAL display a heterogeneity in the expression of various markers, most notably of CD1a and My4 (CD14). This heterogeneity in CD1a and CD14 expression was not simply associated with differences in morphology, enzyme histochemistry or the expression of other immunologic markers: double labeling experiments showed that both $CD1a^-$ and $CD1a^+$ LAF cells expressed HLA-DR, CD68, RFD1 and L25, and that a part of both subpopulations stained for CD14 as well (data not shown). The $CD1a^+$ cells were, however, the most potent accessory cells in the LAF cell population. $CD1a^-$ LAF cells, on the contrary, were potent producers of IL-1 and IL-6, whereas the $CD1a^+$ cells did not or hardly produce IL-1 and IL-6 upon stimulation with LPS.

From these data it was concluded that the $CD1a^+$ cells (both in BAL and in the bronchial epithelium) should be regarded as classic DC, whereas the $CD1a^-$ LAF cells exhibited features of both DC and monocytes. On the nature of the $CD1a^-$ cells several hypotheses should be considered. Firstly, these cells might be precursors of $CD1a^+$ DC. The $CD1a^-$ cells would then gradually upregulate their accessory capability and acquire CD1a expression upon migration into the bronchial epithelium. In the bronchial epithelium, these cells would then be able to pick up inhaled antigens, and subsequently migrate to the lymph node to present antigens to T cells. The fact that most LAF cells show a rounded morphology upon isolation may be either a feature of immaturity or a result of the isolation procedure. Epidermal LC also exhibit a rounded morphology after isolation (R. Troost, personal communication). The $CD1a^-$ LAF cells might, however, also represent precursors of classic macrophages or interstitial macrophages.

Considering this last option, the smaller cells in the BAL (LAF cells are also smaller than AM) have been reported to resemble interstitial macrophages,

and have been hypothesized to have arrived only recently in the lung[5]. Pulmonary interstitial macrophages have been described to display reduced phagocytic functions, but a good antigen-presenting capability[6]. Like LAF cells isolated from BAL and the dendritic-shaped cells in the bronchial wall, interstitial macrophages have also been reported to be either negative or weakly positive for nonspecific esterase and to express high levels of MHC class II molecules[7]. Therefore, the cells described by us would fit well into this description. However, since the CD1a$^-$ cells (from BAL and in the bronchial wall) exhibit the same immunophenotype (except for CD1a) as CD1a$^+$ DC, we feel that the term macrophage is not appropriate for these cells, but that they should be regarded as related to DC. In support of this notion, other investigators tend to assume that strongly MHC class II positive, irregular-shaped cells in the bronchial wall are DC[8,9].

Beside the possibility that CD1a$^-$ LAF cells are precursors of mature macrophages, it can be questioned whether cells that belong to the DC family, such as CD1a$^+$ LAF cells, are able to differentiate into macrophages. This question is intriguing, because the relationship of DC with cells of the monocyte-macrophage lineage is still unclear. It has, however, been hypothesized before that blood-derived DC are able to become macrophages[10].

Here, preliminary evidence (based on two experiments) is presented on the acquisition of macrophage characteristics of FACS-sorted CD1a$^+$ and CD1a$^-$ LAF cells during culture. Both subsets of LAF cells were cultured in RPMI containing 10% FCS and 2 μg/ml LPS. LPS was used since it promotes the differentiation of monocyte-like cells into macrophages and induces phagocytic functions[11,12]. After FACS-sorting, CD1a$^+$ cells were absent in the negative fraction, whereas the positive fraction contained more than 95% CD1a$^+$ cells. Cells were cultured for 3 days in experiment 1, and for 6 days in experiment 2. Cytospin preparations made at the end of the culture periods were stained for CD1a and monocyte-macrophage markers.

As is shown in table 1, CD1a$^-$ cells expressed all macrophage markers tested after culture, and remained negative for CD1a. Of the CD1a$^+$ LAF cells, a part lost their CD1a expression, while all cells gained RFD9, and a part upregulated CD14 expression. In contrast to their CD1a$^-$ counterparts, RFD7 expression was not induced on the CD1a$^+$ LAF cells. After 6 days culture (experiment 2), most cells of the CD1a$^+$ fraction also showed a diffuse and intense APh activity, particularly in those that had become negative for CD1a. In cells that had remained positive for CD1a, various patterns of APh activity could be observed. Some cells exhibiting a massive APh activity still showed a small rim of CD1a reactivity.

	CD1a$^+$			CD1a$^-$		
	fresh	3 days	6 days	fresh	3 days	6 days
CD1a	+ +	80% + +	20% +	—	—	—
CD14	—/±	10% + +	70% + +	—/±	+ +	+ +
CD68	+	+ +	+ +	+	+ +	+ +
RFD7	—	—	—	—	+ +	+ +
RFD9	—	+	+ +	—	+ +	+ +
APh	—/±	—/±	90% + +	—	ND	+ +

Table 1. The immunophenotype of freshly isolated and cultured CD1a$^+$ and CD1a$^-$ LAF cells. Data on fresh LAF cells are derived from chapter 3 and from the double stainings mentioned above. Data on cultured LAF cells represent experiment 1 (3 days culture) and experiment 2 (6 days culture). Data show the staining intensity: -, negative; ±, weakly positive; +, positive; + +, strongly positive. Staining intensity applies to all cells unless indicated else by a percentage positive cells. For details see text.

These observations, although preliminary, support the concept that CD1a$^+$, APh$^{-/spot}$ LAF cells (that meet all criteria for DC) are able to gain APh activity and RFD9 expression, and to downregulate CD1a expression. However, CD1a$^-$ LAF cells were much more apt to express macrophage markers compared to CD1a$^+$ LAF cells, and readily expressed all macrophage markers tested.

Hence, it may be suggested that these CD1a$^-$ LAF cells are the precursors of pulmonary macrophages, and thus represent a pool of immature cells. An important question remains whether these cells are also capable to differentiate into CD1a$^+$ cells with the same accessory capability as the freshly isolated CD1a$^+$ cells. The fact that CD1a$^+$ cells are not present in normal blood and the concept that DC are bone-marrow derived cells imply that these cells must have CD1a$^-$ precursors. As reviewed in the introduction, PBM are able to obtain CD1a expression during culture with low amounts of serum or after incubation with GM-CSF[13,14]. Studies in human skin strongly suggested that CD1a$^-$/CD68$^+$ cells in the dermis (named DC or macrophage) are precursors of CD1a$^+$ LC[15,16]. Since CD1a$^+$ DC have only been observed in the epithelium of skin and airways, local epithelial factors probably play a role in the differentiation of these cells from CD1a$^-$ precursors. The close resemblance in both morphology and marker pattern together with the close physical association of CD1a$^+$ and CD1a$^-$ dendritic-shaped cells in the bronchial wall in histologic sections (chapter 2) supports the hypothesis that the CD1a$^+$ DC are derived from CD1a$^-$ precursors with a similar marker

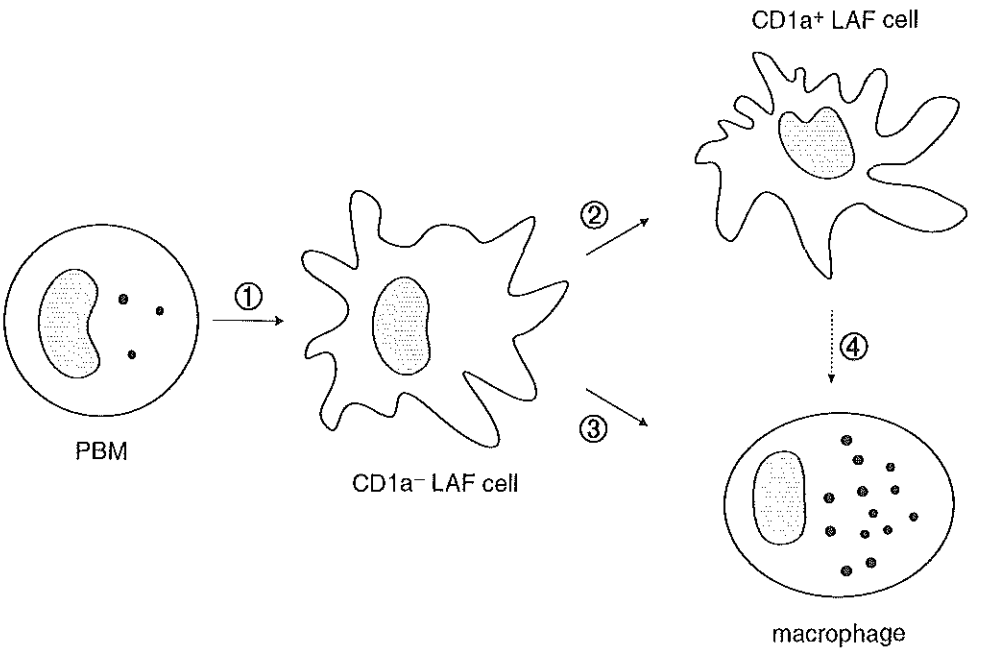


Figure 1. Proposed differentiation pathway for DC and macrophages in the lung. Upon migration into the lung, peripheral blood monocytes (PBM) differentiate into $CD1a^-$ LAF cells. During this transition these cells downregulate their CD14 expression, upregulate HLA-DR expression and gain other markers such as RFD1 and L25 (1). In the bronchial mucosa, under influence of the local conditions (e.g. factors released by bronchial epithelial cells), the $CD1a^-$ LAF cells preferentially differentiate into $CD1a^+$ DC (2). In the alveolar compartment, however, most LAF cells differentiate into macrophages (MΦ) under the influence of alveolar epithelial factors such as surfactant (3). Although $CD1a^+$ DC are thought to migrate into the regional lymph nodes under normal conditions, these cells still have the ability to acquire macrophage characteristics (4).

pattern.

On the basis of the discussion above, we want to propose a concept on the nature of the airway accessory cells described in this thesis and the relationship of dendritic cells with the monocyte-macrophage lineage in general (figure 1). The $CD1a^+$ cells in the bronchial wall and in the BAL are classic DC. The function of this DC is to pick up inhaled antigens and to present their antigenic fragments to T cells. Antigen presentation may take place in the bronchial wall to local T cells or after migration and further maturation of the DC in the draining lymph node. Since DC are bone marrow-derived cells, and since $CD1a^+$ DC do not appear in the blood under normal conditions, these cells probably arise from $CD1a^-$ precursors. A population of mainly

dendritic-shaped cells in the bronchial wall and a population LAF cells in the BAL can be identified with a similar marker pattern as the CD1a$^+$ DC, but negative for CD1a. We suggest that the CD1a$^-$ LAF cells in the BAL represent at least in part similar cells in the bronchial epithelium. These cells compare to monocytes in cytokine production and exhibit a lower accessory capability than the CD1a$^+$ DC. These cells have the potential to differentiate into mature macrophages. We hypothesize that, depending on the local conditions, these CD1a$^-$ cells differentiate into either CD1a$^+$ DC or into mature (alveolar) macrophages.

The ability of CD1a$^+$ LAF cells (true DC) to gain some macrophage characteristics stresses the close relationship between DC and cells of the monocyte-macrophage series, and supports the concept that DC are special members of this lineage.

DENDRITIC CELLS IN AIRWAY DISEASE

In the general introduction (chapter 1), the role of DC in airway disease was reviewed. Here, only additional data derived from our own experiments will be discussed.

Effects of cigarette smoking on DC numbers and function.
With respect to smoking habits, smokers' BAL yielded significantly higher numbers of LAF cells (chapter 3), and higher numbers of cells with the characteristics of DC (chapter 2) were observed in smokers' BAL. These data are in agreement with the higher numbers of cells displaying CD1a or Birbeck granules in the BAL of smokers described by Casolaro and colleagues[17], and the increased numbers of DC/LC in lung tissue of smokers reported by Soler and colleagues[18]. As was discussed previously, the numbers of DC detected in lung tissue specimens studied by us (chapter 2) are probably also influenced by cigarette smoking, since all tissue samples were derived from ex-smokers or current smokers. Much lower numbers of DC have now been observed in bronchial biopsies of a control group of healthy nonsmokers in a study on asthma performed at our department (see below). Hence, it can be concluded that the numbers of DC are elevated in the BAL, in the bronchial mucosa, and possibly also in the alveolar compartment of the lung in smokers. The increase in the numbers of DC contrast with our data showing a decreased T cell-stimulatory capability of BAL cells of smokers compared to nonsmokers, probably due to a lowered T-cell stimulatory function of LAF cells (chapter 3). Previously, a lowered stimulatory capability of smokers' BAL cells was also observed by Laughter[19]. Thus, in contrast with the rise in the numbers of DC in BAL, the accessory cell function of BAL cells is

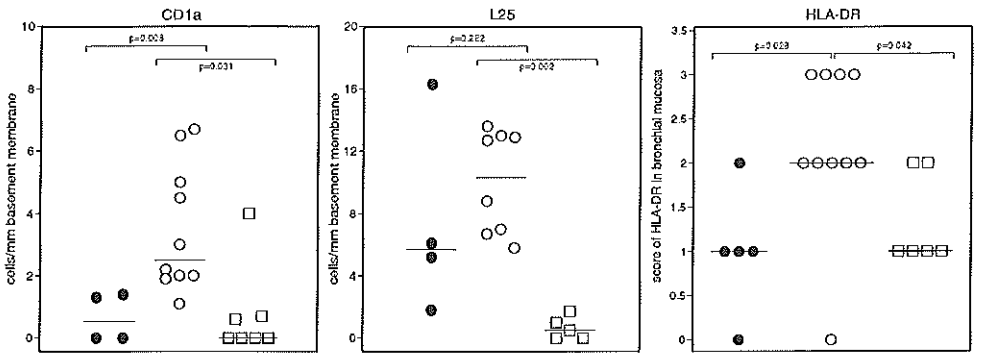


Figure 2. CD1a$^+$ cells and L25$^+$ cells in the bronchial mucosa expressed as the number of positive cells per millimeter basement membrane. HLA-DR expression in the bronchial mucosa scored semiquantitatively on a 0-3 scale (0 = negative, 1 = weak, 2 = strong, 3 = very strong HLA-DR expression). Individual data are depicted of allergic asthmatics using bronchodilators only (○), of allergic asthmatics using bronchodilators in combination with inhaled corticosteroids (●), and of healthy controls (□). Horizontal bars represent the median value. Data from G.M. Möller et al.

decreased in smokers.

DENDRITIC CELLS IN ASTHMA AND COPD

In the general introduction (chapter 1), data were presented from the literature considering the possible role of dendritic cells in allergy and asthma. Most data were derived from atopic dermatitis and allergic rhinitis. Only one study has shown increased numbers of RFD1$^+$ cells (possibly DC) in the bronchial wall in asthma, which were downregulated during inhaled glucocorticoid therapy[20]. To gain a better insight in the kinetics of DC in allergic asthma, a study has been performed at our department (collaboration with G.M. Möller and S.E. Overbeek) on the numbers of DC in the bronchial mucosa in asthma. Three groups were compared: healthy controls, allergic asthmatics treated with bronchodilators alone and allergic asthmatics treated with bronchodilators in combination with inhaled corticosteroids[21]. At the time of bronchial biopsy, the patients - 16 nonsmoking atopic asthmatics - had been treated double blind for 2.5 years. This group was a randomly selected subgroup of the Dutch CNSLD study[22]. Treatment in the bronchodilator group (n=11) consisted of terbutaline in combination with either ipratropium bromide or placebo, whereas patients from the corticosteroid group (n=5) were treated with terbutaline in combination with beclomethasone dipropionate. Corticosteroid treatment coincided with a decrease in asthma symptoms and an improvement in lung function and bronchial

hyperreactivity. Immunohistochemistry showed a significantly higher HLA-DR expression and significantly elevated numbers of $CD1a^+$ and $L25^+$ dendritic-shaped cells in the bronchial mucosa in asthmatics treated with bronchodilators alone compared to the healthy controls (figure 2). Moreover, compared to the bronchodilator group, HLA-DR expression and the numbers of $CD1a^+$ cells were significantly lower in the corticosteroid group (figure 2).
These data clearly demonstrate that the numbers of DC in the bronchial wall are elevated in patients with allergic asthma, and that inhaled corticosteroid therapy reverses DC numbers to levels that compare to healthy nonsmoking controls.

The increased numbers of DC in atopic asthma and the decrease during glucocorticoid therapy correspond with the kinetics of other inflammatory cells in the asthmatic infiltrate, such as eosinophils and activated T cells[23]. We like to hypothesize that the raised numbers of DC in asthma lead to an enhancement of antigen presentation and T cell activation. The latter is thought to play an important role in the pathogenesis of asthma[24]. However, elevated numbers of DC are not specific for asthma. As was discussed above and in the introduction, cigarette smoking also increases DC numbers in the lung, and in response to bacterial stimuli, a rapid influx of DC has been observed in the bronchial wall of the rat[25,26]. Moreover, as was learned from smokers, elevated numbers of DC are not necessarily associated with an increase in T cell stimulatory function. On the other hand, the downregulation of DC numbers during glucocorticoid therapy is probably associated with a decreased accessory function in vivo, since we showed that glucocorticoids are able to suppress T cell stimulatory function of airway DC in vitro (chapter 4). Hence, it is likely that the impairment of DC function by glucocorticoids contributes to the downregulation of the asthmatic inflammation during glucocorticoid therapy.

Except for the activation of T cells, antigen-presenting cells, such as DC, may in addition direct the differentiation of T cells towards a Th1 or Th2 phenotype, which may have direct consequences for the development of allergy. Indeed, data from the literature indicate that DC may skew T cell differentiation towards a Th2 phenotype under certain conditions (reviewed in chapter 1). Therefore, it would be of high interest to investigate the stimulatory capability and the capability to skew T cell differentiation towards Th1 or Th2 of BAL cells and DC from the BAL in atopic asthma.

From a recently started study on COPD patients (G.T. Verhoeven), BAL cells were sorted into LAF cells and AM of 7 included patients. These patients were all smokers with bronchial hyperreactivity and a FEV1 $<70\%$ predicted that improved less than 15% after bronchodilator inhalation. Compared to

healthy smokers, AM of COPD patients produced lower T cell responses at high stimulator-responder ratios ($p<0.01$ at a ratio of 1:1 and $p<0.05$ at 1:2). Total BAL cells and LAF cells of COPD patients induced somewhat

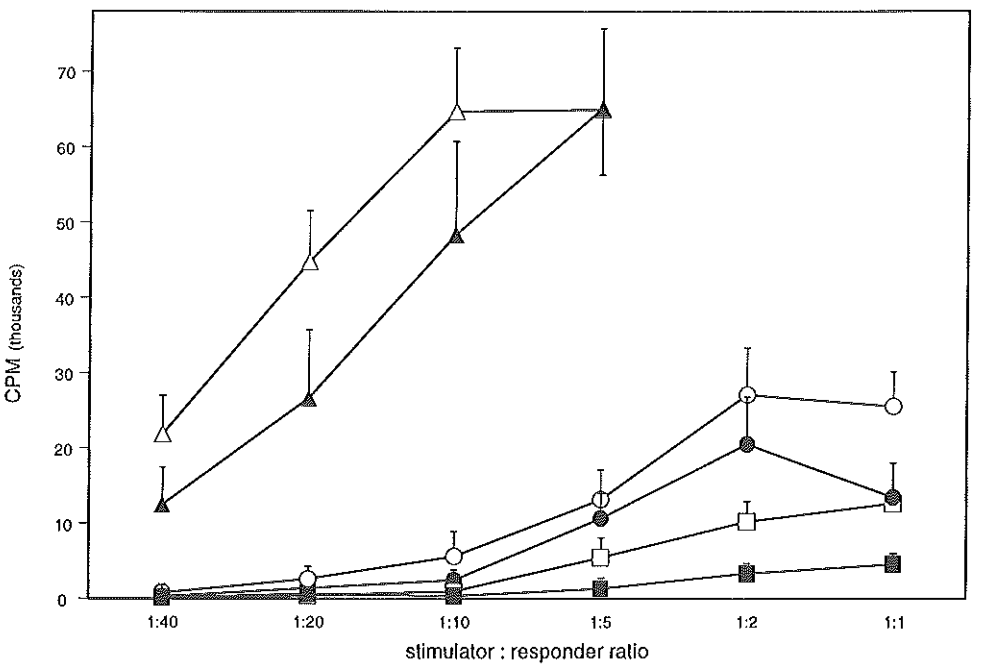


Figure 3. ^{3}H-thymidine incorporation as a measure of allogenic T cell proliferation induced by BAL cells (circles), AM (squares) and LAF cells (triangles) of patients with COPD (all smokers, filled symbols) and healthy smokers (open symbols) in different stimulator to responder ratios. Data represent the mean ± SD.

lower T cell responses. However, these differences did not reach the level of statistical significance (figure 3). In contrast to BALs of allergic asthmatics, the BAL of these COPD patients contained only low numbers of T cells, comparable to numbers in healthy smokers (G.T. Verhoeven, personal communication). Although these are only preliminary data, these observations suggest that enhanced DC function or T cell activation is not a feature of COPD. It is not yet clear how to explain the lowered stimulatory capability of AM from COPD patients.

SIGNIFICANCE OF A DEFECTIVE POLARIZATION OF LEUCOCYTES FOR CHRONIC RECURRENT AIRWAY INFECTIONS

Although it might be more appropriate to investigate the function of local

pulmonary leucocytes (such as AM and LAF cells) than of peripheral blood leucocytes in patients with chronic recurrent purulent infections of the lower airways, local leucocytes are more difficult to obtain since BAL is an invasive technique. Because a reduced polarization of blood monocytes in response to FMLP had been observed in our laboratory before in patients with chronic upper airway infection (chronic purulent rhinosinusitis), we decided to start investigations on the chemotactic responsiveness of monocytes and granulocytes from the peripheral blood of patients with chronic recurrent infections of the lower airways.

We detected a decreased polarization of monocytes and granulocytes in response to FMLP in patients with chronic recurrent infections of the lower airways (chapters 6 & 7). Granulocyte polarization in response to casein was also impaired. Polarization is the change of shape from round to elongated or triangular with marked cytoplasmic extensions. Polarization is mediated by a rapid polymerization and depolymerization of actin microfilaments, and could be completely blocked by cytochalasin B which inhibits microfilament function (unpublished data). Besides microfilaments, microtubules and intermediate filaments may also be involved in cell shape control, although their role in rapid cell deformation seems less prominent than that of actin filaments. Preliminary results suggest that in granulocytes of patients with chronic airway infections and a lowered response in the polarization assay, the depolymerization of actin microfilaments is impaired (dr.A.J. Verhoeven, CLB, Amsterdam, and dr. H. Hooijkaas, personal communication).

It is, however, yet unclear which molecular events cause a defective polarization (and actin depolymerization). The role of calcium in microfilament organization and leucocyte shape changes has been studied extensively[27]. Calcium may regulate many cytoskeleton-associated proteins present in nonmuscle cells, including myosin[28], actinogelin[29], filamin/actin-binding protein[30], fragmin[31], α-actinin[32], villin[33], or fodrin[34].

The exact role of calcium in cell shape changes is, however, not completely clear, since (a) cell extracts behave quite differently from intact cells due to the importance of diffusible actin regulatory constituents, and (b) in intact cells, calcium changes are inseparable from other metabolic processes likely to affect cytoskeleton organization, such as pH, cyclic nucleotides, or phosphatidyl-inositol phosphates[35].

Polarized cells show a similar change in shape as seen in the early phase of chemotaxis. Therefore polarization has also been suggested to be closely related to chemotaxis. Strong correlations in leucocyte performance in assays for polarization and chemotaxis have been reported[36]. In our hands a

positive correlation existed indeed between outcomes of the polarization assay and outcomes in the chemotaxis assay performed on the same leucocyte suspensions in patients with a variety of illnesses (see chapters 6 & 7). It was, however, also evident that clear exceptions existed and that polarization cannot be regarded as synonymous with chemotaxis: we did observe patients with a deeply impaired polarization that were normal in the chemotaxis assay and vice versa.
Besides chemotaxis, an intact microfilament function, possibly reflected by a normal polarization, is also required for phagocytosis and exocytosis. Although gross defects of these functions are rare[37], lowered responses in the polarization assay might be associated with a less effective phagocytosis, especially of poorly opsonized particles.

As was discussed previously in this thesis, DC are closely related to monocytes. The strong motility of the cytoplasmic membrane is thought to be essential for DC function (contacting T cells), and this motility is probably also mediated by microfilaments. In patients with head and neck cancer and in patients with Graves' disease a decreased polarization of monocytes was associated with a decreased cluster capability of blood-derived DC[38]. In future studies on local bronchial leucocytes of patients with chronic purulent infections of the lower airways, it might therefore be of interest to evaluate not only the chemotactic abilities of BAL cells, but also the potency of LAF cells to establish intercellular contacts with T cells.

Do polarization and chemotaxis defects contribute to an increased susceptibility for infections?
If the responsiveness in the polarization assay of monocytes and granulocytes indeed reflect their abilities to migrate to and act in an inflammatory site in vivo, then how to explain the production of purulent sputum with leucocytic debris when leucocyte polarization and migration are disturbed? Although polarization was only lowered (not absent) at the optimal concentration of FMLP, the decrease might reflect a failure of the cells to respond to low levels of chemotaxins and other stimuli present in vivo at the beginning of an infection. The decreased polarization in vitro might then just represent a slower and lazier leucocyte response resulting in an inappropriate delayed infiltration at a stage in which already large numbers of microorganisms are present in the bronchial tree. This will contribute to a raised susceptibility for infection. Moreover, probably more "lazy leucocytes" are required to eliminate relatively small numbers of microorganisms. The increased sputum purulence at later stages of the infection might then just reflect a steady state between aggressors (microorganisms) and defenders (leucocytes), but at a higher level of homoiostasis of leucocyte infiltration (more are needed). This will contribute to the purulence of the disorder.

It must be noted, however, that monocyte polarization and chemotaxis are also decreased in patients with bronchial and laryngeal carcinoma, as well as in patients with endocrine autoimmune diseases (Graves disease, type 1 diabetes)[38]. Since these patients usually do not present with chronic or recurrent infections, a lowered monocyte polarization and chemotaxis alone cannot explain the susceptibility for airway infections. Therefore, either local factors or concomitant immune defects must add to the impaired function of the monocytes and/or granulocytes to cause the enhanced susceptibility for infections.

The possibility should also be considered that a defective polarization and chemotaxis are not causes of and hardly add to the susceptibility for infections. In such a view, a decreased performance of granulocytes and monocytes is secondary to the chronic bacterial infection itself. Bacterial or inflammatory products might alter the sensitivity of the cells to chemotaxins and other stimuli. Alternatively, a redistribution of the granulocyte/monocyte pool via recruitment of chemoresponsive cells into the inflammatory focus might leave a less responsive and "lazy" population in the blood. The detection in the serum of patients of low molecular weight factors (with an antigenic homology to p15E) that hamper monocyte polarization, argues against the concept of redistribution. The detection of immunoreactivity for p15E in the epithelium of chronically infected airways[39], suggests a local production of immunosuppressive factors.
The partial restoration of polarization in vitro by either thymic peptides (monocytes) or G-CSF (granulocytes) together with the preliminary data on the beneficial clinical effects of these substances, however, argue against the concept that a defective polarization is merely a consequence of the infection itself and does not add to the susceptibility for infection.

The background of the lowered responses of the leucocytes to FMLP and casein in the polarization and chemotaxis assays remains hypothetical. Apart from the effect of locally produced immunosuppressive factors, the defective leucocyte function might have a genetic basis, which is, like common variable immunodeficiency, expressed later in life. It is also possible that the expression of a genetic predisposition for a defective leucocytic membrane motility is triggered by a (severe) local bacterial infection (see above). Alternatively, the impaired polarization might be caused by a viral infection: either by a direct infection of the leucocytes themselves or by an infection of other cells (e.g. epithelial cells) via the production of immunosuppressive factors. Many viruses have been shown to suppress immune functions, and an unknown or unrecognized virus might suppress the motility of monocytes and/or granulocytes and induce such susceptibilities for bacterial infections as observed in our patients. For example, a depressed monocyte chemotaxis,

together with other impairments in monocyte function and other immunological defects, has been observed in AIDS[40]. In influenza virus infection, neutrophil chemotaxis is impaired, which has been associated with an increased susceptibility for secondary bacterial infections[41].

Apart from genetic factors or infectious agents, dysregulations in the immune system itself (for instance in autoimmune disease) might also influence leucocyte function. Van de Merwe and Hooijkaas have associated the presence of autoimmune phenomena (in particular resembling Sjögren's syndrome) with chronic recurrent infections and a hampered leucocyte motility[42].

It is clear that further research is needed to establish the relationship between a poor performance of granulocytes and monocytes in the polarization assay and the consequences of these defects for the in vivo function of the cells and the susceptibility for infections. From a clinical point of view the most interesting observation is the beneficial response to thymic peptides and G-CSF. The possible role of these factors in the treatment of patients with chronic recurrent bacterial airway infections merits further attention.

References

1. Havenith, C.E.G., J.M.W. van Haarst, A.J. Breedijk, M.G.H. Betjes, H.C. Hoogsteden, R.H.J. Beelen and E.C.M. Hoefsmit. 1994. Enrichment and characterization of dendritic cells from human bronchoalveolar lavages. *Clin. Exp. Immunol.* 96:339-343.
2. Nicod, L.P., M.F. Lipscomb, G.B. Toews and J.C. Weissler. 1989. Separation of potent and poorly functional human lung accessory cells based on autofluorescence. *J. Leukoc. Biol.* 45:458-465.
3. Havenith, C.E.G., A.J.Breedijk, P.P.M.C. van Miert, N. Blijleven, W. Calame, R.H.J. Beelen and E.C.M. Hoefsmit. 1993. Separation of alveolar macrophages and dendritic cells via autofluorescence: phenotypical and functional characterization. *J. Leukoc. Biol.* 53:504-510.
4. Kradin, R.L., K.M. McCarthy, F.I. Preffer and E.E. Schneeberger. 1986. Flow-cytometric and ultrastructural analysis of alveolar macrophage maturation. *J. Leukoc. Biol.* 40:407-417.
5. Hohan, A., J.H. Dauber, M.S. Diamond and R.P. Daniele. 1983. Separation of bronchoalveolar cells from the guinea pig on continuous gradients of percoll: functional properties of lung macrophages. *J. Reticuloendoth. Soc.* 33:157-164.
6. Lohmann-Mathes, M-L., C. Steinmüller and G. Franke-Ullmann. 1994. Pulmonary macrophages. *Eur. Respir. J.* 7:1678-1689.
7. Lavnikova, N., S. Prokhorova, L. Helyar and D.L. Laskin. 1993. Isolation and partial characterization of subpopulations of alveolar macrophages, granulocytes and highly enriched interstitial macrophages from the rat lung. *Am. J. Resp. Cell Mol. Biol.* 8:384-392.
8. Sertl, K., T. Takemura, E. Tschachler, V.J. Ferrans, M.A. Kaliner and E.M. Shevach. 1986. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma and visceral pleura. *J. Exp. Med.* 163:436-451.

9. Holt, P.G., M.A. Schon-Hegrad, M.J. Philips and P.G. McMenamin. 1989. Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* 19:597-601.
10. Peters, J.H., J. Ruppert, R.K.H. Gieseler, H.M. Najar and H. Xu. 1990. Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage? *Pathobiology* 59:122-126.
11. Ziegler-Heitbrock, H.W.L., W. Schraut, P. Wendelgass, M. Ströbel, T. Sternsdorf, C. Weber, M. Aepfelbacher, M. Ehlers, C. Schütt and J.G. Haas. 1994. Distinct patterns of differentiation induced in the monocytic cell line Mono Mac 6. *J. Leukoc. Biol.* 55:73-80.
12. Ikewaki, N., H. Tamauchi and H. Inoko. 1993. Modulation of the cell surface antigens and regulation of phagocytic activity mediated by CD11b in the monocyte-like cell line U937 in response to lipopolysaccharide. *Tissue Antigens* 42:125-132.
13. Kasinrerk, W., T. Baumruker, O. Majdic, W. Knapp and H. Stockinger. 1993. CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 150:579-584.
14. Rossi, G. 1992. Development of a Langerhans cell phenotype from peripheral blood monocytes. *Immunol. Lett.* 31:189-198.
15. Murphy, G.F., D. Messadi, E. Fonferko and W.W. Hancock. 1986. Phenotypic transformation of macrophages to Langerhans cells in the skin. *Am. J. Pathol.* 123:401-406.
16. Petzelbauer, P., D. Födinger, K. Rappensberger, B. Volc-Platzer and K. Wolff. 1993. CD68 positive epidermal dendritic cells. *J. Invest. Dermatol.* 101:256-261.
17. Casolaro, M.A., J-F. Bernaudin, C. Saltini, V.J. Ferrans and R.G. Crystal. 1988. Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am. Rev. Respir. Dis.* 137:406
18. Soler, P., A. Morreau, F. Basset and A.J. Hance. 1989. Cigarette smoking induces changes in the number and differentiated state of pulmonary dendritic cells / Langerhans cells. *Am. Rev. Respir. Dis.* 139:1112-1117.
19. Laughter, A.H., R.R. Martin and J.J. Twomey. 1977. Lymphoproliferative responses to antigens mediated by human alveolar macrophages. *J. Lab. Clin. Med.* 89:1326-1332.
20. Burke, C., C.K. Power, A. Norris, A. Condez, B. Schmekel and L.W. Poulter. 1992. Lung function and immunopathological changes after inhaled corticosteroid therapy in asthma. *Eur. Respir. J.* 5:73-79.
21. Möller, G.M., S.E. Overbeek, C.G. van Helden-Meeuwsen, J.M.W. van Haarst, E.P. Prens, D.S. Postma and H.C. Hoogsteden. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled glucocorticoids. Submitted.
22. Kerstjens, H.A.M., P.L.P. Brand, M.D. Hughes, N.J. Robinson, D.S. Postma, H.J. Sluiter, E.R. Bleecker, P.N.R. Dekhuijzen, P.M. de Jong, H.J.J. Mengelers, S.E. Overbeek, D.F.M.E. Schoonebrood and the Dutch CNSLD Study Group. 1992. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airway disease. *N. Engl. J. Med.* 327:1413-1419.
23. Wilson, J.W., R. Djukanovic, P.H. Howarth and S.T. Holgate. 1994. Inhaled beclomethasone diproprionate downregulates airway lymphocyte activation. *Am. J. Respir. Crit. Care Med.* 149:86-90.
24. Rochester, C.L. and J.A. Rankin. 1991. Is asthma T cell-mediated? *Am. Rev. Respir. Dis.* 144:1005-1007.
25. McWilliam, A.S., D. Nelson, J.A. Thomas and P.G. Holt. 1994. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* 179:1331-1336.
26. Holt, P.G., S. Haining, D.J. Nelson and J.D. Sedgwick. 1994. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* 153:256-261.

27. Tsien, R.Y. and M. Poenie. 1986. Fluorescence ratio imaging: a new window into intracellular ionic signalling. *Trends Biochem. Sci.* 11:450-455.
28. Christensen, K., R. Santerer and R.W. Merriam. 1984. Role of soluble myosin in cortical contractions of Xenopus eggs. *Nature* 310:150-151.
29. Mimura, N. and A. Asano. 1982. Characterization and localization of actinogelin, a Ca-sensitive actin accessory protein, in non-muscle cells. *J. Cell Biol.* 93:899-909.
30. Stossel, T.P. 1982. The structure of cortical cytoplasm. *Phil. Trans. Roy. Soc. London* B299:275-279.
31. Furuhashi, K. and S. Hatano. 1990. Control of actin filament length by phosphorylation of fragmin-actin complexes. *J. Cell Biol.* 111:1081-1087.
32. Meyer, R.K. and U. Aebi. 1990. Bundling of actin filaments length by phophorylation of fragmin-actin complexes. *J. Cell Biol.* 110:2013-2024.
33. Matsuidara, P.T. and D.R. Burgess. 1982. Parial reconstruction of the microvillus core bundle: characterization of villin as a Ca^{++} dependent actin bundling/depolymerizing protein. *J. Cell Biol.* 92:648-656.
34. Harris, A.S. and J. Morrow. 1990. Calmodulin and calcium-dependent protease I coordinately regulate the interaction of fodrin with actin. *Proc. Natl. Acad. Sci.* USA 87:3009-3013.
35. Zaffran, Y., Lepidi, H., Benofiel, A., Capo, C. and Bongrand, P. 1993. Role of calcium in the shape control of human granulocytes. *Blood Cells* 19:115-131.
36. Cianciolo, G.J. and R. Snyderman. 1981. Monocyte responsiveness to chemotactic stimuli is a property of a subpopulation of cells that can respond to multiple chemotactic stimuli. *J. Clin. Invest.* 67:60-68.
37. Rotrosen, D. and J.I. Gallin. 1987. Disorders of phagocyte function. *Ann. Rev. Immunol.* 5:127-150.
38. Tas, M. 1992. Immunosuppression by retrovirus-related factors. Restoration of p15E-like effects by thymic hormones. Ac. Thesis, Erasmus University, Rotterdam.
39. Scheeren, R.A., R.A.J. Oostendorp, S. van der Baan, R.J. Scheper and C.J.L.M. Meijer. 1992. Distribution of retroviral p15E-related proteins in neoplastic and non-neoplastic human tissues, and their role in the regulation of the immune response. *Clin. Exp. Immunol.* 89:94-99.
40. Smith, P.D., K. Ohura, H. Masur, H. C. Lane, A.S. Fauci and S.M. Wahl. 1984. Monocyte function in the acquired immune deficiency syndrome. *J. Clin. Invest.* 74:2121-2128.
41. Abramson, J.S. and E.L. Mills. 1988. Depression of neutrophil function induced by viruses and its role in secondary microbial infections. *Rev. Infect. Dis.* 10:326-341.
42. Merwe, J.P. van de and H. Hooijkaas. Sjögren's syndrome in patients with chronic bacterial respiratory infections and a diminished chemotactic responsiveness of monocytes and/or granulocytes: treatment with G-CSF. *In* Sjögren's Syndrome - State of the Art, M. Homma, S. Sugai, T. Tojo, N. Miyasaka and M. Akizuki, eds., Kugler Publications. Amsterdam, the Netherlands. pp. 373-376.

SUMMARY

In the introduction (chapter 1) the basics of host defense are explained with emphasis on granulocytes, monocytes, macrophages and dendritic cells (DC). Deficiencies in host defense may lead to an increased susceptibility for infections. Inappropriate immune responses, on the other hand, may also result in disease, for example allergic asthma. For the initiation of immune responses, the presentation of antigens to T cells is an essential step. DC are the most potent antigen-presenting cells (APC), and in contrast to other APC, DC are able to stimulate naive, unprimed T cells. Therefore, DC are thought to play a crucial role in host defense in, a.o., the lung in health and disease. In this thesis we investigated 1.) the distribution, immunophenotype and function of dendritic cells in the normal human lung, and 2.) the function of granulocytes and monocytes in patients with chronic recurrent infections of the lower airways.

Using monoclonal antibodies against dendritic cells, monocytes and macrophages, and enzyme histochemistry, we investigated the distribution and immunophenotype of these leucocytes in human lung tissue (chapter 2). Two major populations could be discriminated: acid phosphatase (APh) negative dendritic-shaped cells and large rounded APh^+ cells.

Dendritic-shaped cells were predominantly observed in the bronchial wall especially in and just underneath the bronchial epithelium. These cells stained for HLA-DR, RFD1, L25 and CD68, but were negative for APh, RFD7 and RFD9. A portion of the dendritic-shaped cells reacted with CD1a (mainly in the epithelium) and/or My4 (CD14). The population APh-positive large rounded cells reacted with HLA-DR, CD68, RFD7 and RFD9. These classic macrophages were predominantly situated in the alveoli and the airway lumen.

We conclude that the dendritic-shaped cells are probably homologous to the potent immunostimulatory DC in the lymphoid organs and to epidermal Langerhans cells. Their immunophenotype, however, also indicates a relationship with monocytes and macrophages.

In chapter 3 we showed via FACS-sorting on the basis of autofluorescence that two completely different cell populations can be obtained from the human bronchoalveolar lavage (BAL). The high autofluorescent cells were 100% typical alveolar macrophages (AM), with the same marker pattern as the AM described in chapter 2. AM appeared poor T cell stimulators, and even suppressed PHA-induced T cell proliferation. The low autofluorescent (LAF) cells displayed a monocyte-like appearance (80% rounded), but 50% developed cytoplasmic protrusions during overnight culture (dendritic morphology). The immunophenotypic properties of the LAF cells were similar to the dendritic-shaped cells described in chapter 2. In contrast to AM, the LAF cells were

potent stimulators of naive T cells, and also formed clusters with T cells (hallmarks of DC).

Our data further showed that smokers' BAL contained more LAF cells than nonsmokers' BAL, but that BAL cells from smokers induced lower T cell responses compared to nonsmokers.

In conclusion, two immunophenotypically and functionally completely different cell populations could be obtained from human BAL: 1.) high autofluorescent AM with immunosuppressive properties, and 2.) LAF cells, which were potent accessory cells, that contained DC and their precursors.

Chapter 4 considers the in vitro effects of glucocorticoids on the function and immunophenotype of LAF cell populations. After incubation with therapeutic concentrations of dexamethasone, LAF cells showed an impaired accessory capability in the allogenic mixed lymphocyte reaction (MLR). This lowered T cell-stimulating capability was associated with a decrease in the expression of B7-1, and - not statistically significant - a decrease in CD11b expression. Glucocorticoids did not affect the expression of several other adhesion molecules, HLA-DR, or other markers on DC. Since B7-1 is an important costimulatory molecule, we suggest that its lowered expression may in part account for the impaired accessory function of DC. During glucocorticoid therapy such a decrease in DC function may contribute to the suppression of T cell activation and downregulation of inflammatory responses.

In chapter 5 we describe the potential of LAF cells to produce IL-1, IL-6 and TNF-α. Both cellular cytokine proteins and biologically active cytokines in the supernatants could be detected in LPS-stimulated LAF cell populations. In contrast to peripheral blood monocytes, LAF cells did not require these cytokines for the induction of T cell proliferation, since neutralizing antibodies against these cytokines did not inhibit LAF cell-induced MLR.

Since we previously observed a heterogeneity in marker expression in the LAF cells, most notably for CD1a and CD14, we also looked for differences in cytokine production in LPS-stimulated $CD1a^{+/-}$ and $CD14^{+/-}$ LAF cells subsets. Double immunolabeling revealed that in contrast to $CD1a^{-}$ LAF cells, $CD1a^{+}$ LAF cells did not (or hardly) react with anti-IL-1, anti-IL-6 or anti-TNF-α. Such differences were not observed in the $CD14^{+/-}$ subsets. These differences between the $CD1a^{+/-}$ subsets were confirmed by assaying cytokine production in the supernatants of FACS-sorted $CD1a^{+}$ and $CD1a^{-}$ LAF cells. Interestingly, the $CD1a^{+}$ LAF cells were more potent T cell stimulators than their $CD1a^{-}$ counterparts.

We conclude that two different subsets of LAF cells occur in the BAL. $CD1a^+$ LAF cells should be regarded as true DC. $CD1a^-$ LAF cells, displaying immunophenotypic and morphologic features of both DC and monocytes, showed a lower T cell-stimulatory capacity, but a high potency to produce cytokines. We hypothesize that the $CD1a^-$ LAF cells represent the precursors of either $CD1a^+$ DC or of mature macrophages.

The investigations on granulocyte and monocyte function in patients with chronic recurrent bacterial infections of the lower airways are described in chapters 6 and 7, respectively. Granulocytes and monocytes from the blood of these patients showed a decreased polarization (change in shape) in response to FMLP compared to the same cells of healthy controls. The performance of patients' monocytes and granulocytes in the polarization assay, however, did not correlate. The superoxide production of granulocytes and monocytes of the patients was normal. The granulocyte polarization was below the range of the healthy controls in 10 of the 18 patients evaluated (chapter 6). Addition of granulocyte colony-stimulating factor (G-CSF) in vitro improved the performance of the patients' granulocytes in the polarization assay.

In monocytes (chapter 7), the polarization was below the range of the healthy controls in 14 of 36 patients. In patients with a lowered monocyte polarization, this polarization could be partially restored by the addition of thymic peptides in vitro (thymopentin [TP5] and thymostimulin [TP-1]). In addition to polarization, monocyte chemotaxis was also lowered in patients with chronic recurrent airway infections. In the serum of some patients low molecular weight factors could be detected that were able to suppress the polarization of healthy donor monocytes. These factors showed an antigenic relationship with the immunosuppressive retroviral protein p15E.

We hypothesize that the hyporesponsiveness of granulocytes and monocytes to chemotactic stimuli contributes to the susceptibility for the airway infections in these patients. Besides the in vitro effects of G-CSF (on granulocytes) and thymic peptides (on monocytes), the in vivo administration of these substances to patients with chronic recurrent airway infections and lowered responses in the polarization assay, also improved the clinical symptoms.

The general discussion (chapter 8) focusses on 1.) the nature and the relationships of the LAF cells from BAL and the dendritic-shaped cells in the bronchial wall, 2.) the significance of these cells in pulmonary disease, and 3.) the meaning of a decreased polarization of granulocytes and monocytes for host resistance.

With regard to the nature of the LAF cells, we conclude that the $CD1a^+$ cells

from BAL and from the bronchial epithelium (probably the same cells) are classic DC, homologous to epidermal Langerhans cells. The $CD1a^-$ LAF cells and the other MHC class II-positive dendritic-shaped cells in the bronchial wall show features of both DC and monocytes. In vitro, the $CD1a^-$ LAF cells readily differentiated into macrophages. In our opinion, these cells are also the precursors of $CD1a^+$ airway DC. Because of the close resemblance of $CD1a^+$ and $CD1a^-$ LAF cells, and because $CD1a^+$ LAF cells were also able to acquire macrophage characteristics, we conclude that DC, monocytes and macrophages belong to the same hematopoietic lineage.

With regard to DC in smokers and lung disease: a.) In smokers, we and other investigators showed that the numbers of DC were increased in BAL and lung. In contrast, the stimulatory capability of BAL cells was decreased in smokers compared to nonsmokers. b.) In bronchial biopsies of allergic asthmatics, DC numbers were elevated compared to healthy nonsmokers. Patients that received inhaled corticosteroid therapy, however, showed DC numbers that compare to control levels. We hypothesize that DC play an important role in T cell activation in asthma and that a downregulation of both numbers and function of DC by glucocorticoids contributes to the clinical effects of glucocorticoid therapy in asthma.

The third part of the discussion considers the significance of the decreased leucocyte polarization for host defense. The polarization of leucocytes depends on the polymerization of actin filaments. Polarization is thought to be closely related to chemotaxis, but defects in polarization might also point to impairments in other cellular processes that require actin polymerization, such as phagocytosis, exocytosis or membrane motility, for the establishment of intercellular contacts. We suggest that an impaired polarization contributes to an increased susceptibility for infections, but local factors or additional disturbances of immune function may factor herein as well. The partial restoration of the polarization in response to G-CSF (for granulocytes) and thymic peptides (for monocytes), together with the in vivo clinical effects of these substances support this hypothesis. The cause of the impaired performance of leucocytes is still unclear, but might include genetic, viral, bacterial and/or autoimmune factors.

SAMENVATTING

In dit proefschrift wordt een aantal onderzoeken beschreven naar de afweermechanismen in de long, waarbij speciaal gekeken is naar het voorkomen en de functie van dendritische cellen (DC) in de long en naar de rol van stoornissen in het afweersysteem bij patiënten met chronische of recidiverende bacteriële infecties van de lagere luchtwegen.

DC zijn gespecialiseerde cellen van het afweersysteem die eiwitbevattende structuren opnemen (antigenen), bewerken en peptides daarvan presenteren aan T lymfocyten. Voor het presenteren van deze peptides gebruiken de DC MHC klasse II moleculen (HLA-DR). Als de T lymfocyten het peptide als lichaamsvreemd herkennen, worden ze geactiveerd door de DC. Dit proces is essentiëel voor het al dan niet op gang brengen van een immuunrespons. In het verloop van deze immuunrespons kunnen verschillende soorten antistoffen worden geproduceerd en kunnen andere T lymfocyten en macrofagen aangezet worden tot het neutraliseren van partikels die deze eiwitstructuur bevatten. Hoewel DC dus erg belangrijk zijn voor het beginnen van een immuunrespons, is er vrij weinig bekend over het voorkomen en de functionele eigenschappen van deze cellen in de long. Een beter inzicht hierin is belangrijk voor een beter begrip van allerlei ziekteprocessen in de long, zoals astma en chronische luchtweginfecties.

In hoofdstuk 2 is het voorkomen van DC en macrofagen in de long bestudeerd m.b.v. immunohistochemie. In en vlak onder het bronchusepitheel bleken relatief veel cellen voor te komen met een dendritische morfologie (onregelmatige vorm en lange uitlopers). Deze cellen kleurden aan met verschillende monoclonale antistoffen tegen eiwitten die vooral voorkomen op dendritische cellen: HLA-DR, RFD1, L25 en (deels) CD1a. Deze cellen kleurden echter ook aan met de macrofagen marker CD68. Een deel van deze cellen was positief voor de monocyten marker CD14. Deze cellen waren echter negatief voor andere monocyt-macrofaag markers (RFD7, RFD9, zure fosfatase en niet-specifieke esterase). In het alveolaire compartiment werden relatief weinig cellen met dergelijke kenmerken gevonden. Typische macrofagen, grote ronde cellen positief voor alle macrofaag markers, werden vooral gevonden in de alveoli en in het lumen van de bronchi, en zelden in of onder het bronchusepitheel. We concluderen dat de cellen met dendritische morfologie een immunofenotype hebben dat vergelijkbaar is met dat van DC, maar dat deze cellen ook enkele monocyt/macrofaag kenmerken vertonen. Waarschijnlijk hebben deze cellen uit de long dezelfde potentie tot het stimuleren van T-cellen als DC in de lymfoide organen en het bloed.

Hoofdstuk 3 beschrijft een techniek om m.b.v. een "fluorescence activated cell sorter" (FACS) cellen uit de broncho-alveolaire lavage (BAL) te scheiden op

autofluorescentie. De sterk autofluorescerende cellen blijken typische alveolair macrofagen (AM) te zijn: grote, ronde, zure fosfatase-positieve cellen met hetzelfde immunologisch markerpatroon als de macrofagen uit hoofdstuk 2. De laag autofluorescente (LAF) cellen hebben hetzelfde immunofenotype als de DC in de bronchuswand. Hoewel de meeste cellen rond zijn, vertoont de helft van de cellen na 16 uur kweken lange cytoplasmatische uitlopers. Behalve het merkerpatroon zijn ook de functionele karakteristieken van deze twee celpopulaties totaal verschillend: de AM zijn zeer slechte T-cel stimulatoren en kunnen zelfs de T-cel proliferatie remmen, terwijl de LAF cellen uitstekend T-cellen kunnen stimuleren. Ook kunnen de LAF-cellen naïeve, rustende T-cellen stimuleren en deze cellen vormen ook clusters met T-cellen gedurende een kweek (beide belangrijke kenmerken van DC). Verder vonden we dat de BAL van rokers zowel absoluut als relatief meer LAF-cellen bevat dan de BAL van niet-rokers. In tegenstelling hiermee waren BAL-cellen van rokers slechtere stimulatoren van T-cellen. We concluderen hieruit dat twee totaal verschillende celpopulaties uit de BAL geïsoleerd kunnen worden m.b.v. een FACS: 1.) de sterk autofluorescente AM bezitten immunosuppressieve eigenschappen, en 2.) de zwak autofluorescente (LAF) cellen zijn uitermate potente T-cel stimulatoren en bevatten DC en precursors van DC.

Glucocorticoïden zijn veel gebruikte medicijnen, o.a. bij astma. Omdat nog niet precies bekend is hoe deze medicijnen werken, werd de invloed van glucocorticoïden op DC-populaties uit de BAL (LAF-cellen) onderzocht (hoofdstuk 4). LAF-cellen die met glucocorticoïden geïncubeerd waren, hadden een veel lagere capaciteit tot het stimuleren van T-cellen. Deze verminderde stimulatoire functie was geassociëerd met een verlaagde expressie van B7-1 (CD80) en (niet significant) met een verlaagde expressie van CD11b. Aangezien B7-1 een belangrijk molecuul is voor de stimulatie van T-cellen, denken we dat de verlaging van de B7-1 expressie o.i.v. glucocorticoïden zou kunnen bijdragen tot het onderdrukken van de stimulatoire functie van DC uit de BAL. Het supprimerende effect van glucocorticoïden op DC in de luchtwegen zou een rol kunnen spelen bij het verminderen van de ontsteking van de longen bij astma gedurende glucocorticoïd therapie.

In hoofdstuk 5 beschrijven we het vermogen van LAF-cellen om de cytokinen IL-1, IL-6 en TNF-α te produceren. In LPS-gestimuleerde LAF-celpopulaties konden zowel intracellulaire als gesecerneerde cytokinen aangetoond worden. In tegenstelling tot monocyten uit het perifere bloed hadden LAF-cellen deze cytokinen niet nodig om T-cellen te stimuleren.

Omdat we in eerdere studies al hadden gezien dat de LAF-cellen heterogeen waren met betrekking tot de expressie van verschillende eiwitten op de celmembraan, m.n. CD1a en CD14, onderzochten we of subpopulaties van LAF-

cellen, die positief of negatief waren voor deze eiwitten, een vergelijkbare potentie hadden om deze cytokinen te produceren. De $CD14^{+/-}$ subpopulaties verschilden hierin niet duidelijk. Echter, in tegenstelling tot de $CD1a^-$ LAF-cellen, produceerden de $CD1a^+$ LAF-cellen vrijwel geen IL-1, IL-6 of TNF-α. Bovendien waren de $CD1a^+$ cellen veel beter in het stimuleren van T-cellen dan de $CD1a^-$ subpopulatie. We concluderen hieruit dat 1.) de $CD1a^+$ LAF-cellen gezien hun fenotype en functie moeten worden beschouwd als echte DC, en dat 2.) de $CD1a^-$ LAF-cellen eigenschappen van zowel DC als van monocyten vertonen.

In hoofdstuk 6 en 7 wordt het onderzoek naar de functie van granulocyten en monocyten bij patiënten met chronisch recidiverende bacteriële infecties van de lagere luchtwegen beschreven. Deze infecties konden niet afdoende behandeld worden met gangbare therapieën, waaronder antibiotica. Vergeleken met cellen van gezonde controlepersonen veranderden granulocyten en monocyten geïsoleerd uit het bloed van de patiënten minder van vorm (polarisatie) na stimulatie. De stoornissen in de polarisatie van granulocyten en monocyten correleerden echter niet met elkaar. De produktie van zuurstofradicalen door monocyten en granulocyten van de patiënten was normaal.

Bij patiënten met een gestoorde polarisatie van granulocyten (10 van de 18 patiënten) verbeterde de polarisatie door toevoeging van "granulocyte colony-stimulating factor" (G-CSF) aan de cellen. Voor monocyten die niet goed polariseerden (14 van de 36 patiënten) was een vergelijkbaar effect waar te nemen als thymuspeptiden (thymostimuline [TP-1] en thymopentine [TP5] aan de cellen toegevoegd werden. Niet alleen de polarisatie maar ook de chemotaxie (migratie door een filter) van monocyten was verlaagd bij patiënten met chronisch recidive van de luchtweg infecties. Bij een deel van de patiënten werden laagmoleculaire factoren in het serum gevonden die een remmend effect hadden op de polarisatie van monocyten.

Onze hypothese is dat de stoornissen in de functie van granulocyten en monocyten bijdragen aan de verhoogde gevoeligheid voor infecties bij de patiënten. Het herstellen van deze stoornissen in het lichaam door toediening van ofwel G-CSF of thymuspeptiden zou dan een gunstig effect op de klinische toestand van deze patiënten kunnen hebben.

De algemene discussie (hoofdstuk 8) gaat vooral in op 1.) de relatie tussen en de aard van LAF-cellen in de BAL en de dendrietachtige in het bronchusepitheel, 2.) de mogelijke rol van deze cellen bij longziekten en 3.) de betekenis van de gestoorde polarisatie van granulocyten en monocyten van patiënten met chronisch recidiverende luchtweginfecties.

De $CD1a^+$ LAF-cellen in de BAL en de $CD1a^+$ DC in het bronchusepitheel zijn

klassieke DC, homoloog aan Langerhans cellen uit de huid. De $CD1a^-$ LAF-cellen en de $CD1a^-$ dendrietachtige cellen in de bronchuswand vertonen eigenschappen van zowel DC als van monocyten. Als deze cellen een aantal dagen gekweekt werden, differentiëerden ze tot macrofagen. Wij denken echter dat deze $CD1a^-$ cellen (onder de juiste omstandigheden) ook tot $CD1a^+$ DC kunnen differentiëren. Vanwege de sterke gelijkenis tussen de $CD1a^+$ en $CD1a^-$ LAF-cellen, en omdat we zagen dat $CD1a^+$ cellen ook bepaalde macrofaag kenmerken konden aannemen, concluderen we dat DC, monocyten en macrofagen tot de zelfde hematopoietische cellijn behoren.

Met betrekking tot DC en longziekten vonden wij een verhoogd aantal dendritische cellen in bronchusbiopten van patiënten met allergisch astma die geen inhalatie corticosteroïden gebruikten. Bij patiënten die wel inhalatie corticosteroïden gebruikten werden aantallen dendritische cellen gevonden die vergelijkbaar waren met die van gezonde controles. Wij denken dat dendritische cellen een belangrijke rol spelen bij de activatie van T-cellen in astma, en dat zowel het verminderen van het aantal dendritische cellen als het remmen van de functie van dendritische cellen door corticosteroïden bijdraagt aan het klinische effect van deze medicijnen.

Het laatste onderwerp in de algemene discussie is de oorzaak van de verlaagde polarisatie van leucocyten bij patiënten met chronisch recidiverende luchtweginfecties en de mogelijke relatie met de verhoogde gevoeligheid voor infecties bij deze patiënten. Polarisatie (vormverandering) is afhankelijk van de polymerisatie van actinefilamenten. Hoewel gedacht is dat polarisatie een eerste stap is voor de migratie (chemotaxis) van leucocyten, zou een stoornis in de polarisatie ook kunnen wijzen op stoornissen in andere processen waarvoor actinepolymerisatie nodig is. Hierbij kan gedacht worden aan fagocytose, exocytose of het leggen van contacten met andere cellen. Wij denken in ieder geval dat een stoornis in de polarisatie van leucocyten bijdraagt aan de verhoogde gevoeligheid voor infecties bij de patiënten. De verbetering van de polarisatie onder invloed van G-CSF (voor granulocyten) en thymuspeptiden (voor monocyten) samen met de klinische effecten van toediening van deze stoffen, steunen onze hypothese. Naast de verlaagde polarisatie zouden echter ook locale factoren of andere stoornissen in het immuunsysteem een rol kunnen spelen bij de verhoogde gevoeligheid voor infecties bij de patiënten met chronisch recidiverende luchtweginfecties. De oorzaak voor de gestoorde functie van de leucocyten is nog onduidelijk. Genetische, virale en/of bacteriële factoren kunnen hierin een rol spelen.

DANKWOORD

Iedereen, die mij de afgelopen 4 jaar op wat voor manier dan ook heeft geholpen met mijn onderzoek en het tot stand komen van dit proefschrift, wil ik daarvoor bedanken. Een aantal mensen wil ik met name danken:

Mijn promotor, Hemmo Drexhage voor zijn begeleiding en vooral voor zijn hulp bij het schrijven, en, al waren we het niet altijd helemaal eens, de samenwerking heb ik als erg prettig ervaren.

Henk Hoogsteden, mijn co-promotor, voor zijn ondersteuning en voor zijn reële en vriendschappelijke opstelling.

Prof. Rob Benner en prof. C. Hilvering voor het in mij gestelde vertrouwen.

Harm, mijn analist, op wie je altijd kunt rekenen en van wie nooit een onvriendelijk woord komt.

Gert Verhoeven, die elke week vroeg opstond voor mijn broodnodige BAL. Heel veel succes met jouw eigen onderzoek.

Joop van de Merwe en Herbert Hooijkaas voor hun belangrijke bijdrage aan de patiëntenstudies.

Het lab: Martha, Meeny, Maarten, Annemarie, Annemieke, Petra, Peter, Pieter, Wilfried, Frans, Paul, Herbert en Belinda. Met biertjes in Dizzy, p15E-bepalingen, het opruimen van mijn troep, gezelligheid, geklets, kritiek en vele andere dingen hebben jullie op zeer gevariëerde wijze bijgedragen aan mijn proefschrift.

De afdeling Immunologie en met name de "longclub" voor de samenwerking en adviezen.

Trudeke, Marella en anderen ook voor de gezelligheid en borrels buiten het werk.

Petra voor haar secretariële en Tar voor zijn grafische ondersteuning, en Sofieke voor de mooie omslag.

De afdeling Longziekten van het Dijkzigt en de perifere longartsen Youke Tan en Paul Hekking voor hun steun en het selecteren van patiënten voor mijn onderzoek.

De afdeling Hematologie voor hun gastvrijheid en het gebruik van de FACS-Vantage.

Mijn paranimfen, Trudeke en Aernout (vooral ook succes met jullie eigen promoties!).

Mijn ouders, de basis zat natuurlijk in de opvoeding.

Hieke, lest best, jouw steun en jouw huidige inspanningen zijn niet met woorden te beschrijven.

ABBREVIATIONS

Acid Phosphatase	APh
Alveolar macrophage	AM
Antigen-presenting cell	APC
Bronchoalveolar lavage	BAL
Bronchus-associated lymphoid tissue	BALT
Cluster of differentiation	CD
Counts per minute	CPM
Chronic obstructive pulmonary disease	COPD
Dendritic cell	DC
Fluorescence activated cell sorter	FACS
Glucocorticoid	GC
Granulocyte colony-stimulating factor	G-CSF
Granulocyte-macrophage CSF	GM-CSF
High autofluorescent (fraction)	HAF
Intercellular adhesion molecule	ICAM
Interferon	IFN
Interleukin	IL
Langerhans cell	LC
Low autofluorescent (fraction)	LAF
Low molecular weight factors	LMWF
Major histocompatibility complex	MHC
Mixed lymphocyte reaction	MLR
Monoclonal antibody	MAb
N-formyl-methionyl-phenylalanine	FMLP
Nonspecific esterase	NSE
Peripheral blood monocyte	PBM
Phorbol-myristate-acetate	PMA
Phosphate-buffered saline	PBS
Phytohemagglutinin	PHA
Serum-treated Zymosan	STZ
Tumor necrosis factor	TNF

CURRICULUM VITAE

Geboren	:	12 september 1961 te Spijkenisse
1973-1979	:	Atheneum B, Alexander Hegius Scholengemeenschap, Deventer
1979-1980	:	propedeuse Scheikunde
1980-1981	:	propedeuse Biologie beiden aan de R.U.Groningen
1981-1986	:	doctoraal Geneeskunde, R.U.Groningen
1986	:	doctoraalassistentschap op het CLB te Amsterdam, onderzoek naar Fc-receptor blokkerende antistoffen bij bloedgroepenantagonisme in de zwangerschap. Begeleider: dr.W.H. Ouwehand.
1987-1989	:	artsexamen (23 februari 1989)
1989-1990	:	assistent Longziekten, Militair Hospitaal dr.A. Mathijsen, Utrecht. Supervisor: A.P. Sips, longarts.
1990	:	AGNIO cardiologie van het Elizabeth Ziekenhuis te Leiderdorp. Supervisor: dr.C. van Rees.
1990-1994	:	A.I.O. op de afdelingen Immunologie en Longziekten van de Erasmus Universiteit en het Dijkzigt Ziekenhuis te Rotterdam. Begeleiders: dr.H.C. Hoogsteden, longarts en prof.dr.H.A. Drexhage, medisch-immunoloog.
1995-heden	:	vooropleiding Interne Geneeskunde, St. Franciscus Gasthuis, Rotterdam (opleider: dr. H.S.L.M. Tjen) in het kader van de opleiding tot longarts (opleider: prof.dr.C. Hilvering).