PULMONARY DENDRITIC CELL TRAFFICKING IN HEALTH AND DISEASE

KARIM VERMAELEN, 2003
Pulmonary Dendritic Cell Trafficking
in Health and Disease

Karim Vermaelen, 2003

Promotor: Prof. Dr. Romain Pauwels

Thesis submitted to fulfill the requirements for the degree of “doctor in medical sciences”
Acknowledgments

I want to dedicate this work to my mom and dad - without their care and support I simply wouldn’t be where I am now. To Veerle and our little boy Driss for being incredibly patient while I was frantically finishing this work. To Romain Pauwels for inspiring this work and provide all the means to bring it to fruition, and whose unique enthusiasm as thesis adviser was a major driving force all the way through.

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Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow-cytometry: methodology and new insights

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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADAM</td>
<td>A Disintegrin And Metalloprotease</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar Macrophage</td>
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<tr>
<td>AT</td>
<td>Angiotensin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncho-Alveolar Lavage</td>
</tr>
<tr>
<td>BALT</td>
<td>Bronchus-Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guérin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma protein 2</td>
</tr>
<tr>
<td>BDCA</td>
<td>Blood Dendritic Cell Antigen</td>
</tr>
<tr>
<td>C3bi</td>
<td>inactive Complement 3b fragment</td>
</tr>
<tr>
<td>CCL</td>
<td>CC-chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC-chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxy-Fluorescein Succinidimyl Ester</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-Forming Unit</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin Gene-Related Peptide</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous Leucocyte Antigen</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DC-CK1</td>
<td>Dendritic Cell-derived ChemoKine 1</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific ICAM-Grabbing Non-integrin</td>
</tr>
<tr>
<td>Der P 1</td>
<td>Dermatophagoides pteronyssinus Protein 1</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-Type Hypersensitivity</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ELC</td>
<td>Epstein Barr Virus-induced molecule-1 Ligand Chemokine</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>Extracellular Matrix Metalloproteinase Inducer</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>Flt-3L</td>
<td>Fms-like tyrosine kinase-3 Ligand</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyleucylphenylalanine</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Monocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leucocyte Antigen</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>ICSBP</td>
<td>Inducible Costimulator Ligand</td>
</tr>
<tr>
<td>ICS-L</td>
<td>Inducible Costimulator Ligand</td>
</tr>
<tr>
<td>Id2</td>
<td>Inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin-</td>
</tr>
<tr>
<td>IM</td>
<td>Intersitial Macrophage</td>
</tr>
<tr>
<td>IPC</td>
<td>Interferon-Producing Cell</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans Cell</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte Function-Associated Antigen</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage-negative</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>Lu-ECAM-1</td>
<td>Lung-endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>MadCAM-1</td>
<td>Mucosal Addressin Cellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>MDC</td>
<td>Macrophage-Derived Chemokine</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed Leucocyte Reaction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
</tr>
<tr>
<td>MRP1</td>
<td>Multi-drug Resistance Protein 1</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>PARC</td>
<td>Pulmonary and Activation Regulated Chemokine</td>
</tr>
<tr>
<td>PDC</td>
<td>Plasmacytoid Dendritic Cell</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PNAd</td>
<td>Peripheral Node Addressin</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-Selectin Glycoprotein Ligand-1</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator of NFkB</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation Normal T-cell Expressed and Secreted</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal-Derived Factor</td>
</tr>
<tr>
<td>Serpin</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>SF</td>
<td>Scatter Factor</td>
</tr>
<tr>
<td>SLC</td>
<td>Secundary Lymphoid organ Chemokine</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TARC</td>
<td>Thymus and Activation Regulated Chemokine</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper lymphocyte</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloprotease</td>
</tr>
<tr>
<td>TLN</td>
<td>Thoracic Lymph Node</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TRANCE</td>
<td>TNF-Related Activation-Induced Cytokine</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic Stromal Lymphopoietin</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase Plasminogen Activator</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VIP</td>
<td>Vaso-Intestinal Peptide</td>
</tr>
<tr>
<td>VLA</td>
<td>Very Late Antigen</td>
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Summary
The lung is a vital organ that packs an enormous surface area exposed to the outside world. As a consequence, immune responses elicited at this level must be both highly efficient and exquisitely regulated. Scientific evidence accumulated over recent years has identified dendritic cells as central orchestrators of innate and adaptive immune responses. According to a prevailing paradigm, DCs are continuously recruited from the blood circulation into antigen-exposed organs. Incoming antigen is sampled and processed by DCs that subsequently migrate towards draining thoracic lymph nodes (LN) where antigen-specific T-cell responses are efficiently induced. Not surprisingly, studies on the pulmonary immune response have increasingly focused on the biology of lung dendritic cells in health and disease.

A main factor defining the extent of pulmonary DC populations is the intensity of DC trafficking to and from the lung. This work was largely devoted to the study of pulmonary DC migration in health and pathological states. First we developed a model allowing to track the migration of DCs from the airways into thoracic lymph nodes by relying on their antigen-transporting capacity. By delivering inert fluorescent macromolecules into the airways of mice, we could reveal a constitutive and rapid transport of antigen into the T-cell zones of thoracic lymph nodes by a specific subset of DCs. These migratory DCs upregulated T-cell costimulatory molecules along the way and induced a powerful antigen-specific T-cell proliferation.

As a next step, we sought to examine the impact of pulmonary inflammation on pulmonary DC trafficking. We applied our DC-tracking strategy in an animal model of allergic asthma, as DCs were shown to play a pivotal role in this disease. The presence of allergen-driven airway inflammation dramatically amplified and accelerated the influx of allergen-transporting DCs into the thoracic LN. In addition, the survival of these cells in the LN was clearly shortened. Maturation of DCs also occurred at an accelerated pace during airway inflammation as these cells upregulated T-cell costimulatory molecules while still residing in the airways.

Leucocyte trafficking involves a myriad of molecules, including cell-cell or cell-matrix adhesion molecules, chemokines and matrix-metalloproteinases (MMPs). MMP-9 mediates the degradation of basement membranes and hence allows the passage of migratory cells across different tissue compartments. Spurred by an earlier study in which we showed how MMP-9 gene deficiency profoundly impaired allergic airway inflammation, we decided to focus on the role of this molecule in pulmonary DC transit. MMP-9 deficiency specifically impaired the recruitment of DCs into inflamed airways. This defect correlated with decreased airway levels of the pro-allergic chemokine CCL17/TARC, a chemokine which appeared to be highly concentrated in lung DCs. As MMP-9 deficiency resulted in decreased peribronchial eosinophil infiltrates and impaired production of allergen-specific IgE, this study unravelled some of the molecular events that link the penetration of DCs into the airways and the maintenance of allergen-driven inflammation. In addition, our data underscores the potential of selective MMP inhibitors as novel treatment options for asthma.

The abovementioned studies required a method to accurately define murine lung DCs. Ideally, this method would eliminate any confusion with a closely related antigen-presenting cell: the pulmonary macrophage. A clear distinction is necessary given previous studies suggesting important functional differences between both cell types. We developed a flow-cytometry-based strategy in order to distinguish DCs and macrophages in mouse lungs without having to rely on lengthy isolation procedures or complex marker
combinations. We could show that the combination of (i) high CD11c expression and (ii) autofluorescence was necessary and sufficient to distinguish cells with morphological, functional and immunophenotypical properties of DC's on one hand and macrophages on the other. In addition, this method delivered some unexpected results with regard to classical notions on the phenotype of these cells in the lung.

In summary, we have [i] established an animal model allowing to describe the trafficking of pulmonary DCs in vivo, (ii) examined the impact of ongoing allergic airway inflammation on this trafficking, (iii) dissected an interaction between matrix metalloproteinases and chemokines in the dendritic cell-mediated maintenance of allergic airway inflammation, and (iv) developed an accurate and simple method for the identification of pulmonary dendritic cells and macrophages.
Samenvatting
De long is een vitaal orgaan waarin een zeer uitgebreide contactoppervlakte met de buitenwereld is omvat. Bijgevolg moet een afweerreactie op het niveau van de long terzelfdertijd extreem efficient, maar ook bijzonder strak geregeld zijn. Over de recente jaren werden dendritische cellen aangewezen als dé centrale spelers in de aangeboren en verworven immuniteit. Volgens een algemeen aanvaard concept worden DC's gerekruteerd vanuit de bloedsomloop naar diverse weefsels die aan antigenen worden blootgesteld. Antigenen worden efficient opgenomen en verwerkt door DC's die vervolgens naar drainerende lymfenodi (LN) migreren. Daar worden antigen-fragmenten gepresenteerd aan T-lymfocyten die hierdoor een krachtige stimulatie ondergaan. Het is dus niet verwonderlijk dat studies over de pulmonale immuunrespons zich meer en meer richten naar de biologie van de long DC's.

Een doorslaggevende factor die de omvang van pulmonale DC populaties bepaalt is de intensiteit van het DC-verkeer van en naar de long. Dit werk werd grotendeels gewijd aan de studie van pulmonale DC migratie in ziekte en gezondheid.

Eerst hebben we een model ontwikkeld waarmee we de migratie van DC's van de luchtwegen naar de thoracale LN konden volgen door beroep te doen op hun antigen-vervoerende capaciteit. Door inerte, fluorescerende macromolecules te instilleren in de luchtwegen van muizen konden we een snel transport aantonen van antigen naar de T-cell zones van de thoracale LN. Dit transport was volledig te wijten aan een specifieke subpopulatie van migrerende DC's, en ging door in afwezigheid van schadelijke prikkels. Na hun migratie verhoogden de DC's hun expressie van T-cell costimulatoire molecules en induceerden een krachtige proliferatie van antigen-specifieke T-cellen.

Als volgende stap gingen we het effect na van een ontsteking thv de luchtwegen op het gedrag van de pulmonale DC's. Wij verkozen om de migratie van luchtweg-DC's te beschrijven in een diermodel van allergisch astma. Het is immers aangetoond dat DC's een belangrijke rol spelen in de pathogenese van deze ziekte. De aanwezigheid van allergische luchtweg-inflammatie veroorzaakte een massief versterkt debiet van luchtweg-DCs (en meegevoerd allergen) naar de thoracale LN. Bovendien was de overleving van de allergen-vervoerende DC's in de LN sterk verkort. De maturatie van luchtweg-DCs was ook versneld tijdens de inflammatie: DC's die nog in de luchtwegen vertoefden verstormden reeds een verhoogde expressie van costimulatoire moleculen op hun oppervlak.

Bij de migratie van leukocyten is een hele waaier van moleculen betrokken: o.a. cel-cel en cel-matrix adhesiën, chemokines en matrix metalloproteasen (MMP's). MMP-9 is een protease die basale membranen aantast en dus migrerende cellen toelaat om doorheen verschillende weefselcompartimenten te passeren. Bijgevolg was het interessant om de rol van deze molecule na te gaan in de transit van long-DC's, in het bijzonder tijdens allergische luchtweginflammatie. Muizen die genetisch deficent waren in MMP-9 veroorzaakten een duidelijk verminderde recrutering van DC's naar ontstoken luchtwegen. Deze verminderde DC accumulatie ging gepaard met gedaalde spiegels van de chemokine CCL17/TARC in de luchtwegen. CCL17 is een pro-allergische chemokine waarvan de productie in de long grotendeels te wijten was aan DC's. Bovendien leidde de afwezigheid van MMP-9 tot een verminderde peribronchiale infiltratie van eosinofielen en tot gedaalde spiegels van het allergen-specifiek IgE. Met deze studie konden we dus enkele moleculaire interacties ontrafelen die de penetratie van DC's in de luchtwegen koppelen aan het onderhoud van de allergische ontsteking. Bovendien wijzen deze gegevens op het potentieel van selectieve MMP-inhibitoren als nieuwe behandelingsopties tegen
asthma.

De hierboven genoemde studies vereisten een methode om tot een accurate definitie van long DC's te komen. Idealiter zou deze methode elke verwarring moeten beletten met nauwverwante antigen-presenterende cellen: de long-macrofagen. Een duidelijk onderscheid is belangrijk in het licht van vorige rapporten die wezen op duidelijke functionele verschillen tussen deze twee celpopulaties. We ontwikkelden een strategie om mbv van flow-cytometrie DCs en macrofagen in muislongen van elkaar te onderscheiden, zonder gebruik te maken van langdurige isolatieprocedures of ingewikkelde merkercombinaties. We konden aantonen dat de combinatie van (i) hoge CD11c expressie en (ii) autofluorescentie noodzakelijk en voldoende was om cellen te onderscheiden met morfologische, functionele en immunofenotypische kenmerken van DC's enerzijds of macrofagen anderzijds. Bovendien leverde deze methode onverwachte resultaten op wat betreft algemeen aanvaarde begrippen over het fenotype van die cellen in de long.

Samengevat hebben we [i] een model ontwikkeld die toelaat de migratie van long DC’s in vivo te volgen, [ii] de impact van allergische luchtwegontsteking op deze migratie nagegaan, [iii] een interactie naar voor gebracht tussen matrix metalloproteasen en chemokines in het onderhoud van de allergische ontsteking door de long DC’s, en [iv] een methode ontwikkeld om eenvoudig en nauwkeurig pulmonale dendritische cellen en macrofagen van elkaar te onderscheiden.
Résumé
Le poumon constitue un organe vital qui renferme une surface considérable exposée à l’environnement extérieur. Par conséquent, toute réponse immunitaire induite à ce niveau doit être tout à la fois très efficace et extrêmement bien contrôlée. Des données scientifiques accumulées au fil des années récentes ont identifié les cellules dendritiques [CDs] comme les chefs d’orchestre des réponses immunitaires innées et adaptatives. D’après un concept prévalant, les CDs sont constamment recrutées vers divers tissus exposés à des antigènes. Les CDs échantillonnent et traitent les antigènes pénétrants et migrent par la suite vers les ganglions lymphatiques [GgL] drainants. Là, les CDs présentent des fragments d’information antigénique aux lymphocytes T qui subissent alors une stimulation puissante. L’intérêt croissant porté aux cellules dendritiques dans le contexte des réponses immunitaires dans le poumon est dès lors peu surprenant.

Un paramètre qui détermine de manière importante l’envergure des populations de CDs dans le poumon est l’intensité du trafic de ces cellules à travers cet organe. Ce travail a été largement consacré à l’étude de la migration des CDs dans le poumon sain et dans des conditions pathologiques.
Tout d’abord nous avons développé un modèle permettant de traquer la migration des CDs des voies aériennes vers les ganglions lymphatiques thoraciques en exploitant leur capacité de transport d’antigène. En introduisant des macromolécules fluorescentes inertes dans les voies aériennes de souris, nous sommes parvenu à révéler un transport rapide et constitutif d’antigène vers les zones T-dépendentes des GgL thoraciques. Ce transport était attribué à une population spécifique de CDs migratoires. Après leur migration, ces CDs augmentaient l’expression de molécules costimulatoires à leur surface et induisaient une prolifération puissante de lymphocytes T de manière antigène-spécifique.
La prochaine étape fut de vérifier l’impact d’une inflammation pulmonaire sur le comportement des CDs. Nous avons entrepris de décrire le trafic des CDs des voies aériennes dans un modèle animal d’asthme allergique, vu que les CDs ont été identifiées comme des actrices fondamentales dans cette pathologie. La présence d’une inflammation allergique des voies aériennes provoquait un influx accéléré et fortement amplifié de CDs transportant l’allergène vers les GgL thoraciques. De surcroît, la survie de ces cellules dans les GgL était nettement écourtée. La maturation des CDs était aussi accélérée pendant l’inflammation vu que ces cellules affichaient une forte expression de molécules costimulatoires tout en résidant encore dans les voies aériennes.

La migration des leucocytes implique une constellation de molécules, entre autres diverses molécules d’adhésion, des chimiokines et des métalloprotéases matricielles [MMP]. La MMP-9 est une protéase qui « digère » les membranes basales et permet ainsi à des cellules migratoires de passer à travers différents compartiments tissulaires. Dans une étude précédente, nous avions démontré que l’absence de MMP-9 pouvait fortement atténuer le développement d’une inflammation allergique des voies aériennes. Il était alors logique d’examiner le rôle de cette molécule dans le transit des CDs pulmonaires. Les souris génétiquement déficientes en MMP-9 affichaient une nette diminution du recrutement des CDs vers les voies aériennes inflammmées. Cet empêchement était accompagné d’une diminution de la production de la chimikine CCL17/TARC au niveau des voies aériennes. CCL17 est une chimiokine pro-allergique qui s’avérait spécifiquement concentrée dans les CDs pulmonaires. Par ailleurs, l’absence de MMP-9 résultait en une diminution des infiltrations péri-bronchiques eosinophiles et une réduction de la production de l’IgE allergène-spécifique. De ce fait, cette étude a permis de lever le voile sur les
événements moléculaires qui lient la pénétration de CDs dans les voies aériennes et l’entretien de l’inflammation allergique. De surcroît, ces données soulignaient le potentiel d’inhibiteurs spécifiques des MMPs dans le traitement de l’asthme.

Les études décrites ci-dessus requièrent une méthode permettant de définir précisément les CDs pulmonaires. De manière idéale, cette méthode éliminerait toute confusion avec des cellules présentatrices d’antigène apparentées : les macrophages pulmonaires. Une distinction nette et claire est nécessaire au vu d’études suggérant d’importantes différences fonctionnelles entre ces deux populations cellulaires. Nous avons développé une stratégie basée sur la cytométrie de flux permettant de distinguer les CDs et les macrophages dans le poumon de souris en évitant de recourir à des procédés d’isolation prolongés ou des combinaisons de marqueurs antigéniques trop complexes. Nous avons pu démontrer que la combinaison de (i) forte expression du CD11c et (ii) autofluorescence était nécessaire et suffisante afin de distinguer des cellules ayant les propriétés morphologiques, fonctionnelles et immunophénotypique de CDs d’une part et de macrophages de l’autre. En plus, cette méthode a produit des résultats inattendus par rapport à des notions établies concernant le phénotype de ces cellules dans le poumon.

En résumé, nos travaux ont (i) établi un modèle animal permettant de suivre le trafic des CDs pulmonaires in vivo, (ii) examiné l’impact d’une inflammation allergique des voies aériennes sur ce trafic, (iii) disséqué une interaction entre les metalloprotéases matricielles et les chimiokines dans l’entretien de l’inflammation allergique par les CDs pulmonaires, et (iv) développé une méthode simple et précise permettant la distinction entre cellules dendritiques et macrophages pulmonaires.


Introduction

General biology of dendritic cells

What is a dendritic cell? A brief history

In 1868 a German medicine student named Paul Langerhans discovered a new type of cell scattered in the basal layers of the epidermis. These cells displayed a "dendritic" morphology, i.e. slender branched processes extending between neighbouring epithelial cells ("dendron" = Greek for "branch"). He used the gold chloride staining technique taken from the repertoire of pioneering neuro-anatomists and he believed his "Langerhans cell" represented a type of epidermal nerve cells (Über die Nerven der menschlichen Haut. Virchows Archiv für pathologische Anatomie und Physiologie, und für klinische Medicin, Berlin, 1868, 44: 325-337). The Langerhans cell remained elusive for more than a century until detailed studies in the 1960’s established a link between Langerhans cells and the leucocytes accumulating in histiocytosis X lesions. Their common feature were mysterious intracellular corpuscles called "Birbeck granules". Soon it was also suggested that the Langerhans cells were related to the macrophage family ¹.

A few years later, a hallmark study was undertaken by Ralph Steinman, a physician and researcher at Zanvil Cohn’s lab, one of the pioneers of the phagocytic leucocyte system. Between 1973 and 1979, Steinman and Cohn produced 5 subsequent papers in which a "novel cell type in mouse peripheral lymphoid organs" was meticulously described in terms of morphology, distribution, in vitro and in vivo properties ². This novel cell appeared to reside primarily in the white pulp of spleen as well as in lymph nodes. Compared to other nucleated cells from these organs, this cell was distinguished by its typical branched cytoplasmic processes, and they were further referred to as dendritic cells (DCs). The studies by Steinman and Cohn also included the first detailed methodology for the ex vivo purification of mouse lymphoid organ DCs, which opened the way for more detailed investigations. The first studies exploiting this technique led to crucial conclusions concerning the main function of DCs. First, DCs were found to bear copious amounts of MHC surface antigens and this expression remained stable for several days in vitro ³. Second, it was found that DCs accounted for most of the T-cell allostimulatory activity present in lymphoid organs: even small numbers of DCs could induce a vigorous T cell proliferation in mixed leucocyte reactions, a response which was stronger than that induced by B cells and much stronger than macrophages ⁴.

Meanwhile, a few reports appeared which for the first time related Langerhans cells to "interdigitating" dendritic cells in the paracortex of lymph nodes. The crucial link was the occurrence of “veiled cells” in the afferent lymph draining the skin. These cells were not only characterized by their high motility and the presence of veil-like cytoplasmic projections, but also Birbeck granules, a typical cytochemical profile and a tendency to cluster with lymphocytes ⁵. Many of these features were shared between Langerhans cells, veiled cells and interdigitating cells alike. The direct link between veiled cells (VCs) and interdigitating dendritic cells (IDCs) was further reinforced by an older report in which surgical interruption of skin lymphatic vessels resulted in a progressive disappearance of IDCs from the draining lymph nodes ⁶. Finally, a number of observations related to contact allergy hinted at the functional significance of the LC-VC-IDC system: (i) LCs in the skin were found to bear surface molecules involved in antigen uptake and presentation ¹⁰⁻¹² and could selectively take up haptens applied on the skin surface ¹³. (ii) The application of contact sensitizers resulted in an increased migration of VCs through the skin’s lymphatic vessels ⁶. (iii)
Interruption of skin lymphatics was already known to prevent the development of contact sensitivity following skin painting [iv]. The final anatomical localization of LC-derived IDCs within T-cell dependent areas made them perfect candidates for the induction of an immune response (it was already known that contact allergies were T-cell dependent as they do not develop in neonatally thymectomized animals [v]).

Thus, the fundamentals were laid for a central paradigm in dendritic cell biology: the main function of dendritic cells in the body appeared to be a sentinel role, as these cells were scattered in anatomical sites prone to antigen exposure (e.g. skin, marginal zones of splenic white pulp). After capture of antigen, dendritic cells would selectively migrate to the T-cell areas of peripheral lymphoid organs and initiate an adaptive immune response. This concept was suggested as early as 1976 by Silberberg-Sinakin et al [v] and today it is still refined, reinforced and exploited by an exponentially growing number of studies from laboratories around the world.

133 years after Paul Langerhans’ discovery, scanner images of a patient’s brain were shown during an international DC symposium showing disappearance of a melanoma metastasis after vaccination with tumor-“fed” autologous DCs. This is but one illustration of how DCs are slowly moving from the field of fundamental cell biology towards clinical application. DCs are nowadays recognized as both crucial participants as well as potential remedies in many pathologies including infectious diseases (including HIV and TBC), allergies, auto-immune diseases and anti-tumor immunity.

Figure 1. Langerhans cells (LC) reside in the parabasal layers of the epidermis and migrate as veiled cells via afferent lymphatics into draining lymph nodes. There, they nestle themselves in the paracortical zones and develop a typical interdigitating cell (IDC) morphology.
Dendritic cell origins and development into subsets

The recent years have seen an impressive expansion of different dendritic cells "species". In addition, the origin of all those dendritic cell subsets has sparked much debate, in which many established notions where later modified or overthrown. Two general concepts for DC development can be put forward, as introduced by Liu and Shortman [1]. In a "functional plasticity model", all DCs differentiate along a single hematopoietic line; the different DC subsets arise late in development under the mere influence of the local environment. In a "specialized lineage model", separate hematopoietic precursors committed to DC generation exist side by side and hence generate different DC subsets.

Despite the high-tech arsenal of tools available to modern-day immunologists, several factors still complicate the study of dendritic cells:

- DCs are rare cells in situ; they represent at most a few percent of the total cell population in a given organ.
- Extensive isolation and purification procedures induce phenotypical and functional changes in DCs. As described below, DCs are exquisitely sensitive to stress signals arising from the environment. The ex vivo extraction of DCs involves a whole sequence of procedures including enzymatic organ digestion, gradient centrifugations and overnight incubation steps. All these interventions can cloud the original state of the DC in situ.
- In contrast to other leucocytes (e.g. B cells), dendritic cells cannot be phenotypically identified using one single antigenic marker. A combination of at least two positive markers is necessary. Alternatively DCs are often defined by the absence of classical "lineage" markers (i.e. the absence of T, B, NK cell, monocyte and granulocyte surface antigens).

Generally, 3 simultaneous criteria must be met in order to "qualify" as a DC:

1. Morphology: typical dendritic cell morphological features include numerous cytoplasmic projections which can be tentacular, branched or veil-like depending on cell source and imaging technique. The nucleus is usually kidney-shaped or lobulated.

2. Function: perhaps the most important criterion is the impressive T-cell stimulatory power of DCs. This is most obvious when B-cells, macrophages and DCs are compared as stimulators in mixed leucocyte reactions or antigen-specific proliferative assays.

3. Antigenic determinants: as mentioned earlier, a combination of markers should be present, mostly molecules involved in antigen presentation (mature DCs are the cells with the highest density of surface MHC class II molecules) and T-cell costimulation (mature DCs upregulate an impressive array of costimulatory molecules). Additional surface or intracellular molecules used to discriminate among DC subsets are discussed below.

Although a lot of insight has been gained from mouse experimental models, conclusions regarding origin, phenotype and function of murine dendritic cells cannot be simply extrapolated to their human homologues.
Mouse dendritic cells

*General immunophenotype*

In general all murine DCs express copious amounts of the CD11c molecule on their surface (CD11c is the \( \gamma \) subunit of the \( \alpha \beta \gamma \) or \( \alpha 150/95 \) integrin). In fact, CD11c-positivity has often become synonymous with "mouse DC" in the literature, which is a deceiving amalgam in some organs such as the lung (as we shall examine below). In addition to CD11c, MHCII and T-cell costimulatory molecules are present on the cell surface in amounts which correlate with the degree of maturation/activation.

*Ontogeny*

For years it appeared that mouse DCs would simply fall into "myeloid" and "lymphoid" lineages. This was based on the exclusive presence of myeloid-related markers such as Mac-1 (CD11b) on the former and lymphoid markers such as CD8\( \alpha \) on the latter (DCs express surface CD8 as an \( \alpha \alpha \) homodimer with unknown function, in contrast to the classical \( \alpha \beta \) heterodimer on T-cells). For a long time this proved to be a useful working model leading to the characterization of important functional differences between CD8\( \alpha + \) and CD8\( \alpha - \) DCs. However this approach appeared quite reductionist with regard to ontogeny, as demonstrated by several studies. Indeed both common myeloid and common lymphoid progenitor cells are capable to reconstitute CD8\( \alpha + \) and CD8\( \alpha - \) DCs *in vivo*. The picture became even more complex with the availability of gene knock-out animals for transcription factors involved in hematopoiesis, such as RelB, Notch-1, Ikaros, Pu.1, ICSBP, Id2 or Id3. These models have generated some insight but even more debate concerning the origin of mouse DCs and their relationship to other leukocyte lineages. Recently, a common precursor of "all" DCs was discovered in mouse blood. This cell was devoid of any classical lymphoid or myeloid regeneration potential but was capable of regenerating all mouse DC subsets described below. It was detected in mouse blood as a mononuclear cell distinct from monocytes, expressing CD11c+ but no MHCII or costimulatory molecules. Remarkably, these cells lacked the growth factor receptors c-kit, IL7R and IL3R as well as early lymphoid and myeloid precursor-specific transcription factors.

*Subsets and tissue distribution*

CD11c+ DCs residing in the interstitium or parenchyma of peripheral organs such as the skin, the lung, the heart or the gut are Mac-1-positive but are devoid of CD8\( \alpha \). They are thought to differentiate from monocytic precursors following appropriate stimuli. For example, dermal monocytes can differentiate *in vivo* into DCs after uptake of foreign material in the dermal matrix and migration towards local lymph nodes. Monocytes which had taken up antigen and remained in the periphery differentiated into resident macrophages rather than DCs. In addition it is possible to generate mouse DCs *in vitro* starting from peripheral blood monocytes. This suggests that local factors (e.g. epithelium-derived cytokines such as GM-CSF) can induce the differentiation of freshly recruited blood monocytes into DCs. It cannot be excluded however that DCs in antigen-exposed organs could be directly derived from a myeloid progenitor upstream of the monocyte.

Intra-epithelial DCs (among which the prototypical epidermal Langerhans cells) seem to form a breed
apart: they bear markers distinct from their sub-epithelial or interstitial counterparts (e.g., langerin, DEC-205) and display a different turnover rate. Although the LC is the oldest known dendritic cell, its origin still remains clouded in mystery. A myeloid origin has long been taken for granted however some studies have suggested a lymphoid pedigree as LCs could be generated in vivo after injection of a CD4-low lymphoid-committed progenitor cell. In addition, LCs display some surface CD8+ upon maturation or homing into lymph nodes. Also, both LCs as well as CD8+ lymphoid organ DCs show a high surface expression of DEC-205, although this molecule is also present on myeloid (Mac-1+) DCs in the lung. Interestingly, mice deficient for Id2 (a repressor of several hematopoietic transcription factors) are devoid of LCs, CD8+ DCs and NK-cells.

The methods to generate LCs in vitro from bone marrow differ from classical protocols for the generation of myeloid DCs in which GM-CSF is the only required growth factor. In some studies, adding TNFα and SCF was necessary to produce DCs with some features of Langerhans cells. Also, the addition of TGF-β1 in this culture model appeared as a key factor pushing the development of murine monocyte/macrophage-like precursors into a more "physiological" type of LCs. Supplementing classical GM-CSF-based murine DC cultures with just TGF-β induces a strong expression of surface E-cadherin (our unpublished observations) and a small subset of DCs becomes Langerin-positive as well (30 and our observations). Interestingly, TGF-β knockout mice are devoid of epidermal LCs, a phenomenon which could be related to the TGF-β-induced upregulation of Id2 in DC-precursors. Although no data is available on DCs from other epithelia (e.g. airways) in TGF-β knockouts, it is tempting to speculate that TGF-β is an important tissue environmental factor driving the local differentiation of DC precursors into intra-epithelial DCs. These precursors have long been considered to be continuously recruited from the circulation, however it has recently been suggested that LCs renew by in situ proliferation. Indeed Merad et al have shown that, in the steady state, LC populations in the epidermis are slowly replaced by local cell division. Recruitment of fresh LC precursors from the blood seemed only to occur after a local inflammatory stimulus.

In mouse spleen, the combination of markers CD4 and CD8 distinguishes 3 main DC subsets which are functionally different.

**CD4+/CD8-** DCs:
- Located in the marginal zones of the spleen white pulp
- Bear the myeloid marker CD11b (Mac-1), no DEC-205
- Preferentially induce T helper 2 responses

**CD4-/CD8+** DCs:
- Located in the perivascular T-cell zones
- Express DEC-205, but no CD11b
- Preferentially induce T helper 1 responses
- Are uniquely responsible for uptake of apoptotic cell fragments and cross-presentation of cell-associated antigens

**CD4-/CD8-** DCs:
- Express CD11b, and are DEC-205 negative
- Produce IFN-γ
These 3 subtypes do not derive from one another but have 3 separate precursor lineages \(^{20}\). They also differ in terms of turnover rate, with the fastest population renewal in the CD8\(\text{+}\) DC subset and the slowest turnover rate in CD8\(\text{-}\)/ CD4\(\text{+}\) DCs (population half-lifes of 1 and 3 days respectively).

Recently a new type of DC was characterized in mouse lymphoid organs which is derived from the mouse interferon-producing cell (IPC) \(^{25}\). The IPC displays a plasmacytoid cell morphology (like it's human homologue – see below) which means it morphologically resembles a plasma cell (antibody-producing B lymphocyte). The murine plasmacytoid dendritic cell displays the following unique surface markers combination: CD11c low / CD11b- / B220+ / Ly6G & Ly6C+. When given the appropriate stimuli (notably TLR9 ligands such as bacterial CpG, viral dsRNA), these cells produce impressive amounts of type I interferon and mature into bona fide DCs with high MHCII expression and potent T-cell stimulatory capacity (these mature mouse PDCs are CD8\(\text{+}\) positive but lack DEC-205 and have a slow turn-over \(^{35}\)). Interestingly, unstimulated PDCs were shown to induce tolerogenic T-cells with immunosuppressive activity \(^{26}\). The origins of the mouse IPC or PDC is still somewhat obscure although it can differentiate from bone marrow cultures in the presence of Flt3-ligand, whereas GM-CSF inhibits its development in favour of CD11b+ DCs \(^{25,26}\). Also, mice knockout for the hematopoietic transcription factor ICSBP have no IPCs/PDCs as well as reduced numbers of CD8\(\text{+}\) DCs \(^{37,38}\). However, the fate of PDCs and CD8\(\text{+}\) DCs is not necessarily coupled in the same direction, as Id2 \(-/-\) mice are severely deficient in CD8\(\text{+}\) DCs but have increased numbers of PDCs. A recent study demonstrated that unlike classical CD8\(\text{+}\) and CD8\(\text{-}\)-splenic DCs, mouse PDCs (along with thymic DCs – cf infra) show evidence of limited Ig-gene rearrangements, suggesting common development steps found in classical lymphoid lineages \(^{39}\).

Murine lymph nodes contain the same 3 subtypes found in the spleen as well as a CD4\(-\)/CD8\(-\) low / CD11b+ DC subset which express high levels of DEC-205 and very high levels of MHCII: these are thought to represent immigrating LCs (in skin LN, these DCs are langerin positive as well) \(^{40}\). PDCs are represented here as well \(^{40}\).

In the thymus, the main DC subset is CD8\(\text{+}\)/CD11b- / DEC-205+. It is the cell involved in negative selection of self-reactive thymocytes in the medulla and the corticomedullar junction (reviewed in \(^{41}\)). The CD8\(\text{+}\) thymic DCs are developmentally related to early thymocytes, in contrast to a CD8\(\text{-}\)-DC subset which enters the thymus at a more differentiated stage and might be involved in positive thymic selection \(^{42}\). In addition, a DC population with phenotypic and functional properties of PDCs is also present in the mouse thymus, but it's in vivo role is still obscure \(^{43}\).

The mouse liver contains precursors with different DC generation potential in vitro, depending on the growth factors used. Hepatic non-parenchymatous cells cultured in GM-CSF +/- IL-4 generate CD11c+/CD11b+ MHCII+ DCs with high allostimulatory capacity, similar to their bone marrow-derived counterparts \(^{44}\). When cultured in IL-3 and CD40 agonist, these liver cells generate CD11c-CD11b-MHCII+ B220+ DEC-205+ DCs. Despite the expression of MHCII and costimulatory molecules, these CD11c- B220+ DCs are poor stimulators in MLRs and induce both T-cell apoptosis as well as regulatory T cells \(^{45}\). Another group could purify CD8\(\text{-}\)/CD11b- and CD8\(\text{+}\)/CD11b+ DCs from murine liver. Both
subsets could be expanded \textit{in vivo} by Flt-3L injection and possessed similar strong allostimulatory potency and lymph-node homing property. In a recent study in which enzymatic tissue processing was avoided, 4 subsets of hepatic CD11c+ DCs were identified: 2 PDC-like subsets (B220+ CD4+/−), 1 myeloid type subset (B220- CD11b+) and one subset containing both myeloid and lymphoid elements (B220- CD11b+).

Figure 2. Developmental pathways of mouse dendritic cell subsets, as derived from the current literature [see main text for references]. HSC: hematopoietic stem cell, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, GMP: granulocytic-monocytic progenitor cell.
Human dendritic cells

Studies of human DCs are obviously complicated by the limited tissue availability. Hence most of the knowledge is derived from in vitro culture systems using a convenient source of DC precursors, i.e. peripheral blood.

General phenotype

Human DCs are commonly identified by the expression of MHC class II (HLA-DR is commonly used) and the absence of T, B, NK cell, monocytic and granulocytic lineage markers (i.e. negative for CD3, CD19, CD56, CD14, CD16). Also, CD4 is expressed on virtually all human DCs known to date. Different DC subsets are then examined within the HLA-DR+, Lin- cells. CD11c is not as ubiquitous as it is on mouse DCs, but is restricted to "myeloid" human DCs. The human plasmacytoid DC [see below] expresses no CD11c in contrast to its murine counterpart. As a somewhat confusing convention, myeloid DCs are often tagged as DC1 and their monocyteid DC precursors as pre-DC1. Meanwhile plasmacytoid DCs are referred to as pre-DC2, which then mature into DC2. This nomenclature originates from a study in which DC1 and DC2 appeared to prime Th1 vs Th2 responses respectively 54, a paradigm which needed to be revised later.

Ontogeny and main subsets

Two main developmental pathways have been extensively described in vitro. In the first, DCs are differentiated from peripheral blood monocytes after 1 week incubation in the presence of GM-CSF and IL-4 (first described by Sallusto et al 55). The resulting differentiated, yet immature DCs bear the myeloid DC markers mentioned above but have relatively low levels of MHCII and T-cell costimulatory molecules. When given an additional stimulus [e.g. TNF-α, CD40 ligation, TLR4 ligands such as LPS], these cells quickly mature and upregulate high amounts of surface MHCII, CD40, B7-1, B7-2. Additional specific markers of mature DCs are the immunoglobulin superfamiliy member CD83 56, the 55 kDa actin-bundling protein fascin 57,58, and the DC-specific lysosome-associated membrane protein DC-LAMP 59. Because of the relative ease with which they can be generated, monocyte-derived DCs are the primary starting material for the increasing number of DC-based immunotherapeutic clinical trials. Recently, a protocol was described in which fully differentiated DCs could be generated from human monocytes within 48h 60. This timeframe might reflect the in vivo differentiation of tissue DCs from freshly recruited monocytes more realistically. Another study described an equally rapid development of DCs from blood CD14+/CD16+ monocytes. This phenomenon occurred within 3 days of culture and did not require the addition of external cytokines, provided the DCs were still in contact with PBMCs 61.

An alternative differentiation system starts from CD34+ hematopoietic stem cells (usually purified from umbilical cord blood) which are cultured in a combination of GM-CSF + SCF + TNF-α (introduced by Caux et al 62). Two CD34+ precursor populations can be distinguished on the basis of CLA expression, a molecule involved in adhesion to skin endothelium. After 12 days, CD34+/CLA+ progenitors differentiate into cells resembling human Langerhans cells or intra-epidermal DCs [especially with the addition of TGF-β in late stages of the culture]. Typical markers of human LCs are langerin, CD1a and E-cadherin, while
CD14 (a monocyte marker) is lost. CD34+/CLA- progenitors differentiate into CD14+ intermediates which probably correspond to peripheral blood monocytes. In the continued presence of GM-CSF and TNF-α, these cells give rise to “interstitial” type DCs. In contrast to LCs, these cells typically express CD68 and coagulation factor Xlla, while they are negative for langerin and E-cadherin.

Next to these “classical” DC culture systems, other studies suggested alternative pathways for DC development. It was found that DCs could be generated starting from CD34+ Lineage- CD10+ human bone marrow progenitors with exclusive potential to generate T-, B-, NK cells and DCs but no myeloid elements. This implies that at least some human DCs could have a “lymphoid”-related origin. In addition, peripheral blood monocytes could be directly differentiated into Langerhans cell-like DCs as well. This seemed critically dependent on the presence of either TGF-β or IL-15 (combined with GM-CSF). All of these cytokines are produced in epithelial environments, which suggests that local factors could drive the differentiation from monocytes into LCs in vivo. This property of monocytes has led to the discovery of a specific CD11c+/CD1a+ peripheral blood monocytic subset which forms a direct precursor pool for LCs. Finally, using an in vitro tissue model, Randolph et al discovered that monocytes could quickly differentiate into bona fide DC by simple migration across an endothelial layer into a subendothelial matrix, followed by a transendothelial migration in the reverse direction. This process simulated the extravasation of monocytes from blood vessels into the extracellular matrix, followed by the entry into lymphatic vessels. Monocytes that did not transmigrate and remained in the subendothelial matrix became resident macrophages. The discovery of this novel mechanism of DC development which primarily relies on cell-matrix interaction stresses the role of the local tissue environment in modulating DC homeostasis.

Human blood also contains a Lin- CD11c- subset of DC precursors with a plasmacytoid morphology. This plasmacytoid dendritic cell expresses surface IL3 receptor (CD123), CD45RA and the novel blood DC markers BDCA-2 and BDCA-4, CD11c+ human myeloid DCs express BDCA-1 and BDCA-3. In addition, human PDCs contain message for pre-TCRβ chain, suggesting a lymphoid origin. However, human PDCs selectively express Spi-B, a hematopoietic transcrption factor which promotes PDC generation but suppresses the development of T, B and NK-cells. The human plasmacytoid cell, which was also found in the T-cell areas of lymphoid organs, was first considered a type of T-cell until Grouard et al discovered its "true" dendritic cell nature. When matured using IL-3 and CD40-ligation, the human PDC developed a typical dendritic morphology and upregulated surface MHCII and costimulatory molecules, correlating with a highly potent allostimulatory capacity. Since then it was discovered that the PDC is activated by a different set of pathogenic stimuli than its myeloid counterpart. This reflects a differential expression of specific TLRs on both DC subsets: CD11c+ human myeloid DCs primarily express TLR2 and TLR4 and mature in response to LPS and mycobacterial cell wall components. In contrast, CD11c- plasmacytoid DCs do not bear TLR4 receptors but typically express TLR3 and TLR9, rendering them sensitive to viral dsRNA and bacterial CpG oligonucleotides respectively. Just like their murine counterparts, stimulated human PDCs produce massive amounts of type I interferon, proving that PDCs are in fact identical to the long-known natural interferon producing cell.

A general concept of human DC ontogeny and development is summarized below.
Figure 3. Developmental pathways of human dendritic cell subsets, as derived from the current literature.
The “sentinel” paradigm of dendritic cell biology

A global picture sketching the physiological role of the dendritic cell system is emerging from experimental evidence accumulated over the years.

In the steady state, DCs or their precursors are constantly recruited from the blood into antigen-exposed tissues at a pace which seems to be organ-specific [slow in the skin, fast at mucosal surfaces]. Resting DCs are directly attracted by a whole array of inflammatory chemokines, complement cleavage products, microbial molecules (e.g. fMLP) and beta-defensins 74,75, which maximizes the targeting of these cells to endangered organs. Nonetheless, the chemokine/chemokine receptor combinations responsible for the non-inflammatory or “steady-state” recruitment of DCs are still poorly defined.

DCs residing in antigen-exposed tissues [e.g. skin, airways, gastro-intestinal tract, marginal zones of spleen] are considered “immature”. This implies a high capacity to sample antigen by endocytosis, but a very low efficiency at processing presented antigen on surface MHC molecules 8. In fact, at this stage most peptide-MHC complexes formed intracellularly are targeted to lysosomal destruction 77. Meanwhile, expression of T-cell costimulatory molecules on the cell surface is typically low as well. Immature DCs express a whole array of endocytic receptors, including Fc-receptors, receptors for opsonizing complement and lectin-like receptors (e.g. mannose receptor, DC-SIGN, DEC-205, Langerin, BDCA-2 – reviewed in 79). In addition, immature DCs display a battery of Toll-like receptors which are primitive sensors for a number of “danger signals”, such as typical microbial signatures as well as products released by cell necrosis and extracellular matrix breakdown (reviewed in 79). When antigen is encountered together with these danger signals, a series of dramatic changes is induced which is commonly referred to as “DC maturation”: (i) the antigen uptake and processing machinery is shut down (endocytic receptors and TLRs are downregulated), (ii) large amounts of MHC-peptide complexes redistribute to the cell surface, along with a whole array of T-cell costimulatory molecules (e.g. B-7 family members) 80. (ii) a radical shift in chemokine receptor expression occurs: DCs loose all responsiveness to CC-chemokines and only respond to chemokines expressed by lymphatic vessels and T-cell areas of draining lymphoid organs (i.e. SLC, MIP-3[@]) 81. The result is a highly immunogenic migratory cell which carries a “snapshot” of one specific dangerous encounter in the periphery and navigates to the very place were optimal reporting to T-cells can occur. The dose of antigen, as well as the nature of concomitant environmental factors present at the time of antigen encounter determine the type of the resulting T-cell response, most notably T-helper 1, T-helper 2 or regulatory T. Importantly, these outcomes are not predetermined by the subtype or lineage of the DC 82,83, which supports the “functional plasticity” concept of DC differentiation mentioned earlier. Bacterial antigens and intracellular parasites usually induce the production of DC-derived IL-12 which results in a T-helper 1 response. In contrast, molecular signatures of extracellular parasites (e.g helminths) cause the DC to direct a T helper 2 response through mechanisms which are not well elucidated 84. Tissue-derived mediators (e.g. PGE2, TSLP) can also push the DC towards preferential Th2-induction 85,86. Viral infections trigger the maturation of plasmacytoid DCs into DC2 with concomitant release of large amounts of IFN-[@] 87. Meanwhile, fragments from dying infected host cells are taken up by DCs. Viral material is then processed and "cross-presented" onto MHC Class I molecules, leading to the priming of virus-specific cytotoxic T-cells. Only DCs possess the necessary intracellular molecular machinery to achieve this unique cross-presentation mechanism (reviewed in 87).

Though it constitutes a very efficient defense system, the sentinel function of DCs carries an inherent
danger. Invasion by pathogens is usually accompanied by tissue destruction and there is a high risk that activated DCs might take up and (cross-)present released self-antigens, leading to the induction of autoimmunity. This is especially worrisome as experimental evidence points to the necessity for maintaining peripheral tolerance. Fortunately, it appears that DCs play an active role here as well. Indeed, apoptotic cell fragments originating from normal tissue turnover are actually taken up and processed by DCs \(^{88-90}\). Those DCs which present self-material and home to the T-cell areas in the steady state lead to the priming of immunosuppressive regulatory T cells or even T cell deletion \(^{91-94}\). In some studies it was suggested that CD8\(^+\) mouse DCs are capable of inducing apoptosis of peripheral self-reactive T-cell clones through a Fas-dependent mechanism, similar to central tolerance induction by thymic DCs \(^{95}\). In summary, the dendritic cell system of leucocytes has evolved into a fine-tuned, highly sensitive sentinel network capable of both igniting as well as shutting down immune responses as appropriate. Central to this task is the ability of DCs to rapidly traffic to endangered tissues and subsequently migrate to T-cell rich organs. In the following chapters, we will describe how this sentinel paradigm applies to the lung, with a special emphasis on the kinetics of pulmonary DC trafficking.
**Figure 4.** The "sentinel" paradigm of dendritic cell biology. Injury to body surfaces induces the release of numerous DC-attracting mediators, mostly inflammatory CC-chemokines. Immature DCs are recruited out of the blood circulation and migrate through interstitial tissues to the site of antigen entry and tissue damage. At this stage, DCs are in an Ag-sampling mode: they express numerous receptors for pathogen recognition and endocytosis, but are inefficient in Ag-presentation. Tissue damage causes the release of secondary danger signals which triggers a series of dramatic changes: (i) DCs downregulate all chemokine receptors except those for chemokine gradients emanating from secondary lymphoid organs, (ii) Ag-capturing receptors are downregulated while high amounts of peptide-loaded MHC and costimulatory molecules appear on the surface. These immunogenic DCs home specifically to the T-cell zones of draining of lymph nodes, where they recruit and activate Ag-specific T-cells.
Biology of lung dendritic cells

General aspects
The lungs represent a vast interface between the body and the outer world. Thousands of liters of air pass through the airways each day, carrying numerous airborne particles. This represents an enormous challenge for the immune system: “firing up” immune responses inappropriately could have devastating results for the fragile gas exchange structures. A delicate balance between tolerance or active immune response must be maintained throughout. Therefore it is not surprising that the lungs are extensively populated with different types of dendritic cells. Sertle et al.\cite{96} was one of the first to describe the presence of DCs in the airway epithelium, lung parenchyma and visceral pleura of human and mouse specimen. These cells displayed a typical dendritic morphology, expressed copious amounts of MHCII on their surface and were potent stimulators in a primary MLR. This finding has spurred numerous studies on the biology of pulmonary dendritic cells in health and disease.

In the next paragraphs we will review the factors which control the homeostasis of pulmonary DC populations.
1. Lung DC recruitment
The "steady-state" situation

The large majority of studies around DC recruitment to the lung has focused on inflammatory states, while little is known about steady-state trafficking. Efficient respiratory immunosurveillance requires a constant pool of pulmonary DCs. Maintaining this pool implies a continuous replenishment as lung DCs permanently emigrate to the mediastinal lymph nodes in the steady state (as will be discussed later).

Kinetics of DC recruitment into non-inflamed lungs

Holt’s group was the first to demonstrate that respiratory tract DCs are continuously replenished by a steady-state bone marrow output. Destroying the production of bone marrow progenitors by gamma-irradiation resulted in a rapid decline in the number of airway DCs, which was supposed to result from the persisting emigration of these cells to the mediastinal LN. The estimated turnover of airway DCs was in the order of 3 +/- 1 days, which was similar to the fast turnover of gut mucosal DCs and much slower than epidermal LCs (several weeks). However, DC turnover seemed to be heterogenous within the lung as well: tracheal DCs “cycled” more rapidly than lung parenchymal DCs deeper in the respiratory tract. Interestingly, despite bone marrow shut-down about 20% of DCs could still be detected in the airways after 3 days and their numbers declined slowly hereafter, which could point to the existence of a long-lived subset of DCs in the airways. After protecting the trachea from the direct effects of radiation and in the absence of bone marrow transplantation, a progressive repopulation of the airways by DCs was observed, suggesting the presence of local pulmonary progenitors with in situ proliferative and DC-generation potential. Such mechanism has been recently reported in the skin, as local cell division is the sole mechanism by which epidermal Langerhans cells appear to regenerate under non-inflammatory conditions. Moreover, steady-state pulmonary alveolar macrophage populations are predominantly maintained by in situ cell proliferation, and to a lesser extent by recruitment of monocytic precursors from the circulation. In contrast, the rapid turnover of pulmonary DCs suggests a mechanism allowing a fast recruitment of DC precursors from the circulation. An interesting finding is that the pulmonary vasculature contains relatively more direct DC precursors than the peripheral blood circulation. This pool of precursors combines a close anatomical relationship to lung tissue as well as the potential to differentiate rapidly in strongly immunogenic DCs.

Factors controlling steady-state recruitment

How does the lung "order" the necessary quantity of precursors from the bone marrow to maintain its average dendritic cell capital? Very little is known, and the factors identified so far do not draw a clean line between inflammatory and non-inflammatory states. With this regard, it can be questioned whether mucosal surfaces can be considered as totally devoid of inflammation in a "normal" real-world situation. Indeed, the constant exposure to inhaled antigen and other non-antigenic stimuli, combined with the delicate physical separation between the outside environment and the underlying tissues could account for a continuous low-level of immunological and inflammatory stimulation. In support of this concept, studies performed in rats have identified two factors playing a role in the steady-state density and distribution of respiratory tract DCs:

Age: Although MHCII+ DCs already colonize the fetal lung mesenchyme, the airway DC network only develops a few days after birth, reaching adult-like density and distribution after weaning. Interestingly,
DCs appear in a “wave” which starts at the nasal mucosa and progressively spreads deeper into the trachea, down to the alveolar walls. Also, the intensity of MHCII staining used to identify the DCs seems to correlate with the proximity to the outside environment.

**Environmental air quality:** animals which are housed in specific pathogen-free but dusty conditions show an increased influx of DCs into the lung. Nevertheless it can be questioned whether both factors are totally independent, i.e. whether the development of the airway DC network after birth is due to the exposure to environmental air. Only a few studies have addressed the molecular events controlling DC recruitment into non-inflamed lung tissue. It is assumed that the extravasation of blood-borne DCs [or their direct precursors] into target tissues follows the same sequence of events as transendothelial leucocyte movement in general, i.e. the classical model of rolling-arrest-diapedesis [see 102 for a review]. Briefly, the initial steps involve “rolling” (i.e. loose adherence under flow) which is mediated by endothelial selectins binding glycosylated receptors on the leucocytes. This slows down the leucocyte allowing it to sense inflammatory signals displayed on the endothelial surface or emanating from the extravascular compartment. Typically, chemokine gradients originating from the subendothelial compartment cause activation of leucocyte \([\beta]_2\) and \([\beta]_4\) integrins, which leads to firm interaction with Ig-superfamily intercellular adhesion molecules on the endothelium and cell arrest. The final steps involve PECAM-1-mediated diapedesis between endothelial cells and breakdown of the endothelial basement membrane by MMPs. The relevance of these molecular events to steady-state pulmonary DC recruitment will be discussed here.

**Adhesion molecules**

It is known that specific adhesion molecules expressed on the endothelium play a crucial role in the tissue-specific homing of leucocytes. This is best exemplified by studies in T-cell trafficking: homing to the skin is mediated by the PSGL-1 isoform CLA which binds E-selectin on skin endothelium; homing to the gut involves \([\alpha]_4[\beta]_1\) integrin binding MadCAM-1 on gut mucosal endothelium; homing to peripheral lymph nodes relies on L-selectin (CD62L) interaction with PNAd expressed on the high endothelial venules. No lung-specific addressin has been documented yet, although memory T-cell homing into BALT structures was critically dependent on \([\alpha]_4[\beta]_1\)-integrin/VCAM-1 interaction. BALT high endothelial venules express high amounts of VCAM-1, which is unique among secondary lymphoid tissues 103. Also, T-cell adhesion to resting bronchial vessel endothelium appeared to be predominantly mediated by PSGL-1/P-selectin interactions 102. The same molecules seemed to be involved in T-cell homing to inflamed human airway endothelium 103. As far as dendritic cells are concerned, steady-state DC numbers in the lung are not affected by E- or P-selectin gene deletion 106, which is in contrast to studies on DC interaction with skin endothelium 107. Rather, CD18-knockout mice showed a decreased number of DCs within resting lung tissue, while ICAM-1 gene deletion had no such impact 104. This suggested that \([\alpha]_l\)-integrins on circulating DCs could interact with the pulmonary vascular endothelium through other molecules than ICAM-1. Still, it is surprising to find that bone marrow-derived DCs, which express copious amounts of \([\alpha]_l\)-integrins and ICAM-1, fail to home to resting or inflamed lung after intravenous transfer 108 and our unpublished observations]. This warrants the search for additional mechanisms of DC transmigration across pulmonary post-capillary vessels. With this regard, a novel DC-specific mechanism of adhesion to
endothelium was recently described by Geijtenbeek et al and involved interaction between DC-SIGN and ICAM-2 \(^{127}\). DC-SIGN is a C-type lectin expressed by circulating blood DCs and intrapulmonary DCs \(^{108}\), while ICAM-2 is a typical LFA-1 ligand widely expressed on the vascular surface. Surprisingly, under flow conditions reproducing the hemodynamic environment of post-capillary venules, DCs appear to use DC-SIGN exclusively rather than LFA-1 for tethering and rolling on ICAM-2-coated surfaces. In addition, DC transmigration induced by SDF-1 (a constitutive chemokine) across resting endothelium is mostly mediated by DC-SIGN and only to a minimal extent by \([\alpha_4]\) and \([\beta_2]\) integrins \(^{107}\). Future studies using targeted gene disruption of a murine DC-SIGN homolog \(^{109}\) or specific agonists will determine whether this molecule plays a role in the recruitment of lung DCs. Another possibility which has not been explored yet is whether the lung-specific endothelial adhesion molecule Lu-ECAM-1 \(^{110}\) plays a role in mobilizing circulating DCs. Although this molecule has been solely studied in the context of lung-homing melanoma metastases \(^{111}\), its possible role in lung-specific leucocyte homing is worth examining.

**Chemokines**

Several pulmonary cells are known to produce chemokines in a constitutive manner; however the specific chemokine signals that direct the steady-state entry of DCs into the lung are largely unknown. Stumbles et al showed that blocking CCR1 and CCR5, both chemokine receptors for RANTES, inhibited about 60% of the DC influx into resting airways \(^{112}\). It was suggested that the remaining 40% could be under control of CCR2 ligands like MCP-1. Interestingly, both RANTES as as well as MCP-1 were reported to be constitutively produced by airway epithelial cells \(^{113,114}\). Although MCP-1 is generally regarded as a chemoattractant for monocytes rather than for differentiated DCs, this chemokine could still play an indirect role by initiating the entry of monocytes which would then differentiate into DCs under the influence of local factors. Also, overexpression of MCP-1 in the basal layers of the epidermis causes increased colonization with Langerhans cells in the absence of inflammation \(^{116}\). The lung is also known to express MCP-4 \(^{117}\), the CXC chemokine SDF-1\(^{118,119}\) and PARC/DC-CK1/MIP-4 \(^{120,121}\) in a constitutive manner. These chemokines can attract immature DCs and could theoretically play a role in the steady-state recruitment of pulmonary DCs. Another candidate in this context could be fractalkine (also known as neurotactin or CX3CL), a chemokine which exists both in a soluble as well as in a membrane-bound form on the surface of endothelial cells and mucosal epithelia \(^{122,123}\). The membrane-bound form mediates strong adhesion of T cells, NK cells and monocytes to the endothelium \(^{124}\), while the soluble form is a known chemo-attractant for the same cells \(^{125}\). Fractalkine is also produced by mature DCs \(^{126,127}\), in addition it exerts a chemotactic effect on these cells \(^{128}\). Although fractalkine-knockout mice have no specific immunological defects \(^{129}\), mice deficient for the fractalkine receptor show a decreased steady-state homing of specific monocytic DC precursors into non-inflamed peripheral tissues including the lung \(^{130}\). Although structurally unrelated to chemokines, the pleiomorphic cytokine TGF-\(\beta\) is a potent chemoattractant for several leucocytes, including monocytes \(^{131}\). It can be effective at femtomolar concentrations through a mechanism of action different from that of chemokines \(^{132}\). Interestingly, TGF-\(\beta\) knockout mice have an absent network of epidermal Langerhans cell \(^{36}\). TGF-\(\beta\) is typically expressed by structural cells and epithelia in many organs In the lung, airway epithelial cells, fibroblasts and alveolar macrophages are important sources \(^{115,133,134}\). The role of TGF-\(\beta\) in the trafficking of pulmonary DCs has not
been investigated yet.

Matrix metalloproteinases (MMPs)

MMPs form a family of extracellular matrix-degrading enzymes \(^{125,126}\) which can be roughly divided according to a certain degree of substrate selectivity: collagenases (MMP-1, -8, -13, -18), stromelysins (MMP-3, -10, -11) and matrylsin (MMP-7) break down several types of collagen, gelatinases (MMP-2, -9) have a higher affinity for basement membrane components (laminin, collagen type IV) and metalloelastases (MMP-12) degrade cross-linked elastin networks. Other members of the MMP family are the membrane-type MMPs (MMP-14 to -17) which are involved in the activation of pro-MMP precursors, and ADAMs (A Disintegrin And Metalloproteinase) which play a role in the shedding of cell surface receptors or tethered ligands. MMPs are under control of tissue inhibitors (TIMPs) so that the net proteolytic activity depends on the ratio between MMPs and TIMPs in the local tissue environment. Finally, a receptor was described which specifically induces the transcription of certain MMP genes, among which MMP-1, -2, -9 and -3 \(^{127,128}\). This Extracellular MMP Inducer [EMMPRIN, Basigin] is a transmembrane molecule belonging to the Ig-superfamily and is frequently expressed in the context of inflammation or tumor invasion \(^{129}\). Indirect experimental evidence suggests that human DCs might express EMMPRIN as well \(^{130}\).

The role of MMPs in inflammatory leucocyte recruitment, tissue remodeling and activation or degradation of cytokines/chemokines has been extensively investigated. However, little insight has been gained on the in vivo role of MMPs in DC biology. Human monocyte-derived DCs have been reported to express and produce bio-active forms of MMP-9, especially after stimulation \(^{131}\). Moreover, differentiation of monocyte-derived DCs in culture was accompanied by an impressive upregulation of MMP-12 mRNA \(^{132}\), while exposure of these cells to live E. coli triggered the upregulation of MMP-7, -12 and -14 \(^{133}\). In the mouse, gene expression profiling of bone marrow-derived DCs revealed a maturation-dependent upregulation of MMP-13, TIMP-1 and EMMPRIN, while MMP-8, -12 and -14 remained unchanged \(^{134}\).

The first ECM component encountered by DCs after their extravasation is the endothelial basement membrane, which can be traversed by means of the gelatinases MMP-2 and/or MMP-9. Further progression of extravasated DCs into the interstitial matrix would be expected to occur through the production of collagen-degrading MMPs, while penetration into epithelial layers would again require gelatinase-mediated basement membrane degradation. Although never verified, MMP-2 would be a good candidate for the steady-state transendothelial and transepithelial recruitment of DCs as it is usually expressed constitutively in leucocytes, whereas MMP-9 is induced by inflammatory cytokines and chemokines \(^{135}\) and reviewed in \(^{114}\). Another candidate would be MMP-12, which in addition to it’s elastin-degrading activity mediates the invasion of macrophages into a basement membrane matrix in vitro and in vivo \(^{136}\). Subsequently, freshly recruited immature DCs might use the uPA/uPAr system to progress into the subendothelial matrix. uPAr has been detected on immature, unstimulated DCs and mediates the invasion of these cells into the ECM \(^{140}\). uPAr binds uPA which converts plasminogen into plasmin. Plasmin in turn can degrade several ECM molecules and activate pro-MMPs. The whole process is focused at the cell’s surface because uPA is mobilized to it’s membrane receptor and membrane-associated plasmin escapes the effects of free plasmin inhibitors in the surrounding matrix \(^{140}\).
Inflammatory recruitment

Kinetics

In a series of hallmark experiments, McWilliam et al reported that DC recruitment to inflamed airways was as fast as the prototypical neutrophil influx [150]. In some cases, such as inhalation of heat-killed *Moraxella catarrhalis*, the wave of DC influx into the airways came ahead of neutrophil recruitment and attained peak levels already 2h after challenge. This acute recruitment of airway DCs was an isolated phenomenon among mononuclear cells as macrophage numbers were not increased and lymphocytes were not present at that time point. The kinetics varied depending on the antigenic stimulus used (dead or live bacteria, virus, allergen) but remained 3 to 4 times faster than the steady-state turnover of airway DCs described above. This surprising discovery has a fundamental importance: it means that the classical view of host defense in which a first phase of neutrophilic influx is gradually taken over by a mononuclear infiltrate is not a universal feature. It indicates that dendritic cell recruitment is an integral part of the early phase of the innate immune response, with the potential to progress towards a powerful adaptive immune response.

Factors controlling DC entry into inflamed lungs

As would be expected, lung inflammation affects all the cellular-molecular events involved in the steady state trafficking of pulmonary leucocytes. A complex storm of cytokine, chemokine and protease interactions gradually builds up in patterns that are more or less specific for a given etiology (e.g. allergy, infection, auto-immune disease or allograft rejection). Those factors which are most relevant to pulmonary DC trafficking are presented here.

Adhesion molecules

There is no direct experimental data available on the role of adhesion molecules in the recruitment of DCs across inflamed pulmonary vessels. First of all, leucocyte extravasation is promoted in a non-specific way as a result of increased vascular permeability, and there is no reason to believe DCs should not be carried along in this process. In addition, it is well known that tissue inflammation, by means of potent cytokines such as TNF-[β], upregulates inducible adhesion molecules on the endothelium, e.g. VCAM-1, E-selectin and P-selectin. Even though selectins appear not to be involved in the entry of DCs into resting lungs, their role in inflammatory DC recruitment could be important (selectin-dependent DC extravasation has been described in inflamed skin [107]). Furthermore, a comparison between the contribution of selectins versus [β]- or [α], integrins in this process is warranted. Studies in which manifestations of allergic airway inflammation were successfully inhibited by [β]-integrin blockade [151] or selectin gene deletion [152,153] have not addressed the specific impact of this intervention on pulmonary DC populations. This is important as dendritic cells are critically required for the initiation and maintenance of the pulmonary allergic response [154,155]. Therefore, the potential existence of dendritic-cell specific endothelial adhesion mechanisms during pulmonary inflammation has important therapeutic implications.
Chemo-attractive factors

In asthmatic subjects, allergen challenge provokes a decline in the number of circulating blood myeloid DCs within a few hours\(^{159}\). At the same time a rapid accumulation of DCs occurs within the bronchial mucosa\(^{157}\). The fact that DCs can be mobilized massively and rapidly to the lung likely relies on two properties: (i) immature/circulating DCs express a whole battery of receptors for inflammatory chemokines and are therefore always “standby”, and (ii) a number of chemokines are probably used both for the inflammatory as well as the non-inflammatory recruitment of DCs depending on the expression levels in lung tissue (e.g. the CCR1, 2, 5 and CX3CR-ligands discussed under “Steady-state recruitment” are strongly upregulated during inflammation). Interestingly, the amplitude of transendothelial DC recruitment was shown to be dependent on the DC subtype\(^{159}\). CD11c+ “myeloid” human DCs were shown to vigorously migrate across vascular endothelium in the absence of any inflammatory stimulus, whereas the spontaneous transendotelial migration of IL3R+ plasmacytoid DCs appeared limited. This difference between both DC subsets was maintained when the transmigration was further stimulated by inflammatory chemokines.

An overview of DC-attracting chemokines expressed in the lung under inflammatory conditions is given in table 1.

Intriguingly, DC recruitment to inflamed lung seems to involve different chemokine receptors depending on the inflammatory stimulus. While the influx of DCs into rat airways exposed to Moraxella catarrhalis was CCR1 and CCR5-dependent, DC recruitment in response to inhaled Sendai virus or aero-allergen challenge was not\(^{159}\). An alternative may be provided by eotaxin and its sole receptor CCR3. Eotaxin is a chemokine produced by many types of cells in the lung\(^{153,159}\) and contributes to the eosinophilic airway inflammation typical of asthma\(^{157}\). Its levels are increased both locally\(^{153,159}\) as well as systemically\(^{164}\) after allergen exposure in sensitized animals. It was shown to be a chemo-attractant for rat respiratory tract DCs\(^{165}\) while its activity on human monocyte-derived DCs was variable\(^{81,166}\). Remarkably, *in vitro* experiments have revealed an unexpected side of eotaxin biology: it was found to stimulate the proliferation and differentiation of bone marrow granulocyte-monocyte colony forming units in a GM-CSF-independent way\(^{164}\). Later it was shown that a CCR3-expressing bone marrow progenitor with DC and eosinophil generation potential was specifically expanded during allergic airway inflammation\(^{157}\)\(^{167}\). It was suggested that eotaxin would mobilize these DC precursors from the bone marrow at an immature stage given that fully differentiated DCs within inflamed lungs were CCR3-negative. The possibility that airway inflammation might somehow signal the bone marrow to increase its DC precursor output was already suggested by our group\(^{159}\). It could be that in addition to its chemotactic activity, eotaxin might lead to increased DC numbers in allergic lungs through the induction of DC-precursor proliferation. The airway-derived eotaxin could exert its effects either remotely at the bone marrow level, or through the local proliferation of putative intrapulmonary DC precursors. MIP-1\(^{8}\) and MIP-1\(^{163}\) are yet other DC-attracting chemokines with an influence on hematopoiesis\(^{165}\). Both molecules can expand GM-CFU, but only in mature progenitors, at high concentrations and in the presence of the relevant CSF. This would mean that the *in vivo* proliferative effects of MIP-1 would only be felt by mature progenitors freshly recruited into inflamed peripheral tissues, i.e. close enough to the chemokine source. In addition, MIP-1\(^{8}\) was shown to amplify uPA-mediated invasion of DCs into the extracellular matrix\(^{165}\). A chemokine typically expressed by mucosal epithelia is CCL20/MIP-3\(^{170}\). It remains controversial
whether MIP-3β is expressed constitutively or only during inflammation. In human airway epithelial cells, MIP-3β expression is strongly enhanced by stimuli such as TNF, IL-1β, IL-4, IL-13 or ultrafine particulate matter. MIP-3β is a selective and potent chemoattractant for immature Langerhans cell-type DCs which express the corresponding receptor CCR6. TGFβ, a prototypical mucosal chemokine, not only promotes LC differentiation from monocyctic precursors but induces surface CCR6 expression as well. The CCR6/MIP-3β system has been implicated in the cellular organization and function of the gut mucosal immune system. Interestingly, CCR6 gene deficient mice fail to develop allergen-induced airway inflammation, but the impact of CCR6 deficiency on pulmonary DC recruitment has not yet been examined. A concept recently put forward implicates a MIP-3β chemotactic gradient in the fine-positioning of dendritic cells to epithelial surfaces when they are already mobilized within the mucosal tissue. In contrast, the recruitment of remote blood-borne DC precursors (which typically respond to CCR1, 2 and 5 ligands) was MIP-3β-independent. This concept was reinforced by the finding of a sequential expression of CCR2 followed by CCR6 in the course of LC differentiation. In addition, a compartmentalized production of CCR2 and CCR6 ligands was observed in inflamed mucosal tissues: MCP-4, a CCR2 ligand, was shown to be expressed in cells adjacent to blood vessels, while MIP-3β was specifically produced by epithelia in contact with the outside environment. It is worth verifying whether a similar sequential DC recruitment model operates in inflamed airways.

Finally, next to the chemokine family of proteins, molecular fragments derived from extracellular matrix breakdown are known to exert a chemotactic influence on leucocytes. Elastin fragments generated through the proteolytic activity of macrophage metallo-elastase (MMP-12) are strongly chemotactic for monocytes. As MMP-12 is expressed in DCs, it is tempting to speculate that the continuous inflammatory transit of DCs through elastin-rich alveolar regions maintains or even amplifies itself by means of chemotactic degradation products. As an indication for such a phenomenon, DC recruitment into inflamed lung interstitium and BAL is decreased in MMP-12-deficient mice (our unpublished observations).

**Cytokines**

It is difficult to discriminate the direct and indirect impact of inflammatory cytokines on DC recruitment. Many effects are probably mediated by the secondary release of tissue-derived chemokines or the upregulation of endothelial adhesion molecules.

Studies on the effect of gamma-interferon on pulmonary DC accumulation have yielded inconsistent conclusions. When given systemically, this product of T-helper 1 responses seemed to cause an increase in the number of MHCII+ cells in the lung parenchyma and airways of rats. However, it was not clear whether this phenomenon was due to de novo recruitment of DCs into the lung, or to the upregulation of MHCII on DCs which were already present. Later, it was shown that systemic IFNγ injection could actually impair the homing of blood-borne DCs towards the lung by activating Küppfer cells and promoting the trapping of injected DCs in the liver. Interleukin 2 could also play a role in the accumulation of pulmonary DCs during inflammation. Intratracheal instillation of IL-2 increases the number of DCs within the lungs of rat. Just like their thymic and epidermal counterparts, lung DCs were found to express low-affinity IL-2R, while in vitro IL-2 appeared to increase the motility of DCs and exert a chemotactic effect as well.
IL-16 is a cytokine with known chemotactic activity on cells bearing the corresponding receptor, CD4. In humans, this includes T-helper cells, eosinophils, monocytes and dendritic cells. IL-16 appears to be an inducible protein and its levels are elevated during allergic airway inflammation\(^1\). IL-16-producing cells in the lung include bronchial epithelial cells, type II pneumocytes and fibroblasts\(^2\)-\(^4\). In addition, mast-cells, eosinophils, activated T-cells, B-cells and dendritic cells themselves produce IL-16\(^5\)-\(^7\). This expression pattern creates an environment likely to amplify dendritic cell recruitment in the context of a Th2-dominated pulmonary inflammation.

**Matrix metalloproteinases**

MMP and TIMP activity are strongly modulated in inflamed tissue\(^8\). In addition to MMP-production by the migratory cells themselves, MMPs derived from activated stromal or epithelial cells could contribute to inflammatory cell invasion according to a cooperative model\(^9\). The effects of pulmonary inflammation (such as allergic airway disease) on MMP expression in DCs, and conversely the requirement of MMPs in the inflammatory recruitment of lung DCs are still largely unexplored. Increased MMP-9 output was observed in macrophages from bronchoalveolar lavage fluid of asthmatic patients\(^10\). However, it cannot be excluded that MMP-9 was chiefly produced by DCs, which are recruited more acutely into the airways than macrophages. As often in these studies BAL cell differentiation is performed on a simple morphological basis, which precludes any accurate distinction between macrophages and DCs. A role for uPA in inflammatory lung DC recruitment was suggested in studies involving lung pathogen clearance. Indeed, uPA-gene deficient mice exhibited a marked inability to clear pulmonary *C. neoformans* infection, a deficit which was accompanied by an impaired recruitment of lymphocytes and Mac-1+ cells into the lung.
<table>
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<th>constitutive / inducible</th>
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<td>CCR6</td>
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<td>-</td>
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<td>- / +</td>
<td>CD4 **</td>
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Table 1. List of chemokines produced in the lung with a known effect on dendritic cells. [*mouse, **human]
2. DCs within the Lung
Different phenotypes and different functions

The concept of one single lung dendritic cell type has proved to be far from reality. Instead, a remarkable heterogeneity in phenotype, function and anatomical position was already manifest in early reports. Anatomically, intrapulmonary DCs are found (i) associated with conducting airways (within or below the respiratory epithelium), (ii) in the connective tissue surrounding bronchi and accompanying vessels, and (iii) within the alveolar septa. The latter are generally referred to as “lung parenchymal DCs”. Some studies have made it possible to separate airway intra-epithelial DCs from the rest of the lung [see below]. However, in most experiments DCs are isolated from whole lung enzymatic digests. These studies mostly address the properties of lung parenchymal DCs, with airway intra-epithelial DCs forming a trace population. Because of their larger number, these “whole lung” DCs can be subjected to more extended investigations. Finally, DCs are present in very small quantities within the airway lumen. These cells can be recovered by standard bronchoalveolar procedures and increase in number in many lung pathologies. Future studies will undoubtedly bring more insight and a higher degree of complexity in our understanding of pulmonary DCs, both in terms of differentiation and relationship to other cells, as well as phenotype and function.

DCs associated with conducting airways

In humans, one of the first studies on pulmonary DCs reported the presence of dendritiform cells with strong HLA-DR positivity scattered within the bronchial epithelium. In this study, bronchial DCs were negative for Mac-1 (C3bi receptor) and the classical Langerhans cell marker CD1a. However later reports have shown that these cells express CD1a, contain Birbeck granules and were even Langerin positive. The number of intraepithelial airway DCs is increased in asthmatic subjects compared to healthy controls, and this number returns to control levels after inhaled corticosteroid treatment. A functional comparison between bronchial epithelial DCs isolated from healthy atopic vs asthmatic atopic donors yielded interesting results: the respiratory epithelium of asthmatic subjects was strongly enriched with CD1a+ DCs, and these cells preferentially induced the production of IL-4 and IL-5 from sensitized autologous T helper cells. Also, CD1a+ airway mucosal DCs from asthmatic subjects displayed increased surface levels of FceRI, the high affinity receptor for IgE which is classically found on the surface of mast cells.

In mice, intra-epithelial DCs (=Ia-positive dendritiform cells) are exclusively found in the upper airways (trachea and main bronchi). In smaller intrapulmonary bronchi, DCs are located just beneath the epithelial basal lamina and our unpublished observations. Mouse tracheal DCs were shown to bear Fc-receptors and perform as effective stimulators in antigen-specific proliferation assays. Further detailed functional studies on murine intra-epithelial respiratory tract DCs are practically absent because of the extremely low number that can be retrieved per animal.

Rats feature one of the best characterized airway intra-epithelial DCs to date. These cells were shown to express MHCII in situ and coexpress the β2 integrin subunit found on intra-epithelial T-cells. Airway inflammation induces an increase in their numbers as well as an upregulation of activation markers such as CD18. By their larger size compared to mice, rats have provided better means to extract and study
the function of respiratory tract DCs. Using a modified epithelial microdissection method, Gong et al managed to "flush out" the epithelium from a whole rat tracheobronchial tree \(^{179}\). Within these cells, they further isolated low-density, transiently plastic-adherent cells with dendritic morphology. These cells expressed MHCII albeit at lower level than DCs within the interstitium, which points to a more immature form [i.e. with a propensity to sample antigen]. Consistent with this idea, rat airway intra-epithelial DCs expressed Fc-receptor, while the co-stimulatory molecules LFA-1 and ICAM-1 were absent \textit{in situ} but were induced after a two-day isolation and purification procedure. Furthermore these cells were good in presenting soluble antigen to primed T-cells, but were relatively weak stimulators in a primary allogeneic MLR.

**DCs in the lung interstitium**

In humans, interstitial lung DCs were shown to have a typical immature DC profile: high endocytic capacity with high expression of mannose receptor, CCR1 and CCR5, low expression of costimulatory molecules (B7-1, B7-2 and CD40) and the mature DC marker CD83 \(^{180}\). In addition, a specific profile of adhesion molecule expression was observed: compared to peripheral blood monocytes, lung DCs displayed lower surface \(\beta_2\) integrins (LFA-1 and Mac-1). In contrast, \(\beta_1\) integrins (especially VLA-5), LFA-3 and ICAM-1 were increased compared to circulating monocytes, with LFA-3 playing an important role in T-cell costimulation.

In mice, DCs extracted from whole lung digests were found to express DEC-205, J11d (a thymic DC marker), the low-affinity IL-2R, Mac-1 and FcRII. Unlike lung macrophages they were non-phagocytic and F4/80 negative \(^{33}\). In a different report, murine lung DCs expressed high amounts of costimulatory molecules (B7-1, B7-2, ICAM-1, CD40L, LFA-1). However, the conclusions in this study are complicated by the lengthy isolation and purification procedures which are known to induce \textit{in vitro} maturation artefacts \(^{180}\). Rat lung interstitial DCs expressed high levels of MHCII and ICAM-1 but were practically devoid of FcR \(^{179}\).

In addition, these cells were poor in presenting soluble antigen to primed T-cells but strong stimulators in an allogeneic MLR. Taken as a whole, these properties strongly suggests that interstitial lung DCs are relatively more mature than DCs at the airway surface. However, the possibility that interstitial DCs are derived from airway DCs is difficult to reconcile with the differences in turnover speed (see section "3. Pulmonary DC emigration"). Functionally, these cells were shown to produce IL-10 in the steady state. When pulsed with antigen and adoptively transferred, they induced the \textit{in vivo} production of IL-4 and Th2-related immunoglobulins \(^{180}\). This prompted the idea that the default DC-mediated immune response to inhaled antigen is Th2-biased. However, experiments regarding the immune response induced in naive T cells should not involve intrapulmonary DCs but DCs isolated from airway-draining lymph nodes, as these are the privileged places of DC-T cell encounter in the priming phase (discussed below).

**DCs within the airway lumen**

The cellular content of the airway lumen (i.e. cells crawling within the epithelial lining fluid film) can be sampled through a bronchoalveolar lavage (BAL) procedure. In the absence of inflammation, quiescent alveolar macrophages account for the large majority of cells recovered, along with a trace population of
polymorphonuclear cells, lymphocytes and DCs. In humans, DCs were identified within a low-autofluorescent fraction of BAL cells \(^{206}\). These cells displayed a monocytoid morphology and acquired typical DC features after overnight incubation. A fraction of these expressed Langerhans cell markers (CD1a, S100) and exhibited potent T-cell stimulatory capabilities in an allogeneic MLR. The implementation of novel DC markers (Langerin, BDCAs) will provide a deeper insight into the human DC populations in the BAL.

A similar strategy based on autofluorescence led to the purification of BAL DCs from rat airways \(^{207}\). These cells, identified as low-autofluorescent MHCII+ cells with a spot-like alkaline phosphatase activity, exhibited a 100-fold increase in numbers after intratracheal instillation of BCG \(^{208}\). Similarly, our group described a massive increase of MHCII+ OX62+ BAL-DCs during allergen-exposure in allergen-sensitized rats \(^{209}\).

The reason for the transepithelial recruitment of DCs is still a mystery. The promotion of leucocyte transmigration in a basal to apical direction might be an inherent property of airway epithelial layers \(^{209}\), a property which can be further amplified by the addition of pro-inflammatory cytokines (TNF-\(\alpha\) or IFN-\(\gamma\)). DCs within the airway lumen have no equivalent in other antigen-exposed organs such as the skin or the gut. In addition, pulmonary immune responses feature an accumulation of lymphocytes along with DCs in the BAL compartment. This, in combination with the immediate exposure to aero-antigen, might provide ideal conditions for locally maintaining the immune response. It is not known whether DCs that have been recruited into the airway lumen can reverse transmigrate across the epithelium, back to the lamina propria, or become eliminated via the mucociliary escalator. \textit{In vitro} cultured DCs transferred into the trachea are capable of transmigration and even proceed towards draining lymph nodes, albeit in small numbers [see below] \(^{206}\). A recent study suggests that this emigration of airway DCs might be strongly delayed during certain immune responses. It was shown that a specific subset of murine BAL DCs (CD11c+ CD11b+ DEC-205+ CD40+ CD80+ Gr-1+) could still present aero-antigen-derived peptides several weeks after the last aerosol exposure, while antigen-presenting activity within the lung tissue rapidly waned over that time period \(^{205}\).

**Interactions with other pulmonary cells**

The transit time of DCs through the lung is likely influenced by interactions with cells encountered along the way. In addition, qualitative and quantitative properties of the lung’s extracellular matrix probably come into play. Pulmonary cells with the greatest known impact on DCs are discussed here.

**Airway epithelial cells**

The factors directing the preferential homing of some DCs into the respiratory epithelial layer are still unknown. As an indication of their homing pattern, rat respiratory tract DCs express the \(\alpha\) integrin subunit [recognized by the OX62 antibody] \(^{207}\). \(\alpha\), in association with \(\beta\), is expressed on veiled cells in rat lymph and on intraepithelial \(\alpha\)-T-cells of skin, gut and lung \(^{208}\). It mediates adhesion to surrounding epithelial cells through a heterophilic, heterotypic interaction with epithelial E-cadherin. Although never verified, homophilic E-cadherin interactions could also “anchor” DCs to the airway epithelium, in a manner similar to E-cadherin-expressing skin Langerhans cells \(^{207}\). A fundamental question remains: how can DCs
penetrate an epithelial barrier without disrupting the layer’s integrity? Indeed the local release of cytokines from stressed epithelial cells would immediately initiate DC emigration towards local lymphoid organs [see below]. An answer could lie in the fact that infiltrating DCs can replace inter-epithelial E-cadherin-mediated adhesion by similar interactions between epithelial cell and dendritic cell E-cadherin or a.b. This concept was confirmed in a recent study involving gut DCs. DCs infiltrating the gut epithelium expressed tight junctions proteins allowing them to open inter-epithelial clefts in a “zipper”-like fashion without disturbing the barrier function. In addition, it was shown that ligation of E-cadherin on DCs inhibits their maturation, which might provide an additional explanation for the retention of DCs in epithelial layers.

Bronchial epithelial cells were found to express a whole range of adhesion molecules constitutively including VLA-1 to –6, ICAM-1, -2 and -3. In addition, TNF- or IL-1 stimulation caused a strong upregulation of ICAM-1 and a de novo induction of VCAM-1. All these molecules can potentially interact with their respective partners expressed on the DC’s surface and could modulate the DC’s pulmonary transit time and/or immune function. An intriguing finding is the expression of Fas-L on the surface of bronchial epithelial cells. It is known that only immature DCs are sensitive to Fas-induced apoptosis. A tantalizing possibility is that bronchial epithelial cells might regulate airway DC homeostasis by providing in situ pro-apoptotic signals, adding an additional layer of complexity to the turnover of pulmonary DCs.

Epithelial cells are an important source for a whole array of soluble mediators with a known effect on DC function. GM-CSF, a crucial DC-poitetic growth factor, is produced at the basolateral side of alveolar type II cell and has been shown to enhance the T-cell stimulatory activity of isolated lung DCs. GM-CSF production is induced by inflammation at all levels of the respiratory tract. Epithelium-derived GM-CSF might affect pulmonary DC homeostasis in several ways: monocytes or immature DC precursors could be induced to differentiate (or maybe proliferate) in situ. As GM-CSF is also a maturational stimulus, the emigration of immunocompetent DCs towards draining lymphatic vessels would be accelerated. In addition, epithelial cells produce a number of mediators which can influence the type of immune response induced during DC-T cell encounters. Prostaglandin E2 can “instruct” maturing DCs to polarize responder T-cells towards a Th2-type response. Recently it was reported that human bronchial epithelial cells produce TSLP, an IL-7-related cytokine which is one of the most powerful stimuli for DC maturation and Th2 induction. Although studied more in detail in the context of atopic dermatitis, it would be interesting to see whether TSLP plays a role in the trafficking and immune function of airway DCs.

Pulmonary macrophages

Significant insight has been gained concerning the co-existence of pulmonary dendritic cells and macrophages. In the lung parenchyma, macrophages in the alveolar lumen and DCs within interalveolar septa are separated by a fraction of a micrometer. It has long been suggested that alveolar macrophages (AMs) inhibit DC functions through the production of soluble mediators. AM-derived nitric oxide was shown to inhibit MHCII expression on lung DC and suppress their allostimulatory activity. In MLRs incorporating lung DCs, bronchoalveolar lavage cells (mostly AMs) and T-cells, such inhibitory effect could be reversed by the addition of indomethacin, catalase and/or TGF- neutralizing antibodies, suggesting a role for AM-derived prostaglandins, H202 or TGF- in suppressing DC function. Other means by which AM could inhibit lung DC effector function have been described and include the production of soluble IL-1R, soluble TNF-R, or IL-10. Together, these data suggest that the immunostimulatory function of
pulmonary DCs is under tight control, which is not surprising given the potentially damaging consequences of inflammation in the lungs. Non-invasive aero-antigens can be taken care of by macrophages crawling over the airway surface, and these cells produce mediators that prevent the triggering of DC activation. However, damage to the surfactant-macrophage-epithelium barrier allows antigen to reach sentinel interstitial DCs and shifts the local cytokine environment in favour of DC activation and the initiation of an adaptive immune response.

Intriguingly, interstitial macrophages (IMs) can exert a supportive influence on pulmonary DC immune function. Enhancement of DC function by IM in the context of an allogeneic MLR has been related to cell contact-dependent mechanisms 218 as well as through soluble mediators 217. Alternatively, conditioned medium from IM cultures exposed to particulate antigen could enable the induction of antigen-specific T-cell proliferation by DCs which were not exposed to the antigen 219. This supports the "co-processor" model of DC-macrophage interaction in which macrophages first take up antigen and subsequently release processed peptides that are loaded on the MHC-rich surface of neighbouring DCs 219,220.

**Mesenchymal cells and extracellular matrix**

A large percent of cells in the lung consists of fibroblasts, so that the chances of encounter between these cells and migratory pulmonary leucocytes are high, especially during inflammation. Respiratory tract DCs, by their anatomical localization, are caught in the continuous cross-talk between fibroblasts and injured/regenerating epithelium which characterizes chronic airway inflammation 221. Recent insights indicate that fibroblasts are not passive players in the immune system (reviewed in 222). During inflammation, fibroblasts express chemokines, cytokines and adhesion molecules that influence the accumulation, activation state and apoptosis of mobilized leucocytes, and DCs should be no exception with this respect. Interestingly, molecular signals that initiate an adaptive immune response normally cause the termination of inflammation-sustaining functions of fibroblasts, which is a prerequisite for the resolution of tissue inflammation 222. Indeed, signalling through the RelB transcriptional activator is known to induce DC activation and maturation 223, while the same molecular signal paradoxically shuts down chemokine production by fibroblasts. This is best exemplified by the phenotype of RelB-knockout mice: stimulation by endotoxin causes a sustained activation of RelB-deficient fibroblasts which leads to the expression of a myriad of inflammatory chemokines, the massive accumulation of inflammatory leucocytes and finally multiorgan failure 224. Fibroblasts and myofibroblasts can express several molecules that are relevant to DC physiology. These include chemotactic factors such as MCP-1, MCP-4, SDF-1β, RANTES, MIP-1β, MDC, IL-16 etc.. 213,160,205,206. In addition it was found that fibroblast-derived factors, most notably TGF-β, induce the expression of CXCR4 on recruited leucocytes, which contributes to their retention in inflamed tissues 227. A similar mechanism might influence pulmonary DC trafficking in the setting of airway remodelling, a complex of pathophysiological changes due to long-standing allergic inflammation and which includes (myo)fibroblast hyperplasia along with increased ECM deposition. Remarkably, fibroblasts express CD40. Engagement of CD40 (e.g. by DCs or T-lymphocytes), upregulates surface ICAM-1, VCAM-1 and PGE2 synthesis 228 which in turn could all influence the behaviour of DCs. In addition, it was found that human lung fibroblasts express high levels of TSLP (cf supra), which provides an additional means to profoundly modulate the biology of pulmonary DCs.

A prominent function of fibroblasts is the synthesis of extracellular matrix (ECM) components. The ECM has a profound impact on the function of immune cells. The lung ECM is predominantly composed of
collagens type I, II and III in the interstitium (about 60% of total pulmonary ECM content). Cross-linked elastin monomers are the next most abundant matrix component, forming an interalveolar scaffold responsible for the mechanical properties [recoil] of the lung parenchyma. The ECM hydration level is regulated by proteoglycan (PGI) macromolecular assemblies with heparan sulphate accounting for all PGI content in the alveolar regions, while conducting airways contain predominantly chondroitin-6-sulphate. Fibronectin is an ubiquitous structural glycoprotein with an important role in modulating immune cell biology. It comes in two forms: a cell surface associated form and a plasma-derived form. The latter is especially increased in the context of inflammatory plasma exudation. Finally, the ECM is "littered" with various matrix-bound cytokines, chemokines and growth factors. These molecules are released from their "web" during inflammation or cellular migration, i.e. events that are accompanied by matrix-degradation. Also, chemokines bound to ECM induce haptotaxis, which is a directional migration of cells along an ECM "scaffold".

Experimental data on the role of ECM components in DC physiology is not abundant. Adhesion of murine DCs on type I collagen has been shown to upregulate surface HSA, the production of IL-1β and IL-6, and enhance T-cell stimulatory capacity. Adhesion to fibronectin or laminin did not produce the same effects. Type I collagen had a similar (TNF-α-dependent) maturation effect in human monocyte-derived DCs, while adhesion to fibronectin was lost during DC maturation. Interestingly, DCs isolated from rat lungs seemed to preferentially bind to fibronectin rather than collagen or laminin, and this adhesion was further promoted by TNF-α or IFN-γ. DCs also adhere to hyaluronan through the CD44 receptor. This interaction is not only necessary for migration [see below], but in addition hyaluronate breakdown products induce DC activation and release of TNF-α. A similar DC activation was observed when DCs were exposed in vitro to heparan sulphate degradation products and peptides. Taken together, these data clearly suggest that the ECM make-up of the lung could affect the phenotype and function of pulmonary DCs.

**Lymphocytes**

Under normal circumstances, lymphocytes are relatively scarce in the interstitial tissues of the lung, [except in BALT zones, which exhibit considerable species-dependent variation in size and frequency]. However, chronic airway inflammation is accompanied by the organization of prominent local lymphoid aggregates, a phenomenon which is probably supported by changes in stromal cell behaviour and the ECM, as described above. The chemokine networks that participate in the construction of such ectopic lymphoid organs have a marked influence on DCs. There is increasing evidence that SLC/60k/CCL21 is critical for the formation of organized lymphoid aggregates, and the same chemokine potently attracts both T-cells and DCs into these structures. DC/T-cell interaction, by means of CD40/CD40L engagement, induces DC maturation which involves an upregulation of CCR7 [the receptor for SLC], leading to more retention of DCs into ectopic mucosal lymphoid aggregates. IL-16 could also be involved in the process, as it is expressed by T-cells, B-cells and DCs alike. In fact, IL-16 was shown to contribute in a very large extent to the DC-attracting activity of activated T-cells or B-cells. Therefore, chronically inflamed airways containing lymphoid aggregates might theoretically affect pulmonary DC transit time by "hijacking" these cells on their way to genuine lymph nodes [a situation described in giant cell arteritis granuloma]. An additional mechanism which could modulate retention vs transit of lung DCs could be the processing of chemokines by lymphocyte-associated peptidases. For instance activated T-cells within
the bronchial mucosa express CD26, also known as dipeptidyl peptidase IV \textsuperscript{240}, an enzyme which was shown to cleave several DC-attracting chemokines such as CCL5/RANTES and CCL22/MDC into less active forms \textsuperscript{241,242}.

In any case, it is unclear whether local tissue inflammation initially induces increased DC efflux towards draining LN, followed by the formation of local DC-lymphocytes aggregates in the chronic phase, or whether both phenomena can continue to coexist. A recent study shed some light on this issue by examining DC-T-cell interactions in the airway mucosa of rats sensitized and exposed to allergen \textsuperscript{243}. Aero-allergen exposure led to an influx of DCs and T-cells into the airway mucosa. Both cells formed clusters, resulting in the upregulation of costimulatory molecules on the airway mucosal DCs. This interaction was followed by an efflux of DCs to the thoracic lymph nodes, leaving activated mucosal T-cells in their wake. Interestingly, the time frame of T-cell activation corresponded to the interval leading to a late phase bronchoconstrictive response typical of asthma.

**Miscellaneous cells...**

The lung contains a myriad of other cell types which could potentially interfere with DC biology. A few examples are given in this section.

**Nerve cells:** An extensive network of non-adrenergic-non-cholinergic (NANC) sensory nerve fibers runs under the basement membrane of the airway epithelium. These nerve endings contain neuropeptides such as substance P (SP), neurokinin A (NKA), calcitonin gene-related peptide (CGRP) and vasointestinal peptide (VIP). Receptor-mediated binding of SP has been reported on skin \textsuperscript{244} and pulmonary DCs \textsuperscript{245}, while it was recently shown that DCs actually express NK1-R, the main receptor for SP \textsuperscript{246}. Although one study reported a suppressive effect of SP on skin LC function in an allo-MLR \textsuperscript{247}, more recent experimental evidence suggests that this neuropeptide supports the immunostimulatory function of DCs \textsuperscript{248}, which is consistent with the fact that SP induces NFκB activation in these cells \textsuperscript{249}. Moreover, SP release contributes to the recruitment of DCs to the lung during a secondary pulmonary immune response, while *in vitro* SP exerts a chemotactic influence on purified lung DCs \textsuperscript{250}. CGRP appears to have a general suppressive effect on DCs: it reduces the antigen-presenting function of LCs *in vivo* and *in vitro* \textsuperscript{251}, it inhibits the LPS-induced release of IL-12 \textsuperscript{252} and decreases the expression of MHCII and T-cell costimulatory molecules \textsuperscript{253}. Just as in the skin \textsuperscript{254}, the close proximity of NANC nerve fibers to airway epithelial DCs allows for a potentially important role for CGRP in restraining airway DC activation. VIP is released during pulmonary immune responses and the corresponding receptors are expressed on infiltrating mononuclear cells in the lung \textsuperscript{255}. The role of VIP in DC biology is still largely unexplored. One study suggested a strong cooperative effect between VIP and pro-inflammatory cytokines such as TNF-\(\alpha\) in promoting phenotypical and functional DC maturation \textsuperscript{256}.

**Endothelial cells:** The lung endothelium is a chief producer of angiotensin (AT). AT is a vasoactive peptide generated from angiotensinogen through the action of angiotensin-converting enzyme (ACE) which is membrane-bound on the pulmonary vascular endothelium. Hence, DCs in close proximity to the pulmonary endothelium (e.g. DCs in the interalveolar septa) are exposed to high concentration of AT, a situation unique for this tissue environment. In a recent report, it was shown that monocytes and monocyte-derived DCs express the two known receptors for AT, and that AT contributes to the proper differentiation and maturation of monocyte-derived DCs through binding with the AT-1 receptor \textsuperscript{257}. Hence, the influence of the ACE-AT system on pulmonary DC function is probably worth investigating.
Mast cells: The lungs form an important reservoir of mast cells (500 – 4000 / mm³ in human lung, 10⁷ – 10⁸ total number in monkey lungs), which are distributed along the respiratory tract in the bronchial submucosa, epithelium, perivascular tissue and bronchoalveolar compartment. Because mast cells share the same anatomical “niche” with DCs (i.e. at the interface between the host and the environment), there is a substantial possibility that both cell types would interact. Histamine, a chief product of mast cell degranulation (typically after allergen-induced crosslinking of surface IgE) was shown to have a significant effect on DC biology. DCs express histamine receptors 1 to 4. Exposure of immature DCs to histamine induced phenotypical and functional maturation, with increased expression of costimulatory molecules (albeit transiently), production of inflammatory chemokines and enhanced capacity to induce memory T-cell proliferation. However, chemokine receptors for homing towards lymphoid organs were not upregulated. Moreover, the pattern of cytokines produced by the DCs was altered: IL-12 secretion is inhibited in favour of IL-10, and histamine-exposed DCs preferentially induced T-helper 2 responses. Finally, histamine exerts a chemotactic effect on DCs. Another major product of pulmonary mast cells is prostaglandin D (PGD). This pro-inflammatory lipid metabolite was shown to have a unique effect on DC biology: PGD. profoundly blocked TNF-α-triggered emigration of LCs towards LN, while phenotypical maturation could proceed normally in situ (for a review of prostaglandin effects on DCs, see ). The discovery that pulmonary mast cells express functional CD40L (which could affect DC migration), constitutively store TNF-α, produce GM-CSF and chemokines such as CCL2/MCP-1, CCL17/TARC and CCL22/MDC suggest additional means to interact with mucosal DCs, especially during allergic inflammation. Recently, it was shown that mast cells release exosomes enriched in exogenous antigen as well as heat-shock proteins. These mast cell-derived exosomes were shown to transfer antigen to DCs and induce their phenotypical and functional maturation. Finally, mast cells appear to be the only hematopoietic cells to produce TSLP, which endows these cells with yet another way to activate DCs and promote robust Th2 responses.

Other DCs: activated DCs are an important source of chemokines which are themselves active on immature DCs. Upon activation, human monocyte-derived DCs rapidly and transiently produce MIP-1α and MIP-1β, while RANTES and MCP-1 are produced in a more sustained fashion. More relevantly, resting respiratory tract DCs from the rat express MIP-1α, MIP-1β, and MCP-1. Whether IL-16 production from lung DCs contributes substantially to further DC recruitment has not been established yet. The production of DC-attracting chemokines by the DCs themselves could have two consequences: (i) a paracrine effect, in which the inflammatory influx of activated DCs would cause the chemotactic recruitment of even more DCs; (ii) an autocrine effect, in which desensitization of chemokine receptors in an autocrine fashion would slow down trafficking or allow the cell to respond to other chemokines and proceed to the next tissue compartment.
Figure 6. Pulmonary dendritic cells can interact with many cell types in the conducting airways and lung parenchyma: 1. bronchial epithelium, 2. pulmonary alveolar macrophages 3. interstitial macrophage 4. (myo)fibroblasts, 5. BALT lymphocytes, 6. NANC nerve endings, 7. capillary endothelium, 8. mast cells.
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<tr>
<td></td>
<td>GM-CSF</td>
<td>survival, differentiation/maturation</td>
</tr>
</tbody>
</table>

Table 2. Non-exhaustive list of main pulmonary cells with a potential effect on dendritic cells [see table 1 for chemokine mediators].
2. Pulmonary DC Emigration
This section explores the final element of the sentinel DC paradigm in the context of the pulmonary immune response: i.e. the transmission of antigenic information from the airways to the T lymphocytes of thoracic lymphoid organs.

**Kinetics**

The progressive disappearance of DCs from the airways of rats after bone marrow destruction already pointed to a steady-state DC emigration towards thoracic lymph nodes. A similar steady-state DC flux has been described in the gut as well: a continuous output of veiled cells could be observed in lymph draining the gut in the absence of any inflammatory stimulus. Again, the steady-state kinetics of DC emigration were the fastest in lung and gut (halflife of a few days) and much slower in the skin (halflife of a few weeks). Similar to DC influx into non-inflamed lungs, the factors triggering the emigration of DCs from resting airways are still unclear. As suggested earlier, mucosal surfaces could be considered in a state of continuous low-grade inflammation. The exquisite sensitivity of DCs in response to environmental stimuli or stress signals in the surrounding tissue might thus account for their fast transit time at mucosal surfaces. The difference in trafficking speed between airway intra-epithelial DCs and lung parenchymal DCs is also subject to speculation. It is not clear at present whether these differences rely on separate lung DC ontogeny, or result from local factors such as a higher exposure to stimuli in the upper respiratory tract compared to the alveolar spaces. Alternatively, there could be a compartmentalized production of cellular mediators which can either stimulate or inhibit DC migration. For instance the close proximity of lung parenchymal DCs to alveolar macrophages exposes the former to AM-derived IL-10 which exerts a restraining influence on DC migration. An additional answer for the slower trafficking of lung parenchymal vs airway DCs could be found in the anatomical distribution of the pulmonary lymphatic system: lymphatic vessels of the lung are predominantly localized around conducting airways and their accompanying blood vessels while they are absent in the alveolar regions. Therefore, parenchymal DCs would have to migrate from the interalveolar septa towards conducting airways in order to reach lymphatics. Alternatively, a tantalizing possibility exists in which interalveolar DCs would emigrate into the bloodstream. Careful examination of some experimental reports suggests such possibility. Pulmonary delivery of FITC-labeled Aspergillus fumigatus resulted in the appearance of FITC+ DCs both in draining thoracic LN as well as in the spleen, with comparable frequency and same kinetics. Moreover, sensitization to aero-allergen can proceed normally in animals devoid of thoracic LN as long as the spleen remained present, suggesting a transport of allergen in an immunogenic form from the lung to the spleen. It would be interesting to confirm whether such blood-bound migratory route exists and what the different immunological outcomes might be after aero-antigen encounter.

The classical migratory route from the airways towards draining thoracic LN was originally studied by intratracheal transfer of exogenous DCs. Havenith *et al* was the first to demonstrate that DCs and not alveolar macrophages were able to actively migrate into thoracic lymph nodes and efficiently sensitize T-cells. The precise immunological outcome of this process was further investigated by Lambrecht *et al*: intratracheal transfer of peptide-loaded DCs led to a rapid induction of peptide-specific T-cell proliferation and recirculation of primed memory T-cells to other lymphoid organs. Moreover, intratracheal instillation of ovalbumin-“fed” bone marrow-derived DCs led to the priming of Th2 lymphocytes in the TLN, and subsequent exposure of these animals to aerosolized ovalbumin induced a pronounced eosinophilic airway inflammation. A kinetics study showed that most instilled DCs remained in
the airway lumen, while a small number was able to cross the epithelium and reached a peak accumulation within the thoracic LN 36 h after intratracheal delivery. Indirect evidence that endogenous DCs might transport airway-antigen towards TLN was provided by Xia et al. DCs isolated from TLN displayed antigen-presenting activity 24 h after intratracheal instillation of soluble antigen. Following a novel approach introduced by our group [vide infra], a few reports appeared in which fluorescein-conjugated particulate antigens (heat-killed *Listeria mono-cytogenes* or *Aspergillus fumigatus*) were instilled, which confirmed earlier observations, i.e. a rapid transport of antigen to the TLN leading to efficient T-cell priming. Yet another method for monitoring pulmonary DC trafficking was introduced recently and involved delivery of the intravital dye CFSE into the airways. Although this method allowed to track the homing of DCs into the TLN as well as identify the migratory subsets, the kinetics were substantially different from all previous studies: in baseline conditions, the number of CFSE+ DCs in the TLN remained relatively constant over a long time period, i.e. no peak accumulation and decline in number could be observed. This is likely due to the long half-life of CFSE in the lung, so that freshly recruited DCs are continuously labeled before proceeding to the TLN. Interestingly, while the accumulation of CFSE+ DCs into the TLN could be amplified after airway viral infection, a second inflammatory stimulus failed to repeat the same phenomenon. This refractoriness might account for the post-viral state of immune-depression which would in turn increase the likelihood of bacterial surinfection.

It is generally considered that emigration of DCs from antigen-exposed sites is paralleled by DC maturation, including downregulation of antigen uptake capacity, upregulation of surface MHCII, costimulatory molecules and chemokine receptors for lymph-node homing [cf The "sentinel" paradigm]. However it is unclear at what stage of migration DCs start to mature, and whether both phenomena are unconditionally tied together. Chemical triggering of LC emigration induced clear signs of maturation while these cells were still residing in the skin: B7-2 was already upregulated on LCs in the epidermis. Although emigrating LCs within "dermal cords" showed signs of even further maturity, invariant chain (a marker for immaturity) was still co-expressed. Also, in a model of skin inflammation, DCs with the fully mature phenotype usually seen after homing into the LN were clustering with proliferating memory T-cells within the dermis. Conversely, DC emigration can occur in the absence of functional maturation, as was shown recently by Geissmann et al. Lymph nodes draining chronically inflamed skin showed an accumulation of LCs which had remained phenotypically immature (Langerin+ CD1a+ CD68+ B7-2-CD83- DC-LAMP-). In the same report, it was shown that TNF-α could induce a peculiar form of LC activation *in vitro*: CCR7 was upregulated, resulting in increased responsiveness to CCL19 and CCL21, while Langerin expression was maintained and costimulatory molecules were not induced.

**Molecular events**

The process of DC emigration towards local LN has been extensively dissected in the context of epidermal Langerhans cells and skin immune responses. Today it is largely unknown whether the factors involved in epidermal LC emigration can be transposed to airway DCs. These factors can be divided in (i) triggering factors, (ii) changes in cell-cell adhesion, (iii) changes in cell-matrix interactions and (iv) changes in chemokine responsiveness.
**Triggering factors**

The presence of danger signals (see section 1) can directly and indirectly trigger the activation of quiescent DCs in antigen-exposed organs [reviewed in 323]. Direct or “exogenous” triggers include microbial products such as endotoxin, CpG oligonucleotides, viral dsRNA, mycobacterial lipo-arabinomannan and fungal wall components which activate their respective toll-like receptor[s] on the DC. Indirect or “endogenous” triggers are damage or stress inflicted on surrounding tissue, resulting in the secondary release of DC-activating mediators, most notably TNF-a, IL-1β, heat-shock proteins released from necrotic cells 72,284 and some ECM degradation products 223,224. Defensins are small molecules released by epithelial cells in the context of pathogenic invasion, which are at the same time activators of DCs [through TLRs] 227 as well chemoattractive (through binding on CCR6) 75. Chemical irritants, contact sensitizers or environmental pollutants might act on DCs both directly as well as indirectly. An interesting example of a molecule which is at the same time triggering factor, antigen as well as modulator of DC-induced immune response is provided by Der P 1, a protein derived from house dust mite fecal pellets. This molecule exhibits proteolytic activity and was shown to cleave epithelial tight junction proteins 291. This would facilitate the delivery of antigen to intra- or sub-epithelial DCs, while the disturbance in the epithelial integrity would likely lead to DC activation. Remarkably, DCs exposed to Der P 1 lead to the preferential induction of a T-helper 2 response characteristic of allergy 290.

Interestingly, some molecules such as prostaglandins or nucleotides can both stimulate as well as inhibit DC emigration depending on receptor usage, DC-subtype, developmental stage etc... For instance, PGE$_2$ produced by epithelial cells after antigen exposure can stimulate DC emigration 293 by means of the EP4 receptor. In contrast, PGD$_2$ exerts an opposite effect 294; a recent study showed that PGD$_2$ or other agonists of the peroxisome proliferator-activated receptor gamma (PPAR-g), an important intracellular mediator of prostaglandin signaling, could inhibit the emigration of airway DCs towards thoracic LN and consequently suppress T-cell priming 291,292. ATP, a nucleotide released during tissue damage, acts as a typical DC danger signal 294,295. However, it was recently shown that ATP could inhibit DC migration as well. This effect was only observed in immature tissue-bound DCs (not in blood-borne DC subsets) and disappeared following DC maturation 296. This implies that tissue damage would cause a transient retention of DCs within endangered tissue to allow thorough antigen-sampling before full activation and emigration towards the LN.

**Changes in cell-cell adhesion**

In the epidermis, LCs are thought to interact with neighbouring keratinocytes through homophilic E-cadherin-dependent interactions 297. Application of a contact sensitizer on skin, a well-known trigger for LC emigration, results in a TNF-α and IL-1β-dependent downregulation of LC E-cadherin 298. A similar effect was observed during CpG-stimulated LC emigration 299, and also when comparing LCs in the skin vs skin-draining lymph nodes in the steady-state 300. In the gut, the introduction of invasive gram-negative bacteria modulates the expression of tight-junction proteins within epithelium-infiltrating DCs: E-cadherin and Occludin are downregulated which allows the DC to detach itself from epithelial cells, en route to the lymphatic network of the lamina propria 299.
Changes in cell-matrix interactions

It appears that proper adhesion of LCs to the basement membrane is required in order to leave the epidermis. Epidermis treated with a blocking antibody against the α4β1 integrin subunit (laminin receptor) leads to a strong reduction in LC migration [when examined in situ, LCs from treated skin retract their dendrites, take on a rounded shape but are unable to leave] 303. The next step is migration across the basement membrane, a process that generally involves MMPs of the gelatinase group (MMP-2, MMP-9). Accordingly, application of chemical sensitizers induces an upregulation of MMP-9 in LCs 304, and LC emigration towards draining LN was shown to be MMP-2 and MMP-9 dependent 305. Whether such mechanisms play a role in the emigration of airway epithelial DCs remains to be established. Important insights have been gained concerning the role of ECM organization in ensuring a unidirectional migration of LCs. It was shown that LCs which were allowed to adhere to the basement membrane constituents laminin and collagen IV could subsequently initiate interaction with collagen I and fibronectin [typical components of a subepithelial interstitium]. However, LCs recovered after adhesion to collagen I or fibronectin were unable to re-adhere to basement membrane proteins, a phenomenon which was accompanied by a downregulation of α4β1 integrin. Obviously, the molecular events described here do not apply to DC subsets already residing in the interstitial matrix, which would only have to proceed towards draining lymphatic vessels. The preferential migration of mature DCs into lymphatic vessels might rely on a differential expression of adhesion molecules on these structures compared to blood vessels. It was shown that blood vessel endothelium expresses CD31/PECAM-1 only on the luminal side, whereas lymphatic capillaries typically displayed CD31 both at the luminal and abluminal side of the endothelium 304. This specific pattern of CD31 expression might enable leucocytes such as DCs to re-enter the vessels in an abluminal-to-luminal direction. However this hypothesis is contradicted by two experimental findings: (i) human DCs downregulate CD31 upon maturation 306 and (ii) the reverse transmigration process proceeds normally under CD31-blockade 306.

Emigrating DCs can also interact with the ECM through CD44, a cell-surface receptor for hyaluronan. It was shown that LC emigration from the skin is accompanied by differential expression of CD44 splicing variants. Particularly, LC emigration into LN is accompanied by the upregulation of epitopes encoded by exons v6 and v9 307. Intriguingly, the same CD44 splicing variants predict an invasive character for tumoral cells 308. In addition, antibodies directed against the v6 epitope could block LC emigration and homing into the T-cell zones of LN, with an impaired capacity to induce a DTH response as a consequence 307. The importance of DC-hyaluronan interaction was further illustrated by the use of a specific peptide inhibitor of hyaluronan which could block LC migration into draining LN and inhibit the sensitization phase of a contact hypersensitivity response 308. The abundance of hyaluronan in the lung makes it likely that migratory DCs interact with this macromolecule, however this remains an unexplored issue. A different ligand for CD44 is osteopontin, an acidic phosphoprotein with several functions: in it’s monomeric form it’s chemotactic for monocytes, in its polymeric form, it is associated with the ECM and can interact with integrins by means of a fibronectin-like RGD-motif 308. It was shown that osteopontin is strongly chemotactic for LCs in CD44 and α4β1-integrin dependent manner. In vivo, it played an active role in the homing of LCs into draining LN and accordingly osteopontin-knockout mice had attenuated CHS responses 310. Recently, it was shown that DC emigration could be induced by scatter factor (SF), also known as hepatocyte growth factor (HGF) 211. HGF is a dimeric glycoprotein with a motogenic, morphogenic and
mitogenic effect on cells \(^{21}\). Binding of HGF on its receptor, a tyrosine kinase encoded by the c-met proto-oncogene, induces a series of changes that are all relevant to the initiation of DC migration, including: (i) downregulation of E-cadherin function \(^{21}\), (ii) cytoskeletal changes initiating cell movement \(^{21}\), (iii) upregulation of \(^{4}\) and \(^{5}\) integrins with enhanced adhesion to interstitial matrix components \(^{21}\), (iv) increased expression of uPA and uPAR \(^{21}\), which initiates matrix proteolysis and the cleavage of pro-HGF into its bioactive form. In addition, TNF-\(\alpha\) and IL-1\(\beta\), typical triggers for DC emigration, induce the release of HGF and the expression of HGFR/c-met \(^{21}\). In the lung, bronchial epithelial cells and fibroblasts produce HGF and a truncated form, NK2, which is only mitogenic \(^{21}\). Taken as a whole, it is likely that the HGF/c-met system plays an important role in the migration of pulmonary DCs.

### Changes in chemokine responsiveness

A key factor in the directional emigration of DCs is a distinct switch in chemokine responsiveness: chemokine receptors for a whole range of inflammatory chemokines are downregulated, while expression is maintained for CXCR4 \(^{21}\) and CCR7 is upregulated \(^{21}\). It is interesting to note that, contrary to the receptors for inflammatory chemokines, CCR7 is remarkably resistant to ligand-induced downregulation \(^{21}\). This ensures a sustained migration towards chemokines expressed in secondary lymphoid organs. Indeed, CCR7 mediates the migration along a CCL21/SLC/6Ckine gradient displayed on the surface of afferent lymphatic vessels. CCL21 is also highly expressed by stromal cells and DCs in the T-cell rich areas of the draining lymph nodes –i.e. the final destination of emigrating DCs. Recently it has been shown that SLC exists in two forms with apparently similar bio-activity: CCL21a is confined to secondary lymphoid organs while CCL21b is found in non-lymphoid organs such as the lung \(^{21}\). CCL21 expression in the lung shows a peribronchial and perivascular pattern, with virtual absence in the alveolar zones \(^{21}\). This pattern probably corresponds to the distribution of the deep pulmonary lymphatic vessel plexus. CCR7 also contributes to the positioning of DCs within T-cell areas of lymph nodes by means of a second ligand, CCL19/ELC/MIP-3\(\beta\). Recent data revealed a novel molecular mechanism controlling DC responsiveness to CCL19. It was shown that intracellular LTC4, pumped out by means of MRP1 (a transporter molecule from the multi-drug resistance protein family), was critical for CCL19-mediated migration towards LN \(^{21}\). Accordingly, MRP1 gene deletion led to a marked decrease in DC homing into LN, a phenotype which was restored by the addition of exogenous LTC4 or its direct metabolite LTD4. Although DCs themselves produce LTC4, other cells in the lung such as bronchial epithelial cells, alveolar macrophages and granulocytes are important exogenous sources of Cys-leukotrienes. Again, the role of MRP1 and cys-leukotrienes in pulmonary DC emigration remains to be verified.

### Function and fate of DCs in the pulmonary lymph nodes

#### T-cell priming vs tolerance

The existence of a steady-state trafficking of DCs in the lung implies that captured innocuous aero-antigens are constantly transported to the draining pulmonary LN. The functional significance of this phenomenon is starting to be elucidated. It was recently found that inert soluble protein antigen delivered into the airways was presented by mature DCs within the draining thoracic LN. These pulmonary lymph node DCs produced IL-10 and expressed high amounts of ICOS-L, both of which favour the induction of antigen-
specific regulatory T-cells with anti-inflammatory potential 324,325. A more recent report by Brimnes et al elegantly showed how a totally inert and tolerogenic protein can become immunogenic when co-inhaled with a respiratory virus 336. Thus, the default response to inhaled antigen in the absence of danger signal is a tolerogenic one, ensuring the protection of the delicate gas-exchange structures from any inappropriate immune response. Moreover, in the absence of inflammation DCs were shown to import self-antigens from normal epithelial cell turnover into draining lymph nodes without provoking an auto-immune response 326,327. It is tempting to speculate that a similar phenomenon might involve migratory pulmonary DCs and cell fragments from airway epithelial turnover, and that some lung pathologies could be attributed to a failure in this process.

The presence of microbial organisms in the lung leads to a different outcome. Only a few studies have provided a direct link between the homing of pulmonary DCs towards TLN and the generation of an active immune response against lung pathogens. As an example, pulmonary influenza virus infection leads to a transient flux of DCs into the TLN and the efficient generation of IFN-ɤ-producing influenza-specific CD8+ T-cells 279. Aspergillus fumigatus delivered into the airways is transported to the TLN by DCs, leading to the priming of CD4+ T-cells 338. Remarkably, the pulmonary DC is able to discriminate among different fungal components: phagocytosis and processing of conidia induced a T-helper 1 response, while uptake of hyphae resulted in T-helper 2 polarization.

**Pulmonary DC survival in thoracic lymph nodes**

It is generally accepted that lymph nodes are a terminal destination for DCs immigrating from the periphery, as no DCs are found within efferent lymphatics 279. The amplitude and quality of an immune response critically depends on the duration of DC-T-cell interactions 337,338, hence several studies have focused on the factors regulating the survival of immigrating DCs within LN.

A model featuring in vivo transfer of antigen-loaded DCs and antigen-specific CD4 T-cells revealed a surprising accelerated disappearance of DCs in the LN only in the context of Ag-specific interactions 339. Recent studies have suggested mechanisms through which DCs can be eliminated during CD4+ responses. An emerging concept considers death signals induced by MHC class II engagement to be counteracted by survival-promoting signals originating from CD40-CD40L binding. Indeed, cross-linking of MHCII 335,336 or Ag-specific interaction with CD4 T-cells 339,340 can induce DC apoptosis in vitro, and this can be prevented by prior CD40-ligation 335. Interestingly this appears to be a major way by which mature DCs, which seem otherwise resistant to Fas-induced apoptosis 335, can be killed. CD40 ligation was shown to rescue DCs from apoptosis through the upregulation of intracellular Bcl-2 339. This molecule was originally described in B cell lymphoma as an oncogene that acts by suppressing apoptosis rather than stimulating proliferation, and it’s role in normal lymphocyte homeostasis has been studied extensively [see 341 for a review]. The importance of Bcl-2 in prolonging DC survival in vivo has been recently illustrated using transgenic animals in which Bcl-2 was specifically overexpressed in DCs 335. Furthermore, induction of full DC maturation [the last step before apoptosis] is associated with rapid downregulation of intracellular Bcl-2 339. Another factor which may control the fate of DCs within secondary lymphoid organs is RANK [receptor activator of NFκB], also known as the receptor for TRANCE [TNF-related activation-induced cytokine]. RANK/TRANCE-R has been shown to enhance the in vivo survival of adoptively transferred bone-marrow cultured DCs 337,338, however no involvement could be confirmed for DCs extracted from peripheral and mucosal lymph nodes 338. Alternatively, an antigen-specific mechanism for
controlling DC numbers in the LN could involve CTL-mediated elimination of DCs \(^3\). This “killing” of DCs \textit{in vivo} appeared to be a rapid process both in the setting of a primary immune response as well as in recently sensitized animals [the kinetics of elimination closely paralleled the kinetics of CD8+ T-cell activation]. As DCs presenting Ag in the context of MHC class I would consequently have a narrow time window for the full activation of CD8+ T-cells, it was concluded that the elimination of DCs would limit the amplitude of anti-viral or anti-tumor immune responses, or provide a negative feedback against an excessive T-cell stimulation. Interestingly, DCs seemed more susceptible to cytotoxic elimination after interaction with T-helper 2 cells \(^3\). Indeed, only prior contact with T-helper 1 cells was shown to induce an upregulation of the protease inhibitor Serpin-6 in the DC, which rendered the cell resistant to the destructive effects of CTL-secreted granzyme-B. The short life of Ag-presenting DCs within LN does not necessarily mean that the immune responses resulting from Ag-exposure \textit{in vivo} are stunted as well. Indeed a combination of other factors should be taken into account, including (i) the half-life of Ag presence in the periphery, (ii) the trafficking speed of DCs through the Ag-exposed peripheral organ, (iii) the possible transfer of Ag in the LN from dying immigrated DCs to other LN-resident DCs.
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Aims of the studies

1. Dendritic cell homing towards lymph nodes is a prerequisite for the initiation of adaptive immunity. Therefore, as a first step, we aimed to develop an experimental strategy which would enable us to evaluate the trafficking of DCs and captured antigen from the lung towards mediastinal lymph nodes as non-invasively as possible. Ideally, this model would allow us to identify which precise lymph node DC population immigrates from the airways along with captured antigen and whether these cells are capable of inducing antigen-specific naïve T-cell proliferation.

2. A major focus of our research is on the pathogenetic mechanisms of allergic asthma. Hence, the next logical step was to examine the impact of experimental allergic airway disease on pulmonary DC phenotype and DC-allergen trafficking in vivo.

3. As outlined in the introduction, numerous molecular interactions are involved in DC trafficking in health and disease. We focused on matrix metalloproteinase 9 as this molecule plays a key role in the recruitment of cells across different tissue compartments. We aimed to address 3 questions by implementing a well-validated model of allergic airway inflammation in MMP-9 gene deficient mice. [i] Could MMP-9 gene deletion affect the pathophysiological manifestations of allergic airway inflammation? [ii] Could these effects be related to altered pulmonary DC trafficking? [iii] What are the precise molecular link[s] between the recruitment of DCs in the airways and the development of the allergic inflammatory cascade in the airways?

4. The research objectives stated above require an accurate definition of DCs in the lung, and an efficient procedure to discriminate these cells from pulmonary macrophages. A review of study reports in this field underscores the need to clearly identify pulmonary DCs. In several studies until now, immunophenotypical criteria used to define lung macrophages could as well apply to DCs, and vice versa. Our aim was to develop a simple yet accurate protocol to discriminate both cells, and to validate this method by comparing both cell types in terms of morphology, T-cell stimulatory function and known antigenic markers.
Publications

1. Specific migratory dendritic cell rapidly transport antigen from the airways to the thoracic lymph nodes
Karim Y. Vermaelen, Ines Carro-Muino, Bart N. Lambrecht, Romain A. Pauwels
The Journal of Experimental Medicine, 2001, 1: pp 51-60

2. Accelerated airway dendritic cell maturation, trafficking and elimination in a mouse model of asthma
Karim Y. Vermaelen, Romain A. Pauwels

3. Matrix metalloproteinase-9 mediated recruitment of dendritic cells into the airways is a critical step in a mouse model of asthma
Karim Vermaelen, Didier Cataldo, Kurt Tournoy, Tania Maes, An Dhulst, Renaud Louis, Jean-Michel Foidart, Agnès Noël, Romain Pauwels
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4. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow-cytometry: methodology and new insights
Karim Y. Vermaelen, Romain A. Pauwels
Cytometry -submitted
Specific Migratory Dendritic Cells Rapidly Transport Antigen from the Airways to the Thoracic Lymph Nodes

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Abstract

Antigen transport from the airway mucosa to the thoracic lymph nodes (TLNs) was studied in vivo by intratracheal instillation of fluorescein isothiocyanate (FITC)-conjugated macromolecules. After instillation, FITC cells with stellate morphology were found deep in the TLN T cell area. Using flow cytometry, an FITC signal was exclusively detected in CD11cmed-hi/major histocompatibility complex class II (MHCII)hi cells, representing migratory airway-derived lymph node dendritic cells (AW-LNDCs). No FITC signal accumulated in lymphocytes and in a CD11cmed MHCIImed DC group containing a CD8a hi subset (non–airway-derived [NAW]-LNDCs). Sorted AW-LNDCs showed long MHCIIbright cytoplasmic processes and intracytoplasmic FITC granules. The fraction of FITC+ AW-LNDCs peaked after 24 h and had reached baseline by day 7. AW-LNDCs were depleted by 7 d of ganciclovir treatment in thymidine kinase transgenic mice, resulting in a strong reduction of FITC-macromolecule transport into the TLNs. Compared with intrapulmonary DCs, AW-LNDCs had a mature phenotype and upregulated levels of MHCII, B7-2, CD40, and intracellular adhesion molecule (ICAM)-1. In addition, sorted AW-LNDCs from FITC-ovalbumin (OVA)—instilled animals strongly presented OVA to OVA-TCR transgenic T cells. These results validate the unique sentinel role of airway DCs, picking up antigen in the airways and delivering it in an immunogenic form to the T cells in the TLNs.

Key words: antigen-presenting cells • endocytosis • fluorescein isothiocyanate • respiratory mucosa • lymph nodes

Introduction

The lungs are extensively exposed to the outside world. On a daily basis, human lungs process >10,000 liters of ambient air. This represents a considerable amount of airborne foreign particles which, depending on their size, deposit at different levels within the airways. Consequently, the respiratory system is one of the most immunologically challenged organs of the body. Therefore, its immunological homeostasis must be kept under tight control to provide adequate defenses while avoiding inappropriate, potentially life-threatening inflammatory damage.

Dendritic cells (DCs) are now widely recognized as controlling the immune response (1) and a dense network of DCs has been described in the airway mucosa (2–4). DCs are the most efficient professional APCs to date and the only ones capable of eliciting a primary immune response. They perform their task through a tightly controlled sequence of events. DC precursors arise in the bone marrow and home to their target organ. At this stage, they are in an “immature” state characterized by high Ag uptake capacity and low expression of surface MHC and T cell costimulatory molecules. Upon contact with Ags, and in the presence of so-called danger signals (5), DCs evolve towards their mature state; the Ag uptake and processing machinery is shut down while Ag-loaded MHC and T cell costimulatory molecules are strongly upregulated on the cell surface. This is paralleled by a migration of the DCs...
Committee.

All in vivo manipulations were approved by the local Ethics Committee. Polyurethane catheters (Insyte-W; Becton Dickinson) were used in sterile conditions using disposable and pyrogen-free 18 GA needles. A macromolecule solution was deposited just above the glottis unilaterally. Transgenic mice (DO11.10 transgenic) were purchased from Harlan. Heterozygous OVA-TCR transgenic (DO11.10 × BALB/c) mice were generated by injecting TK-TG bone marrow cells (M. Moser (University of Brussels, Brussels, Belgium; 18). Thymi- dine kinase transgenic (TK-TG) mice were a donation from Prof. D. Klatzmann (Hôpital de la Pitié-Salpêtrière, Paris, France; 19).

Animals.

Male C57BL/6, DBA/2, and BALB/c mice, 6–8-wk old, were purchased from Harlan. Heterozygous OVA-TCR transgenic mice (DO11.10 × BALB/c) were obtained from Dr. M. Moser (University of Brussels, Brussels, Belgium; 18). Thymidine kinase transgenic (TK-TG) mice were a donation from Prof. D. Klatzmann (Hôpital de la Pitié-Salpêtrière, Paris, France; 19).

For experiments using TK-TG mice, bone marrow chimeric mice were generated by injecting TK-TG bone marrow cells into γ-irradiated DBA/2 mice, as described previously (19, 20). All in vivo manipulations were approved by the local Ethics Committee.

Instillation of Macromolecule Solutions into the Trachea.

Fluorescein-conjugated macromolecules were diluted in sterile PBS to a final concentration of 10 mg/ml: FITC-dextran (FITC-DX; mol wt 40,000, anionic); FITC-OVA (mol wt 45,000; both from Molecular Probes); mannosylated FITC-BSA; and galactosylated FITC-BSA (both from Sigma-Aldrich).

For instillation, mice were anesthetized by intraperitoneal injection of 2.5% avertin, as described previously (15). 70 μl of macromolecule solution was deposited just above the glottis under sterile conditions using disposable and pyrogen-free 18 GA polyurethane catheters (Insyte-W; Becton Dickinson), followed by complete recovery of the animal.

Cryostat Sections of Thoracic LNs.

Paratracheal and parathymic LNs were extracted 48 h after intratracheal instillation of fluorescein-conjugated Ag. 5-μm cryostat sections were fixed in acetone and treated with mouse FcR-block followed by RPE-conjugated anti-B220 mAb. The sections were subsequently examined under fluorescence microscopy.

Buffers and Media for Preparation of Single Cell Suspensions and Immunofluorescent Labeling. Digestion medium consisted of RPMI 1640, 5% FCS (both from GIBCO BRL), 1 mg/ml collagenase type 2 ( Worthington Biochemical Corp.), and 0.02 mg/ml DNase I (grade II from bovine pancreas; Boehringer). EDTA-treated FCS was prepared by passing FCS through a 0.2-μm filter and mixing 1 ml of a 0.1 M disodium EDTA solution through every 10 ml of FCS. FACSS-EDTA buffer contained PBS without Ca²⁺ or Mg²⁺, 0.1% azide, 5% EDTA-treated FCS, and 5 mM EDTA. Tissue culture medium (TCM) was prepared using RPMI 1640 supplemented with 5% FCS, penicillin/streptomycin, 1-glutamine, and 2-mercaptoethanol (all from GIBCO BRL).

Preparation of Lung and LN Single Cell Suspensions. Animals were killed and the pulmonary and systemic circulation was perfused with saline/EDTA to remove the intravascular pool of cells. Paratracheal and parathymic intrathoracic LNs were collected. Lungs were carefully separated from thymic and cardiovascular remnants and removed in toto, including the main bronchi and trachea. Due to the photosensitivity of the FITC material, organs from FITC-macromolecule–instilled animals were protected from direct light throughout the manipulation. Organs were thoroughly minced using irisectomy scissors and incubated for 30 min in digestion medium in a humidified incubator at 37°C and 5% CO₂ according to a modified protocol (21). Organ fragments were resuspended, fresh digestion medium was added, and incubation was extended for another 15 min. After a final resuspension, very few organ debris were left. Samples were centrifuged and resuspended in calcium- and magnesium-free PBS containing 10 mM EDTA for 5 min at room temperature on a shaker. Finally, the cells were subjected to RBC lysis, washed in FACS-EDTA, passed through a 50-μm cell strainer, and kept on ice until labeling. Cell viability after this procedure was consistently >95%.

Labeling of Single Cell Suspensions for Flow Cytometry. mAbs used to identify mouse DC populations were: biotinylated anti-CD11c (N418), FITC- and PE-conjugated anti-IA b (AF6-120.1), and FITC-IE b (14.4–48) (both from BD PharMingen). Additional markers used for phenotyping were (PE-conjugated or uncoupled): anti-CD86/B7-2 (GL-1), and hamster anti–CD80/B7-1 (16-10-A1) (all from BD PharMingen); and rat anti–mouse DEC-205 (NLDIC-145) and F4/80 (A3-1) (both from Serotec) and rat anti–mouse CD54/intercellular adhesion molecule (ICAM)-1 (KAT-1; Caltag). Isotype controls were RPE-conjugated or uncoupled rat IgG2a, rat IgG2b, and polyclonal hamster IgG (BD PharMingen). PE-conjugated goat anti–rat F(ab’)2, fragments (Caltag) were used as second step reagents.

All staining reactions were performed on ice in FACS-EDTA buffer. If possible, cells were preincubated with anti-CD16/CD32 (2.4G2) to reduce nonspecific binding of mAbs. Biotinylated anti-CD11c was revealed by incubation with streptavidin–PECy5 conjugate (Streptavidin Quantum Red™; Sigma-Aldrich). Unconjugated rat mAbs were revealed by PE-conjugated goat anti–rat F(ab’)2 fragments, followed by blocking with normal rat serum and staining with directly conjugated antibodies. Flow cytometry data acquisition was performed on a FACS Vantage™ flow cytometer running CELLQuest™ software (Becton Dickinson). FlowJo software (Treestar) was used for data analysis.

Microscopic Examination of Sorted LNDCs. Animals were instilled with FITC-OVA and after 48 h the two clusters of pulmo-
nary LN cells appearing in the CD11c<sup>+</sup>MHC class II (MHCII)<sup>+</sup> quadrant were sorted on a FACS Vantage™ with a Sort Enhancement Module (SEM; Becton Dickinson). Cytospin slides were prepared from each subpopulation and examined under a fluorescence microscope.

**In Vitro Pulsing of DCs with Fluorescein-conjugated Ag.** Single cell suspensions from thoracic LN (TLN) digests of several C57BL/6 mice were pooled. CD11c<sup>+</sup> cells were enriched by layering the cells on a 14.5% metrizamide gradient (Sigma-Aldrich), as described (15). Cells in the interface were collected, washed three times, and transferred in triplicate to 96-well plates. Subsequently, they were pulsed with graded concentrations of FITC-OVA in TCM and incubated for 30 min on ice or in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Background fluorescence was determined using PBS-pulsed LN cells. After incubation, cells were labeled with anti-CD11c and anti-MHCII (as described above). All labeling steps were performed on ice, in the presence of azide and 5 mM EDTA. FITC mean fluorescence intensity within the CD11c<sup>+</sup>MHCII<sup>+</sup> subpopulations was assessed by flow cytometry.

**Ag-specific Proliferation Assay.** BALB/c mice were instilled intratracheally with either FITC-OVA or FITC-DX and killed 48 h later. TLNs were extracted and pooled, and single cell suspensions were stained with a combination of anti-CD11c and anti-MHCII (I<sub>E</sub>). The two distinct clusters of interest in the CD11c<sup>+</sup>MHCII<sup>+</sup> quadrant were sorted on a FACS Vantage™ with SEM. OVA-specific T cells were isolated from the spleen and LNs of DO11.10 × BALB/c mice. After RBC lysis, T lymphocytes were enriched by magnetic negative depletion of MHCII<sup>+</sup>, B220<sup>+</sup>, and GR-1(RB6)<sup>+</sup> cells (Dynabeads; Dynal). Graded concentrations of CD11c<sup>+</sup>MHCII<sup>+</sup> DCs were added to 2 × 10<sup>5</sup> OVA-TCR transgenic T cells per well, in triplicate. After 72 h of culture in TCM, [<sup>3</sup>H]thymidine (1 μCi/well) was added to the cultures for the final 12 h. Cell proliferation was measured on an automated liquid scintillation counter (Microbeta Workstation).

**Conditional Depletion of DCs.** After bone marrow reconstitution, TK-TG chimeric mice were treated by continuous infusion of ganciclovir (GCV, Cymevene®; Roche) or vehicle (PBS) using subcutaneously implanted miniosmotic pumps (ALZET model 2001; Alza Pharmaceuticals). To selectively eliminate dividing myeloid DC precursors, GCV concentration was adjusted to reach a dose delivery rate of 50–55 mg GCV/kg body wt/d, as described previously (19, 20). After 7 d, the pumps were explanted and 1 d was allowed for any residual drug to wash out. Subsequently, mice were instilled with FITC-OVA and 48 h later TLNs were processed and labeled as described above.

**Results and Discussion**

**Ag Transport from the Airways to the TLNs.** TLN cryostat sections obtained 48 h after intratracheal instillation of FITC-DX or FITC-OVA showed numerous FITC<sup>+</sup> cells that were strictly confined to B220<sup>−</sup> T cell zones (Fig. 1 A). A closer look revealed stellar-shaped collections of FITC material reaching deep into the LN paracortex (Fig. 1 B). Based on the stellate morphology and localization within the T cell area, we hypothesized that DCs within the draining LNs contained FITC<sup>+</sup> material 48 h after instillation of FITC-labeled macromolecules into the trachea.

To address this further, TLNs of FITC-OVA–instilled mice were subjected to collagenase/DNase/EDTA treatment. The use of enzymatic stromal digestion, Ca<sup>2+</sup>-free media, and EDTA has been documented to favor the isolation of as many DCs from lymphoid organs as possible by extracting tightly tissue-bound DC subsets and disrupting DC–lymphocyte rosettes (22). TLN cell suspensions were stained using the DC marker combination MHCII plus CD11c (α<sub>c</sub> chain of the p150,95 integrin abundant on mu-

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**Figure 1.** TLN cryostat section from a DX-FITC–instilled mouse, as seen under fluorescence microscopy. (A) Staining with the B cell marker B220 (red): FITC<sup>+</sup> cells are confined to the B cell-negative, paracortical T cell-dependent zones. (B) High power magnification of the paracortical zone showing numerous infiltrating FITC<sup>+</sup> cells with a stellate morphology.
rine DCs) and examined by flow cytometry, as described in earlier studies (23). The cells were analyzed on bivariate plots of MHCII versus CD11c and examined for FITC positivity (Fig. 2 A). Dead cells and debris were excluded based on light scatter properties. Two main clusters of cells could be distinguished in the MHCII<sup>hi</sup>CD11c<sup>hi</sup> quadrant. One group of cells (group 1) had a very strong expression of MHCII and intermediate to high levels of CD11c (MHCII<sup>hi</sup>CD11c<sup>med-hi</sup>), while group 2 had intermediate expression of MHCII and high level expression of CD11c (MHCII<sup>med</sup>CD11c<sup>hi</sup>). As seen in Fig. 2 B, 24 h after instillation of FITC-OVA into the airways of mice, FITC positivity was exclusively detected in group 1 LNDCs. At that particular time point, the FITC signal was absent in group 2 LNDCs as well as in cells in the CD11c<sup>lo</sup> lymphocyte gate (not shown on this density plot as these cells comprise ~95% of the whole LN cell population).

To further confirm that MHCII<sup>hi</sup>CD11c<sup>med-hi</sup> cells were indeed DCs containing FITC, cytospin preparations of sorted group 1 LNDCs were examined using fluorescence microscopy (Fig. 3, A and B). FITC material appears in yellow-green, whereas MHCII stains red (anti-IA<sub>b</sub>-RPE). Group 1 LNDCs showed a typical DC morphology with long MHCII<sup>bright</sup> cytoplasmic extensions (Fig. 3 A) and FITC material concentrated in intracytoplasmic granules (none on the cell surface; Fig. 3 B). No FITC signal was visible in group 2 LNDCs (data not shown).

To check whether FITC positivity in the LNs may be due to passive leakage of fluorescein-conjugated macromolecules from the airway mucosa to the draining TLNs, we assessed the capacity of both LNDC populations described above to take up FITC-OVA in vitro (Fig. 4). Both groups were equally capable of taking up FITC-OVA in a dose-dependent manner. For instance, pulsing the cells with 0.1 mg/ml FITC-OVA resulted in 86.5 ± 0.1% and 84.0 ± 1.9% FITC<sup>+</sup> cells in group 1 versus group 2 LNDCs. This uptake was partially inhibited by cold, which hints to an active endocytic process. The capacity of group 2 LNDCs to take up Ags in vitro was in sharp contrast to the in vivo situation, in which virtually no FITC signal was seen in these cells after intratracheal instillation of FITC-OVA (Fig. 2 B, bottom). We could therefore exclude the possibility of an important passive leakage of free FITC-OVA from the airway mucosa to the draining LNs, at least in baseline conditions and with healthy animals. In fact, macromolecules such as albumins or dextrans are commonly used in physiological studies to test the integrity of endothelial and epithelial barriers, including the respiratory epithelium (24, 25).

Based on the presence of FITC<sup>+</sup> granular material within group 1 (MHCII<sup>hi</sup>CD11c<sup>med-hi</sup>) LNDCs and the exclusion
of passive leakage of FITC material into the draining TLNs, we believe that these cells have taken up Ags and migrated from the airways, thus representing airway-derived (AW)-LNDCs, whereas group 2 (MHCIImedCD11chi) cells are non–airway-derived (NAW)-LNDCs.

Interestingly, transport of macromolecular Ags by AW-LNDCs appears to be selective. FITC-OVA and FITC-DX were comparable in their ability to be carried to the TLNs (Fig. 5). To verify the possible involvement of the mannose receptor (MR) in this process, we instilled mannosylated FITC-BSA and used galactosylated FITC-BSA as a control neoglycoconjugate which does not bind to the MR. Surprisingly, in vivo Ag uptake was low in both cases. This is in sharp contrast to human cultured DCs (26), but in accordance with recent reports demonstrating the absence of the MR on murine DCs in situ (27). Therefore, other MR-like receptors could be involved in the macromolecule uptake we observed. Although DEC-205, a lectin-like receptor shown to be involved in Ag processing by DCs (28), is expressed on murine pulmonary DCs (29; and our observation below), no glycosylated ligands for DEC-205 have yet been uncovered. Another possible candidate is Langerin, a recently described DC-specific C-type lectin receptor expressed on human DCs (including those within bronchial epithelium [30, 31]) and murine DCs (data not shown).

**Kinetics of Ag Transport by AW-LNDCs.** Having confirmed the nature of migratory AW-LNDCs, a kinetics experiment was conducted (Fig. 6) and showed FITC-carrying AW-LNDCs appearing in the TLNs as soon as 6 h after the instillation of FITC-OVA, with a peak influx reached at 24 h. Similar migration rates were found in our previous studies using intratracheal instillation of carboxy fluorescein succinimidyl ester (CFSE)-labeled exogenous DCs in mice (15), in which we found CFSE+ DCs appearing in the LNs as soon as 12 h after injection. In addition, experiments using intratracheal delivery of soluble protein Ag describe an appearance of strong Ag-presenting activity in DCs of draining LNs 24 h after instillation (17). The kinetics of migration are also in agreement with the early occurrence of T cell activation and proliferation in Ag-specific TCR transgenic T cells when peptide- or Ag-pulsed DCs are deposited in the airways. In these models, some T cells have already proliferated three times 48 h after injection of Ags into the trachea (7). This rapid migration of DCs seems to occur in the absence of airway inflammation and is a fundamental feature of DCs at mucosal interfaces, not observed in skin LCs (32).

A considerable fraction of cells in the AW-LNDC cluster did not acquire Ags after instillation. This could indicate that macromolecules instilled intratracheally do not reach DCs in all lung compartments. Alternatively, FITC− AW-LNDCs could either not be derived from peripheral immigrating DCs (an origin among myeloid resident LNDCs populations has been suggested in the case of skin LC−derived LNDCs [23]) or they could be derived from a subset of lung DCs with a low trafficking rate. Indeed, considerable heterogeneity exists in the turnover rate (and functions) of different pulmonary DC subsets (32–34). Similarly, using TK-TG mice, 7 d of selective myeloid DC precursor depletion resulted in a partial elimination of AW-LNDCs from the TLNs (Table I). This indicates that a substantial fraction of AW-LNDCs has a transit time (from dividing precursor through lung to LNs) longer than 1 wk. In contrast, a similar 1-wk conditional DC depletion in a previous study resulted in >95% disappearance of MHCIImed
FITC airway intraepithelial DCs. The remaining unaffected majority of FITC-OVA–carrying DCs were derived from port (Fig. 7). Taken together, these data suggest that the accompanied by a drastic (86%) reduction in FITC-OVA trans-per, the partial (37%) depletion of AW-LNDCs is accom-
panied by a drastic (86%) reduction in FITC-OVA transport (Fig. 7). Taken together, these data suggest that the majority of FITC-OVA–carrying DCs were derived from airway intraepithelial DCs. The remaining unaffected FITC+ AW-LNDCs could represent Ag-carrying DCs with a lower turnover rate.

In our migration kinetics studies, the aim was to come as near as possible to the description of the steady state flux. DCs are exquisitely sensitive to specific environmental stimuli and maturation/migration can be triggered by very low dose endotoxin, mechanical stress, or damage in surrounding tissue (5). The following precautions were taken to avoid these triggers as much as possible: (a) disposable sterile and pyrogen-free instillation catheters were used; (b) there was no actual penetration of the catheter tip inside the trachea; (c) random testing for endotoxin contamination was performed on the instillates and found to be negative; and (d) low toxicity/high stability of succinimidyl esters of FITC-macromolecules were chosen as opposed to isothiocyanate esters, and free dye was removed through dialysis. Nevertheless, it can be argued that the aspiration of the macromolecule solution, however inert, could represent a local stress signal in itself.

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FITC+ AW-LNDCs could represent Ag-carrying DCs
with a lower turnover rate.

Still, the trafficking rate we observe is of similar magni-
tude as previously described estimates of DC turnover after
irradiation (32). This supports the idea that the FITC-con-
jugates instilled in our model are relatively inert “tell-tales”
for tracking the movement of airway DCs.

Very early after instillation, some FITC signal was ob-
erved within resident LNDCs. However, at most 6% of
these LNDCs became FITC+ (12 h after instillation) and,
in contrast to FITC+ AW-LNDCs, these cells did not fur-
ther accumulate beyond that early time point, reinforcing
the notion that NAW-LNDCs do not import Ag deposited
in the periphery.

As far as the disappearance of FITC+ AW-LNDCs from
the TLNs is concerned, studies will be necessary to deter-
mine which DC apoptosis/survival-related factors are
mainly involved. This is a significant issue, as preliminary
data point towards an altered clearance of Ag-carrying
AW-LNDCs in a model of allergic airway inflammation.

**Table I. Reduction in the Absolute Number of AW-LNDCs and NAW-LNDCs after a 1-wk Systemic GCV Treatment in HSV1-TK Transgenic Bone Marrow Chimeras**

<table>
<thead>
<tr>
<th>Group</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AW-LNDC</td>
<td>37</td>
</tr>
<tr>
<td>NAW-LNDC</td>
<td>30</td>
</tr>
</tbody>
</table>

Values are mean ± SE, n = 6 mice per group. PBS-sys, systemic vehicle treatment; GCV-sys, systemic GCV.

**Figure 6.** Kinetics of DC entry into TLNs. Groups of mice were killed at several time intervals after intratracheal instillation of a 1% FITC-OVA solution. The fraction of FITC+ cells was determined within AW- (○) and NAW- (□) LNDCs (formerly group 1 and group 2 LNDCs, respectively). Each time point is derived from four to seven mice and represents the mean percentage of fluorescein-positive LNDCs ±SE, subtracting background autofluorescence obtained from PBS-instilled mice (dotted line).

**Figure 7.** Treatment of TK-TG bone marrow chimeras with systemic (sys) GCV; impact on FITC-OVA transport within AW-LNDCs. Bars represent mean fraction of FITC+ AW-LNDCs ±SE. The depletion in absolute number of AW-LNDCs was taken into account when calculating the percentage of FITC+ cells in the GCV-treated group.

**Phenotype of Lung and Mediastinal LNDCs.** We compared the phenotype of lung and TLN DCs after collagenase digestion and EDTA treatment of whole lungs and corresponding TLNs. We chose to phenotype cells right after organ digestion in order to avoid artefacts originating from additional DC enrichment and culture procedures. AW and NAW DC subsets in the LNs were outlined using the MHCII/CD11c double staining as described above. To pinpoint DCs in the lungs, we used a strategy based on findings by Havenith et al. (35) in which DCs and monocytc DC precursors were identified within the low auto-
fluorescent cell fraction of rat bronchoalveolar lavage fluid. Additionally, in this study the phenotype of the low auto-
fluorescent cell fraction was found to shift further towards typical mature DC morphology after overnight incubation. We applied this approach to murine lung cells and added the expression of CD11c to the immunofluorescent identifica-
tion criteria (Fig. 8 A). Two peaks of autofluorescence could be distinguished within the CD11c+ gate. When sorted, CD11c+ /low autofluorescence cells showed a predominant monocyte and immature DC morphology. After overnight incubation in cytokine-free, serum-supple-
mented medium this appearance shifted clearly towards typical DCs with numerous cytoplasmic extensions (data not shown). We did not consider high levels of surface MHCII as a DC identification criterion a priori in order to
avoid a bias towards more mature forms. Nevertheless, CD11c⁺/low autofluorescence cells were >90% MHCII⁺. Therefore, these cells are hereafter referred to as “intrapulmonary DCs.” The mean fluorescence intensity of MHCII was even higher for CD11c⁺ TLN cells (Fig. 8 B).

Fig. 9 shows a surface phenotypic comparison between pulmonary DCs and corresponding TLN DC subsets. Compared with their intrapulmonary counterpart, AW-LNDCs were clearly more mature as illustrated by an up-regulation of MHCII (Fig. 8) and the T cell costimulatory molecules CD40, B7-2, and ICAM-1 (Fig. 9 B). This specific pattern of phenotypical shift is also seen after in vitro maturation induction of cultured DCs by such various factors as mechanical stress, LPS, necrotic cells, and a variety of other danger signals (5). Our observations suggest that even in the absence of specific danger signals or Ag, lung DCs undergo phenotypical maturation as they migrate to the TLNs.

Interestingly, we were unable to detect any significant levels of B7-1 on either murine pulm-DCs or AW-LNDCs. In contrast, this costimulatory molecule was clearly detected in a previous work performed on murine pulmonary DCs, albeit at weaker levels than B7-2 (36). In this study however, the DCs were further enriched by an overnight adherence step, which could alter the phenotype. Using the same antibodies, we have previously reported that both B7-1 and -2 are very strongly expressed on cultured bone marrow DCs, illustrating that the absence of B7-1 might be typical of freshly isolated lung DCs (16). Another noteworthy difference between intrapulmonary DCs and AW-LNDCs was the consistent low level expression of CD8α on the latter cells (Fig. 8 B). Moreover, our data have consistently revealed a high level expression of CD8α on NAW-LNDCs, which is not observed in pulmonary DCs (Fig. 8). This suggests that lung DCs undergo a phenotypical shift as they migrate to the TLNs, which could be induced by various danger signals.

In contrast to the CD8αlowDEC-205high migratory LNDCs, the main feature of NAW-LNDCs was a high level expression of both DEC-205 and CD8α. The NAW-LNDC cluster was heterogeneous, however, as it also contained cells expressing the myeloid marker Mac-1. This is
analogous to findings reported by Salomon et al. (23) in which three subpopulations of DCs were described in cutaneous LNs, only one of which (MHCII$^{+}$CD11c$^{med-hi}$) became FITC$^{+}$ after skin painting with FITC. The exact origin of NAW-LNDC subsets and their possible relationship to AW-LNDCs need further investigation.

**AW-LNDCs Efficiently Present Ag Deposited in the Airways.** It was necessary to verify the functional impact of the findings outlined above, namely that AW-LNDC transport Ag from the airways to the TLNs and phenotypically mature while doing so. Therefore, we conducted an in vitro antigen-specific proliferation assay using AW-LNDCs from FITC-OVA–instilled BALB/c mice as APCs, and DO11.10 T cells as OVA-responsive cells. As can be seen in Fig. 10, after FITC-OVA instillation AW-LNDCs induced a very strong OVA-specific T cell proliferation, again stressing the unique role of these cells in the uptake and processing of Ag deposited in the airways. In this setting, FITC$^{-}$ NAW-LNDCs also induced some T cell proliferation, albeit several orders of magnitude weaker than when AW-LNDCs were used as APCs. The possibility of nonspecific T cell stimulation could be ruled out, as AW-LNDCs from FITC-DX–instilled animals induced no T cell proliferation in the same culture conditions. Several explanations exist for the low level induction of T cell proliferation by NAW-LNDCs. (a) Some contamination of NAW-LNDCs by AW-LNDCs may have occurred during fluorescence-activated cell sorting. (b) Alternatively, the minor early uptake of FITC$^{+}$ material in NAW-LNDCs (see kinetics) could suffice to load these cells with very low levels of Ags, generating a low ligand density and a suboptimal T cell response. (c) Another possibility is the transfer of Ag between AW- and NAW-LNDCs. In previous work by Inaba et al. (42, 43), it was shown that Ags from incoming migratory DCs and apoptotic cells can be efficiently presented on (possibly CD8a$^{+}$) DCs residing in the T cell areas. It is possible in our model that NAW-LNDCs (containing a CD8a$^{+}$DEC-205$^{+}$ subset) have phagocytosed and processed apoptotic AW-LNDCs, including their OVA cargo. Alternatively, recent experiments also suggest that preformed MHC–peptide fragments can be transferred from one DC to another (44, 45).

In summary, we have described the kinetics of pulmonary DC migration from the airways to the T cell zones of the TLNs by relying on their Ag-carrying properties. This migration is accompanied by phenotypic maturation and very efficient presentation of the peripherally administered soluble Ag. The novel approach we used allows the study of the kinetics of DC-mediated Ag transport in pathologic states such as allergic airway inflammation. In addition, it offers the possibility of investigating the relative contribution of chemokines, cytokines, metalloproteinases, and integrins and cadherins to the migration of airway DCs, by means of pharmacological blockade and the use of knockout or transgenic animals.

![Figure 10](image)

**Figure 10.** OVA-TCR transgenic T cell proliferation induced by different subsets of TLN DCs. Values represent tritiated thymidine uptake by T cells, expressed as mean counts per minute ± SE derived from triplicate assays. ▼, AW-LNDCs from FITC-OVA–instilled mice; ●, NAW-LNDCs from the same mice; ○, AW-LNDCs from FITC-DX–instilled mice.

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**References**


rat display differences in their abilities to provide accessory co-stimulation for naive (OX-22+) and sensitized (OX-22-)
Accelerated Airway Dendritic Cell Maturation, Trafficking, and Elimination in a Mouse Model of Asthma

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Pulmonary dendritic cells (DC) can induce both tolerogenic as well as inflammatory immune responses in the lung. Conversely, little is known about the impact of ongoing airway inflammation on pulmonary DC biology. In non-inflammatory conditions, expression of T cell costimulatory molecules on mouse airway DCs is low and only upregulated after homing into draining thoracic lymph nodes. In this study, we reveal that ongoing allergic airway inflammation induces a premature upregulation of the T cell costimulatory molecules CD40, B7–2 and intercellular adhesion molecule 1 on DCs still present in the airways. In contrast, high surface expression of inducible costimulator ligand, involved in respiratory tolerance induction is restricted to DCs from noninflamed lungs. In addition, during inflammation the migratory flux of allergen-transporting airway DCs toward draining thoracic nodes increases both in amplitude as well as in speed. Remarkably, migratory DCs from inflamed airways are short-lived in the draining lymph nodes, a finding that is temporally associated with a marked loss of the antiapoptotic protein Bcl-2 in these cells. This study demonstrates the profound effects of ongoing allergen-driven airway inflammation on the dynamics of pulmonary DC physiology, a knowledge that could be exploited in the development of novel DC-based immunotherapies.

Allergic asthma, a disease of the airways with ever-increasing worldwide prevalence, is characterized by an inappropriate T helper 2 type immune response against non-pathogenic inhaled material. Airway dendritic cells (DCs) have come under scrutiny in the search for immunological flaws leading to the asthmatic phenotype. Recent studies suggest an essential role for airway DCs in deciding which pulmonary immune response ensues after contact with respiratory antigen. An emerging concept regards allergic immune responses as a deviation from the normal situation in which DCs capturing inhaled Ag induce tolerogenic, regulatory T cells within pulmonary lymph nodes (1). Under certain circumstances, DCs are capable of actively inducing (2) or maintaining allergic airway inflammation (3, 4). This knowledge is bringing the airway DC to the foreground as a novel cellular target in future anti-asthmatic therapies. However, any specific intervention at this level must come with a perfect understanding of this cell’s physiology in the midst of a pathologic environment. In the steady state, pulmonary DCs are immature in terms of T cell costimulatory molecule expression and continuously sample antigens reaching the airway mucosa (5). Subsequently, these cells transport antigen to the T cell areas of draining thoracic lymph nodes (TLN) and become fully mature (6). This study reveals how experimental allergic airway inflammation profoundly influences some key features of pulmonary dendritic cell biology, i.e., maturation, migration, and terminal fate: the inflammatory milieu induces a strong local activation of DCs as these cells still reside in the airways, while simultaneously driving a massive number of short-lived allergen-transporting DCs into the thoracic lymph nodes.

Materials and Methods
Animals and Immunizations
Mice were subjected to a well-documented allergen sensitization and exposure protocol which results in bronchoalveolar lavage (BAL) eosinophilia, BAL Th2 cytokine production, mononuclear and eosinophilic peribronchial infiltrates, mucus cell hyperplasia, elevated allergen-specific immunoglobulin (IgE), and airway hyperreactivity (7). Briefly, Male C57BL/6 mice, 6–8 wk old (purchased from Harlan, Zeist, The Netherlands) were immunized by intraperitoneal injection of 10 μg ovalbumin (OVA; Sigma, St. Louis, MO) complexed to 1 mg aluminum hydroxide (Al(OH)3). Two weeks later, the animals were exposed for 7 to 14 consecutive days to a daily 30 min of 1% ovalbumin (OVA) aerosol (OVA/OVA groups). Unless otherwise indicated, similar results were obtained in phosphate-buffered saline (PBS)/PBS, OVA/PBS, or PBS/OVA control groups, which is absence of allergic inflammation as verified on BAL cytocentrifuge preparations and absence of OVA-specific IgE in serum samples (not shown).

Intratracheal Instillation of Fluorescent Macromolecules
Fluorescein-conjugated ovalbumin (OVA-FITC; Molecular Probes Europe BV, Leiden, The Netherlands) was diluted in sterile PBS to a final concentration of 10 mg/ml. Intratracheal instillation was performed as previously described using disposable sterile and pyrogen-free polyurethane catheters (Insyte-W; Becton Dickinson, Madrid, Spain).

Preparation of Lung, BAL, and Lymph Node Single-Cell Suspensions
In trafficking experiments, thoracic lymph nodes were prelevated at different time points following OVA-FITC instillation, protected
from light, and processed as outlined below. Alternatively, animals underwent BAL 24 h after the last aerosol exposure, using Hanks' balanced saline solution containing 0.5 mM ethylenediaminetetraacetic acid (EDTA). BAL samples were centrifuged, and the cell pellet was subjected to RBC lysis. Next, right heart catheterization and perfusion with saline–EDTA was performed to remove the pulmonary intravascular pool of cells. Lungs and lymph nodes were prelevated separately and were sequentially incubated in complete medium containing collagenase type 2 and DNase I, followed by 10 mM EDTA, RBC lysis, and finally passed through a cell strainer as detailed previously (6). Lung, BAL, and lymph node single cell suspensions were kept in FACS-EDTA buffer (PBS, 0.5% bovine serum albumin, 5 mM EDTA, 0.1% azide) until cell counting and immunofluorescent labeling.

**Labeling of Single-Cell Suspensions for Flow Cytometry**

Single-cell suspensions were pre-incubated with Fc-receptor blocking antibody (anti-CD16/CD32, clone 2.4G2) to reduce non-specific binding. Monoclonal antibodies used to identify mouse DC populations were: biotinylated anti-CD11c (N418) followed by streptavidin–APC and PE-conjugated anti-IAb (AF6–120.1). In addition, the following panel of antibodies was used: phycocyanin (PE)-conjugated anti-CD40 (3/23), anti-CD86 (GL-1), anti-CD54, anti–inducible costimulator ligand (ICOS-L)-B7RP-1 (HK53), and PE-conjugated isotype controls rat IgG2a and IgG2b. As a last step before analysis, cells were incubated with 7-amino-actinomycin (7AAD or Vapirope) 10 min at room temperature for dead cell exclusion. Intracellular Bel-2 protein levels were detected in fixed and permeabilized cells using PE-conjugated hamster anti-mouse Bel-2 or hamster IgG staining control. All reagents were obtained from BD-Pharmingen (Erembodegem, Belgium) except clone N418 (kindly provided by M. Moser, ULB, Brussels, Belgium), PE anti-mouse CD54 (purchased from Research Diagnostics Inc., Flanders, NJ), and PE anti-mouse ICOS-L/B7RP-1, originally generated by Iwai and coworkers (8), (purchased from eBiosciences, San Diego, CA). Dendritic cells in lung tissues, airway lumen and thoracic lymph nodes were defined as detailed previously (6). Briefly, DCs within lung tissue (pulm-DCs) and BAL (BAL-DCs) were outlined as CD11c Bel-2/low autofluorescent cells. Within thoracic lymph nodes, a CD11c Bel-2/low MHCII hi cluster was found to specifically contain DCs emigrating from the airway mucosa along with captured antigen (= "airway-derived lymph node DCs" or AW-LNDCs). In contrast, the CD11c Bel-2/low MHCII hi LNDC cluster did not acquire and present antigen deposited in the airways (= "non-airway-derived LNDCs" or NAW-LNDC) (6).

**Results**

**Allergic Airway Inflammation Induces In Situ Pulmonary DC Maturation**

A first observation in this study is that ongoing allergen-driven airway inflammation leads to an upregulation of the T cell costimulatory molecules CD40, B7-2, and MHCII on DCs in the lung (Figure 1). This upregulation was especially marked for pulmonary DCs within the airway lumen, sometimes approaching levels seen on terminally mature DCs in the draining TLN (e.g., B7-2). Although DCs are classically considered to mature upon homing into secondary lymphoid organs, only a few in vitro studies have described peripheral maturation of these cells during inflammation. In human atopic dermatitis, increased expression of a novel Th2-promoting inflammatory mediator (thymic stromal lymphopoietin) has been shown to be associated with phenotypic activation/matura-

In situ

of skin dendritic cells in situ (9). In biopsies of giant cell arteritis, locally activated DCs are present with upregulated surface CCR7 and B7-2 (10). More relevantly, both B7-1 and B7-2 were found to be upregulated on pulmonary as well as thoracic lymph node DCs after allergen challenge in sensitized mice (11). In another report, a specific subset of long-lived DCs found in the BAL of allergic mice expressed high levels of B7-1 and CD40 (3). However, the modulations we observed were quantitatively different, which probably reflects differences in cell isolation procedures or the necessity to define relevant DC subgroups more precisely. Unexpectedly, ICOS-L behaved as an exception to the rule of costimulatory molecule upregulation following DC activation. A similar pattern was also observed in experiments in which human DCs were matured with tumor necrosis factor (TNF)-a in vitro (12). Murine ICOS-L (also known as B7RP-1, B7 h, or GL50) binds ICOS, which is induced on T cells upon activation (13). In vivo, ICOS–ICOS-L interaction has been described as an important signal for effector Th2 costimulation, optimal B-cell help (14) or even the interleukin-10-dependent induction of regulatory T cells in the lung (15). We now show that ongoing allergic airway inflammation induces a specific shift in airway DCs from a B7-low/ICOS-L–high to a B7-high/ICOS-L–low phenotype. In a similar model of experimental allergic asthma, blockade of ICOS during the effector phase of the immune response attenuated allergic airway inflammation, although less profoundly than B7-inhibition using CTLA-4-Ig (16). In contrast, blockade of ICOS-L during exposure of naive animals to inhaled antigen annihilated a default pathway of respiratory tolerance induction (15). These contrasting outcomes are probably due to differences in sensitization, antigen delivery (i.e., intranasal versus inhaled aerosol) and the presence of ongoing airway inflammation. Future studies will determine whether the net response after respiratory antigen encounter depends on the ratio between DC B7- and ICOS-L–contributed signals and the presence or absence of inflammation in the airways.

**Allergic Airway Inflammation Amplifies the Speed and Magnitude of Airway DC Trafficking**

Allergic airway inflammation induced a marked increase in absolute DC numbers in the lung interstitium as well as the airway lumen, with the relative increase especially pronounced in the latter compartment (Figures 2A and 2B). Within the thoracic lymph node (TLN), CD11c Bel-2 lymph node (LN) cells were augmented as well (Figure 2C). The elevated number of TLN DCs was primarily due to an expansion of the airway-derived lymph node DC cluster (Figure 3). A massively increased and accelerated influx of allergen-transporting DCs from the airways appeared at the basis of this phenomenon (Figure 4). This was demonstrated by relying on the capacity of airway DCs to endocytose and transport the soluble fluorescent macromolecule OVA-FITC deposited on the airway mucosa (6). In inflammatory conditions (PBS/PBS groups), a peak number of FITC AW-LNDCs was reached 24–48 h after intratracheal OVA-FITC instillation, and all FITC AW-LNDCs had disappeared from the TLN by Day 7 after antigen uptake.
Allergic airway inflammation (OVA/OVA groups) had a profound impact on these baseline kinetics: the peak accumulation of DCs was reached much earlier (8 h after allergen uptake in the airways), whereas the amplitude of this peak influx was increased ~5-fold compared with nonallergic mice. Although allergic airway inflammation could induce leakiness of the epithelial barrier through damage of the respiratory epithelium, passive transfer of OVA-FITC to the TLN still remained a marginal phenomenon. Indeed, uptake by non–AW-LNDCs during full-blown inflammation represented at most 10% of the number of FITC+ migratory AW-LNDC (not shown), while we have previously shown that both LNDC subsets can equally endocytose OVA-FITC when exposed to the same dose in vitro (6). Our findings corroborate a recent report in which the allergic airway environment was found to stimulate the migration of in vitro-cultured, adoptively transferred DCs toward draining thoracic LN (17). The increased emigration toward thoracic LN is not surprising given that DC maturation not only involves increased T cell costimulatory molecule expression, but also a distinct switch in chemokine responsiveness favoring the homing of DCs toward secondary lymphoid organs (18). Further investigations will be necessary to identify the inflammatory mediators underlying this phenomenon. A plausible candidate could be increased local production of TNF-α. TNF-α protein levels are known to be upregulated in the airways of asthmatics (19). In the skin, TNF-α can both stimulate epidermal dendritic cell emigration as well as recruitment (20), which is
in line with our in vivo observations in the lung. Moreover, earlier studies have described an increased trafficking of lung and gut mucosal dendritic cells after LPS stimulation (21, 22), an effect which is known to be largely TNF-α-dependent.

Allergic Airway Inflammation Accelerates the Clearance of Migratory DCs from the TLN

Interestingly, the initial inflammatory rush of allergen-transporting DCs is quickly followed by an accelerated disappearance of these cells from the draining lymph nodes (Figure 4): between Day 1 and 2 after instillation of OVA-FITC, ∼93% of the allergen-transporting DCs were cleared from the LN of OVA/OVA mice compared with less than 50% in PBS/PBS mice. By Day 3, virtually all the allergen-transporting DCs had disappeared from the TLN of OVA/OVA animals. This is very similar to observations made by Ingulli and colleagues using an adoptive transfer model: analysis of DC–T cell encounters within peripheral LN revealed a premature clearance of DCs only in the context of Ag-specific DC-T cell interaction (23). Because migratory DCs in the LN do not progress toward efferent lymphatic vessels (24), their number at any given time point is a balance between cellular influx and the rate of cell death. An emerging concept regarding the terminal fate of DCs within LN confronts death signals induced by antigen-specific MHC class II engagement against survival-promoting signals originating from CD40–CD40L interactions (25, 26). We speculate that a similar mechanism might come into play in our in vivo model. Indeed, CD40 ligation has been shown to rescue DCs from apoptosis with concomitant upregulation of intracellular Bcl-2 (27). The importance of Bcl-2 in prolonging DC survival in vivo has been recently illustrated using transgenic animals in which Bcl-2 was specifically overexpressed in DCs (28); functionally, this resulted in an enhanced T-helper cell–mediated immune response. Furthermore, induction of full DC maturation (the last step before apoptosis) is associated with rapid downregulation of intracellular Bcl-2 (29). Our observations reveal that during full-blown allergic airway inflammation, freshly immigrating allergen-loaded DCs in the TLN have upregulated levels of Bcl-2 compared with migratory DCs from noninflammatory controls (Figure 5). However, further stay in the TLN induces a rapid and massive elimination of these cells that correlates with a marked loss of intracellular Bcl-2.

It has often been suggested that the elimination of DCs within LN acts to prevent unrestrained immune stimulation. However, such kinetics should be integrated over the whole time period of antigen exposure: indeed, in our experiments the net result of ongoing inflammation is a continuous massive presence of fully mature allergen-transporting DCs in the LN, however short-lived these cells may be.
the allergen-exposure regimen used here results in respiratory tolerance induction when prolonged beyond 2–3 wk. It is known that excessive stimulation by high DC densities can induce T cell deletion (30). Therefore, it is tempting to speculate that the increased throughput of allergen-capturing DCs might initiate the development of tolerance in this model by progressively inactivating the pool of antigen-specific central memory T cells within mucosal lymph nodes.

In summary, our study sketches a complex picture of pulmonary DC biology in the midst of allergic airway inflammation. In the airways, we speculate that the local DC maturation, along with the immediate contact with aerosol-laden and the recruitment of effector T and B cells, provide an adequate environment for an in situ maintenance of the chronic airway inflammation. At the same time, the massive influx into mucosal lymph nodes of short-lived, allergen-capturing airway DCs is a finding whose physiologic significance will have to be explored in future studies.

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References

Matrix Metalloproteinase-9-Mediated Dendritic Cell Recruitment into the Airways Is a Critical Step in a Mouse Model of Asthma

Karim Y. Vermaelen, Didier Cataldo, Kurt Tournoy, Tania Maes, An Dhulst, Renaud Louis, Jean-Michel Foidart, Agnès Noël, and Romain Pauwels

Dendritic cells (DCs) have emerged as the most potent APCs and are now recognized as crucial initiators of immune responses in general (1). In the lung, immature DCs are continuously recruited to the airway mucosa where inhaled Ag is sampled, processed, and transported to the thoracic lymph nodes (TLNs), leading to efficient priming of Ag-specific T cells (2). The relevance of DCs in asthma was evident in studies where intratracheal transfer of Ag-pulsed DCs could elicite a Th2-skewed pulmonary immune response (3). In addition, conditional depletion of DCs during allergen challenge completely abrogated eosinophilic airway inflammation (4), suggesting that DCs are necessary APCs during the secondary immune response.

On a molecular level, the cascade of cellular and molecular events leading to the asthmatic phenotype has grown more and more complex. Next to molecules such as cytokines, chemokines, and growth factors, matrix metalloproteinases (MMPs) have recently emerged as critical mediators in this disease. MMPs form a group of proteolytic molecules drawing increasing interest in the field of lung biology (5). MMPs are known for their extracellular matrix (ECM)-degrading activity (6), but they can also modulate inflammation through proteolytic activation or inactivation of cytokines, chemokines, and growth factors (7, 8). We focused on MMP-9 (gelatinase B), a protease involved in the degradation of collagen IV, a major constituent of basement membranes (BM); it therefore constitutes a useful tool, allowing a cell to move from one tissue compartment to another. Other functions of MMP-9 include ECM remodelling and proteolytic activation of inflammatory mediators such as IL-1β and IL-8 (9). Abnormalities in MMP-9 production have been observed in airway secretions (10), bronchial tissue (11), and blood (12) of asthmatic patients. MMP-9 is produced by many cells that accumulate in allergic airway inflammation, including eosinophils (13), neutrophils (14), and alveolar macrophages (15). Broad-spectrum pharmacologic blockade of MMP activity inhibited airway cellular infiltration caused by single aerosol challenge of allergen-sensitized animals (16) and suppressed the pathophysiology in an animal model of occupational asthma (17). In an asthma model featuring chronic allergen exposure, we have recently reported that MMP-9 gene deletion decreases peribronchial inflammation, airway lymphocyte accumulation, airway IL-13 production, and the development of bronchial hyperresponsiveness (18).

In the current study, we sought to investigate which precise cell populations and relevant chemokine networks upstream in the pulmonary allergic cascade are critically affected by the total absence of MMP-9. We show that MMP-9 gene deletion specifically impairs the inflammatory transmigration of DCs into the airways. This was accompanied by a decreased local supply of DC-derived Th2-attracting chemokines and a strongly attenuated allergic airway inflammation.

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Materials and Methods

Animals

MMP-9 knockout mice and their wild-type counterparts were generated as previously described (19) and were kindly provided by Prof. Z. Werb (University of California, San Francisco, CA). Littermates obtained from breeding heterozygous brothers and sisters were used between 6 and 8 wk of age. All in vivo manipulations were approved by the local ethics committee.

Allergen sensitization and exposure

Mice were immunized by i.p. injection of 10 μg of OVA (Sigma-Aldrich, St. Louis, MO) complexed to 1 mg of aluminum hydroxide (Al(OH)3). Two weeks later, mice were exposed for 7 consecutive days to 30 min of 1% OVA aerosol or PBS for control.

Buffers and medium for preparation of single-cell suspensions and immunofluorescent labeling

Tissue culture medium (TCM) was prepared using RPMI 1640 supplemented with 5% FCS, penicillin/streptomycin, l-glutamine, and 2-ME (all from Life Technologies, Rockville, MD). Digestion medium consisted of TCM supplemented with 1 mg/ml collagenase type 2 (Worthington Biochemical, Lakewood, NJ) and 0.02 mg/ml DNase I (grade II from bovine pancreas; Boehringer Mannheim, Brussels, Belgium). FACS-EDTA buffer contained PBS (without Ca2+ or Mg2+), 0.1% azide, 1% BSA (Sigma-Aldrich), and 5 mM EDTA.

Tissue processing and cellular recovery

Twenty-four hours after the last aerosol exposure, mice were anesthetized using i.p. pentobarbital injection, and the trachea was cannulated. One milliliter of HBSS w/o Ca2+ or Mg2+, supplemented with 0.05 mM sodium EDTA, was instilled four times via the tracheal cannula and recovered by gentle manual aspiration. This bronchoalveolar lavage (BAL) fluid was centrifuged, and the cell pellet was subjected to RBC lysis and kept in FACS-EDTA buffer until cell counting and immunofluorescent labeling. Subsequently, blood was collected by cardiac puncture, and mediastinal lymph nodes (LN) were removed. Right heart catheterization and perfusion with saline-EDTA was performed to remove the pulmonary intravascular pool of cells. One lung was clamped, removed, and kept in ice-cold TCM until enzymatic digestion, or snap-frozen in liquid nitrogen for subsequent RNA extraction. The other lung was perfused in situ with 4% paraformaldehyde through the tracheal cannula using a controlled flow-rate syringe pump and later processed for histology.

Morphometric quantification of allergic airway inflammation

Formalin-fixed, paraffin-embedded lung lobes were cut into 3-μm sections and stained with Congo red to highlight eosinophils. Quantification of inflammation was performed in a blinded fashion using a Zeiss (Oberkochen, Germany) KS400 Image Analyzer system running a custom-made morphometry program. For each mouse, digital images of five to eight bronchovascular structures were acquired and processed as follows. First, the following parameters were measured: the total area of a bronchovascular couple (A_{TOT}), the area taken up by the airway lumen (A_{AW}), and the area taken up by the blood vessel(s) (A_{BV}). The bronchovascular tissue area (i.e., the tissue area prone to inflammatory infiltration) was defined as follows: A_{HV} = A_{TOT} - (A_{AW} + A_{BV}). Within each A_{HV}, all mononuclear infiltrate groups were outlined and added up, yielding the total infiltrate area, A_{INF}.

The severity of inflammation was then expressed as A_{GR/AHV}, i.e., the percentage of bronchovascular tissue area taken up by mononuclear infiltrates. In addition, the density of eosinophil infiltration was expressed as the total eosinophil count within the A_{HV}, divided by A_{HV}.

Preparation of lung and LN single-cell suspensions

Prelevated lungs and LNs were minced, using iridectomy scissors, and incubated in calcium- and magnesium-free PBS containing 10 mM EDTA for 5 min at room temperature on a shaker. Finally, samples were subjected to RBC lysis, washed in FACS-EDTA, passed through a 50-μm-cell strainer and kept on ice until immunofluorescent labeling. Lung, LN, and BAL cells were counted using a Z2 Beckman Coulter (Fullerton, CA) particle counter and size analyzer.

Labeling of single-cell suspensions for flow cytometry

Cells were preincubated with FcR blocking Ab (anti-CD16/CD32; clone 2.4G2) to reduce nonspecific binding. mAbs used to identify mouse DC populations were as follows: biotinylated anti-CD11c (N418) and PE-conjugated anti-IAβ (AF6-120.1), followed by streptavidin-APC. As a last step before analysis, cells were incubated with 7-amino-actinomycin (Viapipe) 10 min at room temperature for dead-cell exclusion. All reagents were obtained from BD Pharmingen (Erembodegem, Belgium).

Four-color flow cytometry data acquisition was performed on a dual-laser FACSVantage SE flow cytometer running CellQuest 3.3 software (BD Biosciences, Mountain View, CA). FlowJo software (www.treestar.com/flowjo) was used for data analysis on PowerMac G3 and G4 workstations (Apple Computer, Cupertino, CA).

Morphometric quantification of airway wall DC infiltration

Sections obtained from formalin-fixed, paraffin-embedded lung lobes were subjected to the following immunohistological staining sequence: blocking reagent (Roche Diagnostics, Mannheim, Germany) plus Triton X-100; rat anti-mouse I-A/E (clone M5/114; American Type Culture Collection, Manassas, VA) or rat IgG2b isotype control; goat-anti-rat IgG Alexia 555 (Molecular Probes, Leiden, The Netherlands); avidin/biotin block (Vector Laboratories, Burlingame, CA); blocking reagent plus Triton X-100; biotinylated rat anti-mouse B220 (clone RA3-6B2, BD Pharmingen) or biotin rat IgG2a isotype control; and streptavidin Alexa 488 (Molecular Probes). Stained sections were mounted in GelMount (Biomeda, Foster City, CA). Red (MHC class II [MHCI]) and green (B220) digital images were acquired and combined using a Zeiss KS400 image analyzer platform. DCs, identified as MHCI+ B220- cells, were counted in the tissue area surrounding the airway epithelium (pseudotests with a panel of relevant mAbs and anti-I-A/E and anti-B220 as optimal for use on formalin-fixed lung tissue sections). Results were expressed as cell counts relative to airway perimeter, i.e., as number of cells per millimeter of BM length as outlined by digital morphometry.

Purification of pulmonary DCs

Pulmonary single-cell suspensions were obtained from lungs of which the vasculature was rinsed but no BAL was performed. Cells were first incubated with FcR block, followed by anti-CD11c microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). CD11c+ lung cells were enriched after one passage through a VarioMACS magnetic cell separator (Miltenyi Biotech) according to the manufacturer’s instructions. Subsequently, these cells (70–80% CD11c+ on average) were labeled with PE anti-I-Aβ and biotin anti-CD11c, followed by streptavidin-APC. DCs, defined as low autofluorescent/CD11c+ I-Aβ+, were further sorted on a FACSVantage SE to 99% purity.

Intratracheal instillation of macromolecule solutions into the trachea

Fluorescein-conjugated OVA (OVA-FITC; Molecular Probes) was diluted in sterile PBS to a final concentration of 1 mg/ml. Intratracheal instillation was performed as previously described (2) using disposable and pyrogen-free 18-gauge polyurethane catheters (Insite-W; BD Biosciences).

Bone marrow-derived DC culture

Mouse bone marrow-derived DCs (mBMDCs) were differentiated using a standard protocol (20). Briefly, cells extracted from mouse long bones were subjected to RBC lysis and cultured for 10 days in TCM supplemented with initial doses of 200 U/ml rGM-CSF (generously provided by Prof. K. Thielemans (Vrije Universiteit Brussel, Brussels, Belgium)). Toward the end of the culture, GM-CSF supplementation was gradually decreased to reduce granulocyte contamination. The final yield of differentiation of DCs was found to be similar in MMP-9 knockout and wild-type animals.

DC Matrilig chemotaxis assay

Twenty-four-well Transwell inserts featuring 8-μm pore size and precoated with Matrigel were obtained from BD Labware (Bedford, MA). Each Transwell insert was seeded with 5 × 105 in vitro-cultured MMP-9+/− or MMP-9−/− mBMDC in 200 μl of TCM in quadruplicate. The lower wells were filled with 500 μl of a chemokine solution (R&D Systems, Abingdon, U.K.), Recombinant murine CC chemokine ligand 4 (CCL4, eNOS SANTES), CCL20 (macrophage-inflammatory protein [MIP]-3α), and CCL21 (6Ckine/secondary lymphoid organ chemokine [SLC]/ Exodus) was used at an optimal concentration of 10−5, 10−7, and 10−9 M, respectively. Cells were allowed to transmigrate for 4 h in a cell culture incubator, after which the Transwells were lifted, the underside was washed with 450 μl of PBS/5 mM EDTA, and pooled with the content of the lower well. Fifty microliters (i.e., 50,000) of counting beads (Bangs Labs, Fishers, IN) was added to the transmigrated cell suspension, and this
mixture was acquired on the flow cytometer. The absolute number of transmigrated cells was calculated as follows: percentage of acquired cells × (50,000/percentage of acquired beads). Results were expressed as a relative migration index (MI), i.e., the MI from chemoattractant Transwells relative to the MI of control Transwells. In some parallel experiments, MMP-9+/− and MMP-9−/− mDCs were allowed to migrate across uncoated 5-μm-pore size Transwells (Costar, Badhoevedorp, The Netherlands).

In vivo migration of adoptively transferred DCs

DCs cultured from MMP-9+/− and MMP-9−/− mouse bone marrow were incubated in TC containing 5 μM CFSE (Molecular Probes) for 5 min at room temperature, washed extensively in excess TCM, and resuspended in PBS without Ca2+ or Mg2+. CFSE-labeled DC (10³/mouse) were intratracheally as described earlier (3). Thirty-six hours later, mediastinal LN were retracted and subjected to the above-mentioned enzymatic digestion protocol. The percentage of CFSE+ CD11c+ LN cells was determined by flow cytometry.

Semiquantitative RT-PCR analysis

Total lung RNA was extracted using a cesium chloride ultracentrifugation method (18). Alternatively, cellular RNA was purified using the RNeasy mini-kit (Qiagen, Hilden, Germany). Primer and TaqMan probe combinations for the different target messages were as follows: β-actin, forward, 5’-AGGGAATACTCGTGCGTGAC-3’, reverse, 5’-CAATATGTAAGGCCTGGA-3’, and probe, 5’-CATGGCCGATCCTCTTCTCC-3’; CCL22/ monocyte-derived chemokine (MDC), forward, 5’-GGTCCCTATGGTG CCAATTG-3’, reverse, 5’-CTGGGCGGAGTTTGG-3’, and probe 5’-CCTCTGCCATACGTGTTGAAGGTCTC-3’; and CCL17/thymus and activation-regulated chemokine (TARC), forward, 5’-TCCAGGG GCAAGCTC ATCCTGT-3’, reverse, 5’-TCCAGGG CTTTGGGATG-3’, and probe, 5’-CCCCAAAGACAACATGTGAAAGGCAA-3’.

Chemokine expression was determined using TaqMan RT-PCR. Cycle conditions were as follows: 30 min at 48°C, 12 min at 95°C, and 40 cycles with 15 s at 95°C and 1 min at 60°C. Each reaction was performed in triplicate. In each PCR run, serial dilutions of a positive control sample (RNA from mouse lungs with full-blown allergic airway inflammation) were included separately for both target gene and housekeeping gene to account for possible differences in PCR efficiency between both genes. For semiquantitative analysis, Threshold cycle values were translated into relative concentrations from the standard curves. Relative concentrations of chemokine cDNA thus obtained were standardized relative to housekeeping gene in the corresponding sample. In some experiments, the results were finally expressed relative to a calibrator sample group (i.e., the non-allergic wild-type animals).

ELISA protein measurements

OVA-specific IgE was measured on serum samples using OVA-coated microtitre plates and biotinylated polyclonal rabbit anti-mouse IgE (S. Florquin, Université Libre de Bruxelles, Brussels, Belgium). One unit was defined as a 1/100 dilution of an internal standard serum pool obtained from OVA-sensitized mice.

CCL17 concentrations were determined using a commercially available ELISA kit (R&D Systems).

Statistical analysis

Statistics were performed on Graphpad Instat 3 software (www.graphpad.com). Groups were compared using parametric tests (Student’s t test or one-way ANOVA with posttest) or nonparametric tests (Mann-Whitney U or Kruskall-Wallis with posttest) following standard statistical criteria.

Results

MMP-9 deficiency inhibits allergic airway inflammation

Allergen-sensitized and exposed mice (OVA/OVA groups) demonstrate typical features of human asthma, among which are prominent cuffing of bronchi and bronchopulmonary vessels with mononuclear cells and eosinophils. These features were inhibited in MMP-9−/− mice as revealed by computer-assisted morphometry (Fig. 1, a–c). In addition, synthesis of OVA-specific IgE was impaired in allergen-exposed MMP-9−/− deficient groups (Fig. 1d).

DC recruitment into and around the airways is MMP-9 dependent

Pulmonary DCs were identified by flow cytometry using three simultaneous immunofluorescent criteria: low-autofluorescence, CD11c, and MHCII positivity. Allergen sensitization and exposure caused a considerable increase in DC numbers in the BAL compartment, whereas this increase was strongly impaired in MMP-9−/− animals (Fig. 2a). By contrast, DCs accumulated equally in the lung interstitium of allergen-exposed MMP-9−/− compared with their allergic wild-type counterparts (Fig. 2b). Pulmonary macrophages were identified as high-autofluorescent, CD11c+ cells. In contrast to low-autofluorescent DCs, these cells have a typical macrophage morphology, are MHCII and T cell costimulatory molecule low to negative, stain strongly with MOMA-2 (21), and induce very weak T cell proliferation in MLR cultures.


FIGURE 1. Allergen-induced inflammation in OVA-sensitized MMP-9 wild-type and knockout animals after challenge with either PBS or OVA aerosol. a. Photomicrographs of Congo red-stained sections. OVA-challenged MMP-9+/− mice feature eosinophil-rich inflammation concentrated around bronchovascular structures, whereas only sporadic infiltration can be seen around the airways of allergen-exposed MMP-9−/− mice. b and c. Quantification of bronchovascular mononuclear cell infiltration and eosinophil density obtained from computer-assisted image analysis (see Materials and Methods). d. Measurement of allergen-specific IgE by ELISA. n = 7–8 mice per group. *, p < 0.05 compared with PBS-exposed groups, **, p < 0.05 compared with wild-type OVA/OVA animals.
We hypothesized that DCs attracted toward the airway lumen required MMP-9 to cross the airway epithelial BM, whereas airway macrophage numbers increased moderately after allergen exposure, but this increase was not affected by MMP-9 deficiency (Fig. 2c). Within the lung interstitium, the extent of macrophage populations remained unaffected in all groups (Fig. 2d). In addition, DC infiltration in the airway wall was quantified as well. DCs were identified by a combination of morphology, MHCII positivity, and the absence of B220 staining using a double immunofluorescent tissue staining protocol. As summarized in Fig. 3, there was a significantly impaired accumulation of airway wall DCs in allergen-exposed MMP-9−/− animals compared with wild-type OVA/OVA animals.

Airway DC homing to mediastinal LNs is not MMP-9 dependent

In baseline conditions, intratracheal instillation of the fluorescent macromolecule OVA-FITC leads to a peak accumulation of FITC + migratory airway-derived DCs in the TLN after 24 h (2). MMP-9 deficiency had no significant effect on this trafficking (Fig. 4a). In addition, intratracheal adoptive transfer of CFSE-labeled exogenous DC yielded no difference between the number of MMP-9+/+ and MMP-9−/− CFSE + DCs reaching the TLN (Fig. 4b).

Differential requirement for MMP-9 in the transmembrane migration of immature vs mature DCs

We hypothesized that DCs attracted toward the airway lumen require MMP-9 to cross the airway epithelial BM, whereas airway DCs migrating to the draining thoracic LN do not. We substantiated our in vivo observations by studying MMP-9+/+ and MMP−/− bone marrow-derived DCs in a Transwell chemotaxis assay across a layer of Matrigel (a surrogate BM), using chemokines representative for the afferent and efferent arm of pulmonary DC trafficking. On one hand, we used CCL5 (RANTES) and CCL20 (MIP-3α). CCL5 is a CC chemokine known to attract DCs to the airways (22) and is critically involved in the development of allergic airway inflammation (23), whereas CCL20 is known to recruit immature DCs into inflamed epithelial surfaces (24). In contrast, we used CCL21 (6Ckine, SLC), a chemokine which attracts mature DCs toward lymphatic vessels and T cell areas of draining LN (25). Fig. 5 shows that DC transmembrane migration along a CCL5 gradient was clearly impaired in the absence of MMP-9, a phenomenon which was even more pronounced when DCs were chemoattracted by CCL20. In contrast, DCs attracted by CCL21 were able to migrate normally through the BM in the absence of MMP-9. In addition, migration of MMP-9−/− DCs toward MIP-3α was not impaired when uncoated Transwells were used (not shown), suggesting that MMP-9−/− DCs have no inherent defects in MIP-3α responsiveness.
Impaired production of DC-derived proallergic chemokines in the lungs of MMP-9-deficient allergen-exposed animals

As a next step, we verified whether the pulmonary expression of prototypical Th2-attracting chemokines was affected in allergen-challenged MMP-9-deficient animals. We concentrated on CCL22 (MDC) and CCL17 (TARC). Using TaqMan RT-PCR, we found that both MMP-9+/+ as well as MMP-9−/− animals could up-regulate pulmonary CCL22/MDC expression (~2-fold) after allergen sensitization and repeated challenge (Fig. 6a). In contrast, expression of CCL17/TARC in whole lungs of OVA/OVA MMP-9−/− animals was significantly impaired compared with that of allergic wild types (Fig. 6b). Similarly, MMP-9 deficiency was associated with lowered levels of CCL17 protein in the BAL compartment after allergen exposure (Fig. 6c). Finally, it was found that CCL17 mRNA levels were ~30-fold more concentrated in highly purified pulmonary DCs than in whole-lung extracts (Fig. 6d).

Discussion

The decreased manifestations of allergen-induced airway inflammation in MMP-9-deficient animals led us to investigate the physiology of upstream cellular actors in this disease model. DCs are emerging as key orchestrators of allergic immune responses, including atopic dermatitis and asthma (26, 27). Therefore, we studied the specific impact of MMP-9 deficiency on DC trafficking through the different pulmonary compartments, both in baseline conditions as well as during inflammation. Our experiments suggest an important role for MMP-9 in the recruitment of airway DCs during inflammation only. Airway DC recruitment presumably follows a multistep process involving two subsequent BM barriers: first, DCs have to extravasate from bronchopulmonary venules (21); subsequently, DCs can migrate through the epithelial BM into the airway lumen or BAL compartment (28, 29). DCs within the BAL compartment (BAL-DCs) represent a unique population of cells, with no equivalent in other immunologically challenged organs such as the skin or the gut. BAL-DCs from allergen-sensitized/exposed animals show strong signs of in situ activation, with high surface expression of MHCII and T cell costimulatory molecules, a phenomenon that normally occurs only after homing into draining LN (49). This, in combination with the direct exposure to allergen and the concomitant presence of BAL T cells, makes BAL-DCs prime candidates for locally maintaining the secondary immune response, perhaps even for a prolonged time after allergen exposure (30). Interestingly, whereas the peribronchial accumulation of DCs was impaired in allergen-exposed MMP-9-deficient animals, analysis of enzymatic lung digests indicated that parenchymal DC accumulation was not MMP-9 dependent (Figs. 3 and 2h, respectively). It could be that MMP-9 is especially required for crossing the endothelial BM barrier at the level of peribronchial blood vessels, whereas DC extravasation at the level of the lung parenchyma relies on other MMPs such as MMP-12 (our unpublished observations). Alternatively, there could be differences in BM structure around systemic vs pulmonary blood vessels, or a compartmentalized expression of DC-attracting chemokines, which in turn could be regulated differently by MMP-9 (following a mechanism described earlier (9)).

Although there is accumulating evidence implicating MMP-9 in the emigration of both human as well as murine epidermal DCs toward draining LNs (31–33), our in vivo experiments reveal that analogous DC trafficking in the pulmonary system is not MMP-9 dependent. In particular, airway Ag capture and transport toward draining mediastinal LN does not require MMP-9. It is possible...
that Ag-sampling airway DCs located above the epithelial BM can cross this barrier in an MMP-9-independent fashion. Indeed, MMP-9-deficient DCs deposited in the airway lumen were able to cross the epithelium and home to the TLN. Moreover, our in vitro experiments indicate that MMP-9 only participates in the transmembrane passage of immature DCs toward the inflammatory chemokines RANTES/CCL5 and CCL20/MIP-3α, but not in the transmigration toward the SLC CCL21. One explanation could be that DC maturation induces BM-degrading enzymes other than MMP-9 (MMP-12 is a possible alternative (34)). In contrast, different chemokines might induce different sets of MMPs in DCs through as-yet-unexplored molecular mechanisms. Further detailed investigations will be needed to unravel this complexity in DC-MMP-ECM interactions.

Nonetheless, the presence of DCs scattered just beneath the airway BM (as observed in mouse intrapulmonary conducting airways) might allow for an additional mechanism of MMP-9-independent aeroantigen capture and transport, similar to a recent study on the gut mucosa. Indeed, despite their subepithelial location, intestinal mucosal DCs are perfectly capable of capturing noninvasive luminal Ags by extending interepithelial processes without disrupting the epithelial barrier’s integrity (35). Likewise, aeroantigen sampling could be operated by subepithelial airway DCs in a way that does not require extensive transmigration of the cell body through the BM.

The reduced airway inflammation in MMP-9−/− mice suggested a defect in the upstream network of proallergic chemokines, i.e., chemokines that preferentially attract Th2 cells. Th2 cells typically express the chemokine receptors CCR8, CCR3, and CCR4. CCR8 gene deletion (or neutralization of its ligand) does not affect the full development of allergic airway inflammation (36, 37). CCR3 and its ligand eotaxin were shown to play a prominent role in the early phase of the pulmonary allergic response; however, after repeated challenge such as in our model, CCR4-mediated Th2 recruitment predominates (38). Therefore, we focused on CCR4 ligands, CCL22 and CCL17. CCL22/MDC is a molecule that preferentially attracts chronically activated Th2 cells (39). In addition, CCL22 is part of an amplifying cascade, whereby its expression is further stimulated by the Th2 products IL-4 and IL-13 (39). Similar to previous reports, we observed an up-regulation of CCL22 after allergen sensitization and chronic challenge, but this was only marginally affected by MMP-9 deficiency. Indeed, it has been shown in a murine model of allergic airway inflammation that CCL22 protein is predominantly found in lung myofibroblasts and macrophages (40). Although the former cells are obviously nonmigratory in nature, we now report that both tissue-bound as well as BAL macrophage mobilization is unaffected in MMP-9 knockout mice.

Therefore, we sought to examine a Th2-mobilizing chemokine that is largely produced by cells featuring an MMP-9-dependent recruitment. We focused on CCL17/TARC, another CCR4 ligand with chemotaxtractant specificity similar to that of CCL22 (41). In vivo inhibition of CCL17 activity suppresses bronchial hyperresponsiveness, lung eosinophilia, and BAL Th2 cytokine levels (42). In vitro studies have identified human bronchial epithelial cells, monocytes, macrophages, and DCs as sources of CCL17 (41, 43). In vivo, CCL17 was found to be expressed in human bronchial and nasal epithelial layers as well as tissue mononuclear cells, and this expression was more pronounced in allergic subjects (44, 45). Moreover, CCL17 protein levels are elevated in the BAL of asthmatic patients after allergen challenge (46). Interestingly, CCL17 expression in the mouse respiratory system was found to be concentrated in CD11c+ cells (47) that were scattered in peribronchial regions beneath the airway epithelium (although BAL-DCs were not examined) (48). In these studies, CCL17 expression was shown to be restricted to mouse DCs, with absent expression in macrophages or B cells even after stimulation. Our experiments reveal a marked deficit in pulmonary CCL17 expression and CCL17 protein levels in the airways of allergen-exposed MMP-9 knockouts. We argue that this difference is due to the impaired recruitment of DCs into and around the airways of these mice. In line with previous reports, we found pulmonary CCL17 expression to be highly concentrated in purified pulmonary DCs. Also, pulmonary DCs purified from allergic vs naive lung had equivalent CCL17 expression levels (not shown), suggesting that differences in CCL17 are due to changes in cell numbers, rather than CCL17 modulation per cell. Although we do not exclude airway epithelial cells as CCL17 producers in the allergic mouse lung, we propose that the massively recruited, activated airway DC population constitutes a more direct and relevant source of this chemokine compared with any other structural cell in the lung. Moreover, the functional impact of DC-derived CCL17/TARC far outweighs the consequences of a hypothetical in vivo interaction between epithelial cells and Th2 cells: as demonstrated earlier (4), in this model of allergic airway inflammation, the secondary immune response collapses in the specific absence of DCs.

In summary, our study suggests a mechanism through which MMP-9 gene deletion inhibits allergic airway inflammation: freshly recruited DCs cannot infiltrate the airway mucosa and do not reach the airway lumen in sufficient numbers. The crucial DC-mediated Ag presentation and costimulation to airway memory Th2 cells would come short, and DC-derived Th2-attracting chemokines would fail to accumulate as well. As a consequence, the local deficiency of Th2-derived effector cytokines would limit the amplitude of the allergic cascade. Together, these data emphasize the potential of selective MMP inhibitors in the treatment of allergic asthma, while simultaneously suggesting a critical role for airway DCs in this disease.

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References

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Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow-cytometry: methodology and new insights

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Abbreviations used in this paper:
DC: dendritic cell
Mph: macrophage
AF: autofluorescent
BAL: bronchoalveolar lavage
ICAM-1: intercellular adhesion molecule 1
HSA: heat-stable antigen

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Abstract

Background: The need to accurately discriminate dendritic cells (DCs) and macrophages (Mphs) in mouse lungs is critical given important biological differences. However, a validated flow-cytometry-based method is still lacking, resulting in much confusion between both cell types.

Methods: Single-cell suspensions freshly obtained from collagenase-digested lung tissue were stained with a CD11c-specific monoclonal antibody, detected using a PE-Cy5 or APC-conjugated secondary reagent. Cellular immunophenotype was simultaneously explored using a panel of PE-conjugated markers. The “FL1” or FITC-detection channel was reserved for the assessment of autofluorescence.

Results: CD11c-bright cells were heterogenous and displayed a bimodal distribution with regard to autofluorescence (AF). CD11c+/low-AF cells were lineage-negative and showed features compatible with myeloid DCs. This was confirmed by morphology, potent T-cell stimulatory function in a mixed-leucocyte reaction, surface expression of MHCII and costimulatory molecules, and further immunophenotypical criteria including the expression of Mac-1 and absence of CD8a. In contrast, CD11c+/high-AF cells displayed the features of pulmonary Mphs including typical Mph morphology, very weak induction of T-cell proliferation, low to absent expression of MHCII and costimulatory molecules, and very low levels of Mac-1 as well as F4/80. We also show that only CD11c+/high-AF cells strongly expressed the macrophage marker MOMA-2, while interestingly Mac-3 was expressed at high levels by CD11c+/high-AF and low-AF alike.
Conclusions: This study shows that the combination of CD11c-expression and autofluorescence is necessary and sufficient to accurately separate DCs from macrophage subpopulations in mouse lungs.

Keywords:

lung – mouse - dendritic cell - macrophage - CD11c - autofluorescence
Introduction

Dendritic cells (DCs) and macrophages (Mph) are “professional” antigen-presenting cells (APCs) and both can perform key functions of the innate and adaptive immune response. However, the necessity to clearly discriminate DCs from Mph has already arised from early studies pointing to the DC’s unique ability to elicit a powerful primary immune response, while macrophages often appeared as weak stimulators or even immunosuppressive scavengers (1). These differences are well exemplified in the lung. Pulmonary DCs are sentinel cells with a fast turnover. They continuously sample and transport inhaled antigen to the T-cell areas of thoracic lymph nodes (2,3) where they efficiently initiate a primary immune response (3-5); in contrast, lung Mph appear as poorly migratory cells (6,7) with a slow-turnover (8). Moreover, pulmonary Mph are not only inefficient in stimulating naïve T-cell proliferation, they also display immunosuppressive properties. Pulmonary Mph could actively suppress the APC function of lung DCs in situ through the secretion of soluble mediators such as nitric oxide (9), prostaglandins, H₂O₂, transforming growth factor-beta (10) or IL-10 (11). The separation of DCs from Mph populations is complicated by the fact that neither cell type can be identified by a single antigenic determinant. Rather, a combination of markers is necessary, some of which can be shared by DCs and Mph alike. Autofluorescence has been exploited in attempts to discriminate pulmonary DCs and Mph by flow-cytometry. In rats, Havenith and coworkers showed that broncho-alveolar lavage cells could be divided into a high-autofluorescent (AF) fraction containing almost exclusively macrophages (as assessed by morphology and cytochemical features), and a low-AF fraction containing monocytes, dendritic cells, lymphocytes and granulocytes (12). When extrapolated to human BAL samples, this strategy yielded analogous results, with the low-AF BAL fraction containing cells with morphological,
immunofluorescent and functional features of DCs, while high-AF BAL cells were virtually all Mph with extremely poor APC function or even immunosuppressive activity (13). In the mouse, a similar method has been lacking. It is generally regarded that mouse DCs express high amounts of CD11c, the α subunit of the αβ or p150/95 integrin. However, in this study we show that mouse pulmonary cells with high expression of CD11c do not form a homogenous group, but can be further differentiated into DCs and macrophages by simultaneously considering autofluorescent properties. In addition, we demonstrate using morphological, functional and immunofluorescent analysis that the combination of autofluorescence and CD11c expression alone is sufficient to accurately discriminate pulmonary DCs from Mph in the mouse.
Methods

Animals

C57BL/6 mice, 6-8 week old were obtained from Harlan (Zeist, The Netherlands). All in vivo manipulations were approved by the local Ethics Committee.

Buffers and media for preparation of single cell suspensions and immunofluorescent labeling

Tissue culture medium (TCM) was prepared using RPMI-1640 supplemented with 5% FCS, penicillin/streptomycin, L-glutamine and 2-mercaptoethanol (all from GibcoBRL). Digestion medium consisted of TCM supplemented with 1 mg/ml collagenase type 2 (Worthington Biochemical Corp., NJ, USA) and 0.02 mg/ml DNase I (grade II from bovine pancreas, Boehringer Mannheim, Brussels, Belgium). “FACS-EDTA” buffer contained PBS w/o Ca$^{2+}$ or Mg$^{2+}$, 0.1% azide, 1% bovine serum albumine (BSA; Sigma) and 5 mM EDTA.

Tissue processing and cellular recovery

Mice were sacrificed after intraperitoneal injection of overdosis pentobarbital. Following thoracotomy, right heart catheterization was performed using an 18 GA polyurethane catheter (Insyte-W, Becton Dickinson, Madrid, Spain) and the pulmonary circulation was perfused with saline-EDTA in order to remove the intravascular pool of cells. After carefully discarding the thymus and all thoracic lymph nodes, the lungs were removed and collected in ice-cold TCM. Lungs were minced using fine scissors and incubated in digestion medium in a humidified incubator at 37 °C and 5% CO$_2$. After 30 min incubation, samples were vigorously agitated using glass pipettes, fresh digestion medium was added and incubation prolonged for another 15 min. Subsequently, the samples where agitated, centrifuged and incubated in calcium & magnesium-
free PBS containing 10 mM EDTA for 5 min at room temperature on a shaker. Finally, samples were subjected to RBC lysis, washed in FACS-EDTA, passed through a 50 µm cell-strainer and kept on ice until immunofluorescent labeling. For some experiments, the trachea was cannulated prior to thoracotomy. 1 ml of Hank’s balanced salt solution (HBSS) w/o Ca²⁺ or Mg²⁺, with 0.05 mM sodium EDTA was instilled 4 times via the tracheal cannula and recovered by gentle manual aspiration. This bronchoalveolar lavage fluid (BAL) was centrifuged, the cell pellet was subjected to RBC lysis and kept on ice in FACS-EDTA buffer until immunofluorescent labeling.

**Labeling of single-cell suspensions for flow-cytometry.**

Cells were pre-incubated with Fc-receptor blocking antibody (anti-CD16/CD32, clone 2.4G2) to reduce non-specific binding, unless anti-rat secondary antibodies were used (in which case cells were pre-incubated with heat-inactivated mouse and goat serum). The panel of monoclonal antibodies (mAbs) used is summarized in table 1. Following incubation with primary mAbs, CD11c labeling was revealed using streptavidin-allophycocyanin (SAv-APC). Unconjugated monoclonal antibodies were revealed using phycoerythrin-conjugated goat-anti-rat F(ab’)2 fragments (Caltag, Burlingame, CA, USA). For intracellular markers, cells were first stained with anti-CD11c-biotin followed by SAv-APC. The samples were then subjected to fixation and permeabilization using Cytofix/Cytoperm reagent (BD-Pharmingen) for 20 min on ice. Flow cytometry data acquisition was performed on a FACS Vantage SE equipped with 488 nm and 633 nm lasers and running CELLquest 3.3 software (Becton Dickinson, CA, USA). FlowJo software (www.treestar.com/flowjo) was used for data analysis on PowerMac G3 and G4 workstations (Apple Computer Inc., Cupertino, CA, USA).
**Purification and separation of pulmonary CD11c+ cell subsets**

Pulmonary single cell suspensions were first incubated with FcR-block, followed by anti-CD11c microbeads (Miltenyi Biotec, Bergen-Gladbach, Germany). CD11c+ lung cells were enriched after one passage through a VarioMACS magnetic cell separator according to manufacturer’s instructions. Subsequently, these cells (70-80% CD11c+ on average) were labeled with biotinylated anti-CD11c, followed by streptavidin-APC. On the FACS Vantage SE, a region was drawn around the CD11c-high cluster. Within this region, low- and high-AF cells were delineated on a histogram of FL1. CD11c+/low-AF and CD11c+/high-AF population gates were subsequently separated by fluorescence-activated cell sorting (purity >95%).

**Morphological comparisons**

Cytocentrifuge preparations were prepared from freshly sorted CD11c+/low and high-AF cells and stained with May-Grunwald-Giemsa reagent. Part of the sorted cells were seeded in flat-bottom 96-well plates in 200 µl TCM/well at a concentration of 10⁶ cells/ml, cultured overnight, and harvested for cytocentrifuge preparation.

**Mixed leucocyte reaction**

Stimulator cells were CD11c+/low-AF or CD11c+/high-AF cells sorted from C57BL/6 mice as described above. Responder T-cells were isolated from BALB/c mice. Briefly, spleens were teased through a 50 µm cell strainer in ice-cold TCM. The resulting cell suspension was subjected to RBC-lysis, passed through a 50 µm cell strainer and counted. Splenocytes were incubated with FcR-block (2.4G2) for 15 min on ice followed by anti-Thy1.2 paramagnetic microbeads (Miltenyi Biotec, Bergen-Gladbach, Germany) according to manufacturer’s
indications. Subsequently, Thy1.2+ cells were enriched using a VarioMACS magnetic cell separator. The resulting purity (checked by flow cytometry using an anti-CD3\(\text{e}\) monoclonal antibody) was around 90%. T-cells (2 \(\times\) 10\(^5\) cells/well) were co-incubated in flat-bottom 96-well plates in triplicate with a serial dilution of stimulators for 72h. \(^3\)H-thymidine was added for the final 12h of the co-culture. Cell proliferation was assessed on an automated liquid scintillation counter (Microbeta, Turku, Finland).
Results

CD11c-bright pulmonary cells contain two main subpopulations

Single-cell suspensions obtained from exsanguinated, whole lung lobes of mice contained on average 3.4 +/- 0.2% cells with high CD11c expression. Because non-monocytic cells such as granulocytes or NK-cells can express low-levels of CD11c, clusters of CD11c-low cells were not included in our analysis. A first observation is that CD11c-bright cells contained two distinct subpopulations in terms of autofluorescence as examined in the FL1 channel (fig. 1). It is critical to set PMT amplification levels on the flow-cytometer so that an unstained cell population is completely visible within the first quadrant of fluorescence intensity, i.e. high-autofluorescent cells should not be artificially lowered into the first quadrant (this is best verified on a FL1-FL2 plot using low-autofluorescent cells, e.g. from lymph nodes or spleen). In exsanguinated lungs of naïve mice not subjected to broncho-alveolar lavage, CD11c-high cells consisted of 11.4 +/- 0.7% low-AF and 88.6 +/- 1.1% high-AF cells on average. Co-staining for surface MHC Class II revealed that only CD11c+/low-AF cells express MHCII, with weak to absent expression on CD11c+/high-AF counterparts.

Because BAL cells of naïve mice consist of >95% CD11c+/high-AF cells, a lavage procedure results in a proportional increase of low-AF cells in enzymatic lung digests, which can exceed 30% of all CD11c-bright cells (data not shown). Also, the BAL procedure results in a slight increase of MHCII-negative cells within the CD11c+/low-AF population, suggesting that more immature or precursor forms are present within lung tissue as opposed to airway lumen (data not shown).
CD11c+ / low- and high-autofluorescent cells of the lung display morphological features of dendritic cells and macrophages respectively

Freshly isolated CD11c+/low-AF cells displayed morphological features of monocytes and immature DCs with typical kidney-shaped nuclei, a juxtanuclear clear spot but absent or poorly developed cytoplasmic extensions (fig. 2a). No cells with features of granulocytes, lymphocytes or alveolar macrophages were seen. In contrast, freshly examined sorted CD11c+/high-AF cells uniformly showed a typical macrophage morphology: large round cells with abundant vacuolar cytoplasm and oval-shaped nuclei (fig. 2b). After overnight incubation in FCS-supplemented medium without additional specific cytokines, CD11c+/low-AF cells developed typical morphological features of DCs with long cytoplasmic processes (fig. 2c), while CD11c+/high-AF cells uniformly retained their macrophage morphology (fig. 2d).

CD11c+ / low- and high-autofluorescent cells differ dramatically in their T-cell stimulatory potential

When used in a primary allogeneic MLR, purified CD11c+/low-AF pulmonary cells induced a vigorous proliferation of responder T-cells, while CD11c+/high-AF cells of the lung appeared as very weak stimulators (fig. 3).

Phenotypic differences between CD11c+ / low- and high-autofluorescent cells of naive murine lungs

CD11c+/ low and high-AF cells did not express the lineage markers CD3, CD19, NK1.1 or Gr-1/Ly-6G (fig. 4). CD11c+/low-AF cells expressed high and intermediate levels of CD11b and low levels of intracellular MOMA-2, while CD11c+/high-AF cells displayed virtually no CD11b
and high levels of intracellular MOMA-2. Both CD11c+ cellular subsets expressed high levels of intracellular Mac-3, while surface Mac-3 staining was restricted to low-AF cells. Only CD11c+/low-AF cells expressed substantial levels of surface T-cell costimulatory molecules, most notably HSA/CD24, B7-2/CD86, ICAM-1/CD54, to a lesser extent CD40 and weak levels of B7-1/CD80. In contrast, these molecules were barely detectable on CD11c+/high-AF cells. Also, ICOS-L (Inducible COStimulator Ligand, or B7-RP1), a costimulatory molecule which is critical for the induction of IL-10-producing/regulatory T-helper cells, was predominantly expressed by CD11c+/low-AF cells. Furthermore, CD11c+/low- and high-AF cells did not express CD8α (present on a subset of lymphoid organ DCs) while both subsets displayed surface DEC-205 (a multilectin receptor involved in glycoprotein uptake) and very weak levels of F4/80 (a 7-transmembrane protein expressed by some macrophage subsets and Langerhans cells). Finally, expression of the low-affinity Fc-gamma receptors (FcγII and FcγIII) was restricted to CD11c+/low-AF cells.
Conclusions

This study shows that cells with morphological, functional and immuno-phenotypical features of DCs or macrophages can be identified and separated in mouse lungs solely by means of high CD11c expression, combined with low or high autofluorescence signal. The assumption that CD11c+/low-AF cells are myeloid DCs and/or their immediate precursors, while CD11c+/high-AF cells represent pulmonary macrophages was supported by several experimental findings. Purified CD11c+/low-AF cells developed a typical mature DC morphology after overnight incubation (a manipulation which is often part of classical DC isolation protocols) and were potent stimulators in an allogeneic MLR. When freshly examined they were the only cell type to express substantial amounts of surface MHC Class II as well as a number of crucial T-cell costimulatory molecules. It should be noted that MHCII and T-cell costimulatory molecule expression on pulmonary CD11c+/low-AF cells was relatively low compared to earlier phenotyping studies on pulmonary DCs (14). However, these studies involved overnight adherence and incubation steps which are known to lead to maturation artefacts. Also, expression of these molecules increases constitutively after migration into draining thoracic lymph nodes (3), and is strongly enhanced in situ during allergic airway inflammation (15). The expression of other markers on CD11c+/low-AF cells, including DEC-205, F4/80, low-affinity gamma-Fc receptor is compatible with the murine pulmonary DC as described in an earlier study (16).

In contrast to CD11c+/low-AF cells, CD11c+/high-AF cells appeared uniformly as typical lung macrophages, with no morphological changes after incubation. They performed poorly in MLR experiments, which is in accordance with the literature on pulmonary macrophages and is not surprising given the virtual absence of surface costimulatory molecules we observed on these cells.
cells (note that pulmonary inflammation can induce a weak expression of MHCII and costimulatory molecules on CD11c+/high-AF cells as well –our unpublished observations). CD11c+/high-AF cells expressed virtually no CD11b. CD11b is the α-chain of the β₂ integrin (also known as Mac-1 or Complement receptor 3) and is generally considered a typical myelomonocytic marker involved in intercellular adhesion and phagocytosis (17). It is downregulated when macrophages become resident in many tissues, including the lung (18). The absence of Mac-1 expression on CD11c+/high-AF pulmonary cells, along with the weak surface levels of F4/80 was again fully consistent with the reported phenotype of alveolar macrophages (18). It warrants re-interpretation of studies in which Mac-1 was used as a pulmonary macrophage marker in comparisons with pulmonary DCs (19-23).

Further reinforcing the notion that CD11c+/high-AF cells represent macrophages was the strong expression of MOMA-2, with only weak presence in the CD11c+/low-AF subpopulation. MOMA-2, first described by Kraal et al, is a highly specific intracellular macrophage marker with very low expression on Langerhans cells and dendritic cells in general (24). Another intracellular marker traditionally associated with macrophages is Mac-3, a molecule related to the LAMP family or lysosome-associated membrane proteins. Surprisingly, both CD11c+/low- and high-AF cells express copious amounts of intracellular Mac-3 and surface expression of Mac-3 was detected on CD11c+/low-AF cells only. Therefore, earlier studies in which Mac-3 was used as a specific macrophage marker on lung tissue sections (25-27) or in flow-cytometry (28) should be carefully interpreted: our analysis shows that Mac-3 is expressed by functionally disparate cell populations compatible with pulmonary dendritic cells on one hand and macrophages on the other.
From a technical point of view, the flow-cytometric method presented here has the advantage of being economical in terms of fluorescent channel occupation. This is interesting given the known difficulty in identifying macrophages or DCs using few antigenic markers. First, our data shows that MHC class II expression is a redundant discriminatory parameter, as most CD11c+/low-AF cells are de facto MHCII-positive, while CD11c+/high-AF cells express virtually no MHCII. Also, it was not necessary to use an additional channel for the exclusion of classical lineage-positive cells such as granulocytes, T-lymphocytes, B-lymphocytes or NK-cells, as both CD11c+/low- and high-AF cells do not express these lineage markers. This leaves a channel free for examining specific markers of interest. In many cases, this method can be applied to single-laser systems: autofluorescence in FL1, a marker of interest in FL2 (PE-conjugate), and CD11c expression in FL3 (e.g. CD11c-biotin revealed by streptavidin-PECy5). However, in some experimental conditions (such as tobacco smoke exposure), autofluorescence intensity in lung cells increases substantially and can “spill-over” into the FL3 channel. In such situation it is safer to use a dual laser configuration (488 nm and 633 nm excitation wavelengths), which allows to examine CD11c expression further into the deep-red spectrum (e.g. using APC-conjugates as in the data shown here). In addition, as autofluorescence typically “shines” in equal amounts in both FL1 and FL2 channels, it is perfectly possible to reverse the method and distinguish autofluorescent subpopulations in FL2 while examining a marker of interest in FL1 (i.e. using a FITC-conjugated specific marker) (data not shown). This is useful in cases where a PE-conjugated specific marker does not exist or cannot be used (e.g. in flow-cytometric detection of BrDU incorporation or when tracking CFSE-labeled cells). In most cases however, the brightness of PE-conjugated markers relative to their FITC-conjugated counterparts makes the approach illustrated in this study more useful (i.e. autofluorescence in FL1, specific marker in
In any case, the simultaneous use of FITC- and PE-conjugated antigenic markers for DC or macrophage studies should be avoided when dealing with unfractionated lung cell suspensions, and this knowledge might be extrapolated to other non-lymphoid organs with prominent autofluorescence. Several earlier studies aimed at examining pulmonary DC properties have used DC-related antigenic markers without taking into account autofluorescence (22,28-30). This results inevitably in the inclusion of pulmonary macrophages into DC analyses, and can lead to the artefactual concealment of a marker-specific staining from low-AF DCs within the high-AF background signal of macrophages.

In conclusion, our study provides a method to accurately discriminate pulmonary dendritic cells and macrophages from naïve mice by flow-cytometry using only few fluorescent parameters and without lengthy purification protocols. This method could provide a useful framework for future studies on the regulation of the pulmonary immune response in health and disease.
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Table and figure legends

Table 1.

Panel of monoclonal antibodies used in the study. All antibodies were PE-conjugated except 2.4G2, NLDC-145 and MOMA-2. ¹ULB Institute of Molecular Biology and Medicine, Gosselies, Belgium. ²Erembodegem, Belgium. ³Flanders, NJ, USA. ⁴San Diego, CA, USA. ⁵Oxford, United Kingdom.

Figure 1.

Single cell suspension obtained from collagenase/DNase digested mouse lungs, stained for CD11c. Two peaks of autofluorescence can be distinguished within CD11c-bright cells. Only CD11c+/low-AF cells express MHC Class II (semi-transparent histogram: PE-conjugated isotype control staining; gray histogram: PE anti-I-Ab).

Figure 2.

CD11c+/low- and high-AF pulmonary cells purified using a two-step method involving pre-enrichment of CD11c+ cells by MACS, followed by separation of CD11c+/low-AF and CD11c+/high-AF cells by FACSorting using region combinations as in fig. 1. Photomicrographs of cytocentrifuge preparations stained with May-Grunwald-Giemsa reagent. (a) CD11c+/low-AF cells, freshly examined. (b) CD11c+/low-AF cells, after overnight incubation in cytokine-free medium. (c) CD11c+/high-AF cells, freshly examined. (d) CD11c+/high-AF, after overnight incubation.
**Figure 3.**
CD11c+/low-AF and CD11c+/high-AF cells purified as described above were used as stimulators in a primary allogeneic MLR. Points on the curves represent average cpm +/- SEM of triplicate measurements, after subtraction of background thymidine incorporation from unstimulated T-cells.

**Figure 4.**
Freshly-obtained single-cell suspensions from mouse exsanguinated lungs were stained with CD11c-biotin-streptavidin-APC, and autofluorescence signal within CD11c-bright cells was measured in the FL1 or "FITC" channel. Expression of surface or intracellular antigens within CD11c+/low-AF and CD11c+/high-AF cells was examined using PE-conjugated markers. Dark histograms: specific marker staining; semi-transparent histograms: isotype control staining.
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Figure 1.

Dendritic cells versus macrophages in mouse lungs
Figure 2.
Figure 3.
Dendritic cells versus macrophages in mouse lungs

Figure 4.
References

28. Legge KL, Braciale TJ. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. Immunity 2003;18(2):265-77.
Dendritic cells versus macrophages in mouse lungs
Conclusions – Future directions

From our studies it can be concluded that the pulmonary DC system is inherently programmed for an extremely sensitive immune surveillance of the airway mucosa. Information about inhaled material is constantly reported by migratory DCs to the “central processing units” of the airway immune system, i.e. the thoracic lymph nodes. Remarkably, this reporting can proceed without any breach of the mucosal barrier, and in the absence of any inflammatory stimulus. Although DCs which transport inhaled material to the LN induce a strong T-cell proliferation, it does not necessarily mean that an active immune response is elicited. Future studies will have to demonstrate which type of immune response is specifically initiated by this small population of migratory airway DCs. Is the lung inherently Th2-biased as often suggested, or is the default response to harmless inhaled material a tolerogenic one? In the latter case, is it mediated through regulatory T-cells (Tr1? Th3?), through T-cell anergy or through activation-induced cell death? These issues imply considerable technical obstacles but are undoubtedly worth exploring.

Asthma is a disease of the airways which is currently spreading in almost epidemic proportions. The reason why the pulmonary immune response becomes dysregulated to a point where life-threatening airway obstruction can occur is still obscure. A first question is why does an exacerbated pulmonary Th2 response develop in the first place? Though experimental data clearly demonstrates that transfer of exogenous DCs can initiate this process, it is still unclear how endogenous DCs can switch from a tolerogenic to a pro-allergic mode. Second, why does the Th2 response subsequently spin out of control? Again, DCs have been shown to be crucial for the maintenance of allergic airway inflammation in a semi-chronic allergen-exposure scheme. However, life-like chronic models of asthma are reputedly difficult to establish in animals because prolonged allergen inhalation inevitably seems to lead to respiratory tolerance. Finding a way to circumvent this practical limitation will hint to the possible mechanisms which perpetuate and localize the inflammation at the level of the airways. Such mechanisms may involve an altered behaviour of numerous cellular networks in the lung, including epithelial cells, fibroblasts, organized lymphoid aggregates etc... One thing is sure however: the dendritic cell will have to be part of this challenging equation. We now know that allergic airway inflammation profoundly influences the biology of airway DCs. The throughput of allergen-transporting airway DCs towards the thoracic lymph nodes is massively amplified. T-cell co-stimulatory molecules are upregulated on DCs that are still present in the airways, hinting to a possible local stimulation of T-cells. In support of this idea, airway-borne DCs also provide a strong local supply of Th2-attracting chemokines. Recruited effector Th2 cells would then initiate a cascade of inflammatory mediators which would further promote the in situ activation of DCs. This positive feedback loop would probably focus and maintain the allergic inflammation at the level of the airways. Our studies also show that pulmonary DCs need to interact with the extracellular matrix on their way
to their strategic position. In particular, DCs require MMP-9 in order to penetrate bronchial tissues under inflammatory conditions.

Exciting new technologies such as cell-specific gene knock-ins or knock-outs will help us to further explore the role of pulmonary DCs *in vivo*. In addition, a series of studies could be conceived in which a functional airway mucosal unit would be recreated *in vitro*. This could include co-cultures of dendritic cells, airway epithelial cells, and/or (myo)fibroblasts in a three-dimensional matrix with air-tissue interface. It would allow to switch to human cell material and thus usefully complement animal model data. It would open the door to a limitless number of interventions in a tightly controlled environment. This would include silencing or over-expression of genes in select cell populations, receptor blockade, neutralization of soluble mediators, and application of diverse environmental stimuli (allergens, pollutants, viral particles etc...). Last but not least it could serve as a versatile test platform for next-generation anti-asthmatic drugs.
Figure 4. Schematic overview of our work. A DC trafficking in baseline conditions. Very few DCs transmigrate from the peri-bronchial tissues [1] to the airway lumen [2]. There is a constitutive trafficking of antigen-transporting airway DCs towards the T-cell zones of draining thoracic lymph nodes. B Allergic airway inflammation: DC trafficking to and from inflamed airways is greatly amplified. DCs extravasate in large numbers [3] and subsequently transmigrate across the airway epithelium [4]. Both of these processes are MMP-9-dependent. Airway DCs upregulate MHC II and T-cell costimulatory molecules in situ and produce large amounts of the Th2-attracting chemokine CCL17/TARC. Th2 lymphocytes are recruited towards the airways [5] and produce effector cytokines leading to eosinophilic infiltration [6]. Meanwhile, the throughput of allergen-transporting DCs towards thoracic LN is massively amplified [7]. Although the migratory cells in the LN are short-lived during inflammation, the increased influx leads to a net accumulation in the LN.