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Opportunities and challenges of the pulmonary route for vaccination

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Introduction: The respiratory tract is an attractive target for the delivery of vaccine antigens. Potential advantages of drug delivery by means of the pulmonary route include accessibility, non-invasiveness, ease of administration, and the possibility to reach an elaborate mucosal network of antigen-presenting cells.

Areas covered: This review discusses current pulmonary vaccination strategies and their advantages and disadvantages.

Expert opinion: To improve efficiency of vaccination and develop new strategies, a well-founded knowledge about composition and characterization of antigen-presenting cell populations throughout the respiratory tract is essential. In particular, respiratory tract dendritic cells, as key antigen-presenting cells in the lung, constitute an ideal target for vaccine delivery. Furthermore, particle size is a key factor when designing new inhalable vaccines, as size determines not only deposition in different respiratory tract compartments, but also how an antigen and its carrier will interact with lung tissue components and immune cells. An increased knowledge of different respiratory tract antigen-presenting cell populations and their interactions with other components of the immune system will enable new targeting strategies to improve the efficacy of pulmonary vaccination.

Keywords: dendritic cell, immunity, lung, macrophage, vaccination

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1. Introduction

The respiratory tract is continuously exposed to a broad array of environmental antigens ranging from harmful to innocuous. Through its enormous surface the lung provides a vast interface of interaction between inhaled antigens and the immune system. For this reason, the human lung is equipped with a series of barrier components against inhaled antigens and particulates. This feature is of crucial importance as the large mucosal surface area of the human lung (~ 150 m^2) [1] is the main portal of entry for airborne particulate antigen into the human body. A first line of structural barriers includes the surfactant system [2,3], the lung epithelium endowed with tight junctions [4], an aqueous surface lining layer, and the mucociliary escalator [5]. A second line of defense comprises cells of the innate and adaptive immune systems positioned throughout the respiratory tree. Immune cells are located above and below the respiratory epithelium and their activities are tightly regulated in order to protect the integrity of the airways and the vital gas exchange region [6]. Whereas macrophages show many characteristics that support the innate immune response [7], respiratory tract dendritic cells (RTDCs) are important key players of the adaptive immune response [8-10]. Discrimination between pathogenic and non-pathogenic antigen in the respiratory tract represents a permanent challenge to the local airway mucosal immune system [11]. Exposure to antigens via the airways can induce tolerance, immunity, or allergy, depending on several factors, including antigen dose, form

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Article highlights.

- There is a global necessity for the development of efficacious and reliable vaccine strategies that are readily accessible, non-invasive and affordable.
- Pulmonary vaccination may overcome some of the limitations of current vaccines, providing accessibility, non-invasiveness and the possibility to efficiently induce immune responses against relevant pathogens.
- Challenges of the pulmonary route of vaccination may reside with the fact that within different anatomical compartments of the respiratory tract (i.e., conducting airways versus lung parenchyma) the composition of antigen-presenting cell populations varies. This will ultimately determine the net immune response generated against a vaccine antigen in a targeted lung region.
- Respiratory tract dendritic cells provide a potent target for vaccine delivery because of their strategic positioning throughout the whole respiratory tract and their potent antigen-presenting capabilities that orchestrate adaptive immune responses.
- More studies in human tissue are required in order to define human RTDC subsets.
- Based on current understanding of the mucosal immune system in the respiratory tract, a new vaccine candidate may be required to target primarily the main conducting airways to induce an efficient immune response.
- Alternatively, the lung parenchyma may be targeted to induce immunity against a pathogen, provided that a future vaccination strategy overcomes the net inhibitory immune reactivity (e.g., by specific adjuvant) without compromising vital gas exchange regions by excessive inflammation.
- The size of a vaccine carrier designed for deposition in the lung is a very important factor because it determines the compartment where the vaccine will deposit as well as the ability of the antigen to translocate to the lymphatic system or to interact with important respiratory tract APCs.

This box summarizes key points contained in the article.

and frequency of exposure. Recent growing awareness of the importance of dendritic cells (DCs) to adaptive immunity has focused attention on RTDCs as important regulators of local immune response to airborne pathogens, allergens and antigens. RTDCs have been identified in both the airway mucosa and lung parenchyma of rodents and humans, where they are thought to play different roles in control of immunological homeostasis of inhaled antigens [8,12]. Therefore, understanding mechanisms that govern how antigen is delivered to different RTDC subpopulations would provide new insights into the regulation of protective immune responses and help to design vaccines and pulmonary delivery systems optimally in humans. Pulmonary vaccination has many advantages over conventional vaccination approaches, such as a large surface area for absorption, limited proteolytic activity [13] and non-invasiveness. Furthermore, in lungs the targeted delivery of antigen to specific antigen-presenting cells (e.g., macrophages, DCs, B cells) is potentially feasible in order to induce specific immune responses. In particular, targeting DCs in different compartments of the respiratory tract, such as conducting airways and the lung parenchyma, is particularly appealing owing to different characteristics and functionalities of DC populations depending on the anatomical region considered [11,14]. The potential to exploit DCs for improving immune responses and utilization of these cells as therapeutic agents is equally very promising [15,16]. Moreover, DCs may be able to compensate or substitute for a deficiency of CD4 lymphocytes and directly generate an immune response, as shown previously using DCs as a CD4-independent vaccine in $CD4^+$ T-cell-depleted mice [17]. The entire process of vaccine delivery to appropriate cell types can be broken down into a series of steps, each of which may provide specific obstacles. An ideal pulmonary vaccination approach should integrate appropriate 'strategies' to overcome most, if not all, obstacles that may reduce its efficiency. Recent studies have shown that integration of new strategies, such as nanotechnology and optimized pulmonary delivery, potentially improves targeting, release and therapeutic efficiency of inhalational vaccines by overcoming physicochemical and biological hurdles [18]. This review aims to elucidate the important characteristics of the human pulmonary immune system (with special focus on specific RTDC subpopulations) that, in combination with the pulmonary morphology, provides an excellent basis for a highly specific, effective and non-invasive approach for pulmonary vaccine delivery.

2. Respiratory tract compartments and antigen-presenting cells

The respiratory tract is continuously exposed to large amounts of inhaled airborne antigen that may range from seemingly innocuous substances (e.g., house dust mite allergen) to potentially detrimental pathogens (e.g., bacteria). Several populations of immune cells (macrophages, DCs, B cells, monocytes) in close proximity to the epithelium constitute the immune barrier, which requires stringent regulation to maintain vital gaseous exchange functions [14].

Several factors, such as antigen dose, associated pathogen-associated molecular patterns (PAMPs), as well as type and frequency of exposure, will determine the immune response generated in the respiratory tract, potentially ranging from active tolerance to the induction of immunity. An important body of literature showed that DCs regulate adaptive immunity by controlling local immune responses to airborne pathogens, allergens and antigens [8,12]. The dense DC network in the main conducting airways was visualised and described for the first time in 1991 by analysing longitudinal sections of rat trachea by light microscopy [27]. Utilizing yellow fluorescent protein (CD11c-EYFP) transgenic mice, Veres *et al.* showed that DCs form a tightly meshed network within the entire respiratory tract [19]. With an approach integrating several modalities such as flow cytometry, electron microscopy and functional *in vitro* assays, the authors performed an in-depth

characterization of antigen-presenting cell (APC) populations in bronchoalveolar lavage fluid (BALF), lung parenchyma, main conducting airways and lymph nodes of mice [11,14,20]. In these studies, high expression of CD11c and MHC class II distinguished RTDC from B cells and macrophages (**Figure 1** and **Table 1**). In the main conducting airways, airway mucosal DCs (AMDCs) comprised 2%, whereas in the lung parenchyma, DCs (LPDCs) constituted 1% of the total cells within each compartment (**Figure 1** and **Table 1**) [11,14,20]. The abovementioned respiratory APC populations and their subpopulations are discussed in more detail in the example of the murine respiratory tract in the following three sections.

2.1 Airway and lung conventional dendritic cells

Two major populations of so-called 'conventional', or myeloid origin DCs (cDCs) have been described in the conducting airways of mice: $CD11c^+$ MHC class II^+ $CD11b^+$ $CD103^-$ DCs ($CD11b^+$ DC) and $CD11c^+$ MHC class II^+ $CD11b^-$ $CD103^+$ DCs ($CD103^+$ DC), representing ~ 60 and 40% of total airway DCs, respectively (**Figure 1**) [11,21,22]. In the lungs, these DC populations have also been described, as well as populations of MHC class II^+ $CD11c^-$ $CD11b^+$ DC and MHC class $II^\pm$ $B220^+$ $Ly6C^+$ plasmacytoid DC (pDC) (**Figure 1**) [11,23]. Human RTDCs express MHC class II on their surface and have an immature phenotype with high phagocytic capacity [24,25]. However, in contrast to well-defined blood DC subsets, there is (similar to the murine respiratory tract) a high phenotypical and functional heterogeneity of DCs in the human lung depending on their localization. Intraepithelial RTDCs have been described as expressing Langerin together with features of Langerhans cells, such as characteristic Birbeck granules [26,28]. In the human lung, Masten and co-workers differentiated $CD11c^+$ $CD14^-$ $HLA\text{-}DR^+$ cDC and $CD11c^-$ $CD14^-$ $HLA\text{-}DR^+$ pDC in the low autofluorescent fraction of isolated cells (while the macrophages are showing high autofluorescent properties) [29]. Demedts and co-workers [26] proposed a gating strategy combining low autofluorescent fraction, high HLA-DR expression, with $CD3^+$ and $CD19^+$ exclusion and subsequent analysis of DC markers to identify lung DCs. Using this strategy three main DC subtypes were identified in the lung parenchyma: two cDC subsets defined as $CD11c^+$ $BDCA\text{-}1^+$ HLA^+ and $CD11c^+$ $BDCA\text{-}3^+$ HLA^+ and the pDC subset, defined as $CD11c^-$ $BDCA\text{-}2^+$ 123 bright. One particular aspect of this characterization was that most of the $BDCA3^+$ cells expressed CD11c, although a small fraction were CD11c negative, possibly representing a pDC subset that expresses BDCA-3 [9,26,29].

In the mouse, $CD11b^+$ DCs have been shown to be major producers of chemokines that contribute to T cell, monocyte and polymorphonuclear leukocyte recruitment during airways inflammation, and upregulate antigen processing capacity during the onset of allergic airways disease [14,21]. In draining lymph nodes, other studies in mice have suggested that migratory $CD11b^+$ DCs are specialized for antigen presentation to $CD4^+$ T cells and induction of tolerance to innocuous antigens [30]. On the other hand, $CD103^+$ DCs secrete chemokines that recruit T_H2 cells and have been shown to be dependent on CCR7 expression for migration to draining lymph nodes and exclusively cross-present inhaled antigens to $CD8^+$ T cells [21,30]. This is supported further by studies in influenza-infected mice, in which $CD103^+$ DCs were shown to drive preferentially antiviral $CD8^+$ T-cell responses [31,32]. Murine $CD103^+$ DCs have also been shown to express some tight junction proteins, including Claudin-1, Claudin-7 and ZO-2, suggesting that they interact with epithelial cells for airway lumen sampling of inspired air [22]. Of interest to the current discussion, Jakubzick *et al.* showed in mice that $CD103^+$ DCs preferentially capture inhaled particulate antigens, in the form of antigen-coated 500 nm latex beads, and transport them to the draining lymph nodes (DLNs) for activation of naive $CD8^+$ T cells [33]. In the gut, murine $CD103^+$ DCs have been shown to direct the development of T regulatory (T_{reg}) cells through a TGF-β and retinoic acid-dependent mechanism [34]. Such a role has yet to be directly ascribed to lung-derived $CD103^+$ DCs; however, recent studies in mice have described populations of regulatory myeloid-origin DCs that produce IL-10 and can limit T_H2-mediated allergic airway responses [35-37].

2.2 Plasmacytoid dendritic cells

Plasmacytoid DCs are a specialized population of DCs that were originally identified in the mouse as cells that produce high levels of IFN-α following viral infection [38]. Plasmacytoid DCs appear to provide a rapid type 1 interferon response to viruses at sites of infection, promoting natural killer (NK) cell and cytotoxic T-lymphocyte (CTL) responses and IL-12 production by conventional DCs, and thus were originally thought to be a non-migratory population of DCs. However, in large animals such as sheep and pigs, pDCs have been shown to migrate from skin to draining lymph nodes following TLR9 ligation [39], and in the mouse pDCs bearing inhaled antigens have been identified in lung draining lymph nodes [40]. Plasmacytoid DCs appear to have a dual role in regulating immunity, either through mobilization of innate antiviral T_H1 responses by high-level IL-12 and IFN-α production [41], or by dampening of self-reactive T-cell responses through promotion of systemic T_{reg} activity [42], although Sapoznikov *et al.* identified a productive role for pDCs in naive $CD4^+$ T-cell responses in lymph nodes but not spleen of mice [43]. Rather surprisingly, lung pDCs appear to have a redundant role in the clearance of respiratory viral infections in the mouse [44] but play an anti-inflammatory role in suppressing T-cell proliferation to inhaled antigens in lungs and draining lymph nodes [40,45], and suppress T_H2 cell-mediated immunity and inflammatory cell recruitment [46,47].

2.3 Pulmonary alveolar macrophages

Pulmonary alveolar macrophages (PAMs) represent a significant proportion of potential antigen-presenting cells in

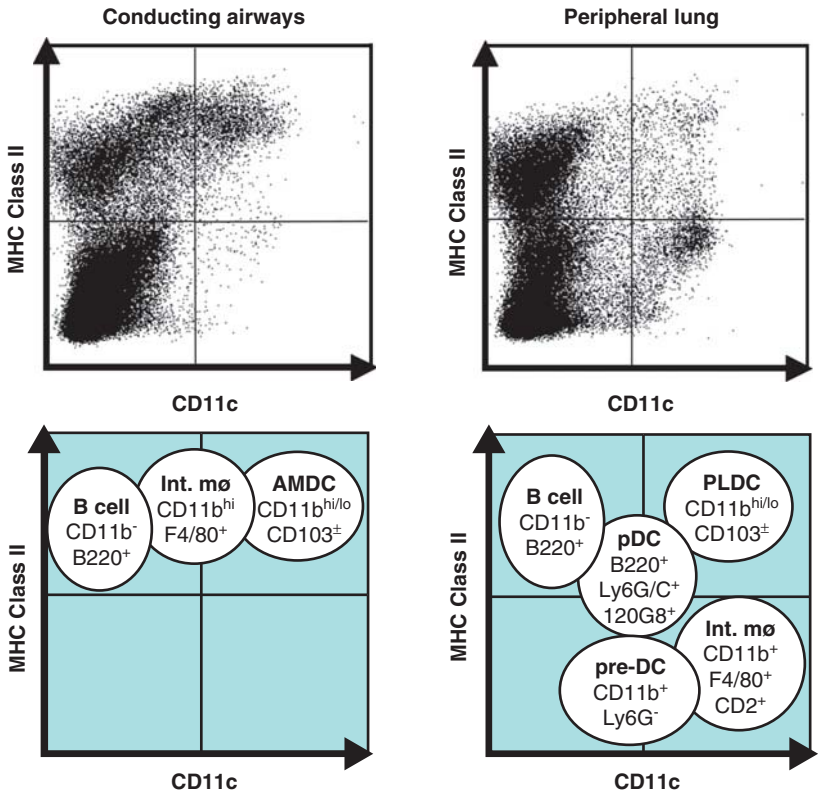

Figure 1. Identification of antigen-presenting cell types in the mouse respiratory tract by flow cytometry. Upper panels: mouse tracheal (conducting airways) or peripheral lung tissue was isolated from normal BALB/c mice after bronchoalveolar lavage, single cell digests prepared and labeled with fluorochrome-conjugated antibodies to CD11c and MHC class II. Fluorescence emission was then detected by flow cytometry after forward scatter and side scatter gating. Lower panels: schematic representation of antigen-presenting cell types that can be identified in conducting airways (left panel) and parenchymal lung (right panel) by flow cytometry using CD11c and MHC class II staining in conjunction with co-labelling for other surface markers.

AMDC: Airway mucosal dendritic cell; Int. mø: Interstitial macrophage; pDC: Plasmacytoid dendritic cell; PLDC: Parenchymal lung dendritic cell; pre-DC: Lung precursor dendritic cell.

the lungs and airways and provide an important level of first-line defense against inhaled microbes and toxins. The importance of PAMs in innate defense against pathogens and particles has long been recognized [48], but the role of PAMs in shaping adaptive T-cell responses to inhaled allergens has remained rather controversial. Early seminal studies in guinea-pigs [49], dogs [50] and mice [51] suggested that PAMs have the capacity to capture inhaled particles and migrate from the alveolar space to draining lymph nodes, a key event in initiation of adaptive $CD4^+$ T-cell responses. These early studies were overshadowed by the subsequent discovery of DCs and suspicions that these early studies were in fact assessing DC migration rather than PAM migration. However, a more recent study Kirby *et al.* in mice revisited this issue and again lends support to the concept that PAMs can capture and transport inhaled particles and pathogens to draining lymph nodes [52]. Similarly, there are some conflicting reports on the role of PAMs in the initiation and progression of allergic asthma: early seminal studies in rats by Holt and co-workers suggested that PAMs were immunosuppressive, inhibiting T-cell proliferation through nitric oxide (NO)-mediated disruption of Jak3/STAT5 signaling in T cells, and that this immunosuppressive activity could be overcome by exposure to GM-CSF [53-55]. Some subsequent *in vivo* studies have shown that PAMs can suppress allergic responses to pathogens and aeroallergens, demonstrated by enhanced immunity to pulmonary Listeria in rats [56] and aeroallergen responsiveness in rats and mice [57-59] following *in vivo* depletion of PAMs, or suppressing bronchial hyper-reactivity by adoptive transfer of resting PAMs into sensitized mice [60,61]. Interestingly, previous activation of PAMs by either allergic sensitization or exposure to microbial products overcomes their suppressive activity following adoptive transfer *in vivo*, consistent with earlier *in vitro* studies described above and contributable to enhanced pro-inflammatory cytokine production by activated PAMs [60,61]. More recently, a subset of macrophages termed alternatively activated macrophages (AAMs) has been

Table 1. Properties of respiratory tract antigen-presenting cell populations.

APC type	Surface markers*					Antigen handling		
	I-A^d	CD11c	CD11b	CD205	F4/80	Uptake‡	Processing§	Presentation¶
CD11b$^-$ RTDC	+++	+++	-	+++	-	++	±	+++
CD11b$^+$ RTDC	+++	+++	+++	+++	++	+++	±	+++
B cell	+++	-	-	+++	-	-	-	+++
Macrophage	+++# ++**	+# +++	+++	+++	+++	+++	±	±

Table summarized and adapted from [10,11,14,20,94,139].

*All populations are uniformly high for CD16/32 and CD54; - < 10%; ± = 11 – 20%; + = 21 – 40%; ++ = 41 – 60%; +++ > 61%.

‡OVA Alexa 647 uptake 2 h after intranasal delivery.

§DQ OVA processing 2 h after intranasal delivery.

¶CD69 expression utilizing an *in vitro* stimulation assay with DO11.10 transgenic T cells.

#Conducting airway mucosa.

**Lung parenchyma.

described in mice and humans that are activated by IL-4 and IL-13 and have been shown to promote T_H2-type responses [62]. Pulmonary AAMs express arginase, IL-13 and chemokines that exacerbate allergic asthma [63], with a recent report suggesting that an increased frequency of AAMs in female mice may contribute to the enhanced susceptibility of females to allergic asthma [64]. Finally, several studies in mice have shown that aeroallergens can persist for several weeks in macrophage or macrophage-like populations of the lung, raising the possibility that chronic low-level antigen presentation may promote the maintenance of effector/memory T_H2 cells in the lung in sensitized individuals [65-67].

2.4 B cells

B cells can be found in the airways of mice and humans, where they can contribute to local immunoglobulin production, including IgE, IgG and IgA, through local germinal center formation [68]. Systemic production of allergen-specific antibodies, and particularly IgE, by activated B cells has long been a mainstay in the paradigm of the association between atopy and allergic asthma [69]. Also, B cells can present antigen by means of MHC class II to CD4$^+$ T cells, but their role in the presentation of aeroallergens is rather more controversial. In mice, one study has suggested a role for B-cell presentation of aeroallergen in the promotion of CD4$^+$ T_H2 responses [70], whereas other studies with mice have proposed a suppressive role for regulatory B cells in aeroallergy [71]. Possibly most convincingly, several studies have reported the development of airway hyper-reactivity and inflammation in B-cell-deficient mice, suggesting that B cells and antibodies are dispensable in the induction of adult mouse models of allergic airways inflammation [72-74]. Nevertheless, targeting antigen for presentation by B cells has been shown to have profound immunomodulatory properties in other settings (rats) [75] and thus may represent a potential therapeutic strategy for allergic airways disease.

3. Respiratory tract dendritic cell biology and pulmonary vaccines

Dendritic cells are specialized APCs that bridge innate and adaptive immunity, therefore occupying a key role in regulating the body's immune responses [76]. Dendritic cells are morphologically characterized by dendrite-like projections and are the most potent APC population able to provide T-cell activation [77]. Body surfaces exposed to the environment, such as the skin, gut and respiratory tract, are endowed with a DC network that constantly scrutinizes incoming antigen and pathogens. Pathogen load is 'sensed' through pattern recognition receptors (PRRs) that interact with PAMPs, triggering innate and adaptive immunity [78]. In DCs, activation through the PRRs leads to upregulation of the chemokine receptor CCR7 (CD197; the ligand is CCL19/ECL), which is essential for DC migration from the site of pathogen encounter to lymph nodes, where activation of naive T cells occurs. When DCs traffic to lymph nodes, differentiation from a so-called maturation from an 'immature' state (high capacity for antigen uptake, low capacity for T-cell activation) to a 'mature' state (low capacity for antigen uptake, high capacity for T-cell activation) occurs.

Advanced imaging techniques by confocal microscopy of conducting airways and lung parenchyma of the mouse following intranasal administration of fluorescent ovalbumin (OVA) showed that RTDCs expressing tight junction proteins continuously sample antigen with intra- and transepithelial dendrites (**Figure 2**) [22]. Flow-cytometric analysis of RTDCs isolated after *in vivo* aerosol or instillation exposure to soluble protein antigen confirmed that antigen capture occurred *in vivo* [11,55,79,80]. A more functional approach in mice with fluorescently labeled OVA i.e. Alexa Fluor 647-labeled OVA (Alexa OVA) and DQ OVA, enabled distinction between uptake and processing of antigen. When collections were made from the main conducting airways and lung parenchyma 2 h following instillation of Alexa

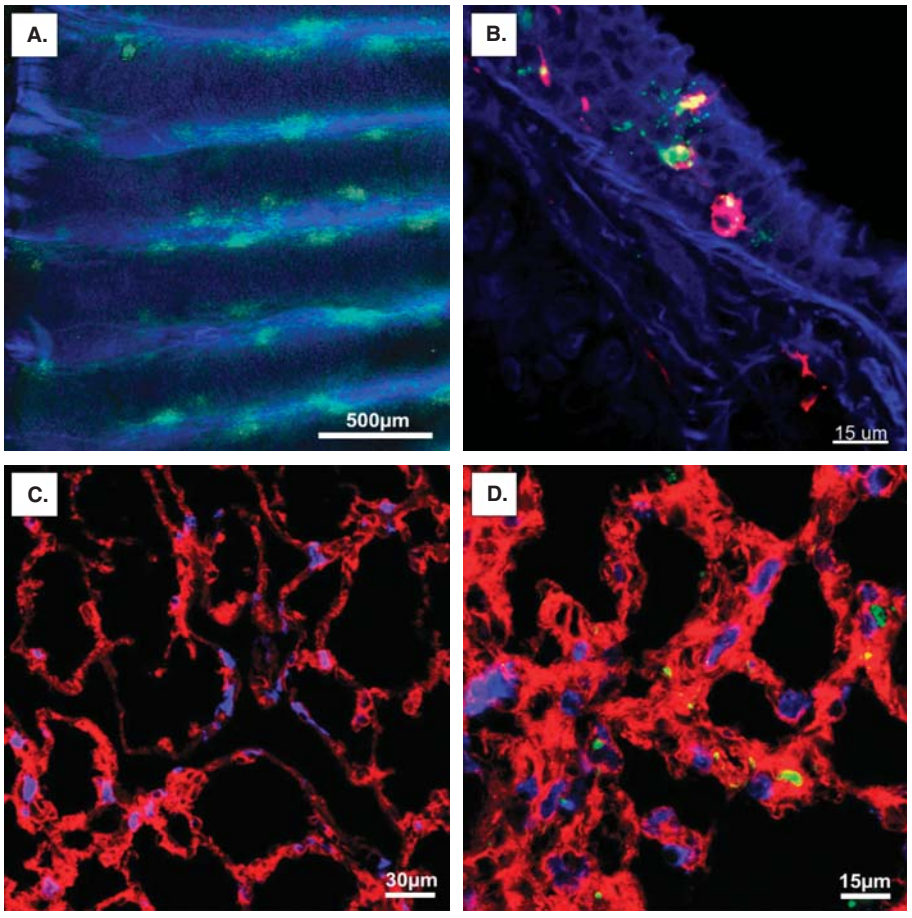

Figure 2. RTDC network and pulmonary antigen uptake visualized by laser scanning microscopy. A. Low power micrograph showing a dense, patchy network of airway mucosal DCs (MHC II, green) in the trachea (F actin, blue). Note that the patches of DCs are mainly localized between cartilage rings that appear as dark horizontal bands. **B.** Antigen uptake (OVA Alexa 488, green) by airway mucosal DCs (MHC II, red) located in the airway epithelium (F actin, blue). **C.** Lung parenchymal DCs (MHC II, blue) within the alveolar walls (F actin, red). **D.** Antigen uptake (OVA Alexa 488, green) from the alveolar space (F actin, red) by lung parenchymal DCs (MHC II, blue). Wholemount (**A**) and 5 µm tissue sections from naive BALB/c mice; Phalloidin Rhodamine: blue in **A** and **B**; red in **C** and **D**; MHC class II: green in **A**, red in **B**; blue in **C** and **D**; OVA Alexa 488: green in **B** and **D**.

DC: Dendritic cell; RTDC: Respiratory tract dendritic cell.

OVA and DQ OVA, LPDC and alveolar macrophages captured considerable amounts of Alexa OVA, whereas $CD11b^+$ AMDC and airways macrophages had intermediate levels, and only very low levels were detected in $CD11b^-$ AMDC and B cells (**Table 1**) [11,14,20]. This finding contrasted with intense DQ OVA fluorescence in LPDC subsets and alveolar macrophages, and only low amounts of processed OVA in AMDC subsets and airway macrophages (**Table 1**) [11,14,20].

To follow the fate of antigen in the respiratory tract, APCs and DCs were characterized by flow cytometry following instillation of fluorescent OVA. Within lymph nodes draining the respiratory tract, several APC and DC populations can be delineated: B cells ($B220^+$, $CD19^+$), $CD8\alpha^{\pm}$ DC, $CD8\alpha^+$ DC and pDC ($B220^+$, $Ly6G/C^+$, $120G8^+$) [11,14,20]. Following intranasal delivery of fluorescent OVA, most fluorescence was associated with $CD8\alpha^-$ DC, followed by a small fraction (< 1%) of $CD8\alpha^+$ DC, and pDC [20]. These data show that the principal antigen-bearing DC subtype in lymph nodes draining the respiratory tract was $CD8\alpha^-$ DC. The discovery and characterization of the human equivalent of mouse $CD8\alpha^+$ DC has recently been reviewed [81]. Four independent studies have characterized a population of $CD1^-$, $CD141^+$, $Clec9A^+$, $Necl2^+$, $XCR1^+$ DCs expressing the pathogen sensors $TLR3^+$, $TLR7^-$ and $TLR9^-$ and showing capacity for IL-12 production, dead cell uptake and cross-presentation [82-85]. Although this subpopulation showed many convincing similarities to mouse $CD8^+$ DC, it has only been possible to characterize their human equivalents in blood, cultures of cord blood stem cells and

humanized mice [81], and not in the periphery (such as the respiratory tract).

Several salient features have emerged on the complexity of different APC and DC populations in the respiratory tract that will need to be taken into account for future developments of pulmonary vaccination approaches. First, though different APC populations and DC subtypes are present within the entire respiratory tract, their relative proportions depend on the anatomical compartment and will determine the resulting net immune response generated (**Figures 3** and 4). In the main conducting airways, AMDCs are largely in excess of interstitial macrophages; therefore, AMDC activity primarily determines the regional immune responsiveness in this compartment. The situation is different in the lung parenchyma, where alveolar macrophages exceeding LPDC by a factor of two to three will principally dominate the parenchymal immune response by an inhibitory activity of macrophages to protect gaseous exchange barriers [10,11,14,20,22].

Second, DCs occupy sentinel positions within the entire respiratory tract mucosa, extending cytoplasmic processes through the epithelium for sampling of antigen and forming a tightly meshed network [10,19,86]. Third, following antigen contact and migration along a chemokine gradient to regional lymph nodes, respiratory tract DCs undergo 'maturation', from an immature state (high antigen uptake capacity, low T-cell stimulatory activity) to a more mature state (low antigen uptake capacity, high T-cell stimulatory activity) [6,87]. In lymph nodes, depending on the cognate interaction by presentation of antigen-derived peptides and the local cytokine milieu, DCs initiate differentiation of naive T cells into various effector T-cell populations (**Figure 4**).

Besides conventional T-cell activation by antigen-bearing RTDCs that migrate to the regional lymph nodes, there is evidence of regional T-cell activation in the airway submucosa, which contains a high T-cell density [88]. This overturns the immunological dogma that primary immune responses are limited to secondary lymphoid organs [89]. Bronchus-associated lymphoid tissue (BALT) has recently been described as a location for local RTDC–T-cell interactions [90]. BALT was initially described by Sminia *et al.* as a follicular-like aggregation of lymphocytes (sometimes containing a germinal center), high endothelial venules, and a specialized lymphoepithelium with intraepithelial lymphocytes and, in some species, non-ciliated M cells [91]. In animals these BALT foci occur particularly at airway bifurcations in the upper airways. Inducible BALT (iBALT), however, has been described as being located in perivascular, peribronchial and even interstitial areas in the lower airways of the lung. Contrary to BALT, iBALT requires antigen to come into being [92]. iBALT therefore appears in the lung only after infection or inflammation and may remain up to 6 months following influenza infection in mice. Furthermore, it shows similarities to Peyer's patches in the gut and isolated lymphoid follicles found in the colon [93]. Therefore, it is possible that inflammatory responses in the lung induce the neoformation of iBALT, which promotes the recruitment, priming and clonal expansion of antigen-specific lymphocytes *in situ*. Thus, iBALT may functionally amplify or even replace respiratory immune responses generated by conventional mucosa-associated lymphoid tissue [90].

Experimental evidence supports the finding that both RTDCs and macrophages within lung parenchyma are endowed with enhanced capacity for uptake and processing of antigen, compared with their counterparts within the main conducting airways. This may in part be related to the experimental approach utilizing intranasal administration, causing fluorochrome to remain in contact with the main conducting airway mucosa for a shorter period of time before a certain degree of accumulation within the lung parenchyma and alveoli. Nonetheless, these findings strengthen the authors' previous broad conclusion that the anatomical location of DCs within different respiratory tract compartments may have profound functional implications on immune responses within the respiratory tract [11,26].

When considering antigen-trafficking from the airways' mucosa to the regional draining lymph node, recent work by Wikstrom *et al.* showed in mice that administration of antigen to the respiratory tract leads to two waves of antigen delivery to the draining lymph nodes. First, an early passive phase of antigen delivery within 4 h, most probably resulting from lymphatic drainage of the respiratory tract, leading to accumulation of the antigen in several DC subsets ($CD8\alpha^-$ and $CD8\alpha^+$ DCs, pDCs) and B cells. Second, a late phase of cell-mediated antigen delivery peaking at 24 – 48 h through influx of $CD8\alpha^-$ DCs from the respiratory tract [94]. With subcutaneous administration of a soluble antigen, Itano and Jenkins showed a similar phenomenon for antigen uptake and presentation in skin draining lymph nodes [95].

Despite accumulating research efforts to characterize APC and DC populations involved in antigen-trafficking within the respiratory tract, several important questions remain unanswered. What role does passive lymphatic drainage play and to what extent do $CD8\alpha^-$ subsets take up passively transferred antigen in the lymph node? Is antigen transfer between different APC populations and DC subsets an important contributing factor? How does a state of inflammation alter the fate of antigen compared with steady-state (i.e., non-inflammatory) conditions? Can specific *in vivo* targeting of particular APC populations or DC subsets modulate antigen trafficking?

Some recent DC studies have unraveled the complexity of the DC network *in vivo*. The salient findings are that not every DC subset is capable of presenting antigen with similar efficacy, and antigen-presenting DCs are not necessarily identical to those that have initially captured the antigen. A precise understanding of the role that each DC subset plays in the net immune response through its capacity for antigen uptake and antigen presentation is particularly important for rational vaccine design. A promising approach may consist of targeting DCs *in vivo*, but a prerequisite is to know the identity of

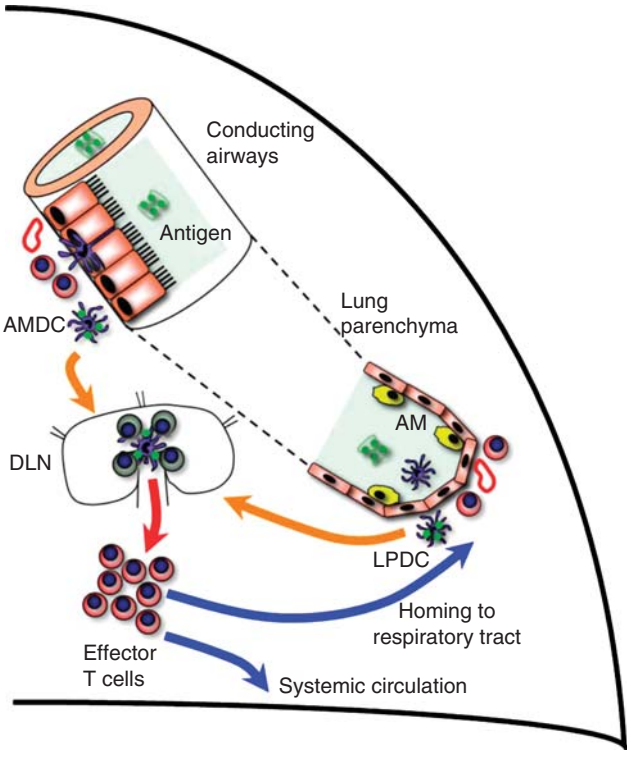

Figure 3. Immunological compartments of the respiratory tract. Antigen-presenting cells such as DCs or macrophages take up inhaled antigen deposited within the main conducting airways or alveolar spaces of the lung parenchyma. Within main conducting airways, AMDCs predominate, whereas in the lung parenchyma there is an excess of AMs exerting an inhibitory effect on LPDCs to protect vital gaseous exchange surfaces. AMDCs and LPDCs relentlessly scrutinize airway lumen and alveolar space for antigen, subsequently migrating via afferent lymphatics to DLNs where they present antigen-derived peptides to naive T cells, causing their differentiation into memory/effector T cells. The latter leave the DLN via efferent lymphatics either to home back to the respiratory tract or to recirculate systemically.

AM: Alveolar macrophage; AMDC: Airway mucosal dendritic cell; DC: Dendritic cell; DLN: Draining lymph node; LPDC: Lung parenchymal dendritic cell.

the target population. Our knowledge of the mouse DC system is rapidly expanding, but identification of the human counterparts remains one of the major challenges.

4. Examples of pulmonary vaccination

Several vaccines administered through the respiratory tract have been developed in the past and some have entered clinical application. Although most of these vaccines are administered intranasally only, the measles vaccine, as an example, has been utilized in an aerosolized form for delivery to the entire respiratory tract, inducing robust regional and systemic immune responses [96,97]. Compared with injectable forms, measles vaccines delivered through the respiratory route are superior at boosting immune responses in seropositive individuals and are less likely to be blocked by maternal antibodies [98]. Aerosolized measles vaccines administered to the respiratory tract were at least as immunogenic and led to similar seroconversion rates in children compared to the identical vaccine delivered subcutaneously [99,100]. Despite much progress and similar rates of adverse events reported for most studies with the aerosolized measles vaccines, significant safety concerns remain to be clarified, especially in high-risk children with asthma and HIV [101]. Furthermore, with the exception of epidemiological data on measles outbreaks in Mexico, there is only limited field experience with clinical effectiveness, as most studies assessed parameters of immunogenicity [102]. Therefore, randomized trials that include disease-related end point on the clinical effectiveness will be a requirement to assess appropriately utility and address safety of aerosolized vaccines.

There are obvious practical advantages that favor the pulmonary route, such as the need of fewer skills than for injection of vaccines, or less risk of unsafe disposal and reuse of syringes in immunization programs. Although aerosolized vaccines might be preferred to injected ones, there is no general acceptability of the respiratory route, as seen with the unexpected low rates of utilization of the intranasal influenza vaccine [103]. This live attenuated influenza vaccine (Flumist®, Medimmune, USA), constituted by cold-adapted viruses containing HA and NA of the target strains, has been available in the US since 2003 [104]. In children, this vaccine strategy offers a greater efficacy than conventional vaccines, but is less effective in adults, possibly related to attenuation of viruses by an immunologically non-naive population [105].

Several other aerosolized vaccines are being developed against Ebola virus [106-108], respiratory syncytial virus [109-113], SARS coronavirus [114], hepatitis B virus [115], human papilloma virus [116], *Streptococcus pneumoniae* [117], tuberculosis [118,119], *Pseudomonas aeruginosa* [120], *Mycoplasma pneumoniae* [121], *Hemophilus influenzae* [122], *Francisella tularensis* [123] and *Listeria monocytogenes* [124]. Although most of these aerosol vaccines are still at an early developmental stage, some have shown promising results in early clinical trials and may represent effective future vaccination strategies against relevant human pathogens. Preferential targeting of specific immunoresponsive areas (e.g., main conducting airways) in the respiratory tract with high densities of DCs may increase the efficacy of a particular vaccine. This will be discussed further in the next section.

5. Differentially targeting lung compartments

The site of interaction between inhaled antigen and immune cells such as DCs within the respiratory tract will determine the specific local and systemic immune response that is generated. It may therefore be of utmost importance to target

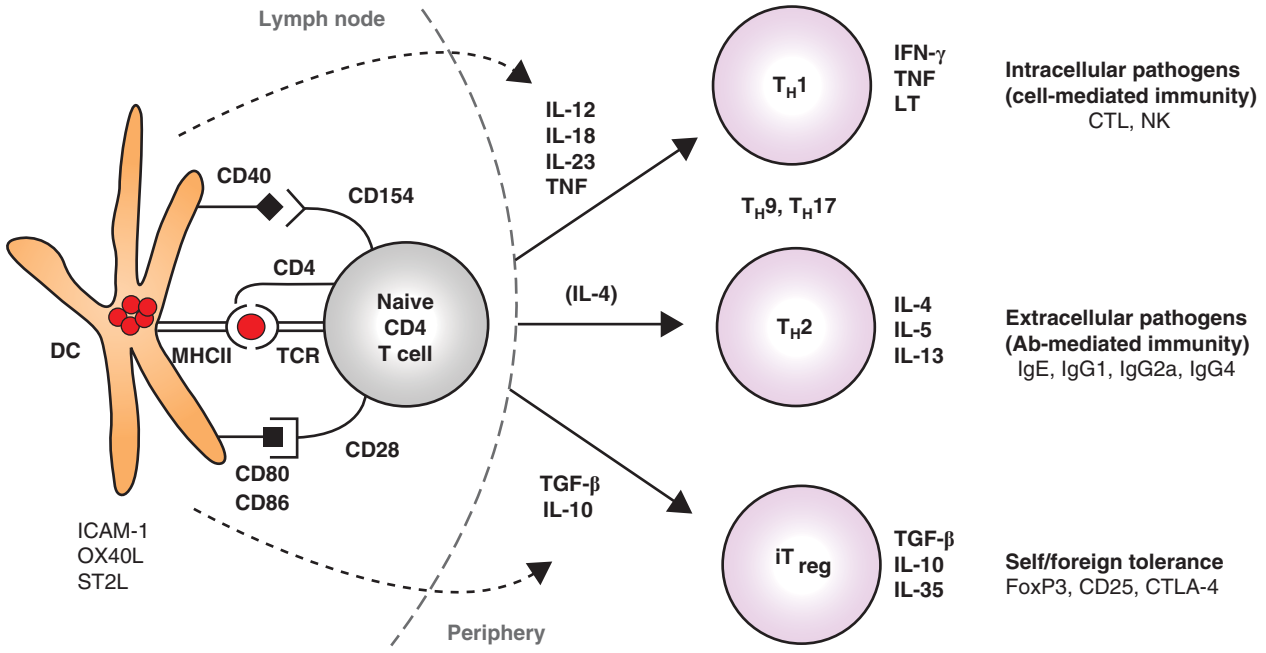

Figure 4. Regulation of naive CD4$^+$ T-cell differentiation into effector cell subsets by DC signaling. Recirculating naive antigen-specific CD4$^+$ T cells interact via their TCR and CD4 co-receptor with DCs expressing cognate MHC class II-restricted antigen in lymph nodes (signal 1). A variety of secondary co-stimulatory signals (signal 2), including CD40–CD40L and CD80/86–CD28 interactions, are then required for initiation of T-cell proliferation and differentiation. In addition, several DC-derived cytokines (signal 3), including IL-12, IL-18, IL-23, TNF, TGF-β and IL-10, then direct the fate of naive T-cell differentiation towards peripheral effector CD4$^+$ T-cell subsets, including: T_H1 cells, producing IFN-γ, TNF and LT responsible for helping cell-mediated cytotoxic clearance of intracellular pathogens such as viruses; T_H2 cells, producing IL-4, IL-5 and IL-13 responsible for helping IgE and IgG production by B cells for clearance of extracellular pathogens such as parasites, but also responsible for allergic inflammatory responses; and iT_{reg} cells expressing FoxP3, CD25 and/or CTLA-4, responsible for maintaining tolerance to self-antigens to prevent autoimmunity and tolerance to foreign innocuous proteins. Other T_H subsets including T_H9 and T_H17 cells have now been identified that appear to have pro-inflammatory roles in a variety of inflammatory diseases, including allergic asthma.

CD: Cluster of differentiation molecule; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; iT_{reg}: Inducible T regulatory cell; LT: Lymphotoxin; MHC II: Major histocompatibility complex class II; TCR: T-cell receptor; T_H: T helper (CD4$^+$) cell.

a specific anatomical compartment within the respiratory tract to obtain a desired immune response to a given antigen. The optimal immune response will differ if tolerance during an anti-allergic treatment is sought, or if immunity against a respiratory pathogen is the aim. For induction of active tolerance, preferential delivery of the antigen to the lung parenchyma, where inhibitory macrophages outnumber LPDCs, may constitute a feasible approach. On the other hand, targeting AMDCs in the conducting airways may represent an attractive option to induce immunity against a respiratory pathogen. However, the task of particle clearance for which the alveolar macrophages are responsible in the lung parenchyma is accomplished in the main conducting airways by the mucociliary escalator [5]: ciliated cells, mucus and surfactant including the opsonizing surfactant proteins SP-A and SP-D together constitute an extremely effective barrier against inhaled and deposited particles. Therefore, directly targeting airway mucosal DCs that are located beyond the mucociliary barrier with particulate carriers might be a challenging task. Targeting airway mucosal DCs with magnetizable aerosol droplets (as discussed below) could probably avoid mucociliary clearance in the main conducting airways. Targeting different respiratory – and immunological – compartments may be achieved by adapting the aerodynamic diameter of particles or aerosol droplets.

In general terms, passive and active targeting of a compound within a tissue can be distinguished. Passive targeting refers to the accumulation of a particular compound based solely on its biophysical properties. In the lung, passive targeting is achieved by size-dependent deposition of aerosol droplets or particles within different lung regions, a feature exploited for delivery of inhalational drugs (**Figure 5**) [125,126]. Deposition of inhaled particles within the respiratory tract occurs by inertial impaction, sedimentation and diffusion. Larger particles (diameter > 5 μm) undergo deposition by inertial impaction in upper airways (i.e., mouth and throat), whereas intermediate size particles (diameter 1 – 5 μm) preferentially deposit in the main conducting airways through

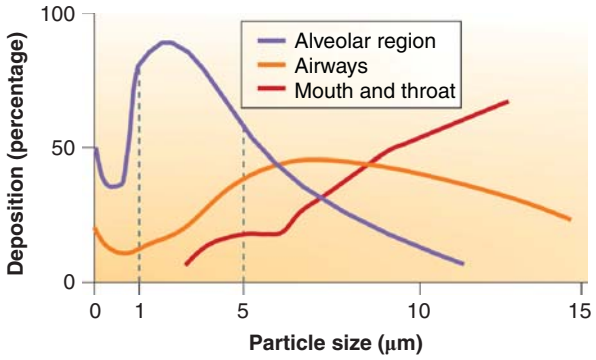

Figure 5. Size-dependent deposition of inhaled particles in the respiratory tract. Larger particles (10 μm) deposit in the upper respiratory tract, that is, mouth and throat, whereas intermediate sized particles (5 μm) reach the conducting airways and smaller particles (5 μm) the alveolar spaces in the lung parenchyma.
Adapted with permission from [126].

both inertial impaction and sedimentation, and small particles (diameter < 1 μm) enter the alveolar space by diffusion, where a certain degree of suspension and exhalation may occur (**Figure 5**) [125,126]. A more active form of pulmonary targeting has been achieved experimentally by directing magnetizable aerosol droplets composed of paramagnetic iron oxide nanoparticles to specific compartments in the respiratory tract [127]. With such an approach it may become feasible to target specifically lung areas of interest with new vaccine formulations based on specifically designed nanocarriers. New nanocarrier-based vaccination approaches in the respiratory tract have been performed experimentally and have achieved sustained antiviral immune responses, for example against respiratory syncytial virus with DNA-chitosan nanospheres [128]. Another study incorporated proteins of the bovine parainfluenza type 3 virus into poly (lactide-*co*-glycolide) (PLGA) and poly(methyl methacrylate) (PMMA) nanoparticles, generating a superior antibody response with the former [129].

6. The importance of size in vaccine delivery

Aerosol droplet or particulate diameter may represent a crucial issue when developing new pulmonary vaccines, either with or without specific carrier molecules. On the one hand, size will determine in which lung region a given particle or droplet will deposit (**Figure 6**), and on the other hand size will determine the fate of the delivered antigen, that is, speed, direction and efficacy of translocation into lung parenchyma, blood circulation, lymphatic system and specific pulmonary APC populations. Deposition of a specific vaccine dosage may seem straightforward in theory, but is a highly challenging issue in reality. **Figure 5** displays the size-dependent deposition of particles in the respiratory tract after a slow single inspiration with a breath hold after the inspiration [126]. To target upper airways regions, particles should ideally be 5 – 10 μm in size. For delivery to peripheral lung parenchyma, particles should not be either too small (increased subsequent exhalation), or too large (deposition in upper airways, mouth and throat). Aerosol particles with an aerodynamic diameter of ~ 1 – 2 μm, if slowly and deeply inhaled, can be deposited in the lungs with efficiencies as high as 90%, with most of the aerosol reaching peripheral alveolar regions [130]. Conventional vaccine antigens vary greatly in three-dimensional size [131,132]. The smallest antigens (< 10 nm) are protein or viral subunit antigen vaccines that are often combined with adjuvants (such as alum and Freund's adjuvant) to form larger particles or aggregates. Supramolecular particulate antigens, such as virus-like particles (VLPs) and nanoparticles, are larger, with antigen sizes 100 – 200 nm. Antigens presented formulated with microparticles, liposomes, water-in-oil emulsions (Freud's adjuvant), oil-in-water emulsions (MF59 adjuvant), mineral salts (alum) and whole-cell vaccines are the largest (100 nm – 20 μm) [133]. Antigen size is also an important determining factor for uptake by APCs. Particulate antigens such as whole-cell vaccines, virosomes and VLPs, or antigens formulated in particulate adjuvants such as liposomes and microparticles, have large surfaces that are endowed with charged, hydrophobic, or receptor-interacting properties. This leads to improved interaction between APCs and particles than between APCs and soluble proteins. Furthermore, many pathogen surfaces are highly repetitive and therefore allow efficient binding of natural IgM antibodies through high-avidity interactions, activating complement cascade through C1q. In addition, artificial hydrophobic surfaces, such as those of pluronic-coated nanoparticles, activate the alternative rather than the classical pathway of the complement cascade. This also results in increased uptake by DCs and macrophages [134]. In general, APCs prefer pathogen-sized particles and protein aggregates rather than smaller particles and protein antigens that are inefficiently taken up by those cells [135,136]. But what is the fate of smaller particles after their pulmonary deposition? As adaptive immune responses are mainly induced in secondary lymphatic organs, trafficking of antigen via the lymphatic system from peripheral tissues to lymphoid organs is crucially important for vaccine design. As lymph fluid is actively pumped against gravity, it is evident that exchange of water and macromolecules between the lymph and lung parenchyma requires regulatory mechanisms. Several factors control the influx of macromolecules into lymphatic vessels, with size being an important determinant. A recent study investigating trafficking of differently sized and charged nanoparticles deposited in the lung of rats showed that the hydrodynamic diameter along with the net surface charge of the nanoparticles are critical in determining the ultimate migration, biodistribution and clearance. The key findings of their study were: particles below a size threshold of 34 nm are rapidly translocated through the

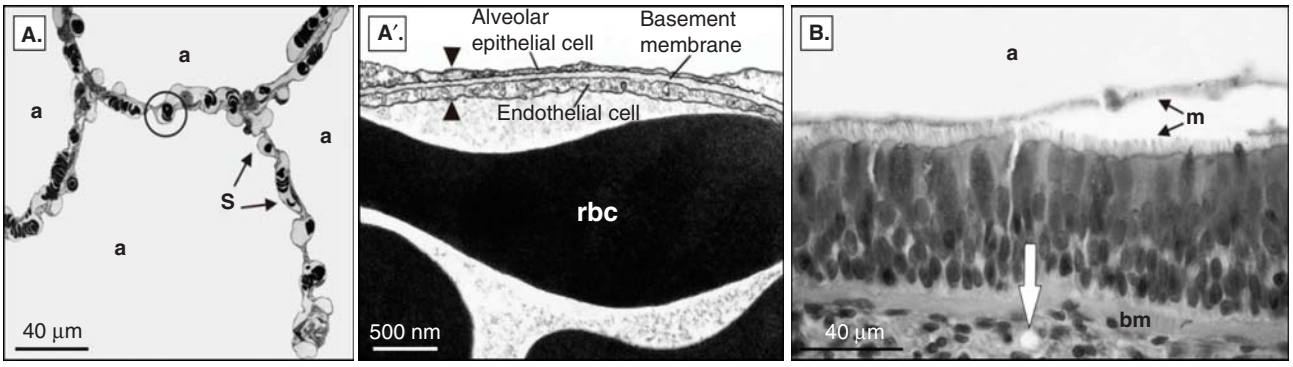

Figure 6. Air–blood barrier and epithelium in different respiratory tract compartments. A. Transmission electron micrograph of the alveolar air–blood barrier. The alveolar airspace (a) is surrounded by the interalveolar septum (s) containing the capillaries that are filled with red blood cells (black rounded structures). **A′.** Higher magnification of a similar location to the one circled in **A**. The thin air–blood barrier (arrowheads) shows a thickness of only a few hundred nanometers at some locations. **B.** Light micrograph of the respiratory epithelium in the trachea that consists of a stratified high-prismatic ciliated epithelium constituting the mucociliary escalator (m), and limited by a thick basement membrane (bm). Consequently, the diffusion distance from the luminal airspace (a) to the blood vessel (arrow) is increased in this compartment.
Courtesy of the Institute of Anatomy, University of Berne, Berne, Switzerland.
rbc: Red blood cell.

epithelium from the alveolar luminal surface to the septal interstitium, followed by rapid routing to regional draining lymph nodes, whereas larger particles require APCs (avidly taken up by such cells) to traffic to lymph nodes. Furthermore, particles with a hydrodynamic diameter < 6 nm and zwitterionic surface charge rapidly passed from the alveolar airspaces to the bloodstream from where rapid clearance occurred by renal filtration. Therefore, for rapid targeting of the lymph nodes the ideal size of a particulate vaccine should lie between 6 and 34 nm [137]. The particle size required to deposit antigen optimally to a specific lung compartment may be different from the ideal size to target specifically a particular APC. This dilemma may be particularly evident for a nanocarrier-based vaccine that may rapidly reach draining lymph nodes without significant deposition in the respiratory tract to interact with APCs. A new approach incorporated nanoparticles into micrometer-scale structures to overcome this issue. With this technique, nanoparticles are formulated into larger hollow or porous structures. These 'Trojan' particles embrace the virtues of both nano- and micrometer-scale particles because the microsized structures are readily deposited in the lung, whereas size-specific characteristics of nanoparticles are maintained after release by the humid environment and the lung lining fluid [138].

7. Conclusion

The respiratory tract provides an enormous surface for interaction between inhaled antigen and immune cells, which renders the pulmonary route versatile and highly promising for new vaccination approaches. Different APC populations are present throughout the entire respiratory system and their anatomically determined composition will determine the net immune responses generated by a given vaccine. Targeting DC populations in the lung parenchyma may consequently limit the efficiency of a vaccine owing to a relative excess of inhibitory macrophages. By contrast, delivery of a vaccine to AMDCs, the most frequent efficient APCs in the larger airways, may enhance immune responses against a pathogen. When targeting specific lung compartments, the size of an antigen with or without a carrier is an important factor determining deposition within the respiratory tract and vaccine efficiency. Smaller particles or aerosol droplets with an aerodynamic diameter of 1 – 3 µm will preferentially deposit in the peripheral lung parenchyma, whereas larger ones (5 – 10 µm) primarily reach the upper airways. Nanoparticles with aerodynamic diameters of 100 nm and less reach the alveolar region but their deposition is limited by exhalation. Size of an antigen and/or its carrier will also determine the interaction with specific APCs and how the antigen is trafficked to draining lymph nodes, where an immune response is generated. In studies with nanosized particles < 30 nm the particles rapidly enter the lymphatic system and passively drain to regional lymph nodes, whereas larger, pathogen-sized particles (from ~ 100 nm to a few micrometers) are efficiently taken up APCs.

Although future strategies for vaccination using the pulmonary route are promising, the field of pulmonary vaccine delivery is still in its infancy and some challenges need to be met before use can be made of successful new vaccination protocols.

8. Expert opinion

As stated in the Conclusion, the field under discussion in this review shows great potential for future approaches that

specifically target respiratory tract compartments and their associated antigen-presenting cell populations in order to generate the desired vaccine-specific immune response. However, there is still much basic research to be done before many of the vaccination strategies discussed have a chance for clinical application.

In this review it has been demonstrated that a large body of knowledge is available regarding the structural morphology of different lung compartments and particle deposition at these sites. In addition, much is known about different antigen-presenting cell populations in the respiratory tract, at least in rodents, cells that are essential in orchestrating innate and adaptive immune responses on interaction with inhaled antigen. Furthermore, an increasing number of potential particulate vaccine carriers have been described in recent years, some of which have been characterized in inhalation studies. But what exactly are the requirements in the near future to bring this knowledge together in order to develop promising pulmonary vaccination strategies? First, a better understanding is needed of basic mechanisms of the pulmonary cellular immune system. For example, several different subpopulations of RTDC have been characterized, yet so far we have limited knowledge of the exact function of these subpopulations in differing pulmonary anatomical locations and how animal studies translate to the human respiratory tract. A need for further knowledge also applies to the interplay of RTDC subpopulations with other important immune cells such as lymphocytes, monocytes, macrophages and cells of the innate immune system (e.g., NK and NKT cells) and how this may influence the strength and class of the immune response to vaccine vectors. Second, in the near future more attention will need to be given to the design and in-depth characterization of biomedical nanoparticles. For the field of pulmonary vaccination to progress, new strategies will be needed, such as nanoparticles that can be targeted to specific compartments in the respiratory tract with high deposition rates and biopersistence tailored according to the specific vaccination approach. Importantly, a new vaccination strategy should induce desired immunity against a given pathogen without compromising the exquisitely fragile air–blood barrier and vital gas exchange by excessive inflammation. Finally, the formulation of vaccine nanoparticles that can be optimally targeted to specific subsets of antigen-presenting cells in specific anatomical locations of the respiratory tract offers great potential for manipulating adaptive immune responses in lungs and draining lymph nodes, and tailoring the appropriate class of immune response required for optimal protection, be it T cell, B cell, or even innate cell responses. In this regard, the emerging field of nano-immunology, which aims to investigate the effects of nanoparticles on the mammalian immune system and how they can be controlled, will become increasingly important in the near and distant future.

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Declaration of interest

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