Identification and characterization of human dendritic cell subsets in the steady state: a review of our current knowledge

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ABSTRACT

Dendritic cells (DC) are generally categorized as a group of rare antigen presenting cells that are to the crucial development of immune responses to pathogens and also of tolerance to self-antigens. Therefore, having the ability to identify DC in specific tissues and to test their functional abilities in the steady state are scientific gaps needing attention. Research on primary human DC is lacking due to their rarity and the difficulty of obtaining tissue samples. However, recent findings have shown that several different DC subsets exist, and that these subsets vary both by markers expressed and functions depending on their specific microenvironment. After discriminating from other cell types, DC can be split into myeloid and plasmacytoid fractions. While plasmacytoid DC express definite markers, CD123 and BDCA-2, myeloid DC encompass several different subsets with overlapping markers expressed. Such markers include the blood DC antigens BDCA-1 and BDCA-3, along with Langerin, CD1a and CD14. Marker specificity is further reduced when accounting for microenvironmental differences, as observed in the blood, primary lymphoid tissues, skin and lungs. The mixed leukocyte reaction (MLR) has been used to measure the strength of antigen presentation by specific DC subsets. Surface markers and MLR require standardization to enable consistent identification of and comparisons between DC subsets. To alleviate these issues, researchers have begun comparing DC subsets at the transcriptional level. This has allowed degrees of relatedness to be determined between DC in different microenvironments, and should be a continued area of focus in years to come.

INTRODUCTION

Dendritic cells (DC) are a specialized form of antigen presenting cell (APC) found in most parts of the human body, including the lymph nodes,[1] skin,[2] blood,[3] spleen[4 5] and lung.[6] Their main role is to sense and process antigens, then dictate the strength, specificity, and direction of the resulting responses by other cell types of the innate and adaptive immune system (hence the name APC). In the past decade, immunotherapy, particularly the use of donor-derived DC against established cancers, has shown efficacy and feasibility.[7–11] However, such treatments may be improved with the use of DC subsets that functionally specialise in clearing cancerous cells. DC have been implicated as a Trojan horse of infection for a number of pathogenic organisms, including the bacteria *Bacillus anthracis*[12 13] and *Burkholderia pseudomallei*,[14] and the fungus *Cryptococcus neoformans*.[15] Dysregulated DC may play a major role in the disease processes of several chronic disorders, including chronic obstructive pulmonary disease,[16] multiple sclerosis[17] and systemic lupus erythematosus.[18] To really know how human DC contribute to the initiation, progression, and/or cessation of various diseases, we first need to understand how DC function in the absence of disease, that is, in the steady state. In fact, understanding the classification and function of DC and their subtypes is likely to result in association of these cells with additional disease entities. The goal of this review is to describe the subsets of human DC present in various tissues, and their characteristics, based on current experimental data. It is recognized that subset classification based on current approaches has its limitations, and thus it will be necessary to describe how these approaches can be optimized in order to develop a lasting, reproducible schema that is widely applicable.

Although the generalized DC population has been identified and characterized for several decades, many of the major developments in the field of DC biology during this time were limited to small animal and in vitro cell culture models. In this review, we try to focus on only work conducted using human primary cells and tissues, while not discounting the contribution of studies using other models towards human medicine. Human DC research, however, will always have some intrinsic constraints. First there is the rarity of DC within the total cell population of all human tissues. In human blood, they represent only about 1% of total peripheral blood mononuclear cells (PBMC),[19] and their percentages are similar in other tissues. The second issue relates to the cost/difficulty in obtaining human tissue. Except for blood obtained from donation, other healthy tissue sources are not readily available and must be obtained following some type of surgical procedure. Finally, DC cannot be tracked in a live person as can be done in animal models. These limitations are exacerbated when trying

to characterize subsets of DC based on surface markers and specialized functions. This situation has been helped by the strong push towards translational research in the 21st century, when more attention has been paid towards comparing animal DC, particularly mouse, to human DC at the transcriptomic level.[20 21 22] Researchers have just begun to realize that DC in different microenvironments can vary significantly in both their specific markers' expression levels and their functional specificity.[23] It can be dramatically seen among the human DC subsets in the blood, lymphoid tissue, skin, and lung, the anatomical sites investigated in this review. After outlining an initial framework for DC identification using surface markers, functional assays, and transcriptomic analyses, along with the constraints of each, we focus on skin and lung DC subsets. These cells constantly interact with environmental antigens and pathogens, play a role in human diseases, and are a subject of active current investigation.

STRATEGIES AND CHALLENGES OF IDENTIFYING HUMAN DC SUBSETS

Identification by surface markers

DCs were originally named based on their morphology, having membrane processes or 'dendrites' for movement and sampling of their environment.[24] In addition, these cells showed a high cytoplasmic to nuclear ratio.[24] However, these morphological details are not enough to specifically identify DC subsets,[25] especially considering that they can change rapidly with minor environmental perturbations that inevitably occur during DC isolation.[26] There has never been a single marker that identifies and separates human DC from their nearest phagocytic relatives, monocytes and macrophages. Even looking at purely DC, a major issue encountered is the absence of a fixed set of surface markers to identify their subsets. As potential new markers are identified, it becomes more difficult to further tease out individual DC subsets. Too broad of a marker does not separate all subsets, while too specific of a marker may ignore certain subsets. As new marker combinations are suggested for specific DC subsets, what was thought to be a single subset a decade ago may now be identified as three separate and even rarer populations. A continuing challenge to this field is to use these markers in a consistent and reproducible manner to define discrete DC subsets with specific functions, rather than to simply identify rare populations without clear physiological roles.

Fortunately, basic human DC identification is relatively standardized by the implementation of certain surface markers which are commonly used to exclude other cell types. These include CD3 (T lymphocytes), CD19 (B lymphocytes), CD20 (B lymphocytes) and CD56 (natural killer cells).[27] After excluding B lymphocytes, APC status in the steady state can be confirmed by major histocompatibility complex (MHC) class II surface expression (specifically human leukocyte antigen (HLA)-DR in humans).[25] This strategy has been accepted as a way to differentiate monocytes/macrophages/DCs from other cell types found in human tissues. Another molecule expressed by most myeloid APC is the integrin CD11c.[28] While it is not found on all myeloid DC (mDC) subsets,[25] its absence has been included in identifying human plasmacytoid DC (pDC).[3 29] Studies of human skin have shown that CD11c is also expressed by Langerhans cells (LC), $CD1a^+$ DC, and $CD14^+$ DC,[30] subsets that are described in more detail below.

In an effort to finally standardize human DC identification, Dzionek *et al*[3] in 2000 generated a wide variety of monoclonal antibodies against various surface molecules on human PBMC. Using flow cytometry and the lineage exclusion strategy described, the blood DC antigens (BDCA) were first characterized.[3] BDCA-1 (CD1c), BDCA-2 (CD303) and BDCA-3 (CD141) were established as viable surface markers for three distinct DC subsets in human blood. BDCA-2 expression correlates with CD123 (IL-3R) expression on the $CD11c^-$ non-myeloid cells which have been identified as pDC.[31–33] pDC show several characteristics of T lymphocytes, including expression of CD4 and the α chain of the pre-T cell receptor.[32] Functionally, pDC express endosomal TLR7 and TLR9 to sense viral and bacterial nucleic acids.[34] In response to viral infection, pDC can rapidly secrete high levels of type I interferon (IFN).[35] The specificity of BDCA-2 as a marker for pDC relates to its function. Antibody ligation of BDCA-2 has been shown to partially, and in some cases completely, inhibit type I interferon production resulting from TLR7 and TLR9 stimuli.[36 37] Therefore, this C-type lectin likely has an in vivo modulatory role in pDC by inhibiting induction of type I interferon and downstream interferon-sensitive genes. In the steady state, pDC are thought to play a tolerogenic role in primary lymphoid tissue,[38] and mechanistically may function through upregulation of inducible costimulator ligand to drive T regulatory cell (T_{reg}) differentiation.[39] However, pDC presence in peripheral non-lymphoid tissues under steady-state conditions has not been well documented. This lack of data likely contributes to the idea that pDC infiltrate the periphery quickly on viral infection, but remain in circulation and in primary and secondary lymphoid tissues during homeostasis.[40] Conversely, pDC have also been reported in human dermis, which could suggest a natural low level occurrence in this tissue type.[41] This observation is likely caused by microenvironmental differences between dermis and other peripheral tissues. The unique combination of interacting cell types found within the dermis allows and maintains a resident pDC population lacking in other peripheral tissues. Unfortunately, while BDCA-2 is indeed specific for pDC, it is rapidly downregulated during in vitro culturing.[3] CD123, therefore, has remained (in combination with lineage exclusion and HLA-DR expression) a specific marker for pDC identification in culture.[3 29]

BDCA-1 and BDCA-3 identify two subsets of mDC from human blood ($BDCA\text{-}1^+$ and $BDCA\text{-}3^+$ DC, respectively), though expression of BDCA-3 is upregulated on $BDCA\text{-}1^+$ DC and pDC with time in culture.[3 29] Thus, combinations of surface markers were developed to separate the known subsets of DC in human blood, though $BDCA\text{-}1^+$ mDC and $CD123^+$ pDC were the only two subsets which could be uniquely identified after being cultured for significant periods of time.[3 29] Group 1 CD1 molecules (CD1a, CD1b and CD1c) are especially important for presenting non-peptide self and microbial antigens to T cells.[42] However, even further specialization likely occurs between these molecules based on their structures and routes of cellular trafficking. CD1c has recently been shown to specifically interact with the T cell receptors of tissue-resident γδ T

cells.[43] Additionally, CD1c traffics through early and late endosomal compartments, which is not true of either CD1a or CD1b.[44] These results collectively suggest that CD1c expression by BDCA-1$^+$ DC may relate to this cell type's function in early defense against a wide range of microbial pathogens. Transit of these internalized pathogens through endocytic compartments allows for processing of non-peptide antigens for detection by γδ T cells with the assistance of CD1c. The implications of the variations in BDCA-2 and BDCA-3 expression seen during culturing are unknown. One possibility is the existence of plasticity within and between the two DC subsets expressing these markers. In that case, upregulation or downregulation of BDCA surface markers would likely coincide with actual functional changes. A direct comparison of functionality in the form of cytokine production or T cell proliferation over time in culture would answer this important question. Alternately, fluctuation in marker expression could be an artifact of isolation and not reflect normal in vivo conditions. Coculturing of the major cell types from source tissue would then result in stabilization of surface markers and would provide a better representation of how these DC subsets exist in a steady-state microenvironment. Of the ~1% of freshly isolated PBMC that are DC, about 90% are equally either CD123$^+$ pDC or BDCA-1$^+$ DC, while the remaining 10% are BDCA-3$^+$ DC.[29] Recently, additional markers have been implicated to further specify human BDCA-3$^+$ DC. These include the C-type lectin CLEC9A[45] and the chemokine receptor XCR1.[46] These markers were seen in BDCA-3$^+$ DC from spleen and blood, respectively, so they may not be helpful in identifying BDCA-3$^+$ DC in non-lymphoid peripheral tissue. Although BDCA-3 is not a unique marker for BDCA-3$^+$ DC, its expression level is higher on this cell type than other DC subsets.[20] The functional consequence of this enhanced expression is unclear. However, CLEC9A is a key regulator in the cross-presentation of necrotic cells, which relates directly to the functional specialty of this DC subset.[47] Finally, XCR1 is a receptor specific for the ligand XCL1, which is produced primarily by CD8$^+$ T cells.[48] Thus, XCR1 facilitates the interaction of BDCA-3$^+$ DC with CD8$^+$ T cells, allowing for cross-presented antigens on the DC to stimulate these T cells. A complete antibody screen of cells isolated from peripheral tissue, as was originally conducted for blood DC, may reveal that BDCA markers are not the best in identifying DC subsets in other tissue types. A diagram summarizing the markers of DC subset identification described above and those still to be discussed can be found in figure 1 for reference.

Identification by mixed leukocyte reactions

At the functional level, the classically identified DC is an APC which has a high stimulatory capacity in the allogeneic mixed leukocyte reaction (MLR). While MLR results alone cannot identify DC subsets, they commonly provide a functional profile for cells expressing a specific combination of surface markers. In this assay, APC are isolated and are exposed to a given stimulus (or not). The cell(s) of interest are then combined with naïve allogeneic CD4$^+$ or CD8$^+$ T cells, and several days later, a function of the T cells is measured and correlated with the strength of antigen presentation.[49] However, there are many variables in this assay that can confound generalizations of a DC subset, or prevent practical comparisons between DC subsets. One issue is the target cell type used in the assay. Conventional MLR tend to focus on CD4$^+$ T cell responses rather than responses of other T cells, especially when the APC in question is exposed to exogenous stimuli. The use of CD4$^+$ T cells in MLR has the obvious advantage that the direction of the immune response (T_h1, T_h2, T_h17, etc) can be determined in addition to the strength of stimulation by the measurement of specific cytokine levels. This protocol is also consistent with the original notion that exogenous proteins were internalized by DC and their resulting peptides solely presented on class II MHC molecules to CD4$^+$ T cells. Conversely, intracellular pathogens like viruses, along with endogenous proteins were thought to yield peptides for class I MHC presentation to CD8$^+$ T cells. However, the discovery of cross-presentation by DC, whereby exogenously-acquired antigens are presented on class I MHC,[50 51] redefined antigen presentation. BDCA-3$^+$ DC have recently been identified as a subset of strong cross-presenting cells.[48] Therefore, standardization of the target cell should involve testing APC mixed with not only CD4$^+$ T cells but also separately with CD8$^+$ T cells. The readout for strength of antigen presentation by a given DC subset in response to exogenous antigens would then encompass the standard class II MHC to CD4$^+$ T cells, as well as the cross-presented class I MHC to CD8$^+$ T cells. A second problem concerns the type of stimulus used, be it bacterial, viral, fungal or chemical. Consistent, reproducible DC subset identification will likely require standardization of the assay by using specific stimuli to serve as benchmarks of each stimulus category. For example, testing a DC subset with *Escherichia coli* for gram negative bacteria, *Staphylococcus aureus* for Gram positive bacteria, and influenza for viruses. That way, responses seen by different groups can be compared. Even if looking for an antigen-specific response, there is still the issue of stimulus concentration. Whenever possible, a dose–response curve should be generated for all stimuli tested. The next difficulty is the ratio of APC to T cells. The ideal experimental design tests multiple ratios, with the best antigen presenting cell-type being the one able to stimulate the most T cells at its lowest concentration. Being able to test multiple ratios becomes difficult with rare primary DC. Finally, the measure of antigen presentation varies greatly between studies. Some groups measure T cell proliferation by [^{3}H]-thymidine incorporation, while others measure it by carboxyfluorescein succinimidyl ester (CFSE) dilution. This is an important point, because although there is usually high correlation between the two methods, their results may diverge in poorly proliferating samples.[52] Such would occur in T cells exposed to DC with low antigen presentation potential. With immunological developments in T cell polarization, measuring certain cytokines such as IFN-γ for T_h1 or IL-4 for T_h2 responses has also become common. However, T cell proliferation and cytokine production do not always correlate, as just a few cells can produce high cytokine concentrations. Including MLR results in functionally defining broad DC types is of current use, but further classification and comparison of DC subsets will likely require tightly standardized methodology.

Despite its flaws, MLR can provide comparative data between APC subsets under a specific set of conditions.

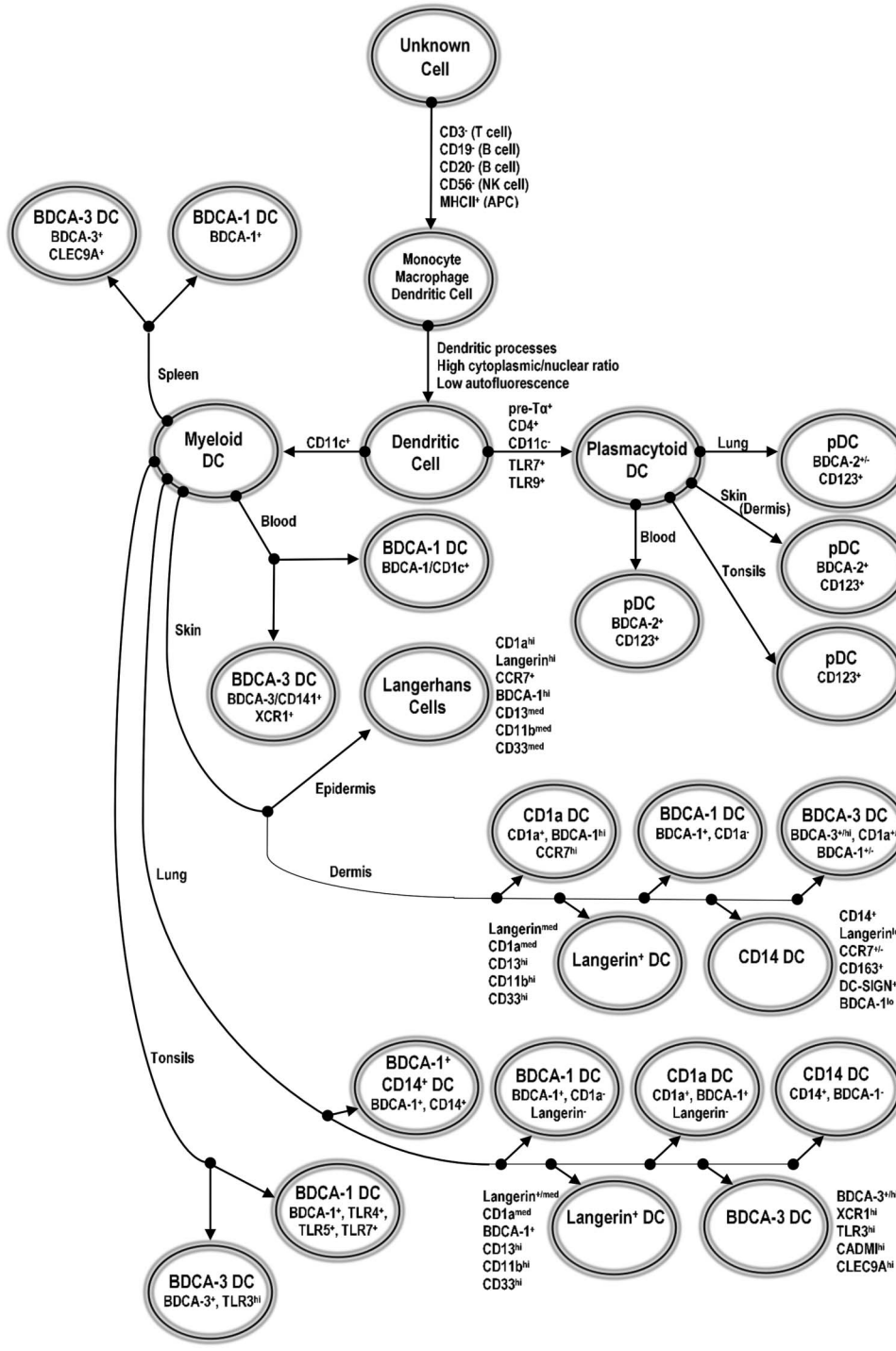

Figure 1 Summary diagram of stepwise human DC subset identification, per markers and tissue type. CADM1, cell adhesion molecule 1; DC, dendritic cell(s); pDC, plasmacytoid DC; BDCA, blood DC antigen; MHC, major histocompatibility complex.

For example, MacDonald *et al*[29] in 2002 further differentiated blood DC subsets by MLR as measured by [^{3}H]-thymidine incorporation. Importantly, they also compared isolated blood DC subsets to monocyte-derived DC (mo-DC) as a standard. Monocyte-derived DC should serve as a cell-type benchmark in MLR due to their accepted techniques of generation and the availability of blood monocytes. McDonald *et al*[29] saw that BDCA-1$^+$ DC were better at T cell stimulation compared to BDCA-3$^+$ DC, which in turn were better than CD123$^+$ pDC. BDCA-1$^+$ DC were also more stimulatory than mo-DC. However, their MLR did not have a defined stimulus, so it was based mainly on the MHC I stimulation of T cells rather than MHC II-presented peptides of a certain antigen. Another issue, which they acknowledged, was a drop in viability of, at least, the pDC, which could certainly have affected the comparative results of the MLR.

These studies yielded very important data for further characterization of human DC subsets. However, they also illustrated that DC subsets were not as unique as some cells (like T cells compared to neutrophils). Rather, they were a heterogeneous mixture of cells that shared many functional characteristics. Surface markers and MLR results were insufficient to further classify DC subsets. As suggested earlier, this observation could also point towards the plasticity of DC subsets. While DC are indeed terminally differentiated, a specific DC subset identified by surface markers could show functional characteristics of multiple subsets depending on culture conditions and stimuli presented. Plasticity between CD123$^+$ pDC and BDCA-1$^+$ DC has been observed in vitro without added stimuli,[53] so it is likely that similar phenotypic changes occur regularly in vivo with microenvironmental fluctuations. With the advent of rapid genomic sequencing and microarray analysis, rare cell populations can now be compared at the transcriptional level. Degrees of relatedness can be determined based on transcriptomes and differentially expressed genes (DEGs), or genes which are expressed at the RNA level in one cell type but not in another, can be identified for downstream function.

Identification by transcriptomic comparisons

In 2005, Lindstedt *et al*[54] were the first to use transcriptomic technologies to investigate and compare human blood and tissue-resident DC. They compared BDCA-1$^+$ DC, BDCA-3$^+$ DC and CD123$^+$ pDC pairwise from blood and tonsils using a strict criteria of >2-fold expression difference to define differential expression. Although they were only able to obtain cells from three donors for blood and tonsils, their high fold expression criteria for classifying differential expression gave credence to their results. Hierarchical clustering between DC subsets showed that pDC in the blood and tonsils were closely related to each other, as would be expected for cells of similar function in the two locations. Surprisingly, BDCA-1$^+$ DC in the tonsils were more closely related to BDCA-3$^+$ DC in the tonsils than they were to BDCA-1$^+$ DC in the blood. Conversely, BDCA-1$^+$ DC in blood were more closely related to blood BDCA-3$^+$ DC than to BDCA-1$^+$ DC in the tonsils. Tonsils are unique lymphoid organs similar to lymph nodes. Their mucosal surfaces are continually exposed to potential pathogens via nasal and oral routes and they can be sites of B-cell proliferation. Blood as a closed system is not regularly exposed to pathogens, and contains a much different spectrum of non-DC cell types compared to tonsils, all at different relative frequencies. DC in tonsils and blood thus exist in greatly differing microenvironments. These microenvironmental differences between the tonsils and blood likely played a significant role in the observed differences in the gene signatures of BDCA-1$^+$ and BDCA-3$^+$ DC in these tissues. While pDC in tonsils and blood showed several hundred DEGs compared to BDCA-1$^+$ and BDCA-3$^+$ DC, these two myeloid subsets had far more DEGs in common than not in both blood and tonsils. BDCA-1$^+$ and BDCA-3$^+$ DC, thus, may arise from a common precursor cell or may even be stages of a common differentiation pathway. Others[20] have challenged these findings by comparing BDCA-1$^+$ and BDCA-3$^+$ DC from blood with their counterparts isolated from human skin and noted a substantial difference between transcriptomes of these two subsets in both compartments and strong transcriptional overlap between the same subsets between compartments. There are explanations for these apparently contradictory results. Tonsil and skin makeup are quite different, with the former being a type of lymphoid tissue and the latter being composed of epidermal and dermal layers. Skin contains afferent and efferent lymphatics, which allow constant bulk flow of lymph, while tonsils only have efferent lymphatics that allow drainage of antibodies and lymphocytes. In their analyses, Lindstedt *et al*[54] focused on BDCA-3$^+$ DC, while Haniffa *et al*[20] specified a BDCA-3^{hi} population. Thus, it is possible that Lindstedt *et al*[54] were investigating a mixed cell population with varying levels of BDCA-3 expression, with some cells that were more susceptible to microenvironmental conditions. Finally, the fold expression differences used to classify DEGs is a major source of variability in transcriptomic studies. Unlike the suggested standardization of surface markers and MLR, an appropriate cut-off for gene expression differences is difficult to determine. Too low of a cut-off allows background noise to cloud results, while too high of a cut-off excludes rare genes with naturally low expression values.

Realizing the limitations of obtaining human tissue-specific DC subsets from other anatomical locations, Robbins *et al*[22] in 2008 used transcriptional profiling to compare lymphoid-resident (spleen) DC from mice to the human blood DC data already available. Their results showed strong overlap between pDC in the two species. More importantly, it gave comparability between species by establishing cell equivalents to the BDCA-1$^+$ and BDCA-3$^+$ human DC (CD11b$^+$ DC and lymphoid-resident CD8α^+ DC, respectively, in mice). In this latter comparison, there was not complete overlap, however, with high genetic distances observed between these suggested cellular relatives from different species. This is not a surprise considering the variable degrees of relationship previously observed between humans and mice at the transcriptional level.[55–57] Haniffa *et al*[20] more recently conducted similar transcriptional profiling of mouse non-lymphoid CD103$^+$ DC and found a closer relationship between these cells and human BDCA-3$^+$ DC. These discrepancies may be explained by the anatomy of lymphoid versus non-lymphoid peripheral tissue. Like tonsils, the spleen does not contain afferent lymphatics. Conversely, human blood DC are generally accepted to be the precursors of most peripheral tissue DC subsets. In peripheral tissue like the skin and lungs, DC continually migrate from the tissue to draining lymph nodes, while being replenished by DC subsets in the blood. Robbins *et al*'s[22] work agrees somewhat with that of Lindstedt *et al*[54] in that the microenvironment of spleen and tonsillar lymphoid tissues (whether in mice or humans) clearly affects the transcriptional profile of resident DC subsets when compared to blood subsets expressing the same surface markers. These transcriptomic studies have provided the DC field with subset comparability in a feasible, live-animal model not restricted by availability as human tissues are.

Although marker definition and functionality are not a panacea for DC subtyping, with the clear transcriptomic differences observed between anatomical DC reservoirs in humans,[54] we suggest that continued attention should

focus more on how primary tissue cells respond to various stimuli in the context of their specific microenvironment. This would entail the important work of making transcriptomic comparisons of DC subsets isolated from the same tissues in humans and other species, such as the mouse.

PATTERN RECOGNITION BY BDCA$^+$ DC SUBSETS

Since DC in general have been most studied in the context of infection, it would seem vital to identify pattern recognition receptors (PRR) expressed by the described human DC subsets.[58] BDCA-1$^+$ and BDCA-3$^+$ DC from tonsils have been analyzed for toll-like receptor (TLR) expression,[59] with important differences seen that could suggest functional specialization. While subsets showed mRNA expression of TLR-1, TLR-2, TLR-3, TLR-6, TLR-8, and TLR-10, only BDCA-1$^+$ DC expressed mRNA for TLR BDCA-4, BDCA-5 and BDCA-7. For specific function of BDCA-3$^+$ DC, the expression level of TLR-3 was much higher compared to BDCA-1$^+$ DC at the mRNA and protein levels. TLR-4 has been well studied as a PRR for the Gram (−) bacterial component lipopolysaccharide (LPS), while TLR-5 senses Gram (−) and Gram (+) flagellin.[60] Both of these TLRs are present on the cell surface, suggesting a specific role for BDCA-1$^+$ DC in sensing a wide variety of bacteria. TLR-3 and TLR-7 are both endosomal PRR, but while TLR-3 senses double-stranded RNA, TLR-7 is specific to single-stranded RNA.[60] Using this information, Jongbloed *et al*[59] discovered a PRR difference between BDCA-1$^+$ and BDCA-3$^+$ DC that likely relates to the specificity of each subset for sensing certain types of viruses. This was supported by their results in a variation of the MLR after BDCA-1$^+$ and BDCA-3$^+$ DC were exposed to human cytomegalovirus, a virus known to produce dsRNA. Only BDCA-3$^+$ DC were able to stimulate CD8$^+$ T cells, which mechanistically requires viral peptide cross-presentation on MHC class I instead of MHC II peptide presentation to CD4$^+$ T cells. Since this work, others have further defined BDCA-3$^+$ DC as functionally and transcriptomically specialized for cross-presentation of exogenous antigens.[20] However, as with the BDCA markers, microenvironmental differences likely affect expression levels of PRR by DC subsets. This has been observed in mice, specifically between CD11c$^+$ antigen presenting cells of lung, colon and spleen mucosal surfaces.[23] Therefore, functional generalizations of the BDCA$^+$ DC subsets are valid to some extent, but must take into account both tissue type and location under steady-state conditions.

CHARACTERIZATION OF DC SUBSETS IN HUMAN SKIN

As the examination of human DC shifted into various peripheral tissues, new DC subsets were identified that did not have definite homologs in the blood. The most well-studied compartment in this respect has been human skin, and we will use this tissue to introduce other characterized DC subsets. It should be mentioned that human peripheral tissues also contain resident macrophage populations (reviewed recently by Davies *et al*[61]). These cells are considered important for removal of dead cells and debris in the periphery under the steady state and also after injury or infection. They are generally regarded as non-migratory and poor at antigen presentation. While not further characterized here, they should be included when discussing DC function due to their continuous interactions with DC subsets in the periphery.

Based on animal studies, DC in the periphery (as compared to blood DC) have a functional characteristic of migration to regional lymph nodes via afferent lymphatics. There they present antigen to T cells to either maintain tolerance under steady-state conditions or to generate an adaptive immune response under inflammatory conditions.[62] Human skin is actually composed of two separate microenvironments with different DC subsets within each. In the outer epidermis are found LC, characterized by their high expression of the non-classical MHC-I molecule, CD1a, and of the C-type lectin, Langerin.[30 63 64] These cells phenotypically also contain specialized rod-shaped cytoplasmic structures called Birbeck granules (BG).[65] Although Langerin is found on the cell surface, it tends to strongly associate with BG intracellularly.[65] The function of BG is yet to be determined, but may be a part of the endosomal pathway (though may not be endocytic structures themselves), as mutations in *langerin* lead to functional LC lacking BG,[66] and antibody ligation of surface Langerin leads to its colocalization with BG.[65] Langerin binds to specific sugar moieties, including certain mannose, glucose, galactose, and fucose residues, all within a single region containing multiple potential interacting groups.[67] Such variation in Langerin ligands likely allows its binding to, and facilitates internalization of, fungal, bacterial and viral pathogens. In the deeper dermal layer of human skin are found CD14$^+$ DC and CD1a$^+$ DC.[63 68] CD14$^+$ dermal DC also express Langerin, but at lower levels compared to LC.[69] Larregina *et al*[69] have suggested that the CD14$^+$ DC are actually precursors to epidermal LC, and showed that addition of transforming growth factor-β1 (TGF-β1) was sufficient to transform CD14$^+$ DC into LC. This may be a physiologically important finding considering TGF-β1 is an anti-inflammatory cytokine and that the epidermal layer of the skin is constantly exposed to non-pathogenic particles to which an inflammatory response is undesirable. Morelli *et al*[70] in 2005 further sought to separate CD14$^+$ DC from their proposed differentiated form, LC, using functional assays. While both cell subsets expressed CCR7, a known homing receptor for DC to tissue draining lymph nodes,[71] they showed contrasting results in MLR using allogeneic CD4$^+$ T cells. LC exposure to known T_h1 stimulants, followed by MLR, resulted in T_h1-biased CD4$^+$ T cells as measured by cytokine production of IFN-γ and T-cell proliferation. In comparison, CD14$^+$ DC only weakly triggered T cell proliferation, while they themselves produced large quantities of the anti-inflammatory cytokines IL-10 and TGF-β. At longer periods of incubations with T cells, CD14$^+$ DC could be skewed towards a T_h1 response of IFN-γ production and T cell proliferation, but only at high APC:T cell ratios. These results went along with the idea that CD14$^+$ DC were indeed immature forms of LC. However, Morelli *et al*[70] grouped LC based on CD1a expression rather than location, so it is possible these cells were a mixture of epidermal LC and dermal CD1a$^+$ DC, as previously described.[63 68] High CD1a expression likely relates to the function of both cell types, but in two different layers of human skin. Unlike CD1c/BDCA-1, CD1a has restricted trafficking from the cell surface to the recycling

endosome (but not the lysosome).[72] Differential trafficking of CD1a would allow pathway-specific processing of lipids from pathogens that reside within this intracellular compartment. Acknowledging that CD1a is expressed on both LC and CD1a$^+$ dermal DC, others have addressed this concern by separating epidermal and dermal sheets of the skin prior to DC subset isolation.[68 71] Angel *et al*[71] further purified the CD1a$^+$ dermal DC by eliminating Langerin$^+$ cells, which were assumed to be contaminating LC, from the dermal preparation. This prevented having to make subjective separation of LC from CD1a$^+$ dermal DC based on levels of CD1a surface expression. In their hands, CD1a$^+$ DC were far more potent in stimulating CD4$^+$ T cells than CD14$^+$ DC were. This work agreed with earlier studies by Nestle *et al*[63] that showed CD1a$^+$ DC were as equally potent in stimulating naïve CD4$^+$ T-cell proliferation as LC were, even down to APC:T-cell ratios of 0.001. Fortunately, as Morelli *et al*[70] and Nestle *et al*[63] had thoroughly tested multiple APC:T-cell ratios to measure antigen presentation capacity of their DC populations, their experimental results are directly comparable.

All these results originally supported the idea that CD14$^+$ DC were potentially immature precursors to LC, but did not eliminate the possibility that they were also precursors to CD1a$^+$ DC. Investigating the causes of graft versus host disease, Haniffa *et al*[68] in 2009 answered this question by using skin transplant studies to show that retention times of recipient dermal CD14$^+$ DC were longer than that of CD1a$^+$ DC. So, while CD14$^+$ DC could be precursors of LC in the epidermis, their slower turnover rate suggested that they were not likely to be precursors of the CD1a$^+$ DC subset in the dermis. CD1a$^+$ DC also expressed higher levels of the costimulatory molecules CD40, CD80, and CD86 than did the CD14$^+$ DC, consistent with their MLR results. Conversely, CD14$^+$ DC have also shown an immature phenotype based on their low levels of the activation/maturation marker CD83 when compared to CD1a$^+$ DC.[73] Interestingly, CD14$^+$ dermal DC show variable expression of the scavenger receptor CD163,[68 74] which is commonly found on monocytes and macrophages, yet, they have also shown specific expression of DC-SIGN, a marker commonly associated with DC.[30 75] More recent transcriptional profiling confirmed the mixed monocyte/DC characteristics of CD14$^+$ dermal DC by showing their overlapping relationships with BDCA-1$^+$ DC and CD14$^+$ blood monocytes.[20] These results collectively suggest that monocytes are the blood precursor of CD14$^+$ dermal DC, without precluding the CD14$^+$ dermal DC as the precursor of epidermal LC.[69] However, no research has suggested that LC carry monocytic characteristics. Data against this relationship has also come from case studies involving patients with either sporadic or familial monocytopenia, which show that LC of the epidermis can still be found in normal frequencies, while both dermal DC populations (BDCA-1$^+$ and CD14$^+$) are absent.[76] In another case, human recipient LC were maintained 10 years after a skin allograft, whereas other DC populations showed donor origin rapidly.[77] These observations suggest that: (1) LC in the steady state can self-renew in the epidermis without the need for a bone marrow-derived precursor, and (2) CD14$^+$ DC may only renew the epidermal LC population under inflammatory conditions. Further in-depth transcriptional analyses push the identity of CD14$^+$ DC towards being more monocyte/macrophage-like than previously thought.[74] Specifically, McGovern *et al*[74] compared other dermal DC subsets with CD14$^+$ DC and dermal-resident macrophages, and showed a distinct gene signature that highly correlated between CD14$^+$ DC and dermal macrophages, but was inversely related to BDCA-1$^+$ and BDCA-3$^+$ dermal DC. Strangely, while CD14$^+$ DC did migrate out of skin explants, they did not express CCR7 (in contrast to work by Morelli *et al*[70]) and could not be localized to lymphatic channels. The differences observed in CCR7 expression may have to do with time of harvest. Morelli *et al*[70] had collected migratory cells at an earliest time point of 24 h after culturing skin explants, while McGovern *et al*[74] had collected cells over a period that included earlier time points. It is possible that it takes up to 24 h for CCR7 surface expression to appear on the CD14$^+$ DC investigated by both groups. Migration aside, McGovern *et al*[74] provided other evidence towards the monocyte/macrophage relationship with CD14$^+$ dermal DC. While not as weak as resident dermal macrophages, CD14$^+$ DC were extremely poor at inducing naïve CD4$^+$ T cell proliferation in comparison to known BDCA-1$^+$ and BDCA-3$^+$ dermal DC. These results collectively would suggest that calling these CD14$^+$ cells 'DC' at all is questionable, and that they may be a type of resident monocyte in the steady state. However, for sake of consistency, we will continue to identify them as CD14$^+$ DC for the remainder of this review.

INCONSISTENCIES AND DISCREPANCIES IN SKIN DC CATEGORIZATION

Human skin is the best-studied peripheral tissue source for DC subsets. Therefore, it best illustrates the issues that arise during identification of unique DC subsets. As suggested by others, too many markers splitting DC subsets can obscure true functional differences,[78] unless the markers used to classify subsets are standardized. Although the following studies (divided into 6 specific topic areas) all give information pertinent to DC taxonomy, function, and distribution, they are cases in point. (1) As several groups have observed, CD1a$^+$ DC and also LC express high levels of BDCA-1.[30 63 68 71] Interestingly, a lower level expression of BDCA-1 was also seen on CD14$^+$ DC by these groups. Perhaps two separate subsets actually exist: CD14$^+$ DC and a lesser population of CD14$^+$ BDCA-1$^+$ DC that has previously fallen under the CD14$^+$ DC nomenclature. Questions arise as to whether BDCA-1$^+$ dermal DC and CD1a$^+$ dermal DC are in fact the same cells, or at least closely related.[30 41] BDCA-1$^+$ DC isolated from human dermis without exclusion of CD1a$^+$ DC do indeed show a strong stimulation of naïve CD4$^+$ T cells comparable to that seen with purified CD1a$^+$ dermal DC.[30 41] In addition, Segura *et al*[1] did not identify a unique population of BDCA-1$^+$ DC in skin after using markers to exclude LC and CD1a$^+$ DC, but did identify a unique BDCA-1$^+$ DC population in all lymphoid organs tested, suggesting that conventional BDCA-1$^+$ DC are not found in the skin under steady-state conditions. These discrepancies will likely be resolved if future DC studies look for LC, CD1a$^+$ DC, BDCA-1$^+$ DC, and CD14$^+$ DC as separate populations. (2) Although LC have shown a strong ability to cross-

present exogenous influenza antigens to naïve CD8$^+$ T cells,[30] they are not likely the epidermal counterparts of BDCA-3$^+$ dermal DC, considering the specialized expression of Langerin and presence of BG associated with LC.[30 63–65] However, both subsets must be consistently purified/analyzed by markers as unique populations prior to cross-presentation assays. (3) CD1a$^+$ DC as studied by Haniffa *et al*[68] expressed high levels of CCR7 after migration out of dermal sheets, while migratory CD14$^+$ DC expressed more variable amounts. This suggests that either CD14$^+$ DC are actually a heterogeneous population themselves, or that their migration is not based solely on CCR7 upregulation, as supported by the work of McGovern *et al*.[74] Both LC and CD1a$^+$ DC migrate along a concentration gradient of chemokines CCL19 and CCL21,[71] the known ligands of CCR7.[79 80] However, even with the low level of CCR7 expression by CD14$^+$ DC taken into account, these cells did not migrate above background in the same chemotaxis assays, suggesting that CD14$^+$ DC do not naturally migrate to draining lymph nodes via afferent lymphatics. Based on migration out of skin explants, LC are about 60% of the total DC fraction, followed by 30% CD1a$^+$ DC, and 10% CD14$^+$ DC;[30] percentages which may reflect the poor migrational ability of CD14$^+$ DC. Another confounding factor of DC studies from skin is the isolation procedure. Digestion of skin is a harsh method that can phenotypically and functionally change DC subsets from their in situ characteristics. Likewise, DC collected on migration out of skin explants will have already matured and, thus, do not accurately reflect resting DC in situ. (4) Segura *et al*[1] analyzed human skin-draining lymph nodes and identified subsets having LC and CD1a$^+$ DC characteristics that were not seen in other lymphoid tissues, including tonsils, spleen and cervical lymph nodes. These DC subsets showed high expression levels of the maturation markers CD83 and CD86, verifying that they were different from resident lymphoid cells. Therefore, LC and CD1a$^+$ DC are likely specific to the skin, or at least to anatomical locations that have epithelial layers, like the gut and lung. However, Segura *et al*[1] were not able to identify a specific lymph node counterpart to skin CD14$^+$ DC based on CD14 expression, although they did note a small population of lymph node CD206$^+$ DC that stimulated naïve CD4+ T cells to take on T follicular cell characteristics based on CXCL13 production. Such T cell skewing has been previously observed with MLR involving skin CD14$^+$ DC,[30] which may indicate that this population can migrate under steady-state conditions, but in the process downregulates CD14. (5) Additionally, Segura *et al*[1] analyzed skin-draining LN and determined that BDCA-3 was not expressed on any migratory DC subsets, but that BDCA-3$^+$ DC were again identified as a unique resident population in the lymphoid organs. If we accept CLEC9A as another specific marker for identifying BDCA-3$^+$ DC,[45] Segura *et al*[1] showed that it too was not expressed on any LN-migrated skin DC subsets. These results suggest that BDCA-3$^+$ DC may be found in the skin in the steady state, but do not migrate to draining LN, as opposed to LC, CD1a$^+$ DC, and possibly CD14$^+$ DC. More directly, Haniffa *et al*[20] have reported a discrete population of dermal DC showing high expression of BDCA-3. This strongly argues against their absence in human skin, even if they do not have the capability of migrating to skin draining lymph nodes. This group ruled out technical differences in isolation procedure as a confounding factor by identifying BDCA-3$^+$ DC in both dermal digests and in collections of migratory cells out of dermal explants. Chu *et al*[81] have also recently identified a dermal BDCA-3$^+$ population that has strong cross-presentation ability and affinity for skin draining LN. They proposed a specific tolerogenic and anti-inflammatory role for BDCA-3$^+$ DC based on their constant secretion of IL-10 and the accompanying induction of T_{reg} during MLR. The ability of BDCA-3$^+$ DC to migrate to skin-draining LN that was observed by Chu *et al*[81] but not by Segura *et al*[1] likely relates to the model system used in each set of studies. The former group used humanized mice in which human skin explants were grafted to the back of mice. This model has the advantage of being a living system tracking human cells, but has the drawback of those cells being exposed to the lymphatics and associated chemokines of mice. Segura *et al*[1] had the advantage of a completely human model, but the drawback of only analyzing lymph nodes. Their results would have been strengthened if they had compared side-by-side cells found in the skin with their suggested equivalents observed in skin-draining LN. (6) Unlike CD14$^+$ DC, CD1a$^+$ DC in the skin show a proinflammatory profile based on IL-1 and IL-6 secretion.[68] This is observed without stimulation and further increases with exposure to the proinflammatory ligands poly-IC and peptidoglycan. In addition, CD1a$^+$ DC cause profound proliferation of naïve CD4$^+$ T cells in MLR after stimulation with anti -CD3 and -CD28 beads. Klechevsky *et al*[30] took this a step further by tracking LC, CD1a$^+$ dermal DC, and CD14$^+$ dermal DC for polarization by initial MLR and T cell restimulation with anti -CD3 and -CD28 beads. They saw that LC are quite efficient at skewing naïve CD4$^+$ T cells towards a T_h2 phenotype, as measured by high T cell secretion of IL-4, IL-5 and IL-13. CD1a$^+$ DC did not induce as strong of a T_h2 response, with less IL-5 and IL-13 secretion and no IL-4 production. However, this weaker response by CD1a$^+$ DC was stronger than that of CD14$^+$ DC. When the skin DC subsets were mixed with naïve CD4$^+$ T cells and then those T cells later mixed with naïve, preactivated B cells, a unique B cell stimulatory profile was elucidated for the CD14$^+$ DC. Through their reaction with T cells, CD14$^+$ DC caused B cells to secrete high levels of IgM and switch isotypes to IgG and IgA. The high IgM and the isotype class switching were specific to CD14$^+$ DC in comparison to LC and CD1a$^+$ DC. Finally, in MLR with naïve CD8$^+$ T cells, LC caused the most robust proliferation, followed by CD1a$^+$ DC and, lastly, CD14$^+$ DC. These results suggest functional specialization of CD14$^+$ DC in generating both general (IgM) and specific (IgG and IgA) B cell responses in the human dermis. Collectively, the above examples show that there are still many holes in our knowledge of skin DC subset markers and functions. Examining all possible subsets with unique and defined surface markers will help eliminate the possibility of overlapping subsets. Functionally, standardizing the cytokines measured for B and T cell responses in MLR would also allow better comparisons between different studies. (For anatomical reference of human skin and its constituent DC subsets described above, see figure 2).

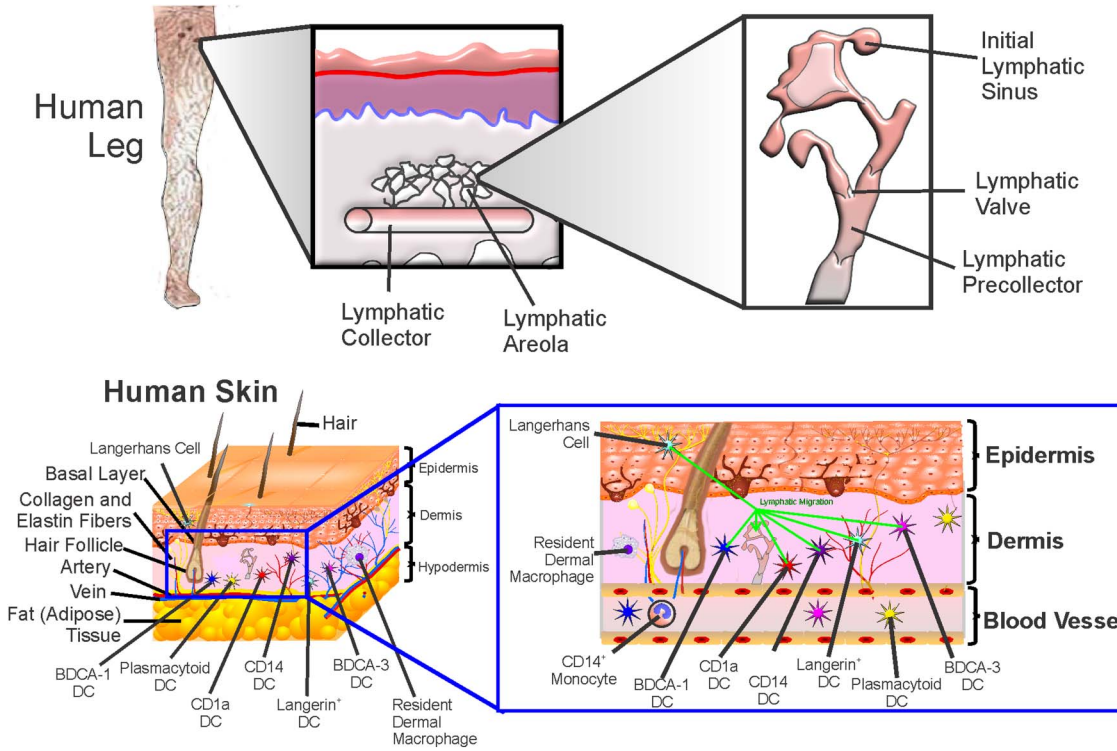

Figure 2 Anatomical schema of human skin. Top: Afferent lymphatics are found in the dermal layer of the skin as open-ended channels through which migratory DC can travel to regional lymph nodes. Bottom: Schematic of the epidermal and dermal layers of the skin, including localizations of DC subsets in relation to other skin components. Distinct Langerhans cells are found in the epidermal layer, while remaining DC subsets are found in the dermal layer. Some of the skin-resident populations originate from cells found circulating in the blood. DC, dendritic cell(s), BDCA, blood dendritic cell antigen.

DC SUBSETS IN THE MICROENVIRONMENTS OF THE HUMAN LUNG

The human lung, and especially its airways, pose a unique environment for DC subsets compared to the rest of the body. At the physiological level, the adult lung averages over 120 m^2 of respiratory area beyond the terminal bronchioles.[82] Stereological analysis has shown that this large surface area is due to the presence of hundreds of millions of open-ended grape-like structures called alveoli.[83] The walls of an alveolus are only a single epithelial cell layer thick and covered externally by pulmonary capillaries. This allows for efficient diffusion of oxygen and carbon dioxide between the blood and air we breathe. This large respiratory surface area must be kept clear and sterile despite constant exposure to airborne particulates. The majority of the alveolar surface (~97%) is made up of large, flat type I alveolar epithelial cells (AECs).[84] There are smaller, but more numerous, type II AECs which serve as stem cells for replacement of type I AECs and also secrete a thin layer of surfactant over the entire alveolar surface.[84] Between individual alveoli are septal holes referred to as the pores of Kohn.[85] These pores allow for movement of liquids, surfactant, and, importantly, immune cells between alveoli.[85 86]

Resident DC have been found within the human bronchiolar and alveolar epithelium under steady-state conditions,[87 88] though their subset identities remain unclear. These DC play a part in the intricate immune network that must maintain tolerance to most inhaled particulates, but also be able to rapidly mount specific responses to viral, bacterial, or fungal invasions of the airways. In mice, DC have been shown to extend processes through the epithelium into the surfactant layer for continuous sampling of inhaled particulates prior to lymphatic migration,[89] and this event is hypothesized to occur in human alveoli as well. While airway and parenchymal DC have been known to exist in humans for nearly 30 years,[6] studies of lung DC subsets have been far more limited compared to similar work in blood and skin. Human lung tissue is not readily available for research, most commonly being acquired through surgical resections.[90–93] A primary drawback of using whole tissue includes the requirement of a lung digestion process to acquire a single cell suspension and the resulting inevitable mixing of residual blood cells with tissue-resident cells. There is also the concern that because resections are usually done for diagnosis of cancer, the resected tissue used for research may not truly represent the steady state at the microenvironmental level. However, it was lung digestion that allowed Nicod *et al*[94] to initially determine that there was a group of low autofluorescent APC in the human lung that were highly capable of stimulating $CD4^+$ T-cell proliferation. It was assumed that these cells were resident to the interstitial compartment, as opposed to the alveolar macrophages (AM) that are found in the airways.

Acknowledging the artifacts induced by tissue digestion, work has also been done to isolate DC subsets directly from the airways using bronchoalveolar lavage (BAL).[95–97]

This technique has the benefits of being conducted on healthy volunteers and also of isolating cells specific to the airway microenvironment. DC have, in fact, been found to exist along the branching airways all the way up to the trachea itself.[88 98] However, lavage does not yield as many cells as tissue digestion, a major concern when studying rare cells like DC. In addition, lavage does not allow for collection of parenchymal cells found below the bronchial or alveolar epithelium.

CORRELATING BETWEEN DC SUBSETS IN LUNG AND THOSE IN OTHER TISSUES

Using lavage, Ten Berge *et al*[96] in 2009 focused on the separation of pDC and mDC from BAL preparations. Their work likely excluded skin-equivalent $CD14^+$ DC, if present, by including CD14 in the lineage mix. They did not further identify the mDC as BDCA-1^+, BDCA-3^+, or $CD1a^+$. Using $CD123^+$ for pDC identification and $CD11c^+$ HLA-DR^+ for mDC, they did show that healthy DC could be isolated from BAL, and that pooled mDC could be obtained in numbers useful for functional studies. By flow cytometric analysis, low autofluorescence was used to discriminate DC populations from the more numerous and highly autofluorescent AM found in BAL fluid. Results showed that lavage-isolated mDC could stimulate naïve $CD4^+$ T-cell proliferation in MLR at a 1:20 ratio, and that proliferation increased after APC exposure to LPS. Interestingly, this group saw elevated levels of IL-4, IL-5, and IL-13 from the T cells after incubation with the lavage mDC. This T_h2-bias suggested the presence of cells equivalent to LC or $CD1a^+$ DC described in the skin by Klechevsky *et al*.[30] Indeed, van Haarst *et al*[99] subsequently identified a small but definite $CD1a^+$ DC subset that caused strong proliferation of naïve $CD4^+$ T cells. However, they did not use Langerin to determine if the $CD1a^+$ cells were in fact LC or a separate $CD1a^+$ DC subset. While not including $CD1a^+$ staining, Tsoumakidou *et al*[95] were able to identify BDCA-1^+, BDCA-2^+, and BDCA-3^+ DC using similar BAL techniques, suggesting that all three subsets reside in healthy human airways. However, without excluding $CD1a^+$ DC or LC, the BDCA-1^+ DC noted by this group may not be a unique DC subset. Segura *et al*[1] were not able to identify unique BDCA-1^+ DC in human skin after excluding $CD1a^+$ DC and LC, although skin and lung epithelial layers are compositionally and functionally different. Standardization of the markers used in human lung airway studies would at least differentiate the multiple DC subsets that are potentially present.

In an attempt to localize DC subsets in human airways, Todate *et al*[88] performed histological studies of human bronchioles and noted separate populations of $CD1a^+$ and BDCA-1^+ DC within the bronchiolar epithelium. While these cells were sparse, they were equally represented in the epithelium. Looking deeper into the submucosal layer, BDCA-1^+ DC were present at higher densities than $CD1a^+$ DC. These results suggest that the $CD1a^+$ DC are the lung-equivalent to LC in the skin based on their higher frequency within the epithelium. However, the equal presence of BDCA-1^+ DC in the epithelium suggests a specific functional role for both cell types along the bronchioles. Also, frequency alone is not enough to verify equivalency of $CD1a^+$ DC with LC recognized in the skin. Cochand *et al*[90] in 1999 had not identified any $CD1a^+$ DC after lung tissue digestion, although this may have been due to scarcity of this subset among the total number of DC obtained. They did see CD14 expression on a large proportion of DC, although levels of this marker dropped substantially after maturation with LPS. This may indicate that digestion of lung tissue introduced $CD14^+$ monocytes from blood into the suspension, thus diluting the percentage of $CD14^+$ DC.

In 2005, Demedts *et al*[92] were the first to test the full repertoire of BDCA markers[3] using flow cytometry in the identification of human lung DC subsets. Indeed, they identified BDCA-1^+ mDC, BDCA-2^+ $CD123^+$ pDC, and BDCA-3^+ mDC from freshly digested lung tissue. These subsets were seen even after CD14 exclusion, meaning that if a $CD14^+$ DC subset is present in the lung, it is separate from the $BDCA^+$ myeloid populations. However, further analysis with CD14 inclusion, revealed a $CD14^+$ population of DC that expressed BDCA-1, and, surprisingly, also a population of BDCA-3^+ $CD14^+$ DC. This suggests that $CD14^+$ cells that are negative for BDCA-1 and BDCA-3 could be the lung equivalent to $CD14^+$ dermal DC. Others have previously excluded $CD14^+$ cells from their studies of lung DC subsets to diminish monocyte contamination.[93] Unfortunately, this exclusion may also be eliminating $CD14^+$ lung DC that have a known counterpart in the skin. When Masten *et al*[93] isolated a mixture of $CD1a^+$ BDCA-1^+ $CD14^-$ DC that were more potent than $CD14^+$ cells at stimulating alloreactive T-cell proliferation, they assumed these $CD14^+$ cells to be monocytes. They did not account for the possibility of $CD14^+$ DC being present in the mixture. These cells have been shown in skin to have lesser stimulatory capacity in MLR compared to $CD1a^+$ DC.[71] Importantly though, Masten *et al*[93] did demonstrate that BDCA-2 was not a reliable marker for pDC in human lung from sample to sample. In agreement with blood results,[3 29] Demedts *et al*[92] saw that BDCA-1^+ and BDCA-3^+ DC expressed the integrin CD11c, while BDCA-2^+ pDC did not. Contrary to the results of Cochand *et al*,[90] they identified a small population of $CD1a^+$ cells from lung digest that also expressed the LC marker, Langerin. While this DC subset was not tested for function, immunohistology demonstrated that $CD1a^+$ $Langerin^+$ cells were present within the epithelial layer of large and small airways, but not present deeper in the parenchyma. These results suggested that an LC equivalent could be present in human airway epithelium. Langerin positivity, along with anatomical location, would be the primary means of differentiating these LC-like cells from BDCA-1^+ DC, as a majority of $CD1a^+$ cells were also BDCA-1^+. Cochand *et al*[90] may not have detected $CD1a^+$ DC because such cells expressed BDCA-1 as well and were thus classified as BDCA-1^+ DC. Demedts *et al*[92] also observed a small number of BDCA-1^+ $CD1a^-$ cells that were obviously not LC-like. In comparing isolated BDCA-1^+ and BDCA-3^+ DC subsets, BDCA-1^+ DC in the lung are better at stimulating naïve $CD4^+$ T cell proliferation, though both subsets are better than pDC.[100]

Recently, Bigley *et al*[101] have challenged the notion that Langerin is even a specific marker for LC. Focusing on skin, they observed the standard LC in human epidermis

characterized by high levels of both Langerin and CD1a, but identified a second population specific to the dermis that expressed lower levels of both markers. Further flow cytometric analysis suggested that these Langerin+ DC expressed higher levels of the surface proteins CD13, CD11c, CD11b, and CD33 than traditional LC. This population was also observed in the lung after tissue digestion, whereas, cells expressing high levels of Langerin and CD1a were not. Therefore, functional characteristics of these human airway Langerin$^+$ cells will have to be investigated under the premise that they might be more like dermal Langerin$^+$ cells than epidermal LC. Unlike human skin, which contains separate dermal and epidermal layers, the respiratory tract has a continuously changing system that begins with the large cartilaginous airways that branch into small non-cartilaginous airways that terminate with alveoli that are a single epithelial layer thick. The function of BDCA-1$^+$, BDCA-2$^+$/CD123$^+$, BDCA-3$^+$, CD14$^+$, CD1a$^+$, and Langerin$^+$ resident cell subsets in the lung airways must, therefore, be further characterized in relation to their specific microenvironments (airways) within microenvironments (whole lung).

With the goal of relating function to specific lung DC subsets, Demedts *et al*[100] later tested the steady-state expression of several TLRs by BDCA-1$^+$ and BDCA-3$^+$ DC. While significant levels of mRNA of TLR-1, TLR-2, TLR-3, TLR-4, TLR-6 and TLR-8 were measured in both myeloid cell types, neither showed transcription of TLR-7 or TLR-9 (pDC showed transcription of both). These results are similar, but not identical, to those observed in the equivalent resident DC subsets in tonsils examined by Jongbloed *et al*.[59] One primary difference seen in lung was a significantly higher level of TLR-4 mRNA in BDCA-3$^+$ DC compared to BDCA-1$^+$ DC.[100] The opposite results were observed when comparing these subsets from tonsils.[59] Another significant difference was lack of TLR-7 transcripts in either subset in lung, whereas in tonsil, BDCA-1$^+$ DC showed specific expression of TLR-7.[59 100] At the protein level, however, BDCA-1$^+$ and BDCA-3$^+$ subsets of the lung showed similar levels of all TLRs assayed.[100] These results provide another example of the likely effects different microenvironments exert on similar cell types. In the microenvironment of the lung, BDCA-3$^+$ DC found in the small airways may need rapid upregulation of TLR-4 when exposed to Gram (−) bacteria, while in tonsils the same DC subset may be more protected from pathogen exposure by surface epithelia. As Lindstedt *et al*[54] suggested by transcriptomic clustering of these DC subsets in blood and tonsils, BDCA-1$^+$ and BDCA-3$^+$ DC in the lung are likely more closely related to each other than to equivalent subsets in other tissues. This is especially true considering the highly specialized microenvironments of the human airways and parenchyma. Therefore, the subsets cannot be completely differentiated by function based only on a few transcripts such as those for TLRs, although certainly these results suggest that BDCA-1$^+$ and BDCA-3$^+$ DC are likely important in bacterial sensing in the airways, while BDCA-2$^+$ pDC are important in viral sensing.

Realizing that lung DC relationships cannot be determined by a few markers and variations in stimulatory capacity of T cells, other groups have sought to differentiate subsets based on limited and complete transcriptomic profiling. Building off the work of Lindstedt *et al*,[54] genomic comparison of human DC subsets from different tissues has been slow to develop, but looks to become an invaluable technology. In 2012, Haniffa *et al*[20] were the first to compare the major skin DC subsets at the transcriptional level to DC subsets in the blood, tonsils and lung. This group chose to focus on a BDCA-3^{hi} population of DC identified in skin and lung digest preparations. They used quantitative RT-PCR with a signature gene set thought to be specific to this BDCA-3$^+$ cross-presenting population, regardless of tissue source: XCR1,[46] TLR3,[102] cell adhesion molecule 1 (CADMI)[103] and CLEC9A.[45] Indeed, high levels of expression were observed for all of these markers in BDCA-3^{hi} DC in all tissue types, a signature not observed from analyzing BDCA-1$^+$ or CD14$^+$ DC subsets. Such a gene signature would be very useful in characterizing this DC subset in different tissues, especially considering that, at least in skin, a majority of BDCA-3^{hi} DC also express BDCA-1 or CD1a.[20] Also by transcriptome analysis, Schlitzer *et al*[21] have recently identified the homologs of BDCA-1$^+$ lung DC in mice as being specific CD11b$^+$ DC dependent on the transcription factor IRF4. Through this animal model, they suggest that BDCA-1$^+$ DC are vital for inducing T_h17-directed immune responses in the lung. However, they acknowledged that they had to use human blood BDCA-1$^+$ DC as the equivalent for lung BDCA-1$^+$ DC due to lack of lung cells required for transcriptomic studies. Thus, their set of DEGs would not be the same had they actually acquired BDCA-1$^+$ DC from the lung, given the accepted vast differences in the microenvironments of these tissue types (For an anatomical reference of the human lung DC subsets described above, see figure 3).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Much progress has been made in identifying and characterizing human DC subsets in different anatomic compartments. Some groups have done this based on the classical DC definitions of surface markers and accompanying induction of CD4$^+$ T cells. Results of MLR can be quite relevant in comparing DC subsets to each other, but only if the assay is standardized and tested over a range of stimuli and APC:T cell ratios. In the past three decades, more and more subsets have been discovered as DC markers change and new markers are identified. However, even with a multitude of different markers, specific DC subsets are still difficult to separate. (For a summary of the basic markers discussed in DC subset identification throughout this review, see figure 1). While it seems that pDC have been characterized across most human tissue types, widely useful mDC characterization has not been achieved. This largely stems from overlapping expression of surface markers such as BDCA-1, BDCA-3, CD1a, CD14, and Langerin. Having a defined set of markers for at least the known human DC subsets would exclude such instances of overlap and allow better comparisons of results between groups, thus facilitating progress in this field.

The most promising solution to the problem of marker specificity currently appears to be the use of transcriptional analysis and hierarchical clustering of subsets to determine overall relatedness and DEGs. Such studies have already identified some genes whose expression may be specific to some DC subsets. More importantly, they have shown us

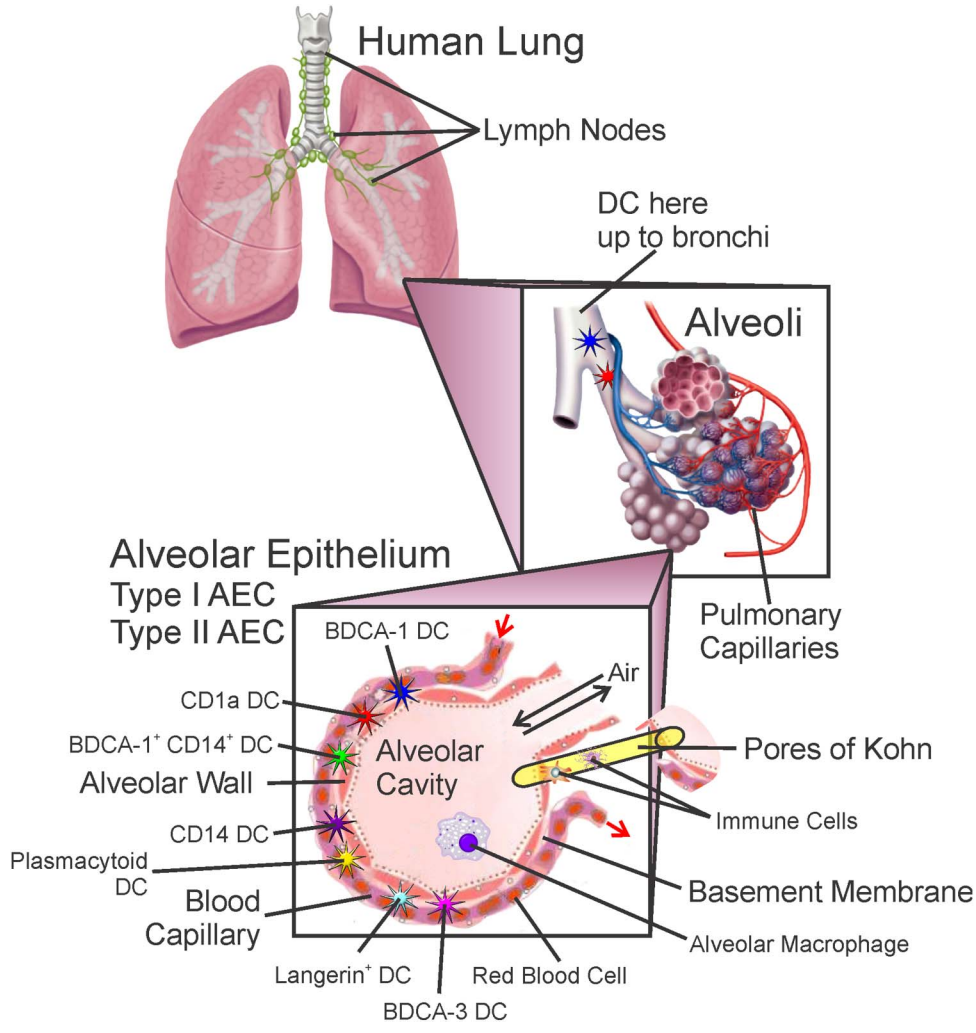

Figure 3 Anatomical schema of human lung airways, including localizations of DC subsets discussed. DC are found imbedded within the alveolar epithelium and just below it in the submucosa, from where they extend processes into the alveolar space to sample antigens. Immune cells, such as DC, can transit between alveoli via channels known as Pores of Kohn. DC, dendritic cell(s), BDCA, blood DC antigen, AEC, alveolar epithelial cell; TLR, toll-like receptor.

that there is significant overlap between DC subsets, and even between DC and other mononuclear APC like monocytes and macrophages. Comparative transcriptomics have recently suggested mouse homologs to several human DC subsets, which allows correlative experiments to be conducted in a live system. These types of comparative analyses for homology should continue in the near-future, but with more importance placed on higher vertebrates such as chimpanzees and baboons. Small rodents are more affordable and easier to handle, but apes present the closest to a human in vivo system in which DC subsets can be investigated. It is recognized that no system is perfect and homology between species is a relative term. While several roadblocks in identifying and characterizing DC subsets have been cleared, there are still many to overcome in order to understand how these populations are maintained in the steady state and what their roles are in maintaining health.

Another avenue that needs to be explored in years to come is the tissue microenvironment. Varying microenvironments, such as those of the skin and lung, play a major role in dictating DC marker expression and functional responses to stimuli. Instead of trying to isolate DC subsets and see how they differ from each other, more emphasis should be placed on determining how a given microenvironment remains in a steady state after accounting for all its resident cell populations. For example, in the human airways, DC subsets must interact with each other and also with resident epithelial cells and macrophages. So even if an isolated DC subset responds in a pro inflammatory or anti-inflammatory manner to a specific stimulus, it does not infer that it will respond that way in situ or in vivo. DC subsets identified by surface markers may not be terminally differentiated in a local microenvironment, but instead have the capability of acting as multiple subsets as a result of the small perturbations that constantly occur in healthy tissue. It is important to note that we use the steady state in this review as the absence of disease rather than a fixed set of conditions within a microenvironment. Plasticity between DC subsets likely occurs in this steady state to sustain tissue function by regulating other cell types, from maintaining their tolerance of self-antigens to stimulating

their rapid clearance of dying cells or microbes. While plasticity makes identifying DC subsets more difficult, it is an advantageous part of the immune response in a microenvironment. It allows resident cells to quickly adapt to instantaneous changes, which would otherwise require cell proliferation or influx from the bloodstream. Specific models of infection, allergy, or autoimmunity are informative but fall short because they are models of large steady state perturbations. The true tests for DC researchers will be in establishing complex models of human tissue microenvironments with all their cellular constituents, and then understanding how DC subsets contribute to these microenvironments remaining in the steady state the majority of the time.

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