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Functional diversity of human vaginal APC subsets in directing T cell responses

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Abstract

Human vaginal mucosa is the major entry site of sexually transmitted pathogens and thus has long been attractive as a site for mounting mucosal immunity. It is also known as a tolerogenic microenvironment. Here, we demonstrate that immune responses in the vagina are orchestrated by the functional diversity of four major antigen-presenting cell (APC) subsets. Langerhans cells (LCs) and $CD14^-$ lamina propria (LP)-DCs polarize $CD4^+$ and $CD8^+$ T cells toward Th2, whereas $CD14^+$ LP-DCs and macrophages polarize $CD4^+$ T cells toward Th1. Both LCs and $CD14^-$ LP-DCs are potent inducers of Th22. Due to their functional specialties and the different expression levels of pattern-recognition receptors on the APC subsets, microbial products do not bias them to elicit common types of immune responses (Th1 or Th2). To evoke desired types of adaptive immune responses in the human vagina, antigens may need to be targeted to proper APC subsets with right adjuvants.

Keywords

dendritic cells; vagina; mucosa; macrophages; T cell responses

INTRODUCTION

Female genital tracts, particularly human vaginal mucosa, have long been attractive as a potential site for mounting protective immunity against sexually-transmitted pathogens,

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DISCLOSURE

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including viruses and bacteria.[1, 2] However, the vaginal mucosa, a site constantly exposed to foreign antigens, is a unique tolerogenic microenvironment that can control unwanted types of immune responses.[1, 3-5] Nonetheless, the immunology of the human vagina remains poorly understood.

Dendritic cells (DCs) are major antigen-presenting cells (APCs). DCs can direct host immune responses toward either immunity or tolerance.[6] Subsets of DCs residing in one particular tissue, as well as DCs in draining lymph nodes, have diverse but specialized functions in directing host immune responses.[7] DCs also display functional plasticity in response to microbial pathogens.[8] Early studies in animals showed that vaginal mucosa is covered with stratified squamous epithelium[2, 3, 9] and shares several common features with the skin. Langerhans cells (LCs), which do not have Birbeck granules,[10] are found in the epithelium and $CD11c^+$ DCs in the lamina propria (LP).[1, 2] $DC\text{-}SIGN^+CCR5^+$ LP-DCs were also reported in animals.[11, 12]. Although the presence of LCs in the human vaginal epithelium has been reported,[13] no further information is available. Moreover, immunological functions of DCs localized in the human vagina remain unknown.

In this study, we first characterized subsets of DCs and macrophages (Mϕ that are localized in the human vaginal mucosa by assessing their phenotypes and morphology. We then tested the functional specialties of the individual subsets of APCs in directing $CD4^+$ and $CD8^+$ T cell responses by measuring the quantity and quality of T cell responses. We also tested the ability of the individual APC subsets for inducing chemokine receptors that are detected on T cells in the vaginal mucosa. To address functional plasticity of the vaginal APC subsets, we first assessed the expression levels of Toll-like receptors (TLRs). We were then able to examine T cell responses induced by the APC subsets activated with different microbial products and their synthetic analogues. Data from this study help us understand how the immune responses in the human vagina are induced and controlled in a steady state as well as during microbial infections. This study also provides fundamental information for the design of effective mucosal vaccines against many sexually transmitted diseases (STDs) that still cause a major public health burden worldwide.

RESULTS

Human vaginal mucosa contains four major subsets of myeloid-originated APCs

Mucosal tissues were enzymatically digested and stained with 7-AAD and the indicated antibodies (Figure 1a). $7\text{-}AAD^-HLA\text{-}DR^+$ cells were separated into $HLA\text{-}DR^+CD207^+$ ($Langerin^+$: I) and $HLA\text{-}DR^+CD207^-$ cells. $HLA\text{-}DR^+CD207^-$ cells were further divided into four subsets based upon CD1c and CD14 expression: $CD1c^+CD14^-$ (II), $CD1c^+CD14^+$ (III), $CD1c^-$ $CD14^+$ (IV), and $CD1c^-CD14^-$ (V). $CD207^+$, $CD1c^+CD14^-$ and $CD1c^+CD14^+$ cells display dendrites (Figure 1b), suggesting their classification as DCs. $CD1c^-CD14^+$ cells contain large vacuoles in the cytoplasm, which is a Mϕ characteristic. Large fractions of the cells in group V express CD34 (77.41% ± 11) and CD54 (59.83% ± 9.3) (Figure 1c and Supplementary Figure 1a online). Only 6.5% of them express either CD66 or pan-cytokeratin that are known to be expressed on epithelial cells. E/P selectin is expressed 31% ± 3.3 of the cells. The expression levels of CD34, CD54, E/P selectin, and CD123 are higher than those of CD66 and E-cadherin (Supplementary Figure 1b online). Therefore, the cells

in group V are mainly composed of endothelial cells, but with epithelial cells that can constitutively express MHC class II.[14, 15]

$CD207^+$ cells are mainly found in the epithelium, while the other 3 subsets ($CD1c^+CD14^-$, $CD1c^+CD14^+$ and $CD1c^-CD14^+$) are in the LP (Figure 1d and Supplementary Figure 2a online). The percentage of HLA-DR^+ cells in total tissues is approximately 10% (Supplementary Table 1 online). Of the HLA-DR^+ cells, $CD1c^-CD14^+$ cells are the most abundant. The frequencies of plasmacytoid DCs, B cells, and $BDCA3^+$ cells were low. Taken together, human vaginal mucosa contains four major subsets of myeloid-originated APCs: $CD207^+$, $CD1c^+CD14^-$, $CD1c^+CD14^+$, and $CD1c^-CD14^+$ cells.

Subsets of the vaginal APCs display shared and distinct surface phenotypes

The 4 APC subsets were further characterized by examining their phenotypes (Figure 1e). $CD207^+$ cells express both CD1a and E-cadherin, which is expressed on LCs.[16] LCs, as well as the $CD1c^+CD14^-$ LP-DCs ($CD14^-$ LP-DCs) and $CD1c^+CD14^+$ LP-DCs ($CD14^+$ LP-DCs), express both CD86 and CD83. $CD1c^-CD14^+$ cells express CD86, but not CD83. In addition, $CD1c^-CD14^+$ cells express CD163, which is expressed on Mϕ[17] Compared to LCs and the other two subsets of LP-DCs, $CD1c^-CD14^+$ cells express lower levels of CD11c, which is in accordance with the classification of these cells as Mϕ. Accordingly, CD1a was detected by immunofluorescence on LCs in the epithelium and on $CD14^-$ and $CD14^+$ LP-DCs (upper panel, Figure 1f and Supplementary Figure 2b online). CD163 was only detected on Mϕ (lower panel, Figure 1f and Supplementary Figure 2c online).

We next tested whether different subsets of the APCs express distinct patterns of chemokine receptors and β7-integrin (Figure 1g). CCR2, a homing receptor for monocytes and Mϕ[18] is expressed on Mϕ but not on the vaginal DCs. CCR5 and CXCR4, co-receptors for HIV, are expressed on LCs as well as on the other three subsets of APCs.[19] Interestingly, LP-DCs and Mϕ express increased levels of CCR5 and CXCR4 compared to LCs. Both LCs and LP-DCs exhibit similar levels of CCR6 that is also expressed on intestinal DCs.[20] β7-integrin is detected only on LCs and LP-DCs, but not on Mϕ. Both CCR4 and CX3CR1 were similarly expressed on the four subsets of APCs. CCR7 and CD103 were not detected on the surface of the vaginal APC subsets (data not shown). Taken together, the 4 subsets of vaginal APCs express shared and distinct patterns of surface receptors, including co-stimulatory molecules and chemokine receptors. This suggested that each subset of the APCs might possess common as well as distinct functions in directing the immune responses in the vagina.

Functional specialties of the vaginal APC subsets in directing $CD4^+$ T cell responses

CFSE-labeled allogeneic naïve T cells were co-cultured for 7 days with different numbers of the APC subsets, and then $CD4^+$ T cell proliferation and intracellular cytokine expression were assessed. Vaginal DCs induced greater $CD4^+$ T cell proliferation than did Mϕ(left panel, Figure 2a). $CD14^-$ LP-DCs and LCs induced similar levels of naïve $CD4^+$ T cell proliferation. However, $CD14^+$ LP-DCs were less efficient than LCs at inducing $CD4^+$ T cell proliferation. The level of $CD4^+$ T cell proliferation induced by monocyte-derived IFNDCs (2×10^3) was similar to those induced by LCs or $CD14^-$ LP-DCs (right panel, Figure 2a).

All 4 subsets of the vaginal APCs induced similar percentages of IFNγ$^+$CD4$^+$ T cells, ranging from 8.7±6% to 14.7±7% of total CD4$^+$ T cells (Figure 2b and Supplementary Figure 3 online). Data generated with cells from different donors are summarized in Figure 2c. Notably, LCs and CD14$^-$ LP-DCs induced greater numbers of CD4$^+$ T cells expressing Th2-type cytokines as well as TNFα than did CD14$^+$ LP-DCs and Mφ. Although Th17 contribute to the protective immunity against mucocutaneous candidiasis[21-23] and skin dermal-DCs are potent Th17 inducers,[24] none of the vaginal APC subsets induced significant levels of IL-17$^+$CD4$^+$ T cell responses (Supplementary Figure 4a online). The 3 vaginal DC subsets displayed similar ability to induce IL-21$^+$CD4$^+$ T cell responses (Supplementary Figure 4b online), while CD14$^+$ skin dermal-DCs were potent inducers of IL-21$^+$CD4$^+$ T cells.[25] Furthermore, vaginal LCs and CD14$^-$ LP-DCs induced similar levels of naïve CD4$^+$ T cell proliferation, whereas skin LCs were superior to CD1c$^+$ dermal DCs at inducing naïve CD4$^+$ T cell proliferation.[25]

The quality of CD4$^+$ T cells induced by the vaginal APC subsets was further analyzed by the Boolean gating strategy. LCs and CD14$^-$ LP-DCs were able to polarize naïve CD4$^+$ T cells mainly toward Th2, whereas CD14$^+$ LP-DCs and Mφ polarized them toward Th1 (Figure 2d). 62% and 21% of the LC-induced CD4$^+$ T cells expressed IL-13 (IL-13$^+$, IL-13$^+$IL-5$^+$, IL-13$^+$IFNγ$^+$, and IL-13$^+$IFNγ$^+$IL-5$^+$) and IL-5 (IL-5$^+$, IL-5$^+$IL-13$^+$, IL-5$^+$IFNγ$^+$, IL-5$^+$IFNγ$^+$IL-13$^+$), respectively. Similarly, 62% and 22% of the CD14$^-$ LP-DC-induced CD4$^+$ T cells expressed IL-13 and IL-5, respectively. In contrast, 79% and 75% of the CD4$^+$ T cells induced by CD14$^+$ LP-DCs and Mφ respectively, expressed IFNγ (IFNγ$^+$, IFNγ$^+$IL-5$^+$, IFNγ$^+$IL-13$^+$, and IFNγ$^+$IL-5$^+$IL-13$^+$). Large fractions of IFNγ$^+$CD4$^+$ T cells induced with LCs and CD14$^-$ LP-DCs expressed Th2 cytokines, whereas the majority of IFNγ$^+$CD4$^+$ T cells induced with CD14$^+$ LP-DCs and Mφ did not express IL-5 or IL-13. The percentage of TNFα single$^+$ cells induced with LCs (25%), CD14$^-$ LP-DCs (31%), and Mφ (28%) were comparable (Figure 2e). However, CD14$^+$ LP-DCs resulted in increased percentage of TNFα single$^+$ CD4$^+$ T cells (42%). LCs (19%) and CD14$^-$ LP-DCs (15%) also induced greater percentage of TNFα$^+$IL-13$^+$ CD4$^+$ T cells than CD14$^+$ LP-DCs (6%) and Mφ did, whereas CD14$^+$ LP-DCs (20%) and Mφ induced greater percentage of TNFα$^+$IFNγ$^+$ CD4$^+$ T cells than LCs (12%) and CD14$^-$ LP-DCs (10%) did.

Taken together, both LCs and CD14$^-$ LP-DCs polarize naïve CD4$^+$ T cells towards Th2, whereas CD14$^+$ LP-DCs and Mφ polarize them toward Th1. Furthermore, CD14$^+$ LP-DCs and Mφ were more efficient than LCs and CD14$^-$ LP-DCs at eliciting TNFα single$^+$ as well as TNFα$^+$IFNγ$^+$ CD4$^+$ T cell responses.

Functional specialties of the vaginal APC subsets in directing CD8$^+$ T cell responses

CD8$^+$ T cell responses induced by the vaginal APC subsets were assessed (Figure 3). Compared to Mφ, both LCs and LP-DCs induced enhanced CD8$^+$ T cell proliferation (Figure 3a). Notably, the 3 vaginal DC subsets were equally potent at inducing naive CD8$^+$ T cell proliferation, although human skin LCs were superior to dermal-DCs at eliciting CD8$^+$ T cell proliferation.[25]

Both LCs and CD14$^-$ LP-DCs showed similar ability to induce IFNγ$^+$ and TNFα$^+$ CD8$^+$ T cell responses (Figure 3b and Supplementary Figure 5 online). However, LCs and CD14$^-$

LP-DCs were more efficient than Mϕ and CD14$^+$ LP-DCs at inducing IFNγ$^+$ and TNFα$^+$ CD8$^+$ T cell responses. Increased numbers of CD8$^+$ T cells induced with LCs and CD14$^-$ LP-DCs expressed IL-5, but not IL-13 (data not shown). Data from 6 (IFNγ and IL-5) and 3 (TNFα) independent experiments are summarized in Figure 3c. The vaginal APC subsets did not induce significant numbers of IL-21$^+$ or IL-17$^+$ CD8$^+$ T cells (data not shown). Figure 3d further demonstrates that large fractions of IFNγ$^+$CD8$^+$ T cells induced by LCs and CD14$^-$ LP-DCs express IL-5. The increased percentages of IFNγ$^+$CD8$^+$ T cells by LCs and CD14$^-$ LP-DCs were mainly due to the increased induction of IFNγ$^+$TNFα$^-$ CD8$^+$ T cells, but not IFNγ$^+$TNFα$^+$ CD8$^+$ T cells (Figure 3e). Taken together, LCs and the 2 subsets of LP-DCs are equal but more potent than Mϕat inducing CD8$^+$ T cell proliferation. Compared to CD14$^+$ LP-DCs and Mϕ, LCs and CD14$^-$ LP-DCs can induce increased level of IL-5$^+$CD8$^+$ T cell responses.

Common and distinct functions of the vaginal APC subsets in the induction of CD103, β7-integrin, and chemokine receptor expression on T cells

Expression of CD103, β7-integrin, and chemokine receptors on the T cells in the vaginal was investigated. Fractions of CD4$^+$ (28.2%) and CD8$^+$ T cells (67.2%) from the vagina (Figure 4a) and T cells in the vaginal mucosa (CD4$^+$ in Figure 4a and Supplementary Figure 6a online and CD8$^+$ in Figure 4b and Supplementary Figure 6b online, and Figure 4c) express CD103 that could allow T cell migration or retention in the vagina.[26-29] More importantly, vaginal DCs and Mϕin a less extent were able to induce CD103 on CD4$^+$ (upper panels, Figure 4d) and especially CD8$^+$ T cells (lower panels, Figure 4d). CD14$^+$ LP-DCs were less efficient than the other 2 DC subsets, but more efficient than Mϕ and IFNDCs at inducing CD103 expression. Fractions of CD4$^+$ and CD8$^+$ T cells from the vaginal mucosa also expressed CCR4 (Figure 4e). All 4 subsets of the vaginal APCs were capable of inducing CCR4 expression on CD4$^+$ and CD8$^+$ T cells (Figure 4f). Notably, the majority of CFSE^{low} T cells induced with Mϕ expressed CCR4. Fractions of CD4$^+$ and CD8$^+$ T cells from the vaginal mucosa also express β7-integrin (Supplementary Figure 7a online) and CXCR3[30] (Supplementary Figure 7c online). All 4 subsets of the vaginal APCs were almost equally capable of inducing β7-integrin (Supplementary Figure 7b online) and CXCR3 (Supplementary Figure 7d online) on CD4$^+$ (upper panels) and CD8$^+$ T cells (lower panels). Taken together, T cells in the vagina express CD103, β7-integrin, CCR4, β7-integrin, and CXCR3. Vaginal APCs can induce these receptors on both CD4$^+$ and CD8$^+$ T cells, but individual subsets could display common and distinct functions at inducing these receptor expressions on T cells.

Microbial products can bias the individual APC subset-driven T cell responses

RNA expression levels of TLRs, melanoma differentiation-associated gene 5 (MDA5),[31] and retinoic-acid-inducible protein I (RIG-I)[32] in the 4 APC subsets were assessed (Figure 5a). Both TLR8 and MDA5 were the most universally expressed in the vaginal DCs. LCs expressed higher level of TLR7 than LP-DCs. CD14$^+$ LP-DCs expressed TLR6 and RIG-I, which were minimally expressed in LCs and CD14$^-$ LP-DCs. Compared to the 3 subsets of DCs, Mϕ expressed increased levels of TLR1, TLR2, TLR4 and TLR6. It was of note that vaginal DCs express increased levels of TLRs for viral products, while Mϕ express TLRs for both viral and bacterial products.

We next tested the effects of bacterial flagellin (TLR5 ligand), R848 (ssRNA: TLR7/8 ligand), poly IC (dsRNA: TLR3, MDA5, and RIG-I ligand) and *Escherichia coli* lipopolysaccharide (*E. coli* LPS: TLR4 ligand), on the vaginal APC-induced $CD4^+$ T cell responses (Figure 5b). R848 enhances LC- and $CD14^-$ LP-DC-induced $CD4^+$ T cell proliferation (Supplementary Figure 8a online), but decreases $IL\text{-}5^+CD4^+$ T cell responses, as previously reported,[33] without altering $IFN\gamma^+CD4^+$ T cell responses. MDA5 was universally expressed in the four APC subsets, but the effect of poly IC was minimal except for the enhanced $CD4^+$ T cell proliferation induced by $CD14^+$ LP-DCs and Mϕ Supplementary Figure 8a online). Flagellin enhanced $CD4^+$ T cell proliferation induced by the four APC subsets. Interestingly, however, flagellin-activated $CD14^-$ LP-DCs decreased both $IFN\gamma^+CD4^+$ and $IL\text{-}5^+CD4^+$ T cell responses, while flagellin-activated Mϕenhanced both $IFN\gamma^+CD4^+$ and $IL\text{-}5^+CD4^+$ T cell responses.

Both poly IC and flagellin enhanced $CD8^+$ T cell proliferation induced by the 4 APC subsets (Supplementary Figure 8b online). However, poly IC did not significantly alter the levels of $IFN\gamma^+CD8^+$ or $IL\text{-}5^+CD8^+$ T cell responses induced by the DC subsets, but enhanced Mϕ-induced $IFN\gamma^+CD8^+$ T cell responses (Figure 5c). TLR4 was highly expressed in Mϕ, but *E. coli* LPS did not significantly alter Mϕ-induced $IFN\gamma^+$ or $IL\text{-}5^+$ T cell responses (Supplementary Figure 9 online). It rather promoted LC- and $CD14^-$ LP-DC-induced $CD4^+$ T cell proliferation (Supplementary Figure 9a online) and LC- and $CD14^+$ LP-DC-induced $CD8^+$ T cell proliferation (Supplementary Figure 9b online).

Taken together, individual subsets of the vaginal APCs display their own functional plasticity in directing $CD4^+$ and $CD8^+$ T cell responses. None of the stimuli bias the 4 APC subsets to induce common types of immune responses. Thus, the immune responses, particularly Th1 vs. Th2, in the vagina can be well balanced by distinct but compensatory functions of the individual APC subsets in the vagina.

Both LCs and $CD14^-$ LP-DCs are potent inducers of IL-22-producing $CD4^+$ T cells

IL-22 induces the secretion of anti-microbial products and contributes to the recovery of epithelial cells.[34-37] As shown in Figure 6a, both LCs and $CD14^-$ LP-DCs display similar ability to induce $IL\text{-}22^+CD4^+$ T cell responses, although LCs in human skins are superior to dermal-DCs[38]. Data from 6 independent experiments are summarized in Figure 6b. $CD14^-$ LP-DC-induced $IL\text{-}22^+CD4^+$ T cells were further analyzed for IFNγ, IL-17, and IL-5 expression (Figure 6c). Summarized data indicate that the overall quality of $IL\text{-}22^+CD4^+$ T cells induced with the 4 APC subsets is similar (Figure 6d), but different from those induced with the control IFNDCs. Compared to the vaginal APCs, IFNDCs induced less numbers of IL-22 $single^+$ $CD4^+$ T cells. Approximately 3-5% of the $IL\text{-}22^+CD4^+$ T cells induced with IFNDCs also expressed IL-17. It was also of note that approximately 40-60% of the $IL\text{-}22^+CD4^+$ T cells induced by the vaginal APCs expressed IFNγ. Although the 3 subsets of vaginal DCs resulted in greater $IL\text{-}22^+CD8^+$ T cell responses than did Mϕ in some experiments, there was no significant difference between the DCs and Mϕat inducing $IL\text{-}22^+CD8^+$ T cell responses Figure 6e). $IL\text{-}22^+CD8^+$ T cells induced with the APC subsets displayed similar patterns of IL-5 and IFNγ expression, the majority of the $IL\text{-}22^+CD8^+$ T cells also expressed IFNγ or IL-5, but not IL-17 (Figure 6f).

We next tested whether microbial products or analogues could alter the vaginal APC-induced IL-22$^+$ T cell responses. Most notably, R848-activated LCs and CD14$^-$ LP-DCs, which can enhance CD4$^+$ T cell proliferation (Supplementary Figure 8a online), enhanced IL-22$^+$CD4$^+$ T cell responses (Figure 6g), but decreased IL-5$^+$CD4$^+$ T cell responses (Figure 5b). Flagellin was able to enhance CD14$^-$ LP-DC-, CD14$^+$ LP-DC-, and Mϕ-induced IL-22$^+$CD4$^+$ T cell responses. The effects of these stimuli on the induction of IL-22$^+$CD8$^+$ T cell responses were minimal, although flagellin-activated Mϕenhanced. IL-22$^+$CD8$^+$ T cell responses (Figure 6h). *E. coli* LPS did not significantly alter the vaginal APC-induced IL-22$^+$CD4$^+$ (Supplementary Figure 10a online) or IL-22$^+$CD8$^+$ T cell responses (Supplementary Figure 10b online).

Taken together, both LCs and CD14$^-$ LP-DCs are potent inducers of IL-22$^+$CD4$^+$ T cell responses that can be further promoted by R848. CD14$^+$ LP-DC- and Mϕ-induced IL-22$^+$CD4$^+$ T cell responses are promoted by flagellin. The ability of the vaginal DCs to induce increased percentage of both IL-22$^+$IFNγ$^+$ and IL-22$^+$IL-5$^+$ T cell responses further distinguish them from DCs in skin[38].

DISCUSSION

This study demonstrate, for the first time, that human vaginal mucosa harbors four major myeloid-originated APC subsets (LCs, CD14$^-$ LP-DCs, CD14$^+$ LP-DCs, and Mϕ) that show distinct phenotypes and functions in directing the immune responses in the vaginal mucosa.

These APCs are further distinguished from those of other human tissues, including skin[24, 25, 38, 39] and intestine[40], by displaying distinct functions in directing both CD4$^+$ and CD8$^+$ T cell responses. These characters of the vaginal APCs support a current notion that human vaginal mucosa is an immunologically unique microenvironment that is different from other tissues or organs.[1, 3, 4]

Th1, Th2, and Th22 are the major types of immune responses induced by the vaginal APCs. They are not potent inducers of IL-10-producing CD4$^+$ T cells (data not shown) or Th17, which contribute to the protective immunity in the vagina.[21-23] All 3 DC subsets are almost equally capable of inducing IL-21-producing CD4$^+$ T cell responses. Foremost, the magnitude of Th1 vs. Th2-type responses can be balanced by distinct but compensatory functions of the individual APC subsets in a steady state. Microbial products and analogues bias the individual subset-driven immune responses. For example, activation of LCs and LP-DCs with R848 resulted in decreased T cells responses, particularly IL-5-producing T cell responses. In contrast, R848 enhanced LCs and CD14$^-$ LP-DC-induced IL-22$^+$CD4$^+$ T cell responses. None of the microbial products tested in this study was able to activate the four APC subsets to induce the same types of T cell responses. Studies have shown that intravaginal administration of vaccines can induce certain levels of protective immunity in both animals[41-43] and humans[44-46]. However, the development of safe vaccines that can establish protective mucosal immunity, particularly cellular immunity which is crucial for the protection against intracellular pathogens, in the human vagina still remains as a challenge.[1, 3-5] Data from this study suggest that vaccines targeting proper vaginal APC subsets with right adjuvants could overcome this challenge.

Vaginal LCs and CD14$^-$ LP-DCs can induce IL-5$^+$CD8$^+$ T cells which do not express IL-13. The physiologic function of IL-5$^+$CD8$^+$ T cells is largely unknown, but CD8$^+$ T cells in the genital tract are thought to be Tc2 or regulatory T cells.[5, 47] For the Th22, both LCs and CD14$^-$ LP-DCs showed a similar ability to induce IL-22$^+$CD4$^+$ and IL-22$^+$CD8$^+$ T cells expressing IFNγ or IL-5, which is distinct from skin DCs and IL-22$^+$ T cells induced with skin DCs.[38] To design more effective vaccines in the future, these features of the vaginal APCs also need to be considered.

αEβ7 (or CD103/β7) integrin expressed on T cells[26, 27] allows lymphocytes to migrate into local mucosal tissues and contributes to their retention within the epithelial layers of the mucosa,[28] especially in the vagina.[27, 29] We demonstrate that fractions of T cells in the human vagina express CD103, CCR4, CXCR3, and β7 integrin. More importantly, vaginal DCs, particularly LCs and CD14$^-$ LP-DCs, can efficiently induce CD103 expression on both CD4$^+$ and CD8$^+$ T cells. All subsets of vaginal APCs are able to induce similar levels of β7 integrin and CXCR3[30] on T cells. Similarly, APCs from the vagina could induce CCR4, a chemokine receptor expressed on Th2-type T cells, on both CD4$^+$ and CD8$^+$ T cells. The numbers of CCR4$^+$ CD4$^+$ T cells induced by LCs or CD14$^-$ LP-DCs were higher than those induced by CD14$^+$ LP-DCs or Mϕ This is consistent with their capacity for polarizing T cell responses toward Th2-type. Taken together, our data support that vaginal APC subsets have the unique capacity to directing immune responses in the vagina by inducing the expression of receptors that could allow effector cells to migrate into the vaginal mucosa.

To get further insight of the immunology of human vagina, future studies need to be performed in humans or animals that have similar features that human vagina has. The microenvironments in the vagina of humans and mice are different. First, the two are constantly exposed with different types of antigens. Second, commensal microorganisms in the two are not the same. Thus, APCs localized in the two immune systems could display distinct functions in directing the immune responses in the vaginal mucosa. This can be applied to the immune responses in a steady state as well as during microbial infections. Future studies also need to consider the effects of female hormones.[48-50]

In conclusion, human vaginal mucosa contains four major subsets of myeloid-derived APCs. Each of the subsets displays common as well as unique phenotypes and functions that direct the immune responses in the vagina. Such functional specialties and plasticity driven by the different subsets of the vaginal APCs may play important roles in shaping the vaginal mucosa as an immunologically unique microenvironment. To elicit desired types of adaptive immune responses in the vagina, antigens need to be delivered to the selected APC subset with adjuvants that can further promote the APC-mediated immune responses.

METHODS

Tissue Samples

Vaginal tissues were obtained from patients (32-88 years old) who underwent pelvic or cosmetic surgeries under a protocol approved by the Institutional Review Board (IRB) of

Baylor Research Institute (BRI). Patients were not infected with HIV, HCV, or TB and did not have inflammation. No other diagnosis information was available.

Enzymatic Digestion of Vaginal Mucosa

Tissues were dissected free from fat, cut in small pieces (1-5 mm^2) and digested for 3h at 37°C with 0.6 unit/ml Dispase II, 2 mg/ml collagenase D (Roche Applied Science, IN), 200 μg/ml DNase I (Invitrogen, CA), 20 units/ml hyaluronidase (Sigma Aldrich, MO) in RPMI 1640 (Invitrogen) supplemented with 25 mM HEPES buffer (Invitrogen), 2 mM L-glutamine (Sigma), 1% nonessential amino-acids (Sigma), 1mM sodium pyruvate (Sigma), antibiotic/antimycotic (Invitrogen), and 5% FCS (HyClone, UT). Cell suspensions were filtered consecutively on 100 μm, 70 μm and 40 μm cell strainers (BD Biosciences, CA) and washed.

Cell phenotypes and Morphology

Cell phenotypes were assessed by flow cytometry analysis. Cells were stained with the following antibodies (Abs) and 7-AAD: anti-HLA-DR-AF700, anti-CD1c-AF647, anti-CD1a, anti-CD11c, anti-CD34, anti-CD54, anti-CD83, anti-CD86, anti-CCR6, anti-E-cadherin from Biolegend (CA); anti-Langerin PE from Beckman Coulter (CA); anti-Langerin AF488 (in-house); anti-CD14-eFluor450 (eBiocience, CA); anti-CD66, anti-CD123, anti-β7 integrin Abs from BD Biociences; anti-E/P selectin, anti-CCR2, anti-CCR4, anti-CCR5, anti-CCR7, and anti-CXCR4 Abs from R&D Systems; anti-CD163 from BMA Biomedicals (Switzerland); and anti-CX3CR1 from MBL International (MA). Phenotypes of vaginal APCs were analyzed by flow cytometry on an LSR II (BD Biosciences). Anti-CD103 and anti-CCR4 Abs used for T cell phenotyping were from eBioscience and R&D Systems, respectively.

Cell morphology was examined after the Giemsa staining of sorted vaginal APC subsets. Giemsa staining of sorted vaginal APCs was done using the Diff-Quik™ Stain Set according to the manufacturer's protocol (Siemens Healthcare Diagnostics, DE). Images were acquired using an Olympus BX60 microscope with Planapo 100x/1.4oil objective and a Nikon DXM1200C digital color camera with Nikon NIS Elements F Version 2.30 software.

APC Isolation

Tissues were cut into small pieces approximately 1 cm^2, and incubated in PBS containing 2 mM EDTA and antibiotic/antimycotic solution overnight at 4°C. Epithelium and LP were then separated. LP was cut in smaller pieces (1-5 mm^2). Epithelial sheets and LP pieces were incubated for 2 days at 37°C in RPMI 1640 supplemented with HEPES buffer, L-glutamine, nonessential amino-acids, sodium pyruvate, antibiotic/antimycotic, and 10% FCS. Migratory cells were recovered, filtered consecutively on 100 μm, 70 μm and 40 μm cell strainers and washed. Cells were stained with 7-AAD, anti-HLA-DR-AF700, anti-Langerin-PE, anti-CD1c-FITC (Invitrogen) and CD14-eFluor450. HLA-DR^+ cells were gated and then $Langerin^+$, $CD1c^+CD14^-$, $CD1c^+CD14^+$, and $CD14^+CD1c^-$ cells were sorted by FACS Aria II (BD Biosciences).

Preparation of T Cells and Monocyte-Derived IFNDCs

Peripheral blood mononuclear cells from healthy volunteers were fractionated by elutriation, under a protocol that has been approved by the Institutional Review Board of Baylor Research Institute. IFNDCs were generated by culturing monocytes in serum-free medium (Cellgenix, Germany) supplemented with GM-CSF (100 ng/ml) and IFNα (500 units/ml) (IFNDCs). The medium was replenished with cytokines on day 1 for IFNDCs. IFNα and GM-CSF were from the Pharmacy at Baylor University Medical Center (Dallas, TX). Naïve ($CD45RA^+CD45RO^-CCR7^+$) T cells (purity>99.2%) were sorted on FACS Aria II.

T Cell Responses

1.5×10^5 CFSE-labeled purified naïve T cells were co-cultured with 2×10^3 or the indicated number of APCs in RPMI 1640 supplemented with HEPES buffer, L-glutamine, nonessential amino-acids, sodium pyruvate, penicillin/streptomycin and 10% AB serum (GemCell, CA). In some experiments, vaginal APCs were activated with R848 (2 μg/ml), Flagellin (200 ng/ml), poly IC (2 μg/ml), or *E. coli* lipopolysaccharide (LPS; 100 ng/ml) 16 h prior to the addition of T cells. All TLR-L are from Invivogen (CA). After 7 days, cells were stained with anti-CD4 APC-Cy7 (Biolegend), anti-CD8 Pacific Blue (Biolegend) and LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen), and T cell proliferation was tested by measuring CFSE dilution. For cytokine expression analysis, T cells were restimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μg/ml ionomycin (Sigma) for 6h in the presence of GolgiPlug (BD Biosciences). They were then stained with anti-CD4, anti-CD8, LIVE/DEAD® Fixable Dead Cell Stain Kit, and anti-IFNγ, anti-IL-5, anti-TNFα, anti-IL-13 Abs (all from Biolegend), anti-IL-17, anti-IL-21, and anti-IL-22 Abs (all from eBioscience). Intracellular staining was performed using BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit according to the manufacturer's protocol.

Immunofluorescence

Cryo-sections were fixed in cold acetone, dried and blocked for non-specific fluorescence with Fc Receptor Block and Background Buster (Innovex Biosciences, CA). Sections were stained with the indicated antibodies and then subsequently stained with DAPI (Invitrogen). Digital images were taken using an Olympus BX51 with a Planapo20/0.7 or Planapo40/0.95 objective, a Roper Coolsnap HQ camera and Metamorph software (Molecular Devices, CA). FACS-sorted $HLADR^+CD207^-CD1c^-CD14^-$ cells were fixed in 4 % paraformaldehyde and stained with the indicated antibodies, and then subsequently stained with DAPI. Confocal images were taken with the Leica SP1 and Planapo63/1.32 objective. Images were acquired using the same exposures for antibody and isotype staining and identical scaling was applied.

Microarray analysis

RNA was purified following the manufacturer's instructions for the ArrayPure™ Nano-scale RNA Purification Kit (Epicentre). Amplification and labeling was carried out following the manufacturer's instructions for the TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre). A total of 750ng of cRNA was used for hybridization of technical duplicates onto the Illumina Human H12v4 BeadChip. Scanning of the BeadChip was carried out on

the iScan (Illumina) using the manufacturer's recommended settings. Microarray data were background subtracted and scaled as per the Illumina GenomeStudio V2011.1 GX1.9.0 User Guide. GeneSpring v7.3 software was used for unsupervised analysis and data filtering (detection $p<0.01$ in at least 2 samples). For each sample, background-subtracted and scaled-intensity values for the probes representing the genes of interest were exported to Excel. Values less than 10 were converted to 10, and then the intensity average was calculated for biological replicates.

Statistical Analysis

Statistical significance was determined using the ANOVA test or paired t-test using Prism 5 software (GraphPad Software Inc, CA). Significance was set at $P<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DD and SO designed experiments, analyzed data and wrote the manuscript. DD, JG, EA, SZ and MD performed experiments. J.B. helped manuscript writing. MC, MB and JS provided tissue samples.

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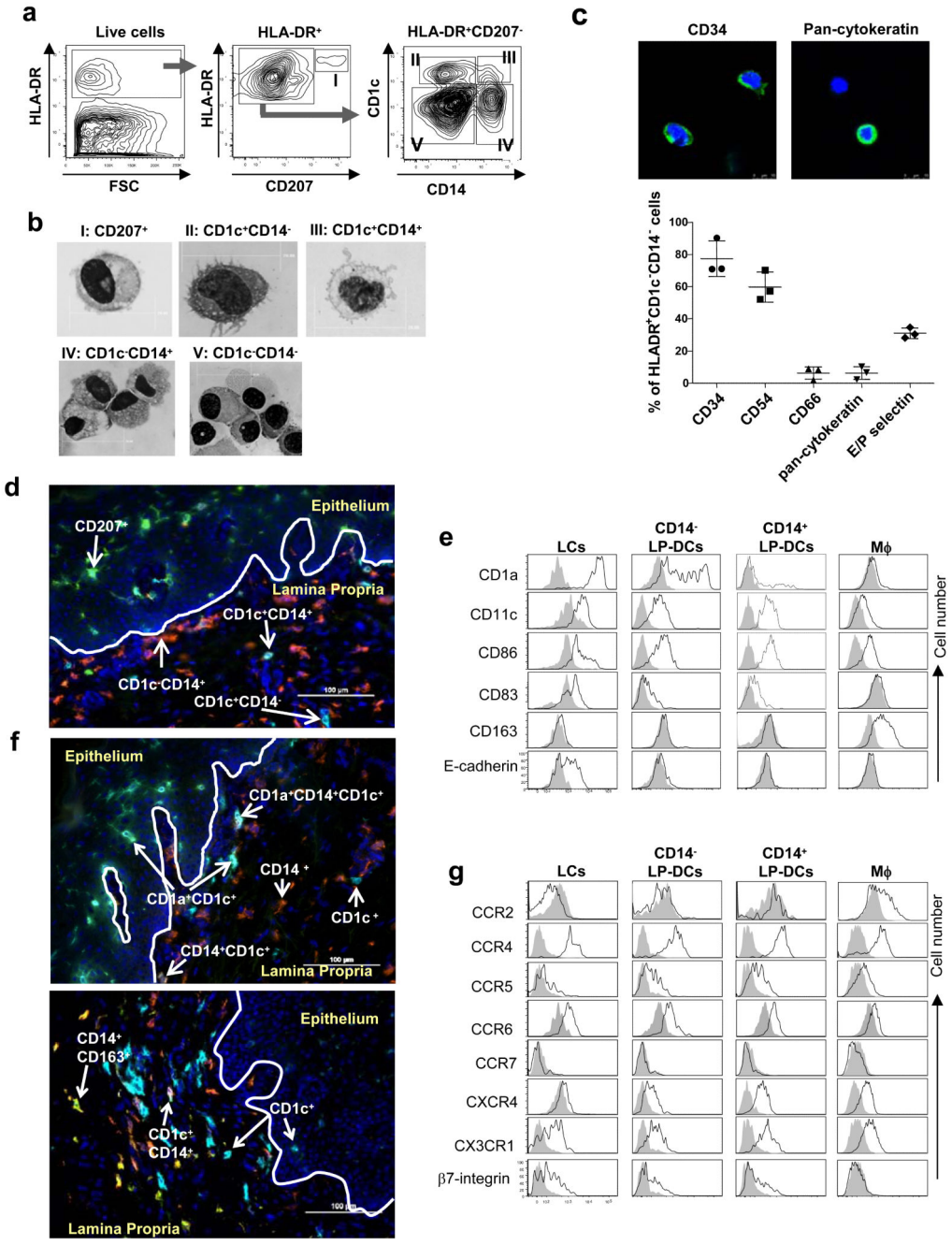

Figure 1.

Human vaginal mucosa contains four major subsets of myeloid-originated APCs. (**a**) Flow cytometry analysis of cells in human vaginal mucosa after enzymatic digestion. Live HLA-DR$^+$ cells were gated (left panel) and CD207$^+$ cells (I) were gated (middle panel). HLA-DR$^+$CD207$^-$ cells were further divided into four groups based on CD1c and CD14 expression (right panel, II: CD1c$^+$CD14$^-$; III: CD1c$^+$CD14$^+$; IV: CD1c$^-$ CD14$^+$; V: CD1c$^-$CD14$^-$). Data are representative of 20 independent experiments. (**b**) Morphology of FACS-sorted subpopulations of vaginal cells (x100, bars are 20 μm). Data are representative

of 4 independent experiments. (**c**) FACS-sorted HLA-DR^{+}CD207^{-} CD1c^{-}CD14^{-} cells were stained for CD34, CD54, CD66, pan-cytokeratin or E/P selectin. One representative data from 3 independent experiments using cells from different donors (upper panel). Percentage of positive cells has been calculated (lower panel). Each dot represents data acquired from one donor. (**d**) Frozen tissue sections were stained for CD207 (green), CD14 (red), CD1c (light blue) and cell nuclei (dark blue) (x20, bar is 100 μm). Data are representative of 6 independent experiments. (**e, g**) Flow cytometry analysis of the vaginal APC subsets. Tissues were digested with enzymes and cell suspension was stained with indicated antibodies and gated as in **a**. Gray histograms represent isotype controls. Data are representative of 10 (**e**) or 6 (**g**) independent experiments. (**f**) Frozen tissue sections were stained for CD1a (green), CD1c (light blue) and CD14 (red) (upper panel) and CD163, (green), CD1c (light blue) and CD14 (red) (lower panel) (x20, bar is 100μm). Data are representative of 6 independent experiments. All of the independent experiments were performed with tissues or cells from different donors.

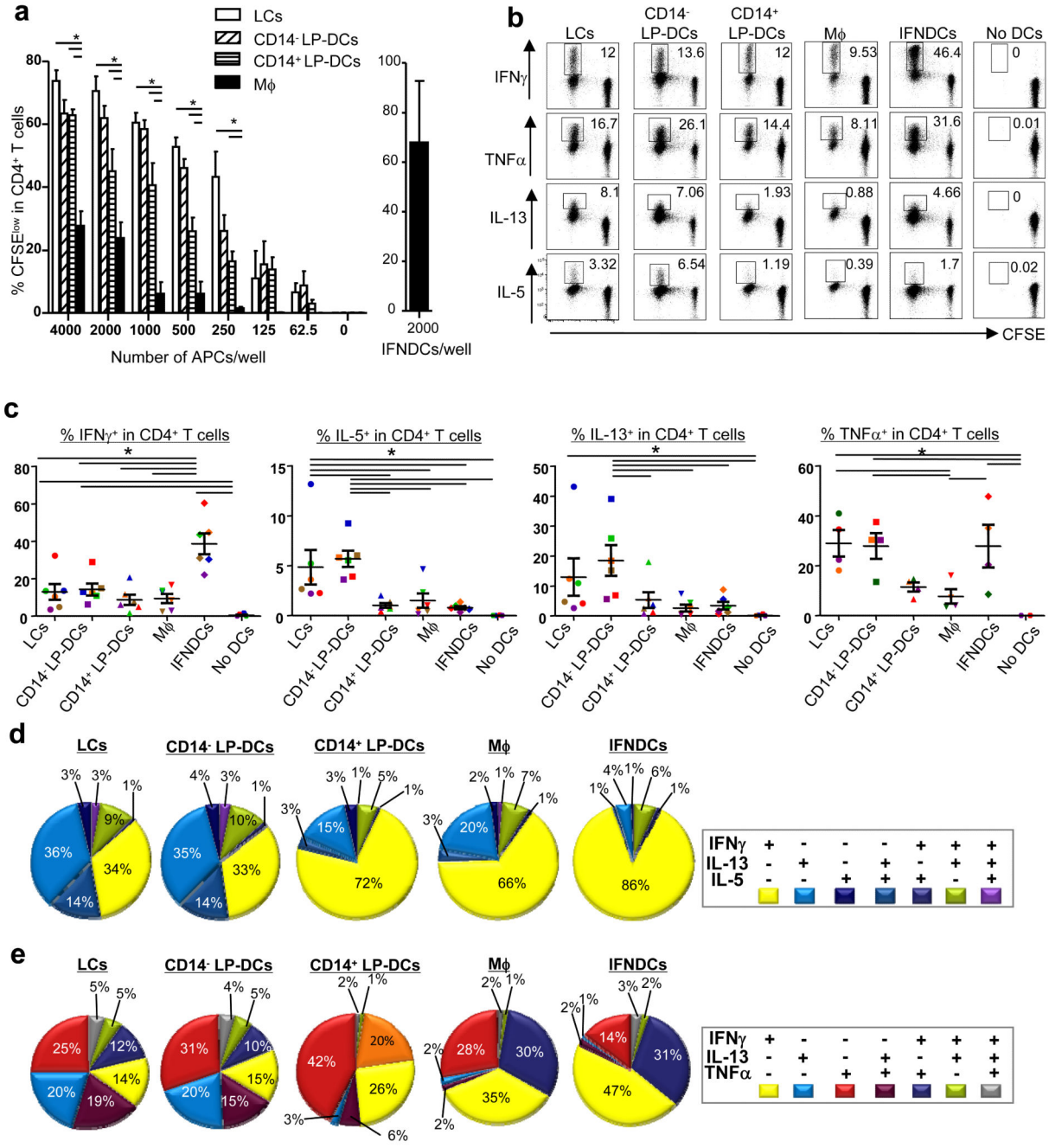

Figure 2.

Functional specialties of the vaginal APC subsets in directing CD4+ T cell responses. CFSE-labeled allogeneic naïve total T cells were co-cultured for 7 days with different numbers of the vaginal APCs or IFNDCs. (**a**) Live CD4+ T cells were gated and CD4+ T cell proliferation was assessed by measuring CFSE dilution. Data are mean ± SD of 3 independent experiments with duplicates. (**b**) After 7 days, T cells were stimulated with PMA/ionomycin in the presence of brefeldin A. Cells were then stained for intracellular IFNγ, IL-13, IL-5, and TNFα. 6 independent experiments using cells from different donors

showed similar data. (**c**) Summary of the data from independent experiments using APCs from 6 donors marked with different colors. (**d-e**) Boolean gate analysis. (**d**) IFNγ^+, IL-13$^+$, and IL-5$^+$ CD4$^+$ T cells (N=6) and (**e**) IFNγ^+, IL-13$^+$, and TNFα^+ CD4$^+$ T cells induced with different subsets of APCs (N=3). * indicates $p<0.05$ by ANOVA test.

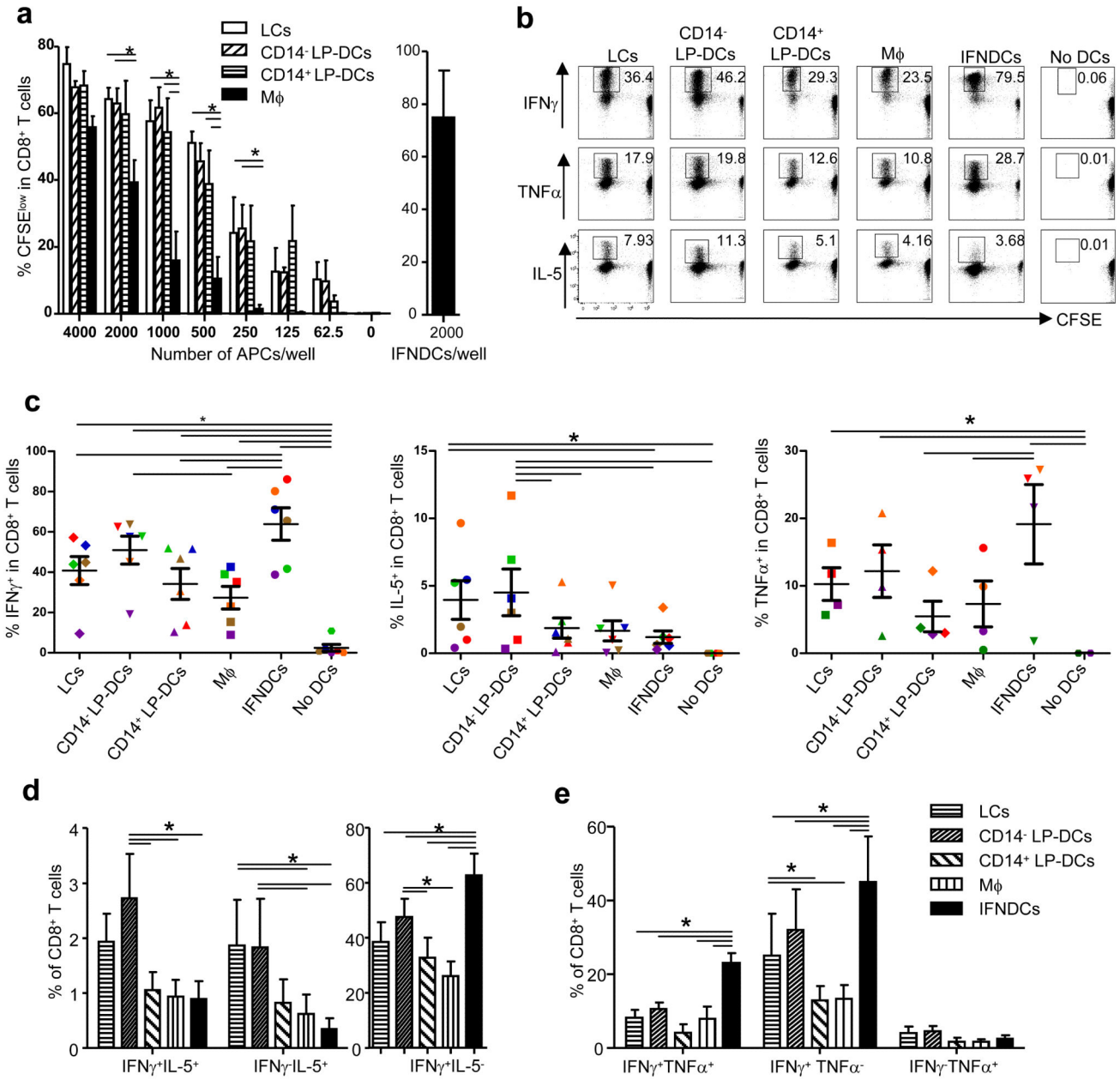

Figure 3.

Functional specialties of the vaginal APC subsets in directing $CD8^+$ T cell responses. CFSE-labeled allogeneic naïve total T cells were co-cultured for 7 days with the vaginal APC subsets or IFNDCs. (**a**) $CD8^+$ T cell proliferation was assessed by measuring CFSE dilution. Data are mean ± SD of 3 independent experiments with duplicates. (**b-e**) After 7 days, T cells were stimulated with PMA/ionomycin in the presence of brefeldin A, and then stained for intracellular IFNγ, TNFα, and IL-5 expression. (**b**) Representative data from 6 independent experiments. (**c**) Summary of the data from 6 ($IFN\gamma^+$ and $IL\text{-}5^+$) and 4 ($TNF\alpha^+$) independent experiments using cells from different donors. (**d**) $IFN\gamma^+$ and $IL\text{-}5^+$ $CD8^+$ T

cells (N=6) or (**e**) IFNγ^+ and TNFα^+ CD8$^+$ T cells induced with different APC subsets (N=3). * indicates $p<0.05$ by ANOVA test.

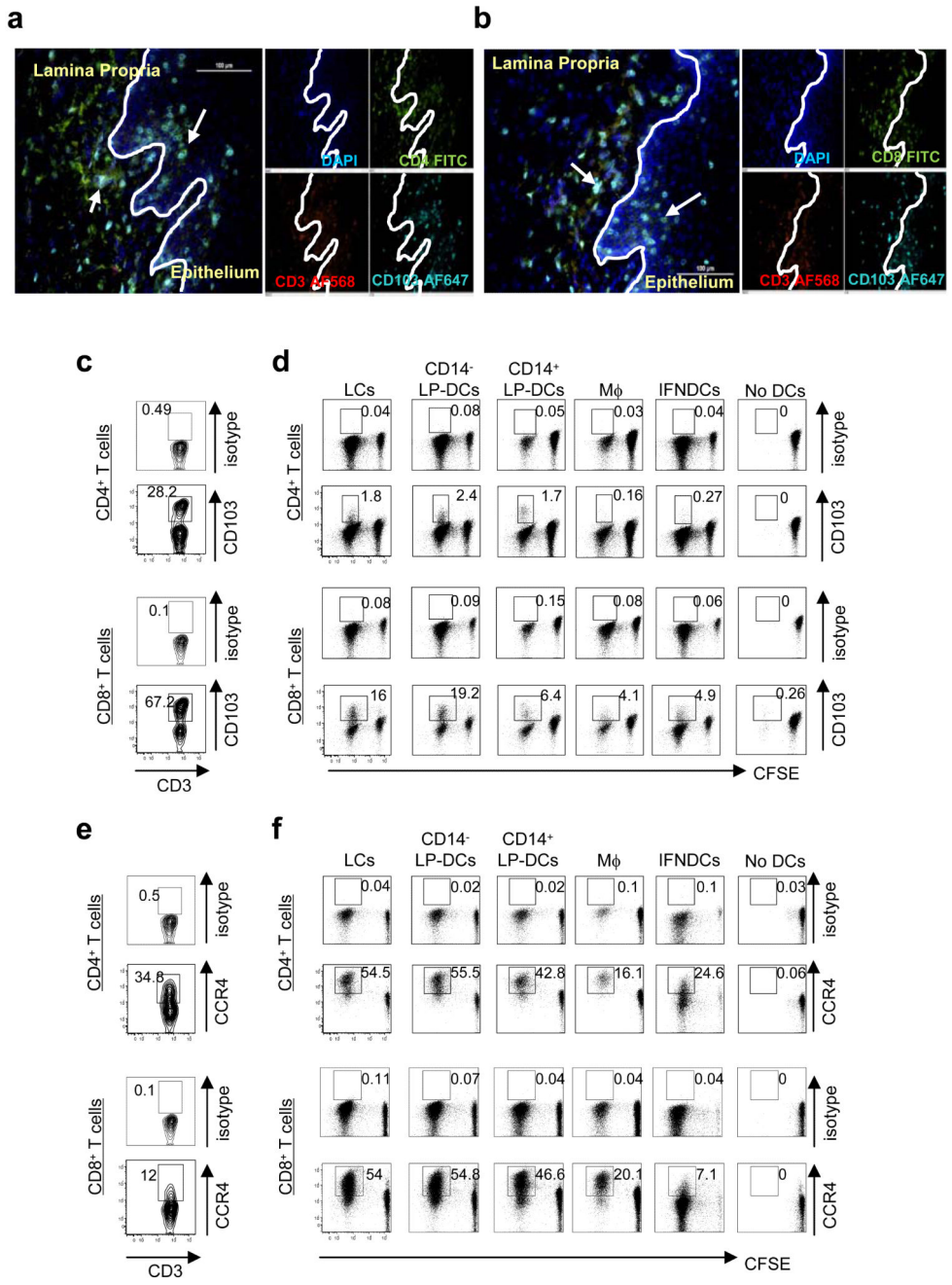

Figure 4.

Vaginal APCs can induce naïve T cells to express CD103 and CCR4 that are found to be expressed in T cells in the vaginal mucosa. (**a**) Frozen tissue sections were stained for CD3, CD4 and CD103 and (**b**) CD3, CD8, CD103 expression (x20, bar is 100 μm). (**c, e**) CD103 (**c**) and CCR4 (**e**) expressions on $CD4^+$ (top panel) and $CD8^+$ (bottom panel) T cells from the vaginal mucosa. (**d, f**) CD103 (**d**) and CCR4 (**f**) expressions on naïve CFSE-labeled $CD4^+$ (top panels) and $CD8^+$ T cells (bottom panels) co-cultured for 7 days with the vaginal APC subsets or IFNDCs. (**a-c and e**) T cells in the vagina from 5 donors showed similar

results. (**d and f**) 4 independent experiments using APCs from different donors showed similar results.

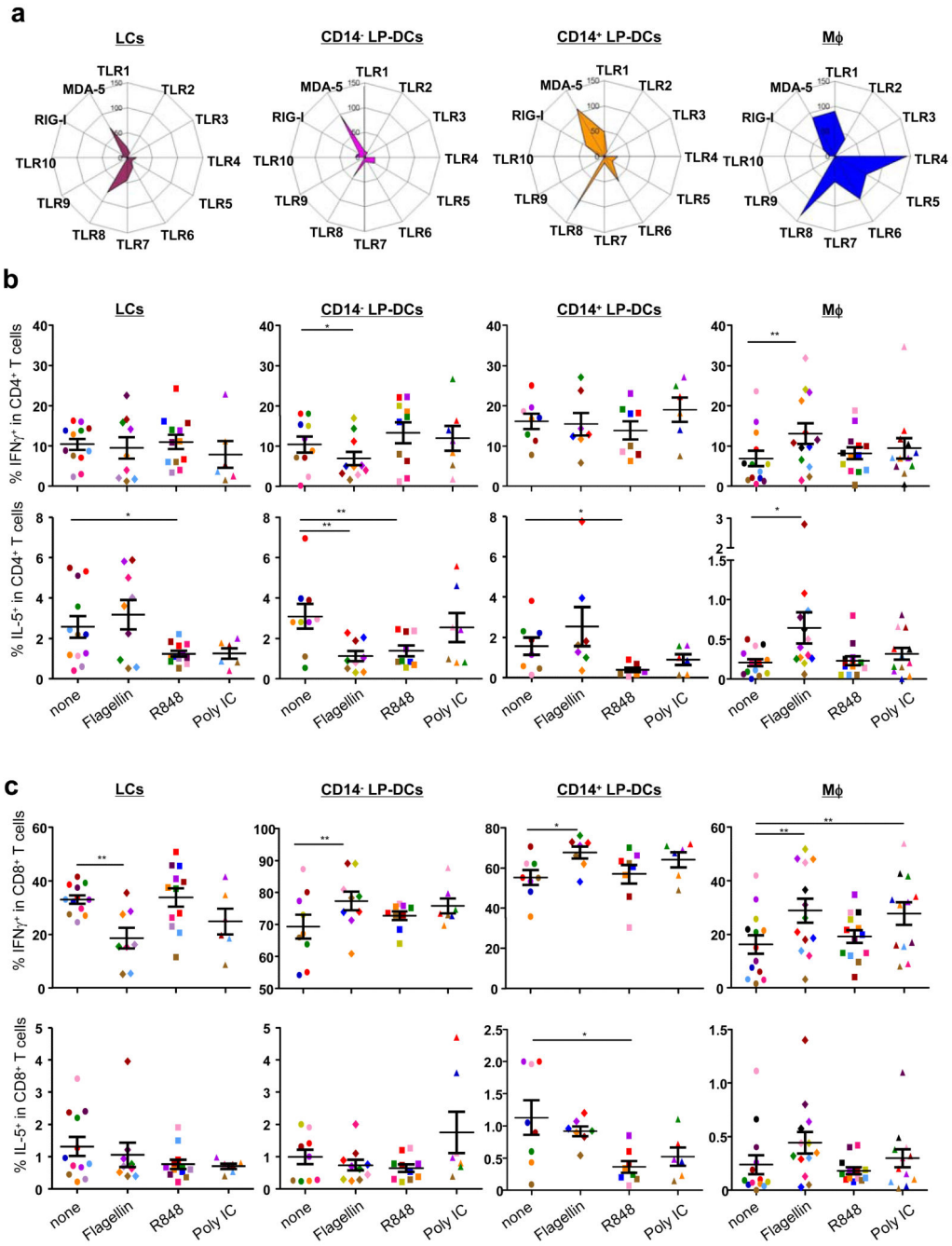

Figure 5.

Expression levels of pattern-recognition receptors and their effects on the APC subset-driven T cell responses (**a**) TLRs, MDA-5 and RIG-I average gene expression in FACS-sorted vaginal LCs (N=2), CD14^{-} LP-DCs (N=3), CD14^{+} LP-DCs (N=2), and Mϕ (N=6). (**b, c**) APC subsets were stimulated with flagellin, R848 or poly IC for 16h and then CFSE-labeled naïve total T cells were co-cultured for 7 days. T cells were stimulated with PMA/ionomycin in the presence of brefeldin A and then stained for intracellular IFNγ and IL-5 expression.

Each color represents the data generated with one tissue donor (* indicates $p<0.05$ and ** indicates $p<0.01$; paired *t*-test). $CD4^+$ (**b**) and $CD8^+$ T cells (**c**).

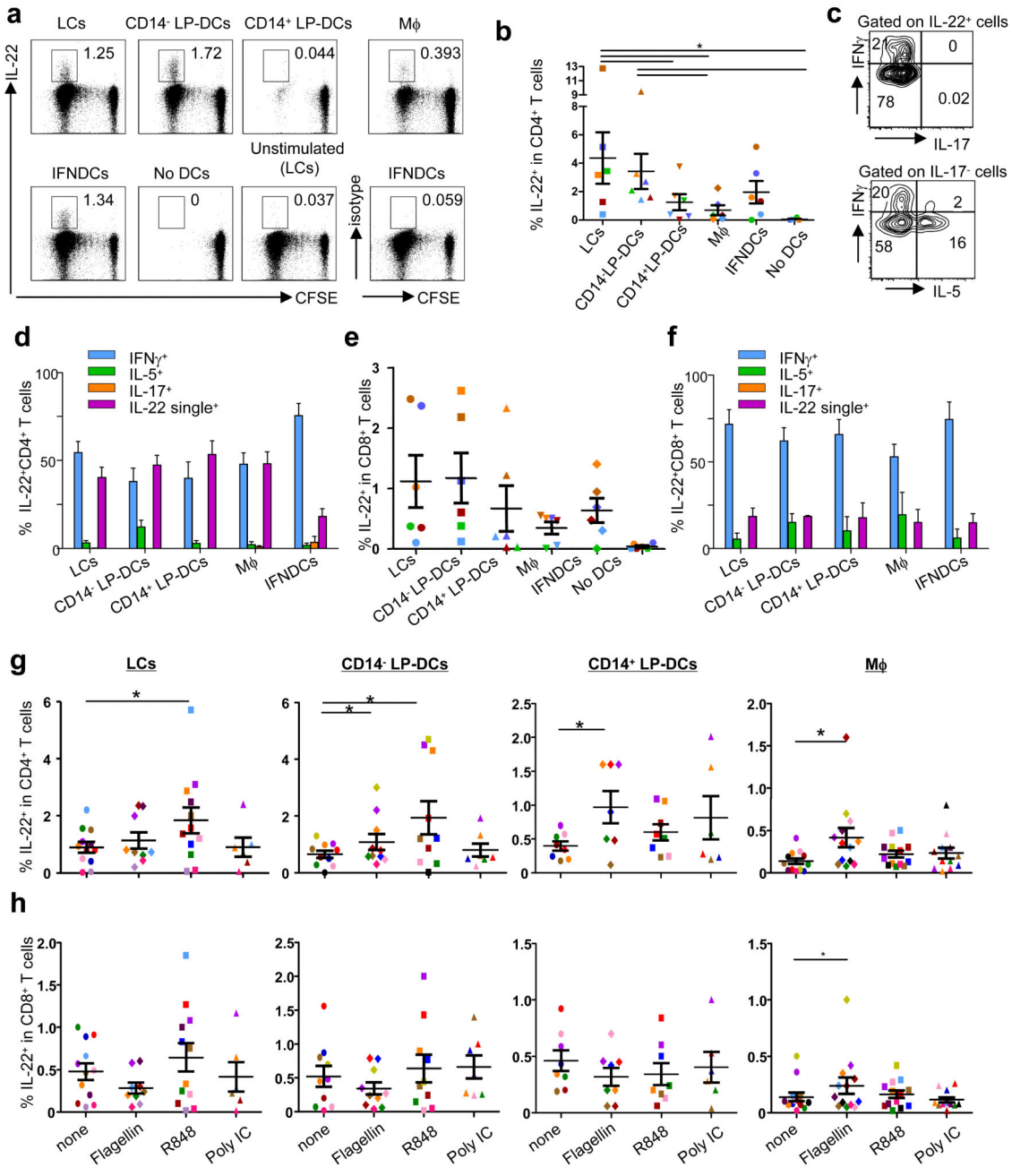

Figure 6.

Vaginal LCs and CD14^{-} LP-DCs can efficiently induce IL-22-producing T cell responses. CFSE-labeled allogeneic naïve total T cells were co-cultured for 7 days with the vaginal APCs or IFNDCs. T cells were restimulated with PMA/ionomycin in the presence of brefeldin A and then stained for intracellular IL-22. (**a-d, g**) Data for CD4^{+} T cells (**e, f, h**) Data for CD8^{+} T cells. (**a**) Representative data from one experiment are presented. (**b, e**) Summarized data from 6 independent experiments (* indicates $p<0.05$; One-way ANOVA test). (**c, d, f**) Frequency of IFNγ^{+}, IL-5^{+} and IL-17^{+} cells among the IL-22-producing T

cells. 5 independent experiments using APCs from different donors showed similar results. (**c**) Representative data from one experiment after co-culture with $CD14^-$ LP-DCs or (**d, f**) Combined data from 5 independent experiments using cells from different tissue donors. (**g, h**) Allogeneic naïve T cells were co-cultured for 7 days with the vaginal APCs non-activated or activated for 16 h with flagellin, R848 or poly IC. T cells were then stained for intracellular IL-22 expression. Data from 7 ($CD14^+$ LP-DCs) to 10 (Mϕ) independent experiments using APCs from different donors are presented (* indicates $p<0.05$ and ** indicates $p<0.01$;paired *t*-test). In (**b, e, g, h**), each color represents the data generated with one tissue donor.