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Retraction notice to "The rapid and sustained responses of dendritic cells to influenza virus infection in a non-human primate model" [Braz. J. Infect. Dis. 18 (2014) 406–413]

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Original article

The rapid and sustained responses of dendritic cells to influenza virus infection in a non-human primate model

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ABSTRACT

Dendritic cells (DCs) are readily infected by influenza viruses and play a crucial role in regulating host innate and adaptive immune responses to viral infection. The aims of this study are to characterize the dynamic changes in the numbers and maturation status of dendritic cells present in the lung and lung-associated lymph nodes (LALNs) in the model of a non-human primate (NHP) infected by influenza A virus (IAV). Cynomolgus macaques were infected with influenza A virus (H3N2) via bronchoscopy. Flow cytometry was used to analyze the DC numbers, maturation status and subsets during the time of acute infection (days 1, 2, 3, 7) and the resolution phase (day 30). A dramatic increase in the numbers of influenza A virus-infected CD11c+CD14− myeloid dendritic cells (mDCs) and CD11c-CD123+ plasmacytoid dendritic cells (pDCs) were observed from day 1 to day 4 and peak up from day 7 post-infection. In lung and lung-associated lymph nodes, the numbers and maturation status of myeloid dendritic cells and plasmacytoid dendritic cells increased more slowly than those in the lung tissues. On day 30 post-infection, influenza A virus challenge increased the number of myeloid dendritic cells, but not plasmacytoid dendritic cells, compared with baseline. These findings indicate that dendritic cells are susceptible to influenza A virus infection, with the likely purpose of increasing mature myeloid dendritic cells numbers in the lung and lung and lung-associated lymph nodes, which provides important new insights into the regulation of dendritic cells in a non-human primate model.

Introduction

Influenza A virus (IAV) is negative-stranded, segmented RNA virus, an important human pathogen that causes worldwide epidemics yearly and pandemics sporadically.[1] The virus is responsible for substantial morbidity and mortality, with an average of over 100,000 hospitalizations and approximately 20,000 deaths annually in the United States.[2–5]

An important paradigm strongly suggests that the lung damage arising from IAV consists of an excessive host response characterized by a rapid, influx of inflammatory

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cells into the lungs. Indeed, the respiratory portal serves as an important entry site for pathogenic organisms, but it is also a primary infection site of all mammalian influenza viruses. Maines et al. have reported that the specific glycan receptors on the apical surface of the respiratory tract were found to bind hemagglutinin of the 2009 A virus (H1N1).[6] Dendritic cells (DCs) are key players in antiviral innate immunity and acquired immune responses development, which can construct a network within epithelium and submucosa of conducting airway as well as in lung parenchyma, where they can be found both on alveolar surfaces and in the vascular compartment of the lung.[7–10] Furthermore, mature DCs efficiently present antigens and initiate adaptive immune response by migrating into lymphoid tissue to present processed viral antigens to T lymphocytes.[11–13] At least two subsets of DCs have been described in humans: the CD11c+ myeloid DCs, and the CD11c-CD123+ plasmacytoid DCs (pDCs), which express a different repertoire of pattern recognition receptors and show a differential response to various microbial stimuli.[14]

At present, pulmonary DCs have been studied in models of bacterial infection and allergic airway sensitization, but their role in respiratory viral infection is still unclear. In a mouse model, IAV infection has been found to increase the number of DC in the respiratory tract, but these were short-lived DCs.[15] Similar increases in the number of lung DCs have also been described following respiratory syncytial virus (RSV)[16] infection and Sendai virus[17] infection in mice. However, there are great differences of DCs characteristics between human and murine. For example, human pDC characteristically express high levels of IL-3 receptor alpha chain (CD123) but in the mouse, pDC do not normally express CD123. However, non-human primate (NHP) DCs have the same markers and functions as human DCs. NHP model, therefore, is helpful for us to reveal the relationship between IVA infection and lung DCs. A better understanding of the interplay of IAV with DCs may facilitate the development of an effective vaccine.

In this study, we have focused on analyzing innate immune response of NHP DCs in response to IAV infection with IAV in lung and lung associated lymphoid node (LALN).

Materials and methods

Animals

Cynomolgus macaques were purchased from a colony located at the Lovelace Respiratory Research Institute (LRRI) animal facilities. All animals (weight: 2.6 ± 1.2 kg) were male and their ages were 6–9 years old when infected. The animals were screened for active RSV, influenza, and parainfluenza infections. All studies were approved by the LRRI Institutional Animal Care and Use Committee.

IAV preparation

Study design

Control animals ($n = 4$) were instilled with sterile media and analyzed at day 4 following instillation. In low dose of IAV infection group (1×10^6 PFU), blood was collected from animals at day 1 ($n = 4$), 2 ($n = 4$), 3 ($n = 4$), 4 ($n = 4$), 7 ($n = 4$) and 30 ($n = 4$) post-infection. Additionally, in high dose of IAV infection group (2×10^6 PFU), scheduled blood test was performed at day 1 ($n = 4$) and 2 ($n = 4$) post-infection.

Bronchoscopic instillation of IAV

Instillations were performed in all groups via a pediatric bronchoscope (FB-10x; Pentax Medical Company, Montvale, NJ). Specifically, each animal was given 10 mg ketamine/kg body weight mixed with 2 mg xylazine/kg body weight intramuscularly. Anesthesia was further induced by 2–3% isoflurane by mask until sufficiently anesthetized. An endotracheal tube was placed into the trachea and 2–3% isoflurane was maintained via endotracheal tube throughout the instillation procedures. Following adequate anesthesia, the bronchoscope was inserted into both right and left caudal lobes for instillation of IAV strain HKx31 (H3N2) (low dose, 1×10^6 PFUs/lobe) diluted in 1 mL of cell culture media. For studies in control animals, both lungs were instilled with 1 mL of media in each lobe.

To facilitate dispersion of the inoculant into the lung periphery, a syringe containing inoculant was expelled into the catheter followed by multiple injections of air. Immediately after inoculation, anesthetic was stopped and the endotracheal tube was disconnected from the anesthesia machine. The endotracheal tube was left in the trachea and the animal was moved to a separate room under observation by the veterinarian or veterinary staff until arousal and the endotracheal tube removed. Animals usually recovered within 20–30 min and resumed normal activities.

Tissue harvest

At each time point following IVA infection and blood collection, the animals ($n = 4$ for each time point) were euthanized by increasing the isoflurane concentration up to 5% as described above and then exsanguination was performed by cardiac or arterial puncture in a bio-hood. A necropsy procedure was performed immediately and the lungs were collected to provide materials for a variety of analyses. Specifically, the middle lobe was inflated with 4% paraformaldehyde and was treated overnight at 4 °C. A part of fresh lung tissues from the lower lobes (about 3–5 g) were collected and immediately placed in 5% RPMI.

Isolation of lung mononuclear cells (MNCs)

Lung mononuclear cells were isolated as previously described.[18] Briefly, lungs were minced and digested with Liberase Blendzymes 3 (Roche, Indianapolis, IN) and DNase I (Sigma, St. Louis, MO) solution for 90 min at 37 °C. After digestion, lung cells were dispersed by shearing through a 20-gauge needle, followed by filtration through a nylon screen cell strainer (70 μm) to remove debris. Lung mononuclear cells (LMNCs) were then separated by a 30% Percoll (Amersham, Piscataway, NJ). Single cell suspensions were washed, contaminating erythrocytes were lysed using lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA), and viable cells were counted by trypan blue exclusion.

Harvest of LALN

LALNs were removed carefully. The LALNs were placed in petri dishes containing RPMI 1640 medium supplemented with penicillin and streptomycin (Sigma Chemical Co., St. Louis, MO) and teased apart with fine-toothed tweezers.

LALN cells were centrifuged at 230 × *g* at 4 °C for 10 min and resuspended in complete medium, which consisted of RPMI 1640 medium supplemented with penicillin and streptomycin (Sigma) and 2% heat-inactivated fetal calf serum (GIBCO-BRL, Gaithersburg, MD). Then cells were resuspended in warm (37 °C) lysing buffer [0.17 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 M EDTA (pH 7.3)] for 5 min to remove erythrocytes, centrifuged, and washed once again. LALN and spleen cells were adjusted to a final concentration of 5 × 106 cells per mL.

Flow cytometry

For investigation of lung DCs surface antigen expression, MNCs were washed and spun to pellet. Lung DCs were then suspended in staining buffer (PBS containing 2% fetal bovine serum) at concentration of 1×10^6/mL. Cells were placed in 200 μL volumes into each tube and again spun to pellet. Fluorescein isothiocyanate (FITC)-conjugated anti-CD14, PE-conjugated anti-CD80, anti-CD83 and anti-CD86, PerCP-conjugated anti-HLA-DR and antigen-presenting cell (APC)-conjugated anti-CD11c antibodies (BD Pharmingen) were used for surface staining together with matched isotype controls. All monoclonal antibody solutions and isotype controls were standardized to a concentration of 10 g/mL by dilution in staining buffer and 50 μL volumes were added to the cell pellets. After vortexing and incubation on ice for 30 min in the dark, cells were washed free of unbound antibody by two washes in staining buffer and subsequently centrifugation. Surface stained lung DCs were resuspended in 0.5% PF/AD solution for analysis.

Statistical analysis

All data were analyzed using SPSS 10.0. Data are expressed as means ± SEM. Statistical analyses was determined by one-way ANOVA or paired *t* test. A value of $p < 0.05$ was considered as statistically significant.

Results

IAV injection can induce prompt and sustained increases of the percentage of lung mDCs in MNCs

The expression of CD11c, HLA-DR, CD14, CD80, and CD86 was assessed by flow cytometry analysis (FACS). Myeloid dendritic cells (mDCs) were detected as the cells expressed CD11c+, CD14−. On day 1 after IAV infection, the percentage of lung mDCs in lung MNCs promptly increased to 25.9 ± 2.9%, which was significantly higher than that in control animals (5.1 ± 2.9%, $p < 0.01$, $n = 4$). Thereafter, the percentages of lung CD11c+CD14-mDCs declined slowly but maintained significantly high level until day 7. On day 30, the percentage of lung CD11c+CD14-mDCs was still higher than that in

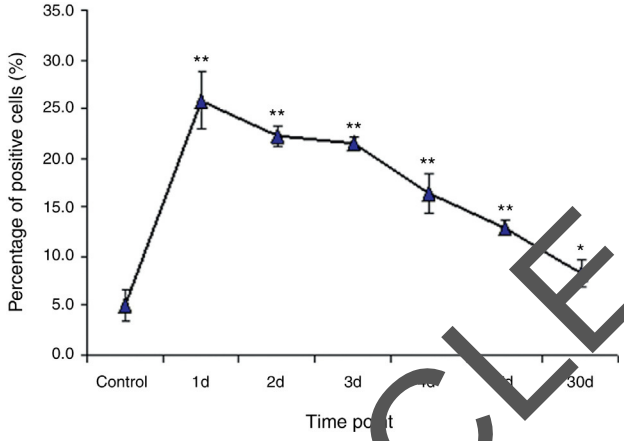

Fig. 1 – CD11c+CD14− DCs rapidly and dramatically increased in the lungs of monkeys following IVA infection. Cynomolgus macaques were infected with IAV (H3N2) via bronchoscopy. The lung cells were isolated and stained for flow cytometry analysis at days 1-4, 7 and 30 following IAV infection ($n = 4$/group). Data are expressed as mean percentages ± SEM. * indicates $p < 0.05$, ** denotes $p < 0.01$ as compared to the control group.

control animals, but this difference did not reach statistical significance (Fig. 1).

IAV infection can induce the maturation of lung mDCs judged by up-regulation of co-stimulatory markers

To assess the maturation status of lung CD11c+CD14-mDCs after IAV infection, the co-stimulatory markers such as HLA-DR, CD80, CD83, and CD86 were analyzed by FACS. In control group, the percentage of HLA-DR expressed from lung CD11c+CD14-mDCs was 14.7% ± 2.4%. After IAV infection, the percentage of HLA-DR increased significantly from day 1 (23.5% ± 2.5%) to day 4 (39.5% ± 3.9%) ($p < 0.01$, $n = 4$). Thereafter, the percentage of HLA-DR dramatically decreased to 15.2% ± 3.8% on d 30 (Fig. 2A).

Furthermore, we determined whether expressions of the CD80, CD83, and CD86 systems are altered after IAV infection. Compared to control group, the expressions of CD80, CD83, and CD86 were up-regulated from day 1, almost peaked on day 4. Then, these co-stimulatory markers were significantly decreased on day 7 after infection. On day 30, the expression of CD83 expression returned almost to the baseline. However, the expression of CD80 and CD86 was still higher than that in control group (Fig. 2B–D).

IAV infection increased the percentage of lung pDCs in MNCs

Here, we further investigated the immune response of lung pDCs after IAV infection, and the percentage of pDCs in lung MNCs was analyzed by FACS. On day 1 after IAV infection, the percentage of lung CD11c-CD123+ pDCs in MNCs increased to 4.1 ± 2.9%, which was higher than that in control group (1.0 ± 2.9%, $p < 0.01$, $n = 4$). The percentages of lung CD11c-CD123+pDCs increased slowly on day 3 and turned to the peak

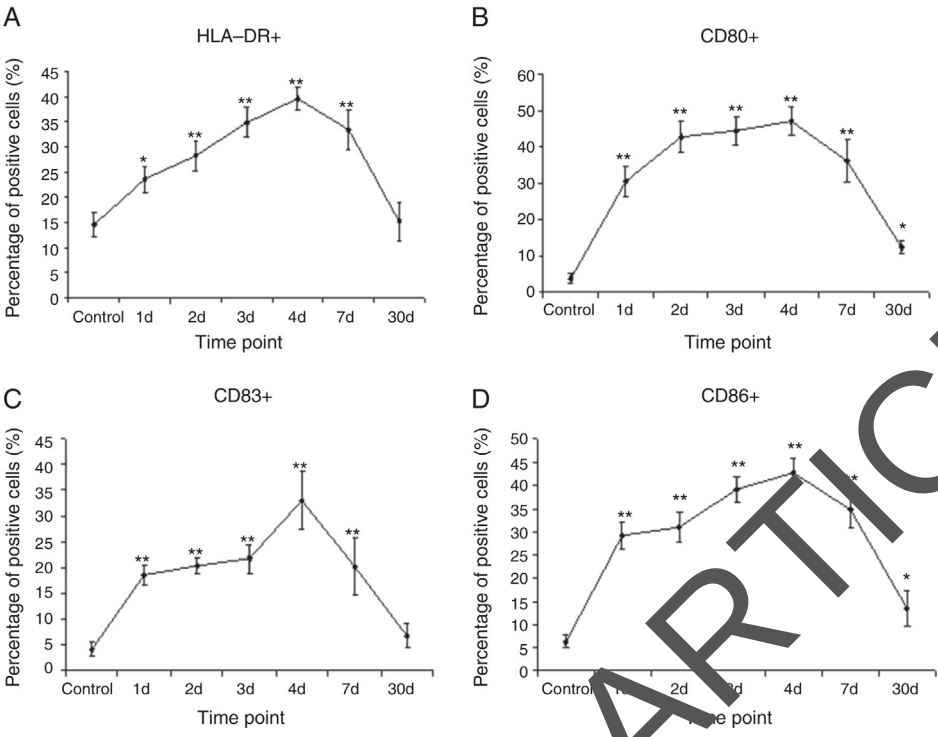

Fig. 2 – IAV infection induced the maturation of DCs in the lungs. The lung cells at different time point post IAV infection were isolated and analyzed for the expression of a panel of surface markers that are related to the maturation status on CD11c+CD14− DC. Mature DCs were analyzed for the expression of HLA-DR, CD80, CD83 and CD by flow cytometry. The percentages of CD11c+CD14-HLA-DR+ cells (A), CD11c+CD14-CD80+ (B), CD11c+CD14-CD83+ (C) and CD11c+CD14-CD86+ (D) were calculated. Data are expressed as mean percentages ± SEM. * indicates $p < 0.05$, ** denotes $p < 0.01$ as compared to the control group.

on day 4. Finally, the percentages of lung pDCs decreased to baseline on day 7 post infection (Fig. 3A and B).

IAV infection can induce gradual increases of the percentage of mDCs in the LALNs of NHPs

We next tested the number of mDCs in LALNs after IAV infection on days 1, 2, 3, 4, 7, and 30, respectively. Of note, the number of CD11c+CD14− mDCs started to increase on day 2, reached peak on day 7 and turned to decline on day 30, which is similar to CD11c-CD123+pDCs observed in LALNs after IAV infection. Unlike CD11c-CD123+pDCs, CD11c+CD14− mDCs did not decrease below baseline amount during the course of infection (Fig. 4A and F).

Expression levels of HLA-DR, CD80, CD83 and CD86 were also observed in LALNs after IAV infection. One day after

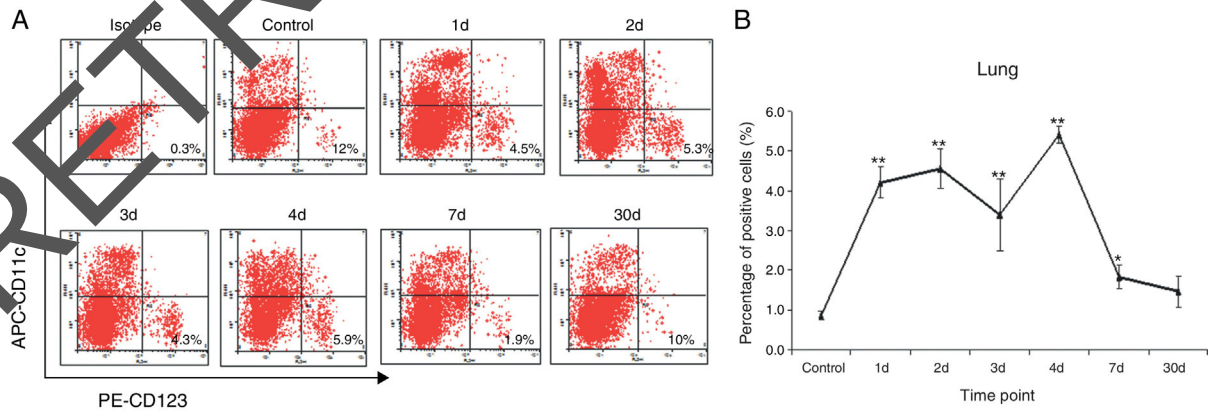

Fig. 3 – IAV infection induced the increases of pDCs (CD11c-CD123+) in the lungs. The lung cells at different time point post IAV infection were isolated and analyzed for flow cytometry. (A) Dot plots of anti-CD123-PE (x-axis; log scale) vs anti-CD11c-APC (y-axis; log scale). Data shown are from representative monkeys in each group. (B) The changes of lung pDCs after IAV infection. Data are expressed as mean percentages ± SEM. * indicates $p < 0.05$, ** denotes $p < 0.01$ as compared to the control group.

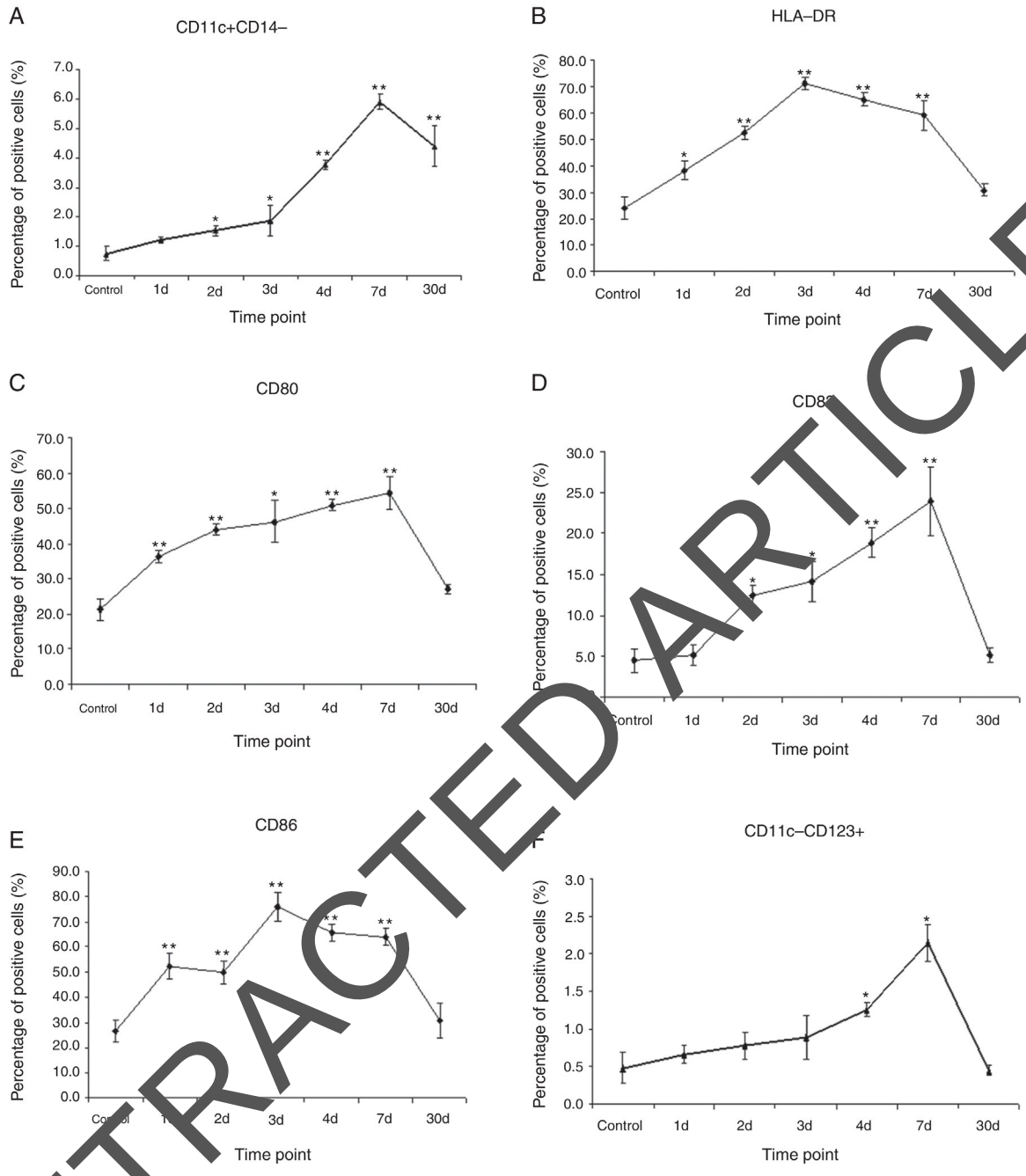

Fig. 4 – The changes of mDC and pDC in the LALNs of monkeys following IAV infection. Cynomolgus macaques were infected with IAV (H3N2) via bronchoscopy. The cells in LALNs were isolated and stained for flow cytometry analysis at days 1–4, 7 and 30 following IAV infection ($n = 4$/group). (A) CD11c+CD14− DCs began to increase on day 2 and sustained high level until day 30 post infection. (B–E) The expressions of co-stimulatory markers such as HLA-DR, CD80, CD83 and CD86 on the CD11c+CD14− DCs significantly up-regulated following IAV infection. The matured DCs increased to the peak on day 7 post infection, but still maintained high level on day 30 post infection. (F) The pDCs (CD11c-CD123+) increased on day 4 and day 7 post infection, then returned to normal level on day 30 post infection. * indicates $p < 0.05$, ** denotes $p < 0.01$ as compared to the control group.

infection, infected DCs showed severely increased HLA-DR, CD80, CD83 and CD86 expression in the LALNs. However, no statistical significant difference between the infected groups and the control group on day 30 has been detected (Fig. 4B–E).

IAV infection can induce the maturation of mDCs in LALNs judged by up-regulation of co-stimulatory markers

Ultimately, we examined whether there was a functional correlation between the IAV amount and the phenotypic changes

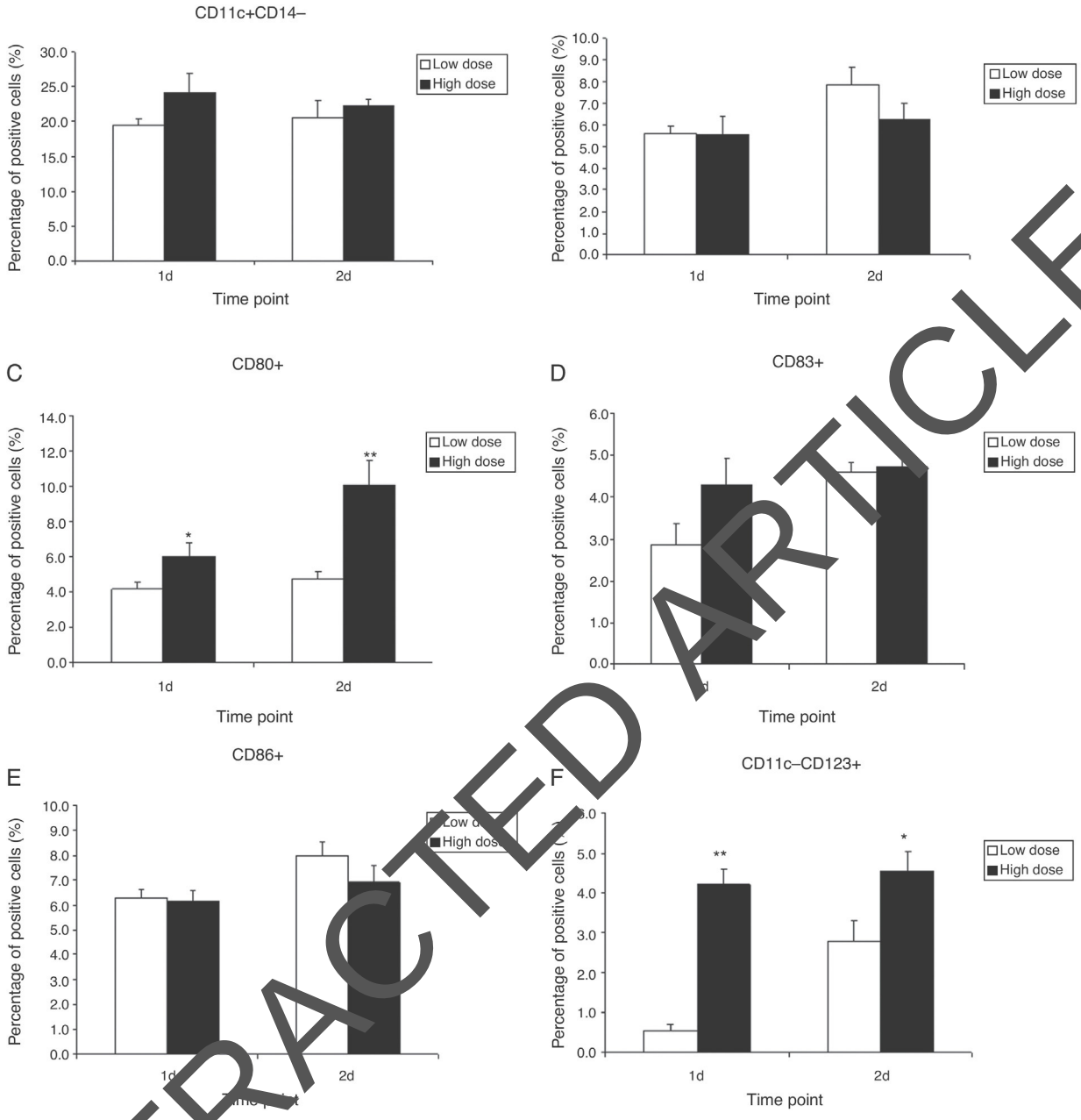

Fig. 5 – The changes of mDC and pDC in the lungs of monkeys following infection with low dose or high dose of IAV virus. Cynomolgus macaques were infected with two dose of IAV (H3N2) via bronchoscopy: low dose is 1×10^6 PFU, high dose is 2×10^6 PFU. The cells in lungs were isolated and stained for flow cytometry analysis at days 1 and day 2 ($n = 4$/group). The results show that both the expression of CD86 on the CD11c+CD14– DCs and the number of pDCs (CD11c-CD123+) in high dose group were significantly higher than those in low dose group. However, the number of CD11c+CD14– and other markers such as HLA-DR, CD80 and CD83 had not any changes. * indicates $p < 0.05$, ** denotes $p < 0.01$ as compared to low dose group.

observed in the infected DCs. As clearly seen in Fig. 5, high dose of IAV can induce greater amount of CD80 and CD83 compared to low dose of IAV by days 1 and 2 after infection. However, other phenotypic changes appear to have no statistical significance between low dose and high dose of IAV infection.

Discussion

Human peripheral blood contains two major DC populations, namely CD11c-CD123+pDCs and CD11c+CD123– mDCs. DCs are APCs that act as a bridge between innate and adaptive

immunity. DCs reside in peripheral tissues in an immature state, where they are on alert for invading pathogens or other danger signals. A contact with nonself structures such as microbes or their structural components activates DC maturation, which is characterized by enhanced expression of co-stimulatory molecules, production of cytokines and chemokines, and a change in the cell surface chemokine receptor expression pattern. Moreover, maturation renders DCs capable for efficient antigen presentation to T cells.[14]

In the present study, we investigated the response of total lung and LALN enriched DC subsets to IAV infection. Our findings demonstrated that a significant increase of DC numbers in IAV infected monkeys compared to the control groups during the acute phase. Furthermore, our preliminary experiments suggested that mDCs were extremely sensitive to the IAV infection, expressing co-stimulatory molecules such as HLA-DR, CD80, CD83 and CD86 in lungs. So in IAV infection, numbers of pulmonary DCs were elevated during the acute phase. Furthermore, mature mDCs were still sustained to day 30 post infection. Two days after infection with IAV, levels of HLA-DR, CD80, CD83 and CD86 were further increased. Strikingly, CD83, a marker of mature DC, appeared on the cell surface after IAV infection.[19] The expression of all these surface markers was then declined at day 4, but is was still higher than those in the control group at day 30 post-infection.

In contrast, RSV infection caused only sustained increases in numbers of mature dendritic cells in the lung.[16] The importance of DC in the initiation and control of innate and adaptive immune responses against influenza infection is well documented.[20–22] So they can effectively take up particulates in the inflamed lung and migrate to the draining lymph nodes.

Meanwhile, following infection with IAV, both DC types produced proinflammatory cytokines and chemokines[23] that can include IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, G-CSF, IFN-γ, TNF-α and so on. The mechanisms by which pDC surface receptors recognize viral products have been widely reported in some previous studies.[24–26] Both retinoic acid inducible gene I (RIG-I) and toll-like receptor 7 (TLR-7) pathways are able to induce production of proinflammatory cytokines and type I interferons (IFNs). RIG-I plays an important role in detecting virus and producing IFNs in infected convention DCs, while pDCs use the TLR-7 pathway for innate immune response against IAV. Interestingly, in this present study, increased numbers of pDCs have also been detected in LALNs after IAV infection, suggesting that pDCs and mDCs can also transport antigens from the infected lung to the draining lymph nodes via the recognition of IFNs.

A hallmark for virus-infected pDCs is the production of large amounts of IFN-α accompanied with the production of other proinflammatory cytokines. Most importantly, type I IFNs possess potent antiviral properties and are critical to the control of IAV infection. IFN stimulation induces transcription of several IFN-responsive genes, many of which can protect and interfere with the establishment of IAV infection in uninfected cells.[27–29] Further, IFN stimulation can regulate innate and adaptive immune responses through its ability to enhance DCs maturation as well as promote the survival and development of effector functions by activated CD8 T cells.[30–33]

Actually, the activation state of DCs plays an important role in the interaction between DC and T cell because non-activated DCs tolerate or delete T cells, whereas activation converts DCs to a stimulatory state that elicits T-cell activation and memory. Besides the role of APCs, the cytokines and chemokines produced by pDCs and mDCs exposed to viruses would exert significant autocrine and paracrine effects in their microenvironments. However, infection with IAV may also cause significant pulmonary immune pathology, because a cytokine storm, with unusually high levels of cytokines in serum and lungs, is possibly correlated with influenza-induced immune pathology and mortality.[34] In this further study, we will concentrate on the study of cytokines and chemokines produced by DCs post IAV infection and analyze their role in regulating the T-cell responses in lungs.

Altogether, we demonstrated that depending on the infecting microbe, the functions of mDCs and pDCs may differ, and may be partially overlapping, which suggests a considerable flexibility of the human DC system. A better understanding of the interplay of IAV with DCs may facilitate the development of an effective vaccine. Our findings may also provide important insights to understanding the immune responses of dendritic cells in response to IAV infection in a non-human primate model.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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