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Flt3 Ligand Generates Morphologically Distinct Semimature Dendritic Cells in Ovalbumin-sensitized Mice

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Abstract

Dendritic cells (DCs) are unique antigen presenting cells that are immature prior to their encounter with an antigen. Exposure to allergens induces the maturation of DCs with changes in morphology and presence of dendrites. Here, we demonstrate that the DCs in the lungs of ovalbumin (OVA)-sensitized and challenged mice are more mature owing to their pronounced dendrites than the DCs in the lungs and spleen of PBS-treated mice, which are immature and possess cytoplasmic veils. Intermediate to these two groups are the DCs in the Flt3 ligand-treated group that exhibit comparatively fewer dendrites and cytoplasmic veils and hence are classified as semimature. Presence of large numbers of well developed mitochondria and rough endoplasmic reticulum in myeloid DCs from both lungs and spleen of OVA-sensitized and challenged mice indicate greater functional activity. Additionally, DCs from the OVA-sensitized and challenged mice also exhibit fat and glycogen stores, which are indicative of a mature population. In addition, treatment of the animals with Flt3 ligand attenuated airway hyperresponsiveness to methacholine in OVA-sensitized and challenged mice. These data suggest that morphological features could be indicative of the maturation and distinct functional state of DCs, and this could be associated with underlying mechanisms of Flt3 ligand-induced immunomodulation in allergic asthma.

Keywords

Airway hyperresponsiveness; Allergy; Asthma; Cytoplasmic veils; Dendritic cells; Flt3 ligand; Immunomodulation; Morphology of dendritic cells

Introduction

Dendritic cells (DCs) are antigen-presenting cells with the unique ability to initiate primary immune responses. DCs are also important for the induction of immunological tolerance as well as the regulation of T cell mediated immune response (Bharadwaj and Agrawal, 2006). DC maturation, which plays a pivotal role in the control of immunity, is a continuous process

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initiated in the periphery upon antigen encounter and/or in the presence of inflammatory cytokines. Factors that induce maturation are presence of lipopolysaccharides (De Smedt et al., 1996), bacterial DNA (Sparwasser et al., 1998), T cell derived signals such as CD40L (Cella et al., 1996) and balance between pro- and anti-inflammatory signals (Reis e Sousa, 2004). In most tissues DCs are present in the immature state. DCs undergo transformation from antigen capturing cells to antigen-presenting cells (APCs) by downregulation of molecules involved in antigen capture (Palucka and Banchereau, 1999).

Maturation of human DCs is accompanied by loss of endocytic/phagocytic receptors, up-regulation of co-stimulatory molecule, CD80, and CD86, change in morphology and increased expression of CD83 (Banchereau et al., 2000). Maturation also results in high levels of CCR7 and CXCR4 expression (Sallusto et al., 1998). Maturation is associated with the activation of NF- κ B pathway (Thurnher et al., 1997). Mature DCs have not only the capacity to prime naïve T cells, but also to induce different types of T cell immune response depending on the type of original maturation signal. Flt3 ligand (FL) is a growth factor that stimulates the development of monocytes, T and B cell lineages (Koski et al., 2001).

FL has the distinct ability to generate functionally mature dendritic cells in the lung and other peripheral sites (Shurin et al., 1998). We have previously reported that FL protects against (Agrawal et al., 2001) and reverses (Edwan et al., 2004, 2005, 2006) airway hyperresponsiveness (AHR) and allergic airway inflammation in murine model of asthma. FL treatment increased the total number of DCs in the lungs together with a reduction in AHR to methacholine. In this study we demonstrate that FL administration generates a population of partially mature DCs, which are morphologically different from DCs found in the lungs and spleen of PBS-treated and OVA sensitized and challenged mice.

One of the important external features of DCs is the presence of numerous membrane processes that extend out from the main cell body analogous to dendrites on a neuron. Important internal features of DC as visualized by transmission electron microscopy (TEM) include the presence of various lengths of cellular projections or surface lacunae and a large cytoplasm containing spherical mitochondria in close association with rough endoplasmic reticulum. We believe that the morphological state of the cell may be directly related to the maturational status.

Materials and methods

Animals

Four to 5 week old female Balb/c mice were purchased from Harlan Laboratories (Indianapolis, IN) and maintained in a pathogen free environment at Creighton University according to IACUC guidelines.

Sensitization and Treatment

The sensitization and treatment protocol followed is shown in Figure 1. Briefly, mice were divided into sensitized and non-sensitized groups. Sensitized groups received 20 μ g of grade V chicken egg ovalbumin (OVA; Sigma-Aldrich, St, Louis, MO) emulsified in 2.25 mg of inject alum (Pierce Biotechnology, Rockford, IL) in a total volume of 100 μ l. Non-sensitized mice received PBS. OVA-sensitized mice were challenged with 1% OVA aerosol for three consecutive days starting with day 28. Twenty four hours after the administration of 5% OVA aerosol on day 32, pulmonary functions were measured to establish AHR. These mice were administered with either PBS or FL (3 μ g per animal per day) for 10 days, followed by OVA challenge and measurement of AHR to methacholine. Lungs and spleen were collected to isolate DCs.

Noninvasive method for measuring pulmonary function

A single-chamber whole-body plethysmograph (Buxco Electronics, Troy, NY) without the use of anesthesia or restraint was used to measure pulmonary functions (Agrawal et al., 2001; Edwan et al., 2004). Mice were challenged with increasing doses of methacholine (Sigma-Aldrich) to measure AHR.

Isolation of dendritic cells from lungs and spleen

The mice were euthanized with 100 μ l of pentobarbital (5mg/20g). The lungs and the spleen were collected and finely chopped and digested with 5ml collagenase D (1mg/ml) in RPMI-1640 containing 1mg/ml DNase (Roche Diagnostics IN) at 37°C for 1 hour. Pellet was resuspended in 1ml of MACS (Magnetic assisted cell sorter) buffer (PBS supplemented with 0.5% BSA) and the cells counted with the help of a coulter counter. For isolation of DCs, cells were first incubated for 5 mins with an Fc blocker (BD Pharmingen, CA) followed by incubation for 30 mins on ice with 100 μ l of CD11c microbeads (Miltenyi Biotech, Auburn, CA) for every 100 million cells. This was followed by a double-positive selection using the POSSELDs program in the AutoMACS (Miltenyi Biotech, CA).

Phenotyping of spleen and lung DCs

Positive fraction of CD11c+ cells were collected and counted. The cells were resuspended in PBS supplemented with 4% FBS (PBS₄) and incubated with the following antibodies CD11cPE (1:100), CD11bFITC (1:200), B220PE-Cy7 (1:200), MHCIIAPC (1:100) for 30 mins on ice (BD Pharmingen, CA). All tubes that received biotin-conjugated primary antibody, received streptavidin conjugated secondary antibody. Samples were washed by addition of ice cold PBS₄ and stored in 1% formalin prior to analysis. However for sorting, the cells were maintained in PBS₄. Sorting was carried out with the use a FACSDiva sorter. The two major populations of DCs that were sorted had the phenotype of CD11c+/CD11b+ myeloid DCs and CD11c+/B220+ plasmacytoid DCs.

Scanning electron microscopy

Cells were fixed and incubated in 3% buffered glutaraldehyde for a minimum of 4 hours. The samples were washed in Sorenson's phosphate buffer. The cell suspension was post-fixed in 1% buffered osmium tetroxide for 30 mins. The samples were washed in Sorenson's buffer several times. This was followed by dehydration in graded concentrations of alcohol. The cell suspension was layered onto a filter paper. The filter paper was carefully folded and placed in a small metal container. The specimen was then dried using the Critical Point Dryer. The critical point for drying is a pressure of 1300 psi and a temperature of 42°C. This is when the liquid CO₂ vaporizes and the specimen is dried. The gaseous CO₂ was carefully bled at approximately 100 psi per minute. The samples were removed from the metal containers. The filter papers were carefully opened and cut to size and placed onto metal stubs that had been coated with carbon paint. The stub together with the filter paper containing cells is coated with gold-palladium particles by using a sputter coater. The samples were viewed under JEOL 240A scanning electron microscope (SEM).

Transmission Electron Microscopy (TEM)

After dehydration in absolute alcohol, the specimen was treated with the transitional fluid propylene oxide. The specimen was centrifuged and then infiltrated with a mixture of half propylene oxide and half embedding resin for 1 hour. The sample was embedded in fresh embedding resin and polymerized in a 60°C oven overnight. The specimen blocks were sectioned to a thickness of 0.5 to 1 μ m on an ultramicrotome using a glass knife. The sections were mounted on a glass microscope slide and allowed to dry on a hot plate. The sections were stained with 0.5% toluidine blue stain in 0.5% borax, cleared with 70 % ethyl alcohol and a

coverslip applied. Sections were viewed on a light microscope for selection of a particular area for further sections. Chosen areas were ultrathin sectioned to be 60–90 nanometers using a diamond knife. The sections were picked up onto 200mesh/inch copper grids and saturated with uranyl acetate in 50% ethyl alcohol for 10 minutes, washed in distilled water and then stained with lead citrate for another 3 minutes and then washed with distilled water again. The sections were viewed under a TEM (JEOL JEM-1011, operated at 60 KV) and representative areas photographed.

Results and Discussion

Administration of FL in OVA-sensitized and challenged animals significantly decreased AHR to methacholine (Figure 2). This data support our previously published results that FL treatment reverses airway hyperresponsiveness to methacholine in OVA-sensitized and challenged mice. Response to methacholine in non-sensitized (PBS-treated) or OVA-sensitized and challenged groups did not change between day 33 and day 44 (Figure 2). Earlier reports demonstrated the expansion of myeloid ($CD11c^+CD11b^+CD8\alpha^-$) and lymphoid ($CD11c^+CD11b^-CD8\alpha^+$) DC subsets following FL administration (Karsunky et al., 2003). However, a recent report showed the preferential increase by FL in the number of functionally active myeloid DCs in the lungs of mice (Masten et al., 2004). We observed a significant increase in myeloid and plasmacytoid DCs in the lungs and spleen of FL treated mice as compared to both OVA-sensitized and challenged mice and PBS treated mice (Figures 3, 4). However, we observed a higher percentage of myeloid and plasmacytoid DCs in the lungs (Figure 4) than in the spleen (Figure 3). These cells also showed an increase in granularity as indicated by the side-scatter plot (Figure 4).

To document whether the differences in size and granularity after treatment induced morphological changes, we sorted DCs and processed them for scanning and transmission electron microscopy. Murine DCs have been classified into three main lineages – myeloid, lymphoid and plasmacytoid DCs with the phenotype $CD11c^+CD11b^+CD8\alpha^{low}$, $CD11c^+CD8\alpha^{high}$ and $CD11c^+B220^+$, respectively. In this study, myeloid and plasmacytoid DCs were differentiated and sorted on the basis of their phenotype. Scanning electron micrographs revealed that myeloid DCs (Figure 4 Panel A) exhibited a more ruffled surface than plasmacytoid DCs (Figure 4 Panel B). DCs possessed either short veils, rounded or smooth veils and long thin veils depending on the stage in the developmental or differentiation pathway (Patterson et al., 1991). This concept is reiterated by studies that have shown that the phenotypic and functional differences among the populations simply represent different maturational stages of the same DC lineage (Traver et al., 2000). Also, there appears to be a difference in surface structures among the DCs isolated from PBS-treated, OVA-sensitized and challenged and FL-treated mice (Figures 3,4). The morphology of DCs is characterized by the possession of numerous long branching processes known as dendrites. Dendrites are elongated spiky arms present on DCs, the main purpose of which is to enable a single cell to contact large number of other cells at a given time. Dendrites confer high surface-to-volume ratio. Dendrites are formed by initial attachment of a cell protrusion to the extracellular matrix substrate followed by retraction of the cell body away from the adhesion site (Swetman et al., 2002). Cells that are either immature or in the process of maturation do not possess dendrites but rather large cytoplasmic veils.

As shown in Figure 4, all groups of cells exhibited the morphology of cytoplasmic veils except for lung myeloid DCs from OVA-sensitized and challenged mice, which appeared to have dendrites. This suggests that lung myeloid DCs from OVA sensitized and challenged mice are the most mature population. We observed comparatively more dendrites in myeloid DCs and OVA-sensitized and challenged DCs than in plasmacytoid DCs or PBS- or FL-treated groups. Although a direct correlation has not been shown in the current study, many previous studies

by others have shown that mature DCs, as defined by their pronounced display of motile dendrites, have a high capacity to induce proliferative responses in resting T cells (Maraskovsky et al., 1996; Gieseler et al., 1997; Thurnher et al., 1997).

Studies suggest that there may be a direct correlation between morphology and maturation. Treatment of monocyte-derived DCs with 1, 25-dihydroxy vitamin D₃ hampered maturation and also led to a reduction in the percentage of cells with cellular protrusions (Canning et al., 2001). On the contrary DCs generated from GM-CSF/IL4 and matured with TNF- α , LPS or CpG have a lot more dendrites (Weigel et al., 2002). These DCs also produced a greater allogenic response.

The dendritic processes play an important role not only in antigen uptake but also in processing and presentation. The signaling pathways that lead to the formation of these processes may also be involved in maturation. Dendrite formation has been shown to be dependent on Rho GTPases (Binks et al., 1998). Expression of Cdc (cell division cycle) 42, a member of the Rho GTPases is involved in the reduction of endocytic activity (Garrett et al., 2000). Maturation is known to induce a dramatic inhibition of endocytosis. Therefore, maturation facilitates reorganization of actin cytoskeleton to form dendrites (Granucci et al., 2003). We observed that myeloid DCs generated after OVA sensitization and challenge exhibited long dendrites (Figure 4 Panel A) and hence it is possible that highly mature population of cells are capable of interacting with T cells. This data agrees with the notion that myeloid DCs are considered to be inherently immunogenic (Anjuere et al., 1999). It would, however, be interesting to examine the effect of FL on the expression of Cdc42.

Matrix metalloproteinase (MMP)-9 plays a major role in the pathogenesis of allergic airway inflammation. MMP-9 is involved in langerhans cell migration and maturation (Kobayashi et al., 1999). Inhibition of MMP-9 prevents dendrite development. Increased AHR in OVA-sensitized and challenged mice may be due to the production of MMP-9 that promotes dendrite development in mature myeloid DCs. It would be important to investigate if FL is involved in the inhibition of MMP-9.

Cytoplasmic veils are sheet-like processes that are present on dendritic cells. They were first observed in large mononuclear cells found in lymph. Upon encounter with allergens, veiled cells in the lymph are involved in contacting and aggregating other cells like neutrophils and mononuclear phagocytes (Drexhage et al., 1979). Langerhans cells display pronounced cytoplasmic veils and are considered to be partially mature (Stoitzner et al., 2002). We observed that the cytoplasmic veils on FL-treated DCs were neither as few as the PBS group nor as dense as the OVA group (Figure 4 Panel A). Therefore, we believe it is a population of cells that is intermediate in maturity, generally referred to as semimature DCs, and maybe responsible for the attenuation of AHR via the reduction of T cell stimulatory activity. However, further studies are warranted to establish the cause or consequence of increase in semimature DCs on the reversal of AHR. If these cells are responsible for attenuating the AHR, it would be important to examine the expression of costimulatory molecules, Rho proteins, matrix metalloproteinases and production of chemokines in these cells. An interesting venture would be to prevent cell shape changes *in vivo* and observe the effect on AHR.

Transmission electron micrographs revealed important differences in the DC subtypes as well as the different treatment groups. Myeloid DCs obtained from the both the spleen (Figure 5) and lungs (Figure 6) of PBS-treated animals appeared to have fewer cellular projections or surface lacunae. The nucleus is made up of thin heterochromatin and a prominent nucleolus. The cytoplasm contains well developed mitochondria in close association with rough endoplasmic reticulum. The cytoplasm of spleen DCs also contains lysosomes and numerous small vesicles which are smooth and coated.

Myeloid DCs obtained from both the spleen and lungs of OVA-sensitized and challenged animals have prominent cellular projections or surface lacunae (Figure 5, 6 Panel A). The nucleus is made up of thin heterochromatin and a prominent nucleolus. The cytoplasm contains several well developed mitochondria in close association with numerous rough endoplasmic reticulum. Lung DCs (Figure 6) show the presence of golgi apparatus and many small vesicles that appear smooth and coated whereas spleen DCs (Figure 5) only show the presence of many small vesicles and some lysosomes.

Myeloid DCs obtained from both the lungs and spleen of OVA-sensitized and challenged and FL treated animals appear to have ultrastructural features similar to those exhibited by PBS treated animals (Figure 5, 6 Panel A).

In comparison to the myeloid DCs, plasmacytoid DCs obtained from both the spleen and lungs of PBS treated animals and OVA-sensitized and challenged animals were devoid of cellular projections or surface lacunae (Figures 5, 6 Panel B). The nucleus is made up of thin heterochromatin. The cytoplasm contains very few mitochondria and rough endoplasmic reticulum. In addition, spleen DCs contained several small coated and smooth vesicles and lysosomes (Figure 5 Panel B).

Unlike plasmacytoid DCs obtained from the non-sensitized and sensitized controls, the lungs of OVA sensitized and challenged and FL treated animals contains some well developed mitochondria and rough endoplasmic reticulum (Figure 6 Panel B). However the spleen plasmacytoid DCs contain very few well developed mitochondria and almost no rough endoplasmic reticulum (Figure 5 Panel B). In addition myeloid DCs from both lungs and spleen of OVA sensitized and challenged mice showed fat and glycogen stores which are typical characteristic of mature DCs (Maroof et al., 2005). Increased presence of mitochondria and rough endoplasmic reticulum indicates heightened cellular activity. This was observed in the case of both DC subtypes from OVA-sensitized and challenged animals (Figure 5, 6 Panel A, B).

In summary, we demonstrate that myeloid DCs generated in the lungs from OVA- sensitized and challenged mice exhibit pronounced dendrites indicating a mature population when compared to DCs obtained from non-sensitized and Flt3 ligand- treated mice. Large numbers of well developed mitochondria and rough endoplasmic reticulum in myeloid DCs from both lungs and spleen of OVA-sensitized and challenged also indicated greater functional activity in these cells.. In contrast, DCs obtained from FL-treated animals exhibited fewer dendrites, mitochondria and rough endoplasmic reticulum, suggesting lower functional activity of these DCs and hence decreased ability to stimulate T cell proliferation. Similar to previously reported data, FL treatment reversed AHR in antigen-sensitized and challenged animals. Though a direct link has not been shown here, decreased T cell proliferation may play a major role in abrogating AHR. These data suggest that generation of a semimature and morphologically different population of DCs in response to FL treatment may play a role in the attenuation of allergic inflammation and AHR in asthma. Obviously, additional studies are warranted which would prove beyond doubt that the two events are not mutually exclusive.

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Figure 1.
Experimental protocol to sensitize and challenge mice with ovalbumin and the treat with Flt3 ligand (FL).

Figure 2.

Effect of FL treatment on AHR to methacholine. Non-sensitized mice received PBS (PBS group). After OVA sensitization and challenge, AHR to methacholine was established on day 33 followed by treatment with PBS (OVA group) or FL (OVA-FL group) (3 μ g per animal per day) for 10 days. On day 44 AHR was measured again and Penh values were recorded. Data are shown as the mean $\pm$ SEM (n= 6 in each experimental group).

Figure 3.

Flow profile and representative scanning electron micrographs (SEM) of spleen DCs treated with PBS, OVA and OVA-FL: After measurement of AHR, dendritic cells were isolated from the lungs and spleen using CD11c microbeads. Briefly, spleen were excised, chopped and digested in 1mg/ml collagenase D for 1 hour at 37°C. CD11c+ cells were isolated by positive selection using AutoMACS column. The CD11c+ cells from the animals of an individual group were pooled and stained with anti-CD11cPE (1:100), anti-CD11bFITC (1:200), anti-MHCApC (1:100) and anti-B220PE-Cy7 (1:200). A fraction was left unstained to be used as a negative control. The proper gates were set and CD11c+/CD11b+ (**Panel A**) and CD11c+/B220+ (**Panel B**) DCs were sorted and collected in eppendorf tubes. The cells were fixed in 3% glutaraldehyde and processed for scanning electron microscopy.

Figure 4.

Flow profile and representative scanning electron micrographs (SEM) of lung DCs treated with PBS, OVA or OVA-FL. After measurement of AHR, dendritic cells were isolated from the lungs and spleen using CD11c microbeads. Briefly, lungs were excised, chopped and digested in 1mg/ml collagenase D for 1 hour at 37°C. CD11c+ cells were isolated on an AutoMACS column by positive selection. The CD11c+ cells from all animals of the individual experimental group were pooled and stained with anti-CD11cPE (1:100), anti-CD11bFITC (1:200), anti-MHCAPC (1:100) and anti-B220PE-Cy7 (1:200). A fraction was left unstained to be used as a negative control. The proper gates were set and CD11c+/CD11b+ (**Panel A**) and CD11c+/B220+ (**Panel B**). DCs were sorted and collected in eppendorf tubes. The cells were fixed in 3% glutaraldehyde and processed for scanning electron micrographs.

Figure 5. Representative transmission electron micrographs (TEM) of spleen DCs treated with PBS, OVA or OVA-FL. Dendritic cells were isolated from the spleen and further sorted using FACS Aria. The sorted spleen cells were processed for transmission electron microscopy. **Panel A** – myeloid DCs, and **Panel B**- plasmacytoid DCs. V- Vesicle (coated and smooth), RER- rough endoplasmic reticulum, Lv- Lysosomes, M- mitochondria, No- nucleolus, Cn- centriole.

Figure 6.

Representative transmission electron micrographs (TEM) of lung DCs treated with PBS, OVA or OVA-FL: Dendritic cells were isolated from the lungs and further sorted using FACSARIA. The sorted lung cells were processed for transmission electron microscopy. **Panel A** – myeloid DCs, and **Panel B**– plasmacytoid DCs. V- Vesicle (coated and smooth), RER- rough endoplasmic reticulum, Lv- Lysosomes, M- mitochondria, No- nucleolus, GA- Golgi apparatus.