A Single Intratracheal Dose of the Growth Factor Fms-Like Tyrosine Kinase Receptor-3 Ligand Induces a Rapid Differential Increase of Dendritic Cells and Lymphocyte Subsets in Lung Tissue and Bronchoalveolar Lavage, Resulting in an Increased Local Antibody Production

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Repetitive doses of the growth factor Fms-like tyrosine kinase receptor-3 ligand (Flt3L) have resulted in increased numbers of dendritic cells (DC), and there is growing evidence for a broad heterogeneity. DC are extremely powerful APCs that are not only found in lymphoid organs, e.g., lymph nodes (LN) and spleen, but also in many nonlymphoid organs such as the skin, gut wall, and respiratory tract (for review, see Ref. 1). DC have been classified based on their stage of maturation and anatomical compartments (2): 1) DC precursors in the bone marrow and blood; 2) recently immigrated, immature DC in nonlymphoid organs such as the tracheal epithelium; 3) maturing DC after Ag uptake and on their way, for example, in afferent lymphatics; and 4) mature, Ag-presenting DC in the draining LN. In respect to their effect on Th1 and Th2 lymphocytes, DC have also been divided into DC1 and DC2 or monocyte-derived and plasmacytoid DC, each characterized by a different set of surface markers (3).

The lung not only contains large numbers of DC, but many basic aspects of DC functions have also been studied in detail in the lung, for example, the short life span, the rapid increase in numbers after viral or bacterial infections, and the suppressive role of corticosteroids (see Ref. 4). Using fluorescent labeling, the rapid Ag uptake in the epithelium and the migratory route to the draining LN have been documented (5). In the LN, the DC can retain MHC class II/Ag peptide complexes for a prolonged period (6, 7). Also, large Ags, such as conidiae and hyphae of fungi, e.g., Aspergillus fumigatus, have been shown to be phagocytosed, cytokine production induced, and DC further matured, while they migrated to bronchial LN to induce specific Th cell immune reactions within the draining LN (8). Most in vivo experiments have been performed in rodents and data on human DC produced in vitro (9, 10) or the SCID mouse model (11). DC in the lung might be of relevance to induce protective immunity, but also tolerance to inhaled Ag by the IL-10 production of DC (12), depending on the stage of maturation of the DC, as recently proposed (13).

Thus, DC play a role in the modulation of immune responses, for example, in tumor immunology (14, 15), as well as in inducing adaptive immune reactions (16) and tolerance (17). It has been stressed that there is an urgent need to study DC functions in vivo, which is much easier now that growth factors such as GM-CSF, G-CSF, and in particular the Fms-like tyrosine kinase receptor-3 ligand (Flt3L) have been described to stimulate the production and mobilization of DC precursors in the bone marrow and blood (18, 19). Obviously, different growth factors stimulate precursors of different DC in vitro (20) or in vivo (21). Some experiments with Flt3L are of particular interest, as repetitive i.p. doses of this growth factor resulted in an expansion of DC in the gut wall. Thus, tolerance was achieved using very low doses of Ag (17). In another mouse model, the expanded numbers of DC in the gut wall after repeated doses of Flt3L resulted in an up-regulated active response to fed soluble Ag (16), indicating a potential application of Flt3L in designing an optimal mucosal immunization strategy.

Having recently described an expansion of DC, macrophages, and lymphocytes after a single intratracheal dose of a synthetic lipoprotein (macrophage-activating lipopeptide-2), which is physiologically produced by mycoplasms in the rat lung (22), and this topically applied compound also resulting in less lung metastasis in a tumor model (23), we wanted to test whether intratracheally applied Flt3L would have an effect on the number of DC, macrophages, and lymphocyte subsets not only in the lung interstitium,
but also in the bronchial space, which is a clinically easily accessible compartment via the bronchoalveolar lavage (BAL). The functional relevance of the local immunostimulatory effect of Flt3L was tested by applying tetanus toxoid as a model Ag, and the IgA and IgG Ab titers were measured in the BAL and serum.

Not only a surprisingly rapid expansion of DC was found with different responses in the lung interstitium and BAL, but also an increase of lymphocyte subsets after this single local injection. Thus, Flt3L seems to affect DC precursor cells in the bone marrow and to induce the expansion and probably also the maturation locally in the lung with an adjuvant effect on local Ab production.

Materials and Methods

Animals

Adult (6–8 wk of age) Lewis rats were obtained from the central animal laboratory of the Medical School of Hannover and were kept under specific pathogen-free conditions. The animals had free access to pelleted food and water and were housed under standardized light/dark cycle. The number of animals per group can be seen in the figures (mean weight dose response: males 217 ± 5.2 g (SEM), time sequence 284 ± 13 g, i.p. dose 228 ± 6.5 g, tetanus toxoid immunization 182 ± 2 g, female animals).

Intratracheal instillation

Under short ether anesthesia, rats were suspended in a hanging position by a rubber band fixed to the incisor teeth of the upper jaw. The trachea was intubated via the oral cavity, a tube (17G; Braun, Melsungen, Germany) was placed in the trachea, the drug or saline was instilled, and 500 μl air was blown into the lung.

Stimulation by Flt3L

Human rFlt3L provided by Immunex (Seattle, WA) was instilled intratracheally at a dose of 1, 10, or 100 μg per animal or i.p. (100 μg).

Immune response to tetanus toxoid

Tetanus toxoid (Tetanol; Chiron Behring, Marburg, Germany) was instilled intratracheally (20 intratracheal-trans, corresponding to 250 μl). The animals were instilled with saline (as a control) or 100 μg Flt3L in a total volume of 250 μl of saline on day 0, received tetanus toxoid on days 3 and 7, and were sacrificed on day 14. The content of IgA and IgG Abs was determined in BAL and serum.

ELISA methods

Single U-shaped wells of nonflexible polystyrol microtiter plates were coated for 2 h with 10 μl tetanus toxoid (3 μg protein/ml; Calbiochem-Novabiochem, Bad Soden, Germany) diluted in 10 mM PBS, pH 7.4. After a washing step with PBS and a blocking step with PBS-BSA (PBS containing 0.1% Tween 20). biotin-labeled goat anti-rat IgG (1/1000; Zymed Laboratories, South San Francisco, CA) was added to the slides for 25 min. Slides were washed and counterstained with hematoxylin (Fluka, Buchs, Switzerland) and mounted in glycergel.

Histology

Samples of the right lung were fixed in 4% buffered Formalin and embedded in paraffin, and the sections (5 μm) were stained with H&E.

Immunohistology

Cryosections of lung tissue (6 μm thick) were made. After air drying, slides were fixed in acetone for 10 min, washed in TBS containing 0.05% Tween 20 (Serva, Heidelberg, Germany), and incubated for 30 min in a moist chamber with the primary Abs (Otx8 and Otx62). They were then incubated with a secondary Ab (rabbit anti-mouse Ig, Z259; DAKO, Hamburg, Germany) and an Ab complex of alkaline phosphatase-antialkaline phosphatase complex, D651; DAKO) for 30 min. After repeating the last two steps for 15 min, 2 mg fast blue Sigma-Aldrich, Munich, Germany), mixed with 4 ml alkaline phosphatase-antialkaline phosphatase substrate (9.8 ml 0.1 Tris buffer (pH 8.2) containing 2 mg naphthol AS-MX phosphate, 200 μl N,N-dimethylformamide, and 1 μl 1 M levamisole), was added to the slides for 25 min. Slides were washed and counterstained with hematoxylin (Fluka, Buchs, Switzerland) and mounted in glycergel (DAKO).

Data analysis

Means, SEs, and the level of significance were determined taking ≤0.05 as statistically significant using SPSS- Windows (SPSS, Chicago, IL) and applying the Mann-Whitney U test.

Results

A single dose of 100 μg Flt3L resulted in an enlargement of the peribronchovascular space and a moderate leukocyte infiltration around small bronchi and branches of the pulmonary arteries, with a maximum on day 3 (Fig. 1). Using immunohistology, the increase of DC in the wall of bronchi could be seen after Flt3L (Fig. 1D). With Abs against MHC class II and Otx62, a double staining was documented for these cells (Fig. 1, G and H).

Comparison of different doses of Flt3L

There were no significant differences between animals injected with 1 and 10 μg Flt3L when the total number of leukocytes, lymphocytes, or DC was compared. However, the dose of 100 μg intratracheally resulted in significantly more leukocytes, lymphocytes, and DC in the lung interstitium, but in the BAL only DC were significantly increased after a dose of 10 μg (Fig. 2). When the DC from the lung interstitium were separated and the MHC class II/Otx62- cells further characterized, other DC markers such as CD80, CD86, CD11c, and CD54 could be documented on different subsets (Fig. 3). The number of neutrophils did not show an
increase at any time after local Flt3L instillation. Rats receiving the same single dose of 100 μg/kg systemically by i.p. injection showed no effect on the total numbers. The relative number of leukocyte subsets did not differ (data not shown).

Time course of Flt3L effects on leukocyte subsets in the BAL and lung interstitium

In the interstitial space of the lung in contrast to the BAL, a significant increase of the total leukocyte number was seen on day 3 after Flt3L instillation (Fig. 4): this increase was not only caused by more DC, but also by lymphocytes and the CD4 and CD8 subsets (Fig. 5). The kinetics of the increase of the different leukocyte subsets differed between the interstitial compartment and the BAL, in which the maximum was seen on day 3, followed by a drop to day 10.

Influence of local Flt3L application on subsequent immunization with tetanus toxoid

Previous Flt3L stimulation resulted in significantly higher IgA antitoxoid concentrations in the BAL compared with controls (Fig. 6). No differences were seen in specific serum IgA. The IgG anti-tetanus toxoid concentrations were also significantly increased after Flt3L stimulation in the BAL fluid. However, in contrast to IgA, there was also a significant increase of specific IgG in the serum, although less pronounced compared with the BAL (Fig. 6).

Discussion

The stimulatory effect of Flt3L on DC maturation is well documented (14, 16, 17), and recently the preferential expansion of IL-12-producing DC type I and memory T cells has been reported in contrast to the GM-CSF-induced expansion of other DC subtypes (25).

To date, Flt3L has only been tested in vitro or after repetitive doses were given systemically (e.g., Refs. 16 and 17). In this study, we show for the first time that a local application resulted in a significant increase of DC, which had a stimulatory effect on local Ab production. Most experimental studies on the effects of Flt3L have been performed in the mouse, and thus the dose applied in rats was extrapolated on a weight basis. It is not known where in the lung the Flt3L is absorbed and how it reaches the interstitial space to influence the DC in this compartment. In future studies, whole mount preparations will be used, as described for the mouse lung, to study the DC in the tracheal and bronchial epithelial layer. The MHC class II/Ox62+ DC expressed other markers for DC of differential maturation stages to a varying extent: few cells were also positive for CD80 and CD86, while others expressed a high density of CD11c and CD54 (Fig. 3) with a pattern comparable to that shown for rat DC previously (26, 27).

The increase in the number of DC after the local Flt3L instillation into the trachea may be caused by local proliferation, maturation, or reduced emigration into the draining LN. The bone marrow as a source of these DC, as is likely after repetitive doses systematically applied (17), can be excluded because the same single dose applied i.p. did not result in any effects in the lung. The differential results regarding the number of leukocytes and their subsets in the lung interstitium and the bronchoalveolar space support our concept that the BAL should not be automatically considered as representing the situation in the lung as a whole (28). After Flt3L treatment, the DC have to be characterized further by using other markers, because the evidence is continuously growing.
that DC are extremely heterogeneous in surface Ag expression and functional aspects.

The increase of lymphocytes after a local Flt3L instillation was surprising, as to date reports of the effects have focused on DC (18, 19, 21). The lymphocyte subset composition differs between the various compartments of the lung, and little is known about regulatory factors, e.g., adhesion molecules, chemokines, or their receptors, to direct the lymphocytes from one compartment of the lung to the other. However, cytokine production by lymphocytes, e.g., IFN-γ (for review, see Ref. 29), differs between lung compartments in immune reactions (30). It has to be stressed that only in the lung do lymphocytes traverse a body surface, return later to

**FIGURE 2.** Total numbers of leukocytes, lymphocytes, and DC in the lung interstitial tissue and the BAL on day 3 after different doses of Flt3L. The asterisk indicates significant differences.

**FIGURE 3.** Cells of the lung tissue were separated and investigated by flow cytometry ($n = 5$). The MFI of CD80, CD86, CD11c, and CD54 expression of double-positive cells for MHC class II and Ox62 DC markers was determined after correction of the isotype control.

**FIGURE 4.** Comparison of the total number of leukocytes in the lung tissue and BAL at different times after 100 µg Flt3L intratracheal (i.t.) instillation, saline instillation, or application of the same dose of Flt3L i.p. The asterisk indicates significant differences ($\pm$ SEM).
the organ, and migrate finally to the draining, i.e., the bronchial LN (31). Recently, we proposed a concept of a unique compartment in the lung, the perivascular space (32). It was surprising to find an enlargement of this space after the intratracheal Flt3L application, with a maximum on day 3 (Fig. 1). The functional consequences of this phenomenon have to be studied in future. Flt3L might stimulate certain cells in the lung, which then secrete cytokines to modify the local microenvironment to attract other leukocyte sub-sets. If the increase of lymphocytes were caused by factors produced by DC, one would expect different time kinetics with an initial increase of DC, followed by the increase of lymphocytes. That was not the case (Fig. 4). Obviously, the local regulation is more complex. By serial analysis of gene expression of gut intraepithelial TCRγδ+, and TCRαβ+ lymphocytes, a moderately increased expression of Flt3L was documented (33). Similar mechanisms might also play a role in the lung.

The increased local Ab production in the lung after local Flt3L stimulation may be of great clinical relevance. IgA as the typical Ab of mucosal organs (34) will prevent the adhesion of bacteria to the epithelium of the trachea and bronchi. IgA is produced by plasma cells in the lamina propria and transported to the bronchial lumen by the poly Ig receptor. In the peripheral parts of the lung, IgG is of great relevance. IgG produced by local plasma cells in the lamina propria and probably also in the draining bronchial LN reaches the serum via the lymphatics and can also diffuse into the air space, from where it was recovered by the BAL.

The previous experiments with repetitive systemic doses of Flt3L resulted in an increased production and emigration of DC to all lymphoid and nonlymphoid organs, which can be advantageous for certain diseases. However, in most situations, it would be preferable to induce an immune response (either protective and tolerogenic) at a specific site in which an Ag as a vaccine would be applied, and not affecting all Ags that gain access to the immune system at that time, e.g., microbial Ags of the gut and other mucosal sites. Future studies have to clarify whether repetitive doses of Flt3L or other growth factors, such as GM-CSF, would be even more effective in inducing tolerogenic or immunogenic protective responses. After it became obvious that the DC subset composition in the blood of children differs with age (35) and how important the local stimulation of the lung by environmental factors is, e.g., LPS.
in early childhood for the development of asthma, one might speculate on prophylactic stimulation of the lung by inhaling stimulatory factors for lung DC using compounds such as Flt3L. A further application might be to induce antitumor activity by the combination of Flt3L and immunostimulatory DNA or a tumor Ag, as suggested by Merad et al. (36) recently.

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