ONLINE SUPPLEMENT

Distal Respiratory Tract Viral Infections in Young Children Trigger a Marked Increase in Alveolar Mast Cells

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Material and methods

Animal Model of Influenza A Infection and Allergic Inflammation in Infant Mice

Fifteen-day pregnant (female) BALB/c mice were purchased from Charles River Laboratories (Ottawa, ON, Canada) and housed under specific pathogen-free conditions and maintained on a 12-h light–dark cycle with food and water *ad libitum*. Upon birth, mothers were housed with their litters in light-protected cages until completion of the study (or weaning at 4 weeks of age). All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, ON, Canada).

Influenza A infection and sensitization protocols

Separate groups of 8-day-old BALB/c mice were infected with influenza A/PR8 virus or given PBS solution without anesthesia. Influenza type A virus strain A/PR/8/34 (H1N1) was prepared as described previously. The viral stock suspension (10^9 PFU ml^{-1}) was diluted 6,000-fold and a sublethal dose of ~1 PFU administered intranasally in 15 μl PBS.

Allergen administration: HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in sterile saline (Sal) at a concentration of 2.5 μg (protein) per μl and 10 μl (25 μg dose) was administered to lightly isofluorane-anesthetized 2-week-old mice by intranasal delivery. According to the manufacturer, the levels of endotoxin in HDM extracts range between 25 and 100 EU ml^{-1} extract; this corresponds to 0.25–1 EU per dose of allergen per day or 0.1–1 ng per 25 μg dose. These levels of lipopolysaccharide are significantly lower than the 100 ng dose of lipopolysaccharide required to promote Th2 responses in OVA models of allergic disease.

Concurrent influenza A infection and allergen exposure in early life: Groups of 8-day-old mice were infected with influenza A or PBS and then 7 days later, groups of mice were exposed to either HDM or Sal, 5 days a week for a total of 3 weeks. The immune-inflammatory response

and structural changes were evaluated 3 days after the last challenge (Fig 1A).

Airway inflammation and remodeling in adulthood: To investigate whether MC changes persisted, the above protocol was recapitulated and, after the last allergen challenge, mice were allowed to rest for a period of 3 weeks (Fig 1A).

The inflammatory and remodeling responses were re-evaluated and the impact on lung mechanics determined as previously described[21].

Collection and Toluidine Blue Staining of Mouse Lungs

Lung tissues were collected at the time of sacrifice. The lungs were inflated with 10% formalin at constant pressure of 20 cm H_2O and, then, fixed in 10% formalin for 48–72 h until further processing. To evaluate the total number of mast cells in the mouse tissue, 3 μm sections where prepared as described above. Briefly, the sections where deparaffinized in xylene and rehydrated in decreasing concentrations of EtOH. Sections were placed in a 1% solution of Toluidine Blue (70% EtOH) for one hour, thereafter rinsed in 99,6% EtOH (3x1 min) and xylene (2x5 min) and mounted in Pertex (Histolab). All mast cells per section (purple staining) were counted and related to tissue area using (ImageScope, v10.0.36.1805, Aperio).

Subjects

Post-mortem lung tissue from a group of totally 21 children (15 males) with age (median (range)): 4 (5-16) months that died from fatal LRTIs was autopsied at Hospital Roberto del Río, Santiago, Chile. Lung tissue samples from cases of fatal RSV (n=5), ADV (n=10) and Influenza A (n=6) infections were processed for haematoxylin and eosin staining and viral infection were confirmed by immunohistochemistry. Days ill before death were 4 (<1-8) (median (range)). Two pathologists who were unaware of the virological diagnosis reviewed all tissues. In all

cases, the cause of death was determined to be bronchiolitis (typical sloughing of bronchiolar epithelium, plugging of the terminal bronchioles, and infiltration of the airway wall and of the alveoli macrophages and neutrophils). No evidence of bacterial infection was identified, and no cases had immunodeficiency as determined by the clinical history and the general autopsy findings. Children included in the study had not been subjected to prolonged mechanical ventilation or to the use of anti-inflammatory agents or antivirals. Control lung tissues were obtained from 10 children (4 males) with a median age of 2.9 (0.4-28) months who died from non-respiratory causes with no evidence of viral infection and were autopsied at Dept. of Pathology, Sao Paulo, Brazil and at the Institute of Legal Medicine of the Medical School of the Hannover University between 1995 and 2004. Eight of the children died of natural causes (congenital right ventricular hypoplasia, congenital metabolic disease, streptococcus agalactiae meningitis, gastroschisis, metabolic disorder, acute hepatic failure and 2 of malnutrition and/or dehydration). All had normal lungs upon macroscopic and histological analysis performed by experienced pathologists. Two of the children autopsied in Hannover died of accidental or inflicted causes, and also had normal lungs. No difference in age was found between children with LRTIs and controls ($p=0.5$). This study was approved by the institutional medical ethical committee Sao Paulo, Brazil and at the Institute of Legal Medicine of the Medical School of the Hannover University. All tissue samples were collected with informed parental consent.

Processing of Tissue and Histological Procedures

Human and murine lung tissue was dissected, placed in 4% buffered formaldehyde, dehydrated, embedded in paraffin and sequential 3 μm sections were generated. An initial screening of haematoxylin-stained sections was performed to evaluate gross pathological changes.

Immunohistochemistry

All antibodies used have been extensively validated for staining of human tissue in research and routine clinical diagnosis (Table 1). Staining was absent in sections using isotype-matched control antibodies (Dako, Glostrup, Denmark).

Double Immunohistochemical Staining of MC_{TC} and MC_T

A double staining protocol was used for simultaneous visualization of MC_{TC} and MC_T cells [21-23]. The staining was performed by an automated immunohistochemistry robot (Autostainer; Dako, Glostrup, Denmark) with EnVision™ G|2 Doublestain System (K5361, Dako). After rehydration and antigen retrieval, chymase-containing mast cells were detected with a mouse anti-chymase antibody, a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody, and the non-permeable chromogen DAB. A double-stain blocking reagent (K5361, Dako) prevented further recognition of anti-chymase antibodies (by chemically destroying the antigenicity of previously applied antibodies); the remaining MC_T subclass was visualized with a mouse anti-tryptase antibody, an alkaline phosphatase (AP)-conjugated anti-mouse secondary antibody, and Permanent Red chromogen. Sections were stained with Mayer's haematoxylin for visualization of background.

Immunohistochemical Identification of CD34 positive Mast Cells

Immunofluorescence double staining was used to visualize CD34 positive mast cells (for details, see Table 1). After antigen retrieval and a blocking step with serum-free blocking (Dako), the CD34 immunoreactivity was visualized with a goat anti-mouse Alexa Flour 555 secondary antibody (Invitrogen, Molecular Probes, Oregon, US). Next, mast cells were stained with a mouse anti-tryptase antibody that was pre-labeled with AlexaF-488 fluorochrome (Invitrogen, Zenon IgG1 labeling kit, Molecular Probes). Nuclei were visualized with Hoechst (H33342, AppliChem GmbH, Darmstadt, Germany) that labels all DNA.

Immunohistochemical Identification of CD34, ITGB1, ITGA4, Ki-67, 5-LO, and IL-6 positive Mast Cells

Immunofluorescence double staining was used to visualize CD34, ITGB1, ITGA4, Ki-67, 5-LO, or IL-6 positive mast cells.

Double Immunohistochemical Staining of TLR-7 and Mast Cells

A double staining protocol was used for visualization of TLR-7 positive mast cells. The staining was performed by an automated immunohistochemistry robot (Autostainer; Dako), with EnVision™ G|2 Doublestain System (K5361, Dako).

Immunohistochemical Identification of VCAM-1

A single staining protocol (EnVision™ Detection system, K5007, Dako) was used for visualization of VCAM-1. Briefly, VCAM-1 was detected by a mouse anti-VCAM-1 antibody (Invitrogen) and secondary antibodies conjugated with peroxidase polymers.

Tissue Analysis

Quantification of Density of Mast Cell Subtypes

High-resolution images of sections double-stained for MC_{TC} and MC_T were generated through a 20x microscope lens by an automated digital slide-scanning robot (Scanscope CS™, Aperio, Vista, CA). All mast cells of each subpopulation per tissue compartment were quantified manually and related to the perimeter of the basal membrane (small airways and pulmonary vessels) or alveolar parenchyma tissue area in randomized, blinded images of lung compartments using ImageScope (v10.0.36.1805, Aperio) [23]. The density of MC_T and MC_{TC} where quantified in three small airway walls, three pulmonary vessel walls and three 20X

magnification areas (533159 μm^2) of the alveolar parenchyma per subject. In total 96 small airways, 96 pulmonary vessels and 96 alveolar parenchyma tissue were analyzed. To consider the tissue remodelling in the alveolar parenchyma, tissue area was measured in two ways (including and excluding airspaces. Tissue area of the alveolar septa (excluding air) was measured and alveolar mast cells were related to tissue area in the same 20X regions described above.

Quantification of CD34, 5-LO, IL-6 and TLR7 positive Mast Cells

After immunofluorescence staining, tryptase-positive mast cells (488 nm) and expression of CD34, 5-LO and IL-6 (555 nm) was analyzed and the proportion (%) of positive mast cells was calculated, *i.e.* mediator positive mast cells divided with all tryptase positive mast cells times 100. All mast cells double positive for TLR7 (red and brown) per tissue compartment were counted manually and related to the tissue area in randomized, blinded images of lung compartments using ImageScope. The analysis was performed in sequential sections and corresponding compartments as for the mast cell double staining.

Quantification of VCAM-1 immunoreactivity

The immunoreactivity per mm^2 tissue area of VCAM-1 or the number of cells double positive for tryptase and TLR-7 as well as the tissue area in the walls of small airways and pulmonary vessels and in the alveolar septa was calculated using Visiomorph™ (Visiopharm, Hoersholm, Denmark). The area of interest was delineated through manual cursor tracing in blinded sections and the image analysis program calculated the area, excluding air spaces so that only tissue (*i.e.* bronchial and vessel wall or the alveolar septa) was measured. The analysis was performed in sequential sections and corresponding compartments as for the mast cell double staining.

RESULTS

Increased mast cell density in alveolar parenchyma in children who have died following LRTIs

To investigate the density of mast cells during viral infection in the lung, mast cell-populations were quantified in the small airway walls, pulmonary vessel walls and alveolar parenchyma in children who died of LRTI and compared to control children. There was a significant increase in mast cell numbers in the alveolar parenchyma. The increase was significant for both MC_T and MC_{TC} subpopulations and present in RSV, ADV and influenza A infection. To consider the tissue remodelling in the alveolar parenchyma, tissue area was measured in two ways (including and excluding airspaces). However, the analysis showed the same increase in mast cells independent of analysis method.

TABLE E1. Mast cell densities in children with LRTIs compared to controls

Feature	Controls	LRTIs	p-value
Total MC (cells/mm^2)			
*Alveolar Parenchyma**	0.6 (0-4)	12.5 (0-78)	0.0005
Alveolar Parenchyma#	1.4 (0-13)	48.2 (0-100)	0.003
MC_T (cells/mm^2)			
*Alveolar Parenchyma**	0.6 (0-3)	11.3 (0-58)	0.0005
Alveolar Parenchyma#	1.3 (0-11)	15.8 (0-75)	0.004
MC_{TC} (cells/mm^2)			
*Alveolar Parenchyma**	0.0 (0-1)	2.8 (0-20)	0.0008
Alveolar Parenchyma#	1.4 (0-13)	4.2 (0-25)	0.1

Data presented as median (range). *Mast cell number related to alveolar parenchyma area including airspaces. #Mast cell numbers related to tissue area of alveolar parenchyma excluding airspaces.

No difference in TLR7, 5-LO or IL-6 mast cell expression in infected children compared to controls

No differences in mast cell expression of TLR-7, 5-LO and IL-6 were found between children with LRTI and non-infected age-matched controls (see Table E2), or between non-infected children and healthy adult (data not shown)[18 22].

TABLE E2. Mast cell mediator expression

Feature	Controls	RSV	Adenovirus	Influenza A	p-value
TLR-7 (positive MC,%)					
Small Airways	12 (7-56)	7 (0-13)	21 (0-45)	19 (18-34)	0.3
Pulmonary vessels	20 (15-40)	20 (0-25)	25 (0-40)	17 (12-30)	0.5
Alveolar Parenchyma	13 (0-43)	0 (0-13)	11 (0-43)	10 (0-20)	0.8
5-LO (positive MC,%)					
Small airways	23 (0-50)	33 (25-50)	55 (25-71)	41(30-50)	0.3
Pulmonary vessels	60 (50-100)	50 (33-67)	50 (13-80)	76 (63-100)	0.1
Alveolar Parenchyma	30 (0-75)	48 (25-80)	20 (18-40)	33 (20-100)	0.3
IL-6 (positive MC,%)					
Small airways	28 (0-50)	15 (14-20)	16 (0-25)	10 (0-13)	0.4
Pulmonary vessels	71 (20-83)	40 (33-50)	45 (13-67)	41 (14-60)	0.1
Alveolar Parenchyma	25 (0-50)	8 (0-14)	15 (0-50)	8 (0-25)	0.7

Data presented as median (range).