Factors influencing severity and treatability of allergic airway inflammation

Ph.D. theses

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Severity and treatability of allergic airway inflammation persisting in the background of asthma may be influenced by the innate immunity. Exposure to lipopolysaccharide (Lps) – or other natural immune stimulatory agents – as well as pregnancy [1] can induce the activation of innate immune processes. To further clarify the role of these factors the following examinations were performed:

ANIMAL EXPERIMENTS

Introduction and aims

Lipopolysaccharide (bacterial endotoxin) is a ubiquitous environmental pollutant present in air, household and organic dust. Lps inhalation has been considered to contribute to both the induction of airway allergy and the worsening of asthma symptoms. According to previous investigations, the co-exposure to low concentrations of Lps was indispensable for the induction of airway allergy in mice, and it was shown, that without Lps binding protein (necessary for Lps recognition) allergized mice did not develop airway hyper-responsiveness and showed no increase of airway peroxynitrite, which is a highly pro-inflammatory agent. Lps could exacerbate airway inflammation in individuals with established allergic asthma, who have also proven to be more sensitive to the bronchoconstrictive effects of Lps. In addition, inhalation of Lps decreased to 1/5th the PD20 of allergens in asthmatic patients. Purely allergic mechanisms of inflammation respond quite well to glucocorticoids, but among patients with bronchial asthma, there is a great variability of steroid responsiveness, whose cause remains mostly unknown. Of note, inflammation and bronchoconstriction induced by Lps may be hardly treatable by glucocorticoids in humans, as well as mice. During recent years, several experimental studies on airway effects of Lps plus allergen exposure-induced inflammation have been published, but none of them have investigated how Lps co-administration modifies treatability of allergic airway inflammation and hyper-responsiveness.

Aims

1. Establishment and characterization of a mouse model of airway eosinophil inflammation and airway hyper-responsiveness (AHR) as induced by priming with Lps and subsequent challenges by inhaled ovalbumine (Ova) in previously sensitized mice.

2. Comparison of the effects of dexamethasone (Dex) between mice with Lps+allergy and others with pure allergic airway disease (without Lps priming).

Bronchial NO is a major mediator of both allergic and non-allergic airway inflammation and inducible NO synthase (iNOS) inhibitors are also potential antiasthma agents. Considering this, our third aim was:

3. Measurement of nitrite (NO_2) – the primary metabolite of NO – in bronchoalveolar lavage (BAL) fluid and testing the effects of 1400W, a new, highly selective inhibitor of iNOS.

Methods

Animals

Six- to eight-week-old, female, specified pathogen-free BALB/c mice were used.

Sensitization and airway challenge

Mice were sensitized by intraperitoneal injection of 20 μg Ova emulsified in 2.25 mg aluminium hydroxide (Alum) in a total volume of 100 μL phosphate-buffered saline (PBS) on days 1 and 14 (Fig. 1). Non-sensitized animals received Alum and PBS only. On days 28, 29 and 30, mice were placed in a plastic chamber and challenged with 1% Ova aerosol (in PBS) or PBS only for 20min by a compressor nebulizer. Lung resistance (R_L) measurements and sample collections were timed on day 31, 24 h after the last challenge.

Lps priming

Mice were treated with Lps on day 27, 1 day before the first Ova (or PBS) challenge (4 days before measurements of lung resistance, BAL and sacrifice, Fig. 1). After anaesthesia by diethyl ether in a vapour-filled jar, 10 μg Lps (167 μg/mL) from Escherichia coli (serotype 055:B5) was instilled intranasally. Sixty microlitres of Lps solution was placed onto the nares in three 20 mL drops with a repeater pipette. Non-primed animals received 60 mL PBS.

Steroid treatment

Two groups of mice were treated with dexamethasone sodium phosphate (Dex) on days 29 and 30. Dex was diluted in normal saline and 5 mg/kg/day was injected subcutaneously in a total volume of 100 μL. Other groups received 100 mL normal saline as placebo.

Treatment with 1400W, a selective inhibitor of iNOS

N-(3-(Aminomethyl) benzyl) acetamidine (1400W), a highly selective inhibitor of iNOS, was administrated subcutaneously at 2 mg/kg dose, 2 h before R_L measurements on day 31 in two groups of mice. 1400W was dissolved in PBS and injected in a total volume of 100 μL. Other groups received PBS as placebo.

Measurement of R_L

On day 31 (4 days after intranasal Lps or PBS instillation and 24 h after last Ova or PBS challenge), mice were anaesthetized, the left jugular vein was cannulated and tracheostomy was performed. Then, the animals were placed into a whole-body plethysmograph and mechanically ventilated. R_L was registered continuously during the infusion of MCh in threefold increasing concentrations to assess AHR. The starting dose of MCh was 43 μg/kg, and the consecutive doses were administrated in intravenous bolus at 2 min intervals, but only after R_L normalized or showed new baseline (horizontal trace) at ≤120% of pre-MCh baseline. This was a non-cumulative administration, and peak responses of R_L during each infused dose of MCh were considered as representative.

Bronchoalveolar lavage

BAL was performed in each mouse via a tracheostomy tube using 600 μL PBS three consecutive times. The recovery of BAL fluid (BALF) was 81.9±0.7%. Total cell count was determined under a light microscope using a Bürker haemocytometer. The cell suspension was diluted and centrifuged in a cyto-chamber. Slides were stained with a hematological dye, and differential cell counts were determined under a light microscope by counting at least 300 cells.

Histology

Lungs were inflated with a fixative (4% phosphate-buffered formaldehyde, pH 7.4) and removed from the thoracic cavity. The left lung was immersed in a fixative for 24 h, and then dehydrated, embedded in paraffin block and 5 μm sections were stained with haematoxylin and eosin for light microscopy examination. The density of perivascular and

peribronchial eosinophilic infiltration was evaluated by a semi-quantitative score.

Nitrate and nitrite measurements

Nitrate and nitrite levels of BALF supernatants were assayed by capillary electrophoresis with transient isotachophoresis preconcentration. Animals had free access to food containing nitrate and tap water, and thus, the nitrate concentrations were equally high in BALF of all groups. BALF nitrite concentration, however, was considered as a marker of pulmonary NO synthesis, as the primary decomposition product of NO in an aqueous solution containing oxygen is nitrite.

Cytokine measurements

The levels of interleukin (IL)-4, IL-5, IL-13, interferon (IFN)-γ, tumour necrosis factor (TNF)-α and granulocyte-monocyte colony-stimulating factor (GM-CSF) in BALF supernatants were assessed by commercially available ELISA kits. The assays were performed according to the instructions of the manufacturers.

Design of the experiment

1. Effects of Lps priming on airway inflammation, AHR and steroid responsiveness in allergized mice (n = 12–14/ group).

Groups of mice:

C: control, non-sensitized, non-primed and PBS challenged

LPS: non-sensitized, Lps-primed (96 h before assessment of R_L and sample collection) and PBS challenged

OVA: Ova sensitized, non-primed and Ova challenged

LPS/OVA: Ova sensitized, Lps primed and Ova challenged

OVA+DEX: Ova sensitized, non-primed, Ova challenged and treated with Dex (on the second and third days of Ova challenges)

LPS/OVA+DEX: Ova sensitized, Lps primed, Ova challenged and treated with Dex (on second and third day of Ova challenges)

All OVA and LPS/OVA mice were injected with normal saline subcutaneously (as placebo) at the time of Dex treatments of OVA+DEX and LPS/OVA+DEX mice. Cellular build-up, cytokine, nitrite and nitrate concentrations of BALF and lung histology were examined in all mice. AHR was assessed in half of the mice in each group (randomly selected, n = 6–8).

2. Short-term effects of 1400W on the AHR.

(n = 6–8/group).

Groups of mice:

C, **LPS**, **OVA** and **LPS/OVA** groups as described above (1.), except that the latter two groups received placebo (PBS) at the time of 1400W treatments.

OVA+1400W: Ova sensitized, non-primed Ova challenged and treated with 1400W (2 h before assessment of R_L and sample collection)

LPS/OVA+1400W: Ova sensitized, Lps primed, Ova challenged and treated with 1400W (2 h before assessment of R_L and sample collection).

Cellular build-up of BALF was examined and AHR was assessed in all these mice. There were no significant differences between either BALF cell counts or AHR results of C, LPS, OVA and LPS/OVA groups of the two experiments, respectively. Therefore, these data were drawn together.

Statistical analysis

Parametric results are expressed as mean±SEM, and nonparametric data (histological scores) as median and interquartile range (in parentheses). One-way analysis of variance (ANOVA) was used for parametric and the Kruskal-Wallis test for non-parametric variables to determine overall difference between groups. Pair-wise comparisons were performed by Neumann-Keuls and Dunn's post hoc test as appropriate.

Results

Airway inflammation

In OVA mice, 1 day after the Ova challenge, a marked increase in BALF eosinophil and macrophage cell counts was observed. In LPS/OVA mice, the presence of macrophages and lymphocytes, but especially eosinophil granulocytes, became more pronounced than in OVA mice. This indicated that Lps instillation 1 day before a 3-day Ova challenge significantly potentiated the eosinophil character of airway inflammation (Fig. 1). The semiquantitative assessment of lung eosinophil infiltration showed that in LPS mice, some eosinophils occurred around airways and blood vessels, but the scores were not significantly different from controls (C: 0.5 (0–1); LPS: 2 (1–2); $P>0.05$ C vs. LPS). In OVA and LPS/OVA

groups, marked eosinophil infiltrations were found (OVA: 3.5 (3–4); LPS/OVA: 4 (3–4); P<0.05 or less vs. both C and LPS).

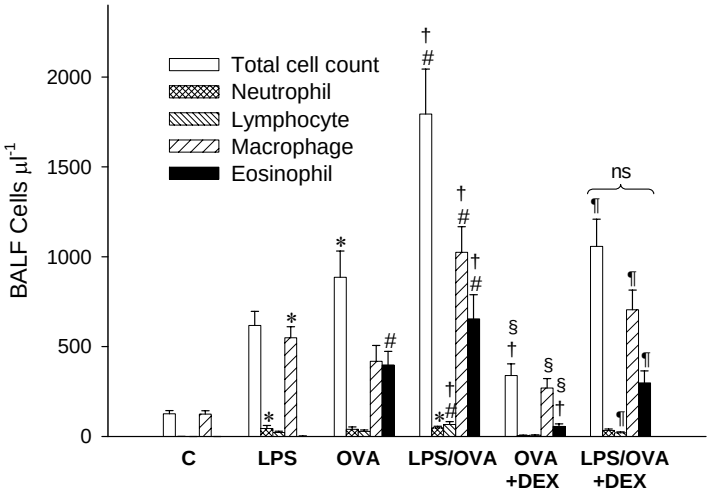

Figure 1.: Total and differential cell counts in bronchoalveolar lavage fluid (BALF). *: p<0.05 vs. C; #: p<0.01 vs. C and LPS; †: p<0.05 vs. OVA; §: p<0.05 vs. LPS/OVA+DEX; ¶: p<0.05 vs. LPS/OVA; ns: p>0.05 vs. OVA in all cell populations. (n=18-20 in C, LPS, OVA and LPS/OVA; n=12-14 in OVA+DEX and LPS/OVA+DEX).

Nitrate and nitrite

The concentrations of nitrite were found to be different among the groups of experiment. NO_2 was somewhat elevated in LPS, more increased in OVA and the highest in LPS/OVA mice (Fig. 2). Therefore, Lps priming exaggerated NO_2 formation in the lung after Ova challenges.

Cytokines

As expected, IL-4, IL-13, as well as IL-5 concentrations were markedly increased in OVA mice (Fig. 3). In LPS/OVA counterparts, however, IL-4 and IL-13 were reduced relative to OVA animals, while IL-5 remained unchanged. BALF concentration of IFN-γ, GM-CSF and TNF-α remained below the detection limit in all groups.

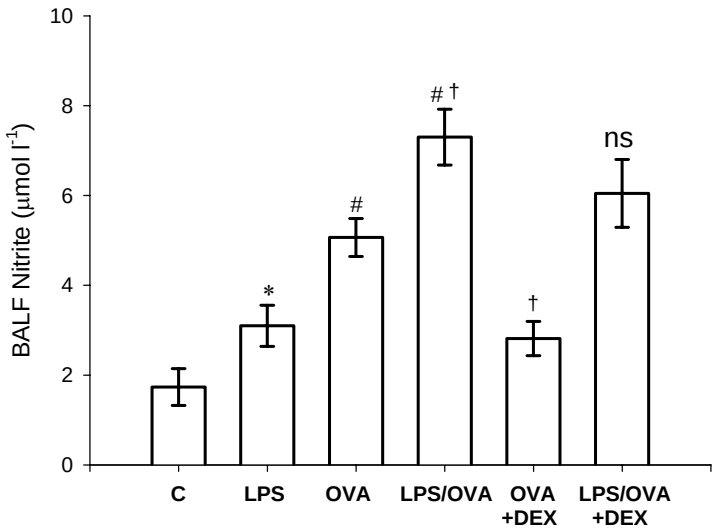

Figure 2.: Nitrite concentration of bronchoalveolar lavage fluid (BALF). *: p<0.01 vs. C; #: p<0.01 vs. LPS; †: p<0.01 vs. OVA; ns: p>0.05 vs. LPS/OVA (n=12-14).

Airway hyper-responsiveness

As compared with C, there was no increase in airway responsiveness to intravenous infusion of MCh 96 h after Lps treatment in the LPS group (Fig. 4A). AHR in OVA mice was, however, striking, as R_L increased four times more than in C or LPS animals after the two largest doses of MCh. This demonstrated the development of the known AHR of mice undergoing the described protocol of sensitization and challenge. AHR became reduced by Lps priming in Ova-sensitized and challenged mice, as elevation of R_L during infusion of MCh was observed to be midway between OVA and C (or LPS) mice (Fig. 4A).

Effects of dexamethasone

Dex effectively reduced eosinophil inflammation of OVA mice (OVA+DEX, Fig. 1). In LPS/OVA animals, treatment with Dex also led to reduced cellular inflammation, but the absolute number of eosinophils and macrophages remained significantly higher than in OVA+DEX mice.

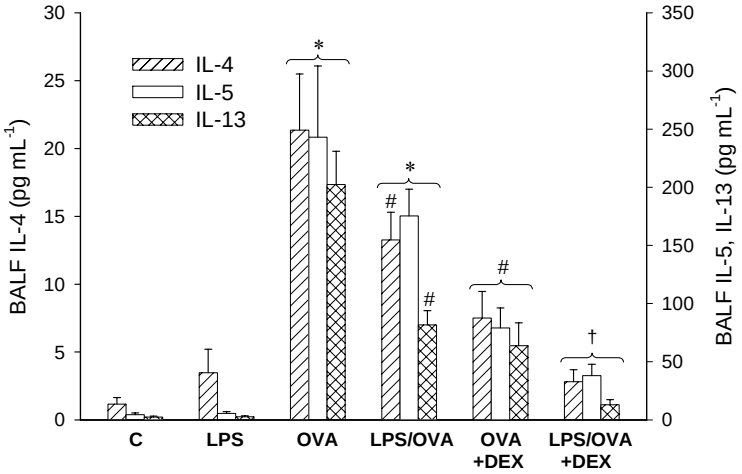

Figure 3.: Concentrations of Th2 cytokines in bronchoalveolar lavage fluid (BALF) supernatants. *: p<0.05 vs. C and LPS; #: p<0.05 vs. OVA; †: p<0.05 vs. LPS/OVA (n=12-14).

Of note, in LPS/OVA+DEX animals, the size of these inflammatory cell populations was as high as in untreated OVA animals. The semi-quantitative eosinophil infiltration score of histological preparations supported the observations made in BALF (OVA+DEX: 1 (1–1); P<0.001 vs. OVA; LPS/OVA+DEX: 2 (2–2); P<0.05 vs. C and LPS/OVA). Similar eosinophil infiltration scores were revealed in LPS/OVA+DEX and OVA animals (P<0.05). The NO_2 concentration in BALF was effectively reduced by Dex in OVA+DEX mice (P<0.05 vs. OVA), while no significant effect was revealed in LPS/ OVA+DEX animals (P>0.05 vs. LPS/OVA, Fig. 2). In contrast to cellular inflammation and nitrite levels, the concentration of all three Th2 cytokines became proportionally reduced by Dex in both OVA+DEX and LPS/OVA+DEX mice (Fig. 3). Dex markedly inhibited AHR of mice with pure allergic inflammation (OVA+DEX), while there was only a non-significant trend towards benefit of Dex on AHR of LPS/OVA+DEX (Figs 4B and C).

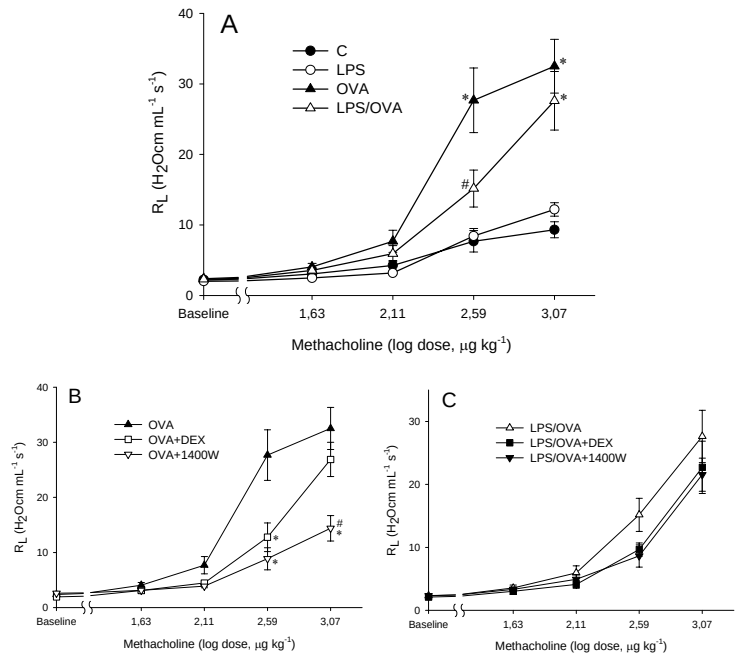

Figure 4.: A. Effects of lipopolysacharide (Lps) priming on AHR. *: p<0.001 vs. C and LPS; #: p<0.01 vs. OVA. B. Effects of dexamethasone (Dex) and 1400W (inhibitor of iNOS) on airway hyper-responsiveness (AHR) of Ova-sensitized and challenged (OVA) mice. *: p<0.05 vs. OVA; #: p<0.05 vs. OVA+DEX. C. Effects of Dex and 1400W on AHR of Lps-primed Ova sensitized and challenged (LPS/OVA) mice. (n=14-16 in C, LPS, OVA and LPS/OVA; n=6-8 in OVA+DEX, LPS/OVA+DEX, OVA+1400W and LPS/OVA+1400W).

Effects of 1400W

Figures 6b and c also depict R_L data of OVA+1400W and LPS/OVA+1400W mice. These animals received one dose of 1400W, just 2 h before measurements. This compound was found to be a more effective inhibitor of AHR than Dex in OVA mice (Fig. 4B). In LPS/OVA animals, however, AHR was as refractory to 1400W, as to Dex (Fig. 4C). Cell counts in BALF were not influenced by 1400W in either groups of mice (data not shown).

Summary and conclusions

In an experimental investigation [2], we have developed a model of eosinophil airway inflammation and airway hyper-responsiveness (AHR), which was induced by Lps and allergy together. Allergen challenge, preceded by Lps-priming resulted in more severe eosinophil inflammation and higher nitrite formation in sensitized BALB/c mice, than allergen provocation alone. After Lps priming, AHR and concentrations of Th2 cytokines in bronchoalveolar lavage fluid were decreased, but still remained significantly higher than in controls. Probably, some accessory – non-Th2 – immune-mechanisms might have been activated by Lps, during the development of allergic inflammation. Eosinophil inflammation was partially, while nitrite production and AHR were observed to be largely dexamethasone resistant in Lps-primed allergized animals. Thus, an animal model of steroid-resistant asthma was established in mice. The role of inducible nitric oxide synthetase (iNOS) in AHR was also assessed. Our results demonstrated that 1400W – a selective inhibitor of iNOS – effectively and rapidly (within 2 hours) reversed AHR in allergized mice. This effect of 1400W was, however, absent after by Lps priming. In conclusion, Lps inhalation may exaggerate eosinophil inflammation and reduce responsiveness to anti-inflammatory treatment in allergic airway inflammation.

HUMAN STUDY

Introduction and aims

The severity of bronchial asthma may change during pregnancy. Although the direction of change is quite unpredictable, during the first 3 post-partum months, asthma was reported to revert to its pre-pregnancy severity in 73% of women. There is a concordance between the course of asthma during subsequent pregnancies. The negative influence between pregnancy and asthma may be mutual, as – according to population-based studies including thousands of subjects – even mild and moderate asthma increases the occurrence of fetal growth retardation, pre-term labour, pre-eclampsia and other pregnancy complications. Although recent reports suggest that seven out of every 100 pregnant women suffer from asthma, interactions between asthma and pregnancy remain largely unstudied.

Aims

Based on these considerations, we hypothesized that:

1. Pregnancy-induced immune alterations may modify allergic mechanisms of asthma.

2. Immune responses maintaining asthma may modify fetal development.

As T cell synergism or antagonism may develop in pregnant asthmatics, IL-4^+ and IFN-γ^+ subpopulations of circulating T lymphocytes were determined and correlated with maternal lung function, as well as with the birth weight of their babies.

Methods

Subjects

Forty-eight asthmatic pregnant women (2nd trimester: n=12, 3rd trimester: n=36) were recruited in this study. Three groups of control subjects were also included: healthy pregnant women of similar age and gestational age at pulmonary examination and blood withdrawal (n=18, four women in the 2nd and 14 women in the 3rd trimester), and healthy (n=8) and asthmatic (n=13) non-pregnant women. During their visit at the Asthma Ambulance, they were asked about pre-existent atopy, the start and severity of asthmatic symptoms and anti-asthma medications. Pregnancy-related changes in asthma symptoms were also recorded. Venous blood was drawn for routine laboratory tests and also into heparinized test tubes for flow cytometric (FACS) analysis of lymphocytes. Spirometry, wholebody plethysmography and arterialized capillary blood gas and acid–base were analysed. On first visit after delivery, data were collected about asthma control during pregnancy, as well as about pregnancy-related complications and newborns.

Analysis of lymphocyte subsets

Mononuclear cells were separated from anti-coagulated peripheral blood via density-gradient centrifugation. In-vitro cytokine production of separated lymphocytes was measured. Cells were acticated by phorbolester and ionomycin in the presence of brefeldin A. Intracellular expression of IL-4 and IFN-γ was stained by fluorescent isotiocianate- or phycoerthyrin-conjugated monoclonal antibodies after labelling of cell surface CD3 molecules. Measurements were performed in a flow cytometer

Results

Peripheral blood of healthy pregnant controls contained measurably increased IL-4^+ and IFN-γ^+ subpopulations of CD3 cells. This finding indicated proliferation (activation?) of some T subsets during normal pregnancy. Our asthmatic pregnant patients, however, had much higher T cell counts. IFN-γ-synthesizing T cells were found in greatly increased numbers, although the IL-4^+ fraction were also increased. These women had about 20-fold higher number of IFN-γ producing T cells than non-pregnant women suffering from the same disease of comparable severity. The IL-4^+ subset also increased proportionally with that of the IFN-γ^+ subset, but in a smaller magnitude. Therefore, pregnancy in asthma seems to lead to a culminating cytokine response, which is of mixed phenotype, but, considering absolute numbers – dominated by IFN-γ. Although pregnant asthmatics had high pro-inflammatory cytokine production, their symptoms were not more severe than the symptoms of non-pregnant asthmatics. However, significant negative correlations were revealed between the IFN-γ^+ T cell counts and PEF rates, as well as between IL-4^+ counts and PEF rates. Furthermore, patients on higher doses of inhaled steroid maintenance therapy had significantly higher numbers of IL-4^+ and IFN-γ^+ T cells In addition, a significant negative correlation was observed between maternal IFN-γ^+ T cell counts and birth weights of newborns in the asthmatic group. Three pregnancies ended up in pre-eclampsia. Our data proposed that exceedingly high IFN-γ + T cell numbers of pregnant asthmatics may be associated with pre-eclampsia.

Summary and conclusions

In our human study [3], the immunological interferences between asthma and pregnancy were examined. The IFN-γ^+ and IL-4^+ T cell counts – determined by flow cytometry – were markedly increased in peripheral blood of asthmatic pregnant women. Significant negative correlations were revealed between the sizes of these cell populations and maternal peak expiratory flow, as well as birth weight of their newborns. Thus, the culminating proliferation of IFN-γ^+ and IL-4^+ T lymphocytes may potentially impair fetal development as well as maternal airway symptoms.

PUBLICATIONS

In the theme of thesis

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