Early life exposure to toxic environments: effects on lung and immune cell development in mice and men
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Chapter 1

General introduction
1. Developmental responses to environmental stimuli

Organisms have the ability to respond to environmental cues during development. They have evolved appropriate adaptive responses to specific environmental conditions during development, which either give them the advantage for immediate survival or long-term benefit for fitness. For example, under low oxygen conditions, in the fetus more blood will flow to the brain and the heart than to the muscle and the kidney to ensure oxygen supply to the vital organs, resulting in fetal survival [1]. In addition, the freshwater crustacean Daphnia offspring has been described to be born with a defensive helmet because of maternal exposure to trace levels of chemicals from a predator during pregnancy [2], which was not present in the offspring from mothers that had not been exposed to this predator’s chemical signal.

Unfortunately, not all environmental stimuli can induce the evolved adaptive responses during development. For example, environmental toxicants such as heavy metals, tobacco smoke and persistent organic pollutants are novel challenges for some species, to which they have not yet evolved suitable adaptive responses during development. However, the organisms can still plastically respond to environmental toxicants in a dose-dependent manner. Very strong stimulation by environmental toxicants usually induces disruptive responses, which results in disturbance of developmental processes in a specific organ or tissue, and subsequently causes death or a structural defect. A relatively moderate stimulation by environmental toxicants may induce an immediate adaptive response, which helps to buffer acute stress and damage in cells or tissues resulting in survival. However, such immediate adaptive responses are stochastically and usually are at the expense of normal cell or tissue function in later life although no obvious developmental deficit existed at birth. For example, prenatal exposure to environmental heavy metal lead (Pb) had been associated with the lower cognitive ability in children [3]. In some conditions, environmental toxicants are able to indirectly induce the evolved adaptive responses in the developing organisms. For example, Cadmium accumulation in the placenta was shown to prevent nutrient transfer to the fetus [4], which lead to intrauterine under-nutrition. In the fetus this would initiate the evolved response of reducing growth and result in lower birth weight.
The window of exposure to environmental stimuli during development is pivotal for induction of an adaptive response. Each organ/tissue has its own critical window during development, during which cells are under dynamic proliferation and differentiation and more vulnerable for environmental perturbation. The organ or tissue is less sensitive to the environmental influence once it has finished the key developmental processes [5]. Except for the brain, lung and immune system, most organs finish its developmental processes during the fetal stage [6, 7]. Environmental toxicants may still interact with the developing processes of brain, lung and immune system after birth and may induce the adaptive responses in these organs in early postnatal life.

The adaptive response (except for the disruptive response) to environmental stimuli during development may persistently affect the health of the offspring by two ways. Firstly, the immediate adaptive response may alter specific gene expression through epigenetic mechanisms, and cell dysfunctions in offspring will be manifest under pathophysiological conditions or primed by a stimulus later in life. For example, cord blood methylation levels of the proximal enhancer methylation sensitive region in the Perforin-1 gene were associated with the risk of lower respiratory tract infections in infants [8]. This variant methylation levels of target regions of the Perforin-1 gene reflected maternal environmental exposure during pregnancy, such as maternal smoking [8]. Secondly, the environmental stimuli may indirectly induce an evolved adaptive response, which promotes the fetus to develop a phenotype that could adapt to the predicted future environmental condition based on current environmental cues. The mismatch between the anticipated environment and real condition after birth will lead to the increased risk of diseases. For example, intrauterine under-nutrition had been shown to give a signal to the rat fetus that the outside world was scant of nutrients, and induced metabolic alterations in the fetus for the predicted environment after birth. However, the offspring suffered from obesity, hyperphagia, hyperinsulinemia if they were fed a high-fat diet after weaning [9].
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2. Prenatal exposure to environmental toxicants: adaptive responses from developing airway epithelial cells and lung health risk in later life

Airway epithelial cells in the conducting airways include ciliated cells, club cells, goblet cells, basal cells and neuroendocrine cells. In the mouse, goblet cells appear mainly in the trachea and the largest bronchi. As during the fetal stage dynamic proliferation and differentiation processes give rise to a functional airway structure at birth, this is a highly sensitive period for the developing airway epithelial cells to be exposed to environmental toxicants.

Developing airway epithelial cells are able to adaptively respond to environmental stimuli. Some environmental toxicants can directly act on receptors expressed on developing airway epithelial cells. For example, nicotine that crossed the placenta and bound to nicotinic acetylcholine receptors (nAChRs) on airway epithelial cells of the rhesus monkey fetus induced more expression of α7 subunit of nAChRs and increased the number of neuroendocrine cells in neuroepithelial bodies [10]. This was accompanied by an upregulated collagen expression around the airways as well as an increase of vessels in the area with increased α7 subunit expression [10], suggesting the altered lung cell development in the monkey fetus. Prenatal nicotine exposure also increased glutamatic acid decarboxylase (GAD) and gamma aminobutyric acid A (GABA_A) receptor mRNA and protein levels, resulting in mucus overproduction in bronchial epithelium of new-born rhesus monkey fetuses [11]. Additionally, cigarette smoke extract exposure at a nontoxic concentration widely suppressed genes related with cilia-genesis during basal cells differentiation into ciliated cells in vitro, resulting in the shortened cilia in these ciliated cells [12]. Together, these data indicate that developing airway epithelial cells interact with environmental toxicants which can lead to morphological or functional alterations.

The adaptive response of developing airway epithelium to environmental stimuli can have long term effects on lung health later in life. Ciliated cells and secretory cells form the mucociliary system to clear inhaled pathogens and particles [13]. Mucus secreted by secretory cells traps inhaled particles [14]. The
coordinated beating of cilia then removes the particles [13] and keep the airway clean, protecting the lung from invasion of toxicants into the lung tissue. Excessive mucus production and shorter cilia length may be induced by environmental stimuli and may contribute to failure of particles/pathogens clearance and airway obstruction, the features in asthma [15] and chronic obstructive pulmonary diseases (COPD) [16].

*In utero* exposure to cigarette smoke is one of the most common threats to fetal lung development. Although numerous epidemiological studies have demonstrated an association between prenatal smoke exposure and lower lung function and the risk for development of lung diseases in later life [17-21], the underlying molecular mechanisms are still elusive. Studies in nicotine-exposed rhesus monkey fetuses have provided important insights into the effect of prenatal exposure to nicotine on airway epithelial function and offspring lung health [11]. However, many other toxic chemicals other than nicotine are present in cigarette smoke. For example, Cadmium also is present as a main type of heavy metals in cigarette smoke. Cadmium can accumulate in the placenta by maternal smoking and prevent nutrient transport to fetus, which may induce an adaptive response that would affect lung development with different mechanisms.

Our lab has previously demonstrated that maternal smoking during pregnancy was associated with increased airway responsiveness to methacholine and elevated goblet cell numbers after house dust mite exposure in BALB/c offspring [22]. In this thesis, we set up a new smoke exposure protocol, using a new smoke machine, new cigarettes and a different mouse strain to further explore the underlying molecular mechanisms that promote goblet cell metaplasia in prenatally cigarette smoke exposed mice. In addition, we explored the effect of prenatal smoking on susceptibility to cigarette-smoke-induced lung inflammation and airway remodeling in adult offspring.

### 3. Maturation of immune function in early postnatal life: threat from environmental toxicants

After birth, the neonatal or infant immune system is still developing. During this
period, the immune system is fine-tuning a variety of key functions, in the face of direct stimulation from environmental signals not previously encountered during fetal life. The response patterns “learned” during this period persist into adult life [23]. Regarding the innate immune system, blood monocytes from neonates produced less interferon-α (IFN-α), IFN-γ and interleukin-12 subunit p70 (IL-12p70) after Toll-like receptor (TLR) stimulation than monocytes obtained from adults [24]. However, production of these cytokines was markedly upregulated at 1 or 2 years of age [24]. The production of IL-12p70 and the percentage of dendritic cells in neonatal peripheral blood mononuclear cells were also lower than in adults [25, 26], a difference that remained until at least 12 years of age [26]. This suggests a long term functional maturation of the dendritic cells.

The adaptive immune system in neonates or infants is also subjected to maturation processes. T lymphocytes from neonates or infants are able to mount immune responses. However, the quantity and quality of responses are different from that in adults. For example, neonatal T lymphocytes were shown to have a limited ability to produce IFN-γ or IL-4 after in vitro stimulation with anti-CD3 and anti-CD28 when compared with adult naïve T cells [27]. The production of IFN-γ and IL-4 increased along with age in infants and children [28, 29], indicating the gradually maturation of T cell cytokine responses after birth. In addition, Th1 responses were found to be elevated while Th2-biased responses in fetus/neonates were decreased along with age [23, 30, 31]. With respect to the B cell function in neonates or infants, the duration of an infant antibody response was shorter and the antibody affinity was lower than that in adults [32]. Meanwhile, naïve B cells from neonatal mice and humans were not able to effectively develop germinal centers until at 4 months old in humans and approximately 3 weeks old in mice [32].

The matured immune system is capable to gain immune memory. However, development of immune memory in neonates or infants is defective. It was shown that dendritic cells produce less IL-12 and more IL-10 after TLR stimulation [33, 34], which resulted in insufficient stimulation signals for T cell activation and differentiation and subsequently diminished development of
memory T cells. Lower numbers of dendritic cells in neonates exacerbated impairment of memory development. In addition, neonatal T cells were shown to be less able to develop immune memory. In a recent study in an animal model [35], equal numbers of CD8$^+$ T cells with an identical T cell receptor (TCR) were isolated from neonatal and adult mice and were subsequently transferred to the same host mice. The study showed that all transferred CD8$^+$ T cells responded to a bacterial infection in host mice. However, the transferred CD8$^+$ T cells from neonatal mice expanded more rapidly than CD8$^+$ T cells from adult mice and quickly took a fate into shortly-lived terminally differentiated effector cells, while the CD8$^+$ T cells from adult mice differentiated into a pool of effector and memory cells at the same time. Moreover, CD8$^+$ T cells from neonatal mice that were isolated from host mice 7 days after the primary infection displayed gene profiles that enhanced effector rather than memory cell fate, indicating an intrinsic defect of signals in neonatal CD8$^+$ T cells that did not facilitate development of immune memory. However, a series of studies have demonstrated that the absolute number and percentage of memory T cells increased with age [36-39], suggesting that neonatal or infant T cells are able to mature enough to develop immune memory in later life.

Environmental toxicants can induce adaptive responses in immune cells after birth, which may quantitatively or qualitatively affect immune function. In an in vitro study it was demonstrated that development of bone-marrow-derived dendritic cells was suppressed when Pb (10 mM PbCl$_2$) was added into the culture medium [40]. In that study, CD80 expression on the Pb-exposed bone marrow-derived dendritic cells was lower than in non-exposed bone marrow-derived dendritic cells after stimulation with lipopolysaccharide (LPS) [40]. Moreover, Pb-treated dendritic cells produced less proinflammatory cytokines IL-6, IL-12p70, and TNF-α than non-Pb-treated dendritic cells did, although synthesis of all these cytokines increased after stimulation with LPS [40]. Other studies showed that Pb could directly disrupt protein biosynthesis of IFN-γ in Th1 cells in vitro [41], and promoted Th2 differentiation in vivo [42]. Additionally, low levels of Pb (~1 microM) were demonstrated to promote antigen-specific CD4$^+$ T cell proliferation probably through targeting at antigen presentation cells and affecting peptide: MHC interaction [43]. Considering that
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the strength of peptide: MHC interaction is essential for memory T cell differentiation, it is possible that Pb exposure may also disturb development of immune memory in early postnatal life.

Many other environmental chemicals such as arsenic, cadmium and polycyclic aromatic hydrocarbons are additionally able to interact with immune cells and affect maturation of immune function. They have been shown to suppress ontogeny of innate immune cells after birth, promote misbalance of Th1/Th2 responses, and disturb peripheral immune cell homeostasis and immune memory development. The combined exposure to these environmental toxicants in early life may even more widely affect maturation of immune function in later life. In this thesis, we tested T cell and NK cell differentiation and the functional related parameters in preschool children from an e-waste-contaminated area and evaluated influence of early childhood exposure to environmental toxicants on development of immune competency in these children. These studies will help to better understand immune dysfunction caused by exposure to environmental chemicals in early life and predict susceptibility to immune-related diseases in later life.

4. The scope of this thesis

The aim of this thesis was to investigate the effects of early-life exposure to environmental toxicants on lung and immune cell development in mice and men, which helps to understand why early-life exposure to toxicants is associated with disease risk in later life.

In chapter 2, the effect of early-life exposure to a variety of environmental toxicants on lung and immune cell development and function, as well as the related disease risk in later life has been reviewed. In chapter 3, the effect of maternal smoking during pregnancy was investigated on lung epithelial development in 1-day-old mouse pups. Additionally, expression analysis of genes that control goblet cell metaplasia has provided us an explanation for the previously observed effect of prenatal smoke exposure on the house dust mite-induced increased susceptibility of goblet cell metaplasia in offspring. In
**chapter 4**, the effect of maternal smoking during pregnancy on lung inflammation, airway remodeling and lung senescence in adult offspring was explored. We observed that maternal smoking during pregnancy resulted in persistent lower expression of anti-inflammatory genes and anti-senescence genes in adult offspring lung. However, maternal smoking during pregnancy did not further enhance lung inflammation and tissue remodeling in the C57BL/6 adult offspring with chronic cigarette smoke exposure. In **chapter 5**, studies were continued in a group of preschool children that were exposed to e-waste in early postnatal time. In this study, characteristics of T cell subset distribution in blood, levels of helper T cell cytokines and helper T cell differentiation-related cytokines were evaluated in preschool children and linked with blood Pb levels. This study provided new information about T cell memory development and helper T cell differentiation in preschool children from an e-waste-contaminated area and helped us to predict T cell-mediated immunity or disease risk in these children. In **chapter 6**, the NK cell numbers and levels of cytokines/chemokines that were related to NK cell differentiation and cytotoxic activity were analyzed in preschool children from an e-waste-contaminated area and again related to blood Pb levels. This study provided new information about the effect of e-waste exposure on NK cell-mediated innate immune function in preschool children.
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