Which subtype Toll-like receptor is important for the signaling of farm dust?

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Abstract

Background: The incidence of allergic disorders is rapidly increasing, especially in the western countries. This brought a renewed interested in the hygiene hypothesis. Epidemiological studies show an association of microbial exposure and infections with the risk of developing allergic diseases. But these studies give little insight in the molecular mechanism behind the hygiene hypothesis.
Objectives: We investigated the role of TLR family members in directing the adaptive immune system to mediated the protective effect of farm dust exposure.
Results: There were some conflicting results, in some studies it was suggested that TLR 2 and TLR 4 activation induces Treg and Th17 responses, while other studies showed that they induce pro-allergic cytokines.
Conclusions: TLR 2 and TLR 4 are most likely to be involved in mediating the protective effect of farm dust exposure. However, the molecular mechanism is still controversial.

Abbreviations: Airway hyper responsiveness (AHR); Dendritic cell (DC); House dust mite model (HDM-model); Lipopolysaccharide (LPS); Pathogen associated molecular patterns (PAMPs); Regulatory T-cell (Treg); Thymic stromal lymphopoietin (TSLP); Toll-like receptor (TLR)

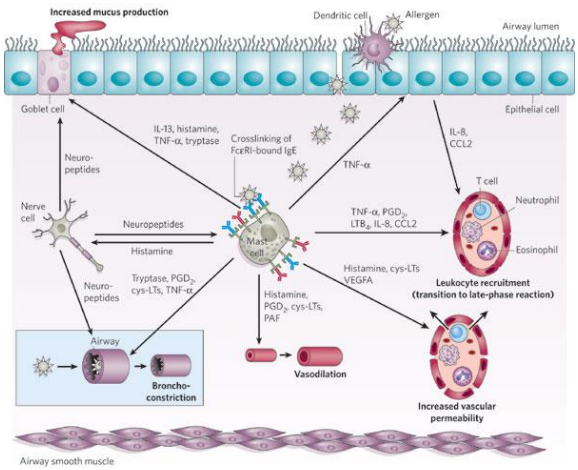
Introduction

The incidence of allergic diseases, including asthma, is worldwide rapidly increasing. In search of an explanation for this growing problem, there is a renewed interest for the hygiene hypothesis. According to the hygiene hypothesis, exposure to microbial compounds and infections during early childhood may have a protective effect against sensitization and atopic diseases later in life. The hygiene hypothesis was for the first time described by David P. Strachan in 1989 as a possible explanation for the observation that eczema and hay fever were less common in large size families (Strachan DP, 1989). There are also higher incidence and increasing prevalence of allergic diseases, including asthma, in developed countries (Keller MB, 2002). Many epidemiological studies were performed to investigate this theory. The studies analyzed the prevalence of allergic disorders in different family sizes and found a protective effect of large families (Wickens KL et al., 1999). The presence of pets was also found to have a protective effect against sensitization (Plaschke P et al., 1999). In studies that compared the incidence of allergic diseases in non-developed versus developed countries, concluded that developed countries have a higher incidence. (Horner AA, 2006) Growing up in an urban surrounding, in contrast to a farm surrounding, was found to be a risk factor for the development of allergies (Ernst P et al., 2000; Klintberg B et al., 2001; Von Ehrenstein OS et al., 2000). These epidemiological studies show that microbial exposure and infections are associated with risk for developing atopic disorders. However, the causal correlation as postulated by the hygiene hypothesis, cannot be proved by epidemiological research. To investigate whether the hygiene hypothesis is right, we reviewed mice model studies and looked at the molecular mechanism behind the hypothesis. In search for a possible molecular

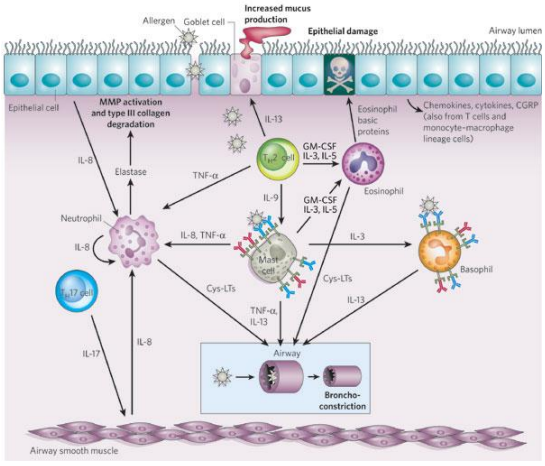
mechanism that explains how microbial exposure would protect against atopic disorders, we have to look at the pathophysiology of allergies.

The development of an allergy always begins with allergic sensitization. When encountering the allergen for the first time, the specific T-cell that reacts to this allergen differentiates from a naive phenotype into a Th2 cell. Th2 lymphocytes produce, among other cytokines, IL-4 and IL-13 to activate B cells and promote immunoglobin-class switching of the antigen-specific antibody to an IgE-subset. The constant region of the IgE antibody binds to the high-affinity Fcε receptor on the mast cells and the basophils. Until this moment the person has no allergy symptoms because there is not yet an inflammatory reaction against the allergen. But the IgE-loaded basophils and mast cells are now ready to react if the person encounters that specific allergen for the second time, this is called sensitization.

The response upon subsequent allergen exposure is further divided into two stages: the acute phase and the late phase reaction. The next time there is contact with the allergen, cross-linking of IgE activates mast cells and basophils, inducing these cells to undergo degranulation and release chemical mediators, like histamine, proteases, chemokines and heparin. These mediators, cause an influx of lymphocytes, neutrophils, macrophages and eosinophils to the site of allergen exposure. The release of the chemical mediators will induce the acute-phase response. The secreted mediators can induce bronchoconstriction and an increased mucus production which cause an obstruction in the airway and lead to wheezing and a running nose. The vasodilation is the cause of the local erythema, (reddening of the skin). An increased vascular permeability results in swelling of the contact site. The mediators also stimulate nociceptors of sensory nerves of the nose, skin or airway, which leads to the well-known symptoms of allergy: sneezing, itching and couching.



Figuur 1 early phase response
Stephen J. Galli, Mindy Tsai & Adrian M. Piliponsky, Nature 454, 445-454(24 July 2008)



Figuur 2 Late phase reactions
Stephen J. Galli, Mindy Tsai & Adrian M. Piliponsky, Nature 454, 445-454(24 July 2008)

The influx of inflammatory cells causes the late phase response. Approximately two hours after the allergen exposure, the late phase reaction begins. Th2 cells migrate to the initial site and release cytokines, like IL-5 and IL-13. The cytokine IL-5 plays an important role in the growth, differentiation, proliferation, migration and activation of eosinophils and IL-13 is, among others, responsible for the airway remodeling and inflammation (Hamelmann E et al.,

1999). The release of these and other cytokines is associated with long term effect of allergic airway hyper responsiveness (AHR). Other symptoms of the late-phase response are warmth, oedema, pain and erythema on the entry side. If the initial site is located in the lungs, airway narrowing and mucus hypersecretion are common symptoms which lead to tightness of the chest (Galli SJ, 2008).

But before these symptoms appear, the patient must first be sensitized to the allergen, which is dependent on T-cell differentiation to the Th2 phenotype and IgE production by B cells to allow activation of the mast cells by the specific allergen. The phenotype of T cells that have switched to a certain T cell subset is very stable, so the specific T-cell reaction to an allergen is difficult to change after sensitization. This is the reason that the hygiene hypothesis only focused on the environment during early childhood when the adaptive immune system is still developing.

Reduced exposure to microbial compounds and minimal infections early in life may modulate the development of the adaptive immune system. This could be the biological explanation for the hygiene hypothesis. In an environment with reduced exposure to bacterial and viral infections during childhood, there are less Th1 mediated immune responses. This can cause an imbalance in the Th1/Th2 responses of the adaptive immune system later in life. In subsequent years, another hypothesis was added to explain the simultaneously increasing prevalence of Th1 mediated immune diseases (Cooper GS et al., 2003; Bach JF, 2002). This hypothesis claims that a decreased number of infections during early childhood, leads to a lack of regulatory T-cell (Treg) responses. A decrease in Treg cells is responsible for the increasing Th1 mediated immune diseases because Treg cells suppress both excessive Th1 and Th2 responses. Research has indeed found that there is a decreased number of Treg cells in people with asthma (Orihara K et al., 2007; Lin YL et al., 2008). Not only reduced incidence of infections are the cause of the decreased Th1 responses during early childhood. Environments with reduced levels of microbial compounds, like an urban environment and developed countries are also modulating the adaptive immune response. Infections are not necessary to help prevent allergic diseases, exposure to distinct microbial compounds, pathogen associated molecular patterns (PAMPs), is enough to trigger the Th1 immune response.

As already known, certain Toll-like receptor subsets belonging to the group of pattern recognition receptors (PRR), are involved in the recognition of PAMPs. The epidemiological studies showed a protective effect of a farm environment against allergic reactions. This farm environment is characterized by high exposure to microbial compounds. TLR 2 and TLR 4 ligands are present in farm dust and could contribute to directing the adaptive immune system (Boasen J et al., 2005). These receptors are located on cells belonging to the innate immune system and can be responsible for directing the response of the adaptive immune system. TLR 2 ligands are a wide array of microbial molecules of gram-positive and gram-negative bacteria, mycoplasma and yeast. Lipopolysaccharide (LPS), the major component of the gram-negative bacteria membrane, is known to be the main ligand of TLR 4. The different TLR subsets have different ligands and signaling, therefore it is possible that only some subsets are involved in the farm dust induced decrease of allergic diseases.

In this review, we focused on farm dust as a possible protective agent against allergies. Our hypothesis is that the receptor subset recognizing the farm dust molecules during allergen exposure is important for the reaction against the allergen and modulates the T-cell differentiation into an effector subset. We aim to find out in this study which Toll-like

receptor is important for the protection against allergies and by which molecular mechanism this is mediated.

Results

Epidemiological studies described the relation of farm dust exposure in childhood and the incidence of allergies in different countries. A study in Sweden (Klintberg B et al., 2001) examined the prevalence of respiratory allergy, eczema and atopic sensitization in farmers versus non-farmers children (7-8 yrs), born and raised on the island of Gotland. They used a questionnaire to investigate the incidence of symptoms related to atopic diseases en the lifestyle factors that may contribute to the development of allergies, like smoking, farming and horseback riding. The atopic sensitization for 15 allergens was measured by a skin-prick test. The authors concluded that living in a farm environment protects against the development and symptoms of asthma, allergic rhino-conjunctivitis and atopic dermatitis but not against atopic sensitization. This suggests that the protective effect of a farming environment is only on the clinical symptoms and independent of sensitization. In Canada (Ernst P et al., 2000), a decrease in atopic asthma prevalence in children (12-19 yrs) growing up on a farm was also found. These authors defined asthma as atopy, current wheeze and hyper responsiveness to methacholine. The results could not be explained by current smoking or family size. Other published articles reported similar results of reduced risk of hay fever and current wheeze in Germany (Von Ehrenstein OS et al., 2000), and a protection against the sensitization and symptoms of allergies in Austria and Switzerland (Riedler J et al., 2000; Braun-Fahrlaënder C, 1999) among children raised on a farm. So the beneficial effect of farm dust on allergic sensitization is still controversial. The protective effect against the symptoms of asthma was found in all studies, but only some studies found a protection with regard to sensitization. (Riedler J et al., 2000; Braun-Fahrlaënder C, 1999; Klintberg B et al., 2001).
There are epidemiological studies who suggest that there is an increased beneficial effect of exposure to farm dust, if there are livestock present on the farm where the children are raised (Riedler J et al., 2000; Von Ehrenstein OS et al., 2000). This could be explained if the levels of microbial compounds are increased on farms with livestock present. In dust gathered on a farm with livestock, endotoxin levels are higher than in farm dust without the presence of animals (Gehring U et al., 2002). Endotoxin consists mainly of LPS, a TLR 4 ligand (Broad A et al., 2006). This could be an indication that TLR 4 activation is involved in the mechanism which allows farm dust to protect against allergic disorders.

To investigate the role of TLR 2 and TLR 4 in the protective effect of farm dust, the expression of CD14, TLR 2 and TLR 4 genes in blood samples of farmer children and non-farmer children were measured with a real-time quantitative PCR. The blood samples came from the Swiss participants that also enrolled in the cross-sectional survey of J. Riedler (2001). The results of the study were that the expression of TLR 2 gene and CD14 gene markedly higher was in farmers` children. In contrary TLR 4 expression was slightly but not significant reduced in farmers` children. They concluded that TLR 2 and CD14 gene expression is influenced by the exposure of microbial compounds such those found in farm dust. Therefore it might be possible that the adaptive immune response is altered in presence of a high TLR 2 ligand environment (Lauener RP et al., 2002). Consistent with these results the authors also measured in their *in vitro* experiment a significantly increased expression of TLR 2 after exposure to endotoxin, just like the expression of its co-receptor CD14 (Lauener RP et al., 2002). Although, they did not mention the cell type that they used. This results suggest that endotoxin present in farm dust is responsible for TLR 2 activation and so is

responsible for the protective effect of farm dust. One possible mechanism for this protective effect mediated by TLR 2, is directing the adaptive immune reaction towards the Th17 and Treg responses. To investigate the effect of farm dust on the adaptive immune system the following *in vivo* mice models for asthma were used.

There are two different methods that studies can use as an *in vivo* asthma mice model: The classical OVA/Alum model and the house dust mite model (HDM model). In the classical OVA/Alum model the mice are sensitized by an intraperitoneal injection with ovalbumin precipitated on aluminium hydroxide (alum) and the control group with aluminum hydroxide alone. During the sensitization the mice are treated with the test substance, in this case farm dust. After about two weeks the mice are challenged by aerosolized ovalbumin and it is tested whether the response against this allergen is altered. With this model a strong and systemic sensitization phase is used, which gives a strong Th2 response but is not reflecting the human pathology. In the HDM-model the mice are exposed to aerosol HDM and simultaneously sensitized against the test allergen. There is no strong, systemic sensitization, so this mice model is more reflective of the human disease. In this alternative model the mice are only stimulated intranasally, first with the test substance followed by HDM.

The first experimental mouse model (Peters M. 2006) that investigated the beneficial effects of farm dust used the OVA/Alum mice model. Intraperitoneal injections with ovabumin/alum on day 1 and 14 induced allergic sensitization, and during this time the mice were also treated with aerosolized solution of farm dust or PBS as a control. After two weeks the mice were treated with two ovabumine aerosol inhalation challenges. The authors found that the mice that were sensitized and challenged with OVA recruited eosinophils, lymphocytes and macrophages in the lungs and developed AHR, in contrast to the non sensitized control group. This proved that the asthma model worked. Treatment with farm dust inhalation during the sensitization phase resulted in a loss of responsiveness to methacholine almost to the level of the non-sensitized control group and no recruitment of eosinophils and lymphocytes. The authors concluded on the basis of these results that inhalation of dust extract was protective against the induction of eosinophilic airway inflammation and AHR development. They also found that the Th2 response was not down regulated by an increased Th1 response. This is contradicting with the initial explanation of the hygiene hypothesis that is the balance between Th1 and Th2 responses. If an increased Th1 immune response is not responsible for the decreased allergic Th2 response, then an increased Treg and Th17 response might be responsible for the Th2 suppression. However, the authors did not investigated this theory in their study.

Another *in vivo* mice experiment (Blacquière MJ et al., 2010) evidence was found that Treg and Th17 might be involved in the modulation of the adaptive immune response away from the Th2. The mice were intranasally exposed to farm dust or saline control followed by house dust mite (HDM) or PBS. The cytokines, inflammatory cells and goblet cell hyperplasia were measured in the lung tissue and serum. Exposure to HDM led to higher levels of eosinophils in lung tissue, more total and HDM-specific IgE in serum, higher number of goblet cells in the airways and a higher responsiveness to methacholine. In general, the mouse group treated with farm dust and HDM showed data that were more at the level of the control groups. The cytokine profiles in lung tissue showed an decrease in Th2 cytokines IL-5 and IL-13, but not in IL-4. Furthermore an increase in Foxp3 positive Treg cells in lung tissue and higher levels of the Th17 cytokines IL-17, IL-1beta and IL-6 after stimulation with farm dust was found in lung tissue of farm dust-treated mice. The authors concluded from this experiment that the

farm dust-induced down regulation of Th2 responses is probably mediated by the observed increase in Treg and Th17 responses.

It is shown that *in vitro* activation of TLR 4 and TLR 2 on human peripheral blood mononuclear cells, promote the production of cytokines that activates T cells and directs their maturation toward the Th17 phenotype (Kattah MG et al., 2008). It could be that this same mechanism is responsible for directing the adaptive system toward a Th17 and Treg phenotype in airway epithelial cells. One possible component in farm dust that can cause this direction is chitin a component of fungi and parasitic nematodes, which is a ligand of TLR 2 (Da Silva CA et al., 2008). This polysaccharide has been suggested to be an immune modulator and induces macrophage IL-17 production and enhanced expression of IL-17 receptor via TLR 2. This is an example of component present in farm dust that could direct the immune response via TLR 2 towards the Th17 phenotype (Da Silva CA et al., 2008). This conclusion is in line with the results of the mentioned study of Lauener RP (2002), who measured an increase in TLR 2 expression in farmers' children.

TLR 2 and TLR 4 are not only present on innate immune cells but also on barrier airway epithelial cells (Saito T et al., 2005; Berndt A et al., 2007; Hammad H et al., 2009). In the next mouse model study of Blacquière MJ et al. (2010), they investigated the effect of exposure to farm dust on the TLRs on epithelial cells in the airway. TLR 2 and TLR 4 were down regulated in the epithelium of the mice lungs, after intranasally exposure with farm dust. The authors observed lower levels of thymic stromal lymphopoietin (TSLP) in lung tissue of farm dust treated HDM sensitized mice, compared to control treated HDM sensitized mice. Exposure to farm dust reduced the effect of HDM exposure mice on TSLP, almost to the level of the non-sensitized control group. TSLP is a cytokine excreted by activated epithelium cells. This cytokine is known to attracts T-cells and is involved in their maturation (Al Shami A et al., 2004). It was also recently found that TSLP contributes to the development of allergic airway inflammation (Zhou BH et al., 2005). This suggests that the preventive effect of exposure to farm dust on allergic sensitization and airway inflammation could be explained by the down regulation of TLR 2 and TLR 4. This reduced number of TLRs on the membrane leads to a reduced TSLP secretion by the epithelium cells after stimulation with farm dust. A lack of TSLP itself leads to a decreased attraction and maturation of T-cells which protects against Th2 mediated allergic diseases, and most likely reflects a decreased activation of the airway epithelium.

A study by Hammida Hammad investigated the effect of TLR 4 on airway epithelial cells and concluded that TLR expression on the airway epithelium is not only sufficient but even necessary to activate a dendritic cell (DC) induced inflammatory response. (Hammad H et al., 2009) Bone-marrow chimeric mice were used to investigate the role of TLR 4 expression on the lung structural cells versus the hematological compartment on the cellular influx as a response on stimulation with LPS. TLR 4 deficient mice reconstituted with a wild-type bone marrow failed to recruit neutrophils, monocytes and DCs into the lungs. Triggering of TLR 4 on the airway epithelium of wild-type mice caused production of pro allergic cytokines like TSLP, IL-25 and IL-33. Inhalation of a TLR 4 antagonist leaded to diminished symptoms of asthma, including AHR. This study showed that activation of TLR 4 expressed on the airway epithelium causes the cellular influx and the pro allergic cytokine production that leads to the DC activation and induces the allergic inflammation and a Th2 directed adaptive immune response. This result is consistent with the conclusion of the mice model study of Blacquière MJ, mentioned above. Taken together, these studies suggest that the TLR 4 ligand can be responsible for the induction of an allergic reaction. Upon activation of the airway epithelial

cells by TLR 4, the cells start to produce pro allergic cytokines like TSLP to activate and attract DCs and T-cells and direct the adaptive response to the Th2 phenotype. Confirming the relevance of TLR 4 on airway epithelial cells for allergic sensitization. Farm dust protects against the allergic responses by down regulating the number of TLRs on the epithelial cells.

Discussion

In this review we investigated the role of different TLR subsets in the protective influence of exposure with farm dust on allergic reactions. The main finding of our research is that TLR 2 and TLR 4 are involved in the protective effect of farm dust, but we cannot yet exclude the role of other TLR family members.

The first study from Blacqière MJ et al. (2010) showed a protective effect of exposure to farm dust against IgE production, eosinophilic airway inflammation and AHR development, mediated through Th17 and Treg responses. Other studies suggested that TLR 2 and TLR 4 could be responsible for directing the adaptive immune system toward a Treg and a Th17 reaction (Da Silva CA et al., 2008; Kattah MG et al., 2008). Therefore, one possible explanation for the protective effct of farm dust exposure, is the induction of Th17 and Treg responses mediated by TLR 2 and TLR 4 signaling.

Hammad H et al. (2009) showed that activation of TLR 4 on airway epithelial and dendritic cells, leads to a increased production of pro allergic cytokines, like TSLP. These cytokines are known to activate and attract DCs and T-cells and direct the adaptive response to the Th2 phenotype (Al Shami A et al., 2004). This suggests that TLR 4 ligand is responsible for directing the adaptive immune response towards an allergic reaction. This conclusion in combination with the findings of the second study from Blacqière MJ et al. (2010), could provide another explanation for the protective effect of farm dust mediated by TLR family members. This study showed a down regulation of TLR 2 and TLR 4 on airway epithelial cells. If the TLRs are down regulated on the antigen presenting cells (APCs), than there is less T cell co-stimulation for the Th2 direction because the APCs are not receiving the danger signal.
Taken this all together, continuous stimulation of the TLRs might not only lead to a Th17 and Treg response, but could also induce down regulation of the expression level of the involved TLRs, most notably TLR 2 and TLR 4. This will make the cells less sensitive to the ligand, because the quantity of the receptor on the membrane is down regulated. Such a negative-feedback regulation on the level of receptor expression in the presence of high levels of the ligand, is a well known phenomenon in cell biology and protects the cells against over stimulation.

Polymorphisms of TLRs subsets may contribute at varies levels with the protective effect of farm dust exposure. Recent investigations explored the possibility that the conflicting findings of previous studies could be explained genetically (Gern JE et al., 2004). A study by GB Marks et al. (2006) demonstrated that only in farmers' children with a specific CD14 polymorphism, farm dust had a protective influence. This could explain the conflicting results that are found in all the years that the hygiene hypothesis is investigated. The interaction between genetics and environment may be the reason that most correlations could not be found by other researchers in replication studies. Further investigations should focus on both the environmental influences as the genetic factors and their interactions. Using a factor analysis, it might be possible to unravel the most important environmental and genetic factors

involved in the hygiene hypothesis. A possible method for this investigation is the nonparametric Bayesian variable selection with applications to multiple quantitative trait loci mapping with gene-environment interaction (Zou F et al., 2010).

The hygiene hypothesis is definitely not so simple to explain as the original theory described. There are most likely multiple molecular mechanisms and complex gene- environment interactions involved in the development of allergic diseases.

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