

Received Date : 12-Feb-2016

Accepted Date : 21-Feb-2016

Article type : Review

Manuscript - Review paper for Pediatric Allergy and Immunology (PAI)

Title:

“Allergy protection by cowshed bacteria - recent findings and future prospects”

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Running title: Allergy protection by cowshed bacteria

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Pediatr Allergy Immunol

Abstract:

Since the first publication in 1999 numerous epidemiological studies provided strong evidence that frequent contact to a traditional farm environment in early life protects children from the development of allergic airway diseases. These consistent findings prompted enormous efforts to identify and characterize the potential causative factors and the underlying immunological mechanisms in experimental studies. The cumulating evidence for the role of the cowshed associated bacterial flora led to enhanced efforts not only to identify the relevant species but also to examine their specific immunomodulatory capacity, the bacterial components involved, and particularly the cellular and molecular mechanisms of their interaction with the immune system. We review here the methods

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi:

10.1111/pai.12559

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applied to identify relevant bacterial species, the species which emerged thereof and the similarities and differences in their mode-of-action as revealed so far. We further consider the impact of the current knowledge on worthwhile clinical application and reflect on the required next steps to foster the translation of the encouraging scientific progress which has been made in recent years.

Key words: allergic sensitization, allergic airway disease, asthma, cowshed bacteria, innate immunity, prevention, Toll-like receptors

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Introduction

Since the first report of a decreased prevalence of hay fever and allergic sensitization in farmer's children compared to their peers living in the same rural community by Braun-Fahrlander et al. in 1999 (1) a lot of research on this topic has been conducted and repetitively has shown that exposure to a farm environment in early childhood protects against the development of allergic diseases. Based on the increasing amount of data several reviews on the health-protective effects of traditional farming environments have been published in the last five years (2-5). In these reports, varying emphasis was put on specific topics such as the biological nature of the farm exposure (2), gene-environment interactions (3), and differentiation between specific disease indications or disease phenotypes (4).

The epidemiological findings prompted enormous efforts to identify and characterize the potential causative factors and the underlying immunological mechanisms in experimental studies. Complementary to a review in this journal by Schröder et al. in 2015 covering mainly regional aspects (5), here we review the results obtained for different cowshed bacteria as these have not been summarized in detail so far. Special emphasis will be put on recent findings regarding the mode-of-action analysed by means of diversified *in vitro* and *in vivo* experimental settings.

The specific nature of the farming environment

The term 'farm exposure' is not uniformly defined but a traditional farming environment is in any case an environment characterized by an extraordinary biological diversity. Mostly, presence of several animal species arising from livestock, pets and associated wildlife (mice, birds, insects) is combined with diverse plant material from fodder, litter, and wild flowers, herbs, trees growing around the farm buildings and sheds. This mixture is further enriched by the myriad of microbes colonising these habitats. Accordingly, various farm-associated exposures were differentially analysed in association studies (6) and components of all these sources have been taken into account in the search for potential causative substances. Starting from extracts of cowshed dust (7) such differing substances

as arabinogalactan (a high molecular weight plant sugar) (8) and a serine protease from the mealworm (the larva of the beetle *Tenebrio molitor*) (9) have been demonstrated to protect from allergic sensitization and/or inflammation in appropriate mouse models. The inverse relation of endotoxin levels in children mattress dusts to the occurrence of hay fever, atopic asthma, and atopic sensitization (10) spurred studies focussing on the microflora of the farming environment. A model for the immunobiology of farming has been developed (2) trying to summarize and disentangle the various pieces of evidence known to that date. Recent association studies underpinned both the impact of the diversity of microbial exposure and the relevance of distinct bacterial species (11-13). However, these findings are not necessarily contradictory as high microbial diversity may also more likely cover the relevant protective bacterial species (12).

The cumulating evidence for the role of the cowshed-associated bacterial flora led to enhanced efforts not only to identify the relevant species but also to examine their specific immunomodulatory capacity, the structural components involved, and particularly the cellular and molecular mechanisms through which they interact with the immune system in controlled experimental settings in recent years.

Bacterial candidate identification

Mainly, two techniques were employed to identify the relevant species that were involved in allergy protection from the vast cowshed associated bacterial flora. In one approach the farming environment was examined by culturing of dust samples collected from farms in Bavaria, Germany, which were included in the German arm of the epidemiological Allergy and Endotoxin (ALEX)-cohort study (14). As result of the culturing on non-selective agar plates more than 100 different bacterial isolates could be distinguished based on standard microbiological differentiation methods in subsequent analyses. For further investigations concerning the potential allergy-protective properties of bacteria from the farming environment isolates were chosen against which antibodies were found in sera of children living on these farms and which represent relative abundant species in cowshed microflora. Strain denomination was derived from the study internal coding of the precise origin (farm code, sampling location on farm, number of agar plate). *Acinetobacter lwoffii* F78 (15), *Bacillus licheniformes* 467 (16), and *Lactococcus lactis* G121 (15) were identified this way and further examined with respect to potential allergy-protective properties *in vitro* and in animal models.

In another approach mattress dust samples which were collected in the cross-sectional PARSIFAL (Prevention of allergy - Risk factors for sensitization in children related to farming and anthroposophic lifestyle) study (17) were analysed by a combination of PCR (polymerase-chain reaction) and SSCP (single-strand confirmation polymorphism) electrophoresis technique. To assess the bacterial diversity and specificity in the samples the DNA was extracted and the highly variable bacterial 16S rRNA gene was amplified by PCR. PCR products were digested to single strand DNA and differentiated by SSCP gel electrophoresis (18). This type of electrophoresis allows for discrimination of DNA fragments not only by size but also by their nucleic acid composition even if sequence differences are marginal. Resulting gel bands of interest, i.e. associated with a specific environmental exposure or disease, were excised and sequenced for determination of bacterial genus/species (12). Amongst others, *Staphylococcus sciuri* emerged from this analysis and was subject to further immunomodulatory characterization (19). The gel bands containing *Lactobacillus spp.* and *Jeotgalicoccus sp.* also showed a significant inverse association with childhood asthma and are currently under investigation in our group for their experimental protective capacity. The PCR-SSCP method also confirmed the relevance of *Acinetobacter lwoffii* which was already shown before.

For analysing the immunomodulatory and potential allergy-protective properties a vast array of molecular and cell biology methods has been used by the different scientific groups including imaging and flow cytometric techniques. However, the complex immunological mechanisms leading to sensitization and allergic disease cannot be covered by *in vitro* experiments. Hence, *in vitro* findings have to be supplemented with results from animal models to achieve a relevant preclinical proof-of-

concept. Mainly mouse models were applied as i) the species shares the main characteristics of allergen sensitization and allergen induced airway inflammation with the human situation, e.g., the development of allergen specific IgE antibodies, the influx of eosinophilic granulocytes (eosinophils) into the lung, and the increase in cytokines as IL-5 and IL-13 in the lung; ii) appropriate husbandry is commonly established in many research institutions. The mouse models which are well approved for preclinical testing of potential allergy protective effects differ amongst other in the experimental allergen which is used and the route of administration for sensitization. Typically, ovalbumin (OVA) or house dust mite extract (HDM) are used as experimental allergen and sensitization is induced by intraperitoneal (i.p.), subcutaneous (s.c.), or intranasal (i.n.) administration. Each model has its specific advantages and restrictions (20, 21) imposing examinations in more than one model for a comprehensive preclinical assessment of a bacterial candidate. Revelation of dose response relationships for crucial experimental parameters is a further step in preclinical prequalification for later clinical application. As an example, the dose-response relationship for *Lactococcus lactis* G121 as assessed in a mouse model with experimental sensitization against OVA and intranasal administration of LL G121 during the sensitization and early challenge phase (22) is shown in Figure 1. An overview of the experimental results on the immunomodulatory characteristics of all the cowshed derived bacteria examined so far is provided in Table 1. If not stated otherwise, lyophilized bacterial cells were suspended in appropriate buffer solution or cell culture medium and added to the cell cultures *in vitro* or applied intranasally *in vivo*.

Table 1: Summary of immunomodulatory characteristics* of different cowshed bacteria

Bacterial species/strain	Experimental model	Immunomodulatory characteristics	References
<i>Acinetobacter lwoffii</i> F78	Human moDCs	CD40, CD80, CD86, MHC II ↑ Delta-4 ↑, Jagged-1 ↓ IL-12, TNF-α, ↑ IFN-γ ↑	(15, 23)
	Human moDC/T cell coculture		
	Balb/c mice, OVA i.p.	AHR ↓ Eosinophils in BAL ↓ Lymphocytes in BAL ↑ Neutrophils in BAL ↑ Eosinophils in lung tissue ↓ Peribronchial/-vascular inflammation in lung tissue ↓ Goblet cell hyperplasia in lung tissue ↓	(15)
	Maternal (prenatal) microbial exposure of Balb/c mice and C57BL/6 mice, OVA i.p. in offspring	in exposed mothers: TNF-α, IL-6, IL-12 in BAL ↑ in offspring: AHR ↓ Eosinophils in BAL ↓ Inflammation score in lung tissue ↓ Goblet cell hyperplasia in lung tissue ↓	(24)
<i>Lactococcus lactis</i> G121	Human moDCs	CD40, CD80, CD86, MHC II ↑ Delta-4 ↑, Jagged-1 ↓ IL-12 ↑	(15)
	Balb/c mice, OVA i.p.	AHR ↓ Eosinophils in BAL ↓ Neutrophils in BAL ↑ Eosinophils in lung tissue ↓ Peribronchial/-vascular inflammation in lung tissue ↓ Goblet cell hyperplasia in lung tissue ↓	(15)
	Balb/c mice, HDM i.n.	Total cells in BAL ↓ Eosinophils in BAL ↓ Neutrophils in BAL ↑ Eosinophils in lung tissue ↓ Goblet cell hyperplasia in lung tissue ↓	(22)

<i>Bacillus licheniformes</i> 467	Human moDCs Mixed lymphocyte reaction	TNF- α , IL-10 $\uparrow$ IL-5, IL-13 $\uparrow$	(16) ^s
	Balb/c mice, OVA i.p.	Eosinophils in BAL $\downarrow$ Neutrophils in BAL $\uparrow$ (absolute numbers 20 times higher than with <i>A. lwoffii</i> or <i>L. lactis</i>) IFN- γ , IL-10 in BAL $\uparrow$ (spores) Peribronchial inflammation in lung tissue $\downarrow$ Goblet cell hyperplasia in lung tissue $\downarrow$ Multinucleated giant cells in lung tissue	(16) ^s
<i>Staphylococcus sciuri</i> W620	Human moDCs	CD40, CD80, CD86, MHC II $\uparrow$ IL-23 $\uparrow$ IFN- γ $\uparrow$	(19)
	Human moDC/T cell coculture		
	Balb/c mice, OVA s.c.	AHR $\downarrow$ Total cells in BAL $\downarrow$ Eosinophils in BAL $\downarrow$ Lymphocytes in BAL $\downarrow$ IL-5 in BAL $\downarrow$ Inflammation score in lung tissue $\downarrow$ Goblet cell hyperplasia in lung tissue $\downarrow$ OVA-specific IgE in serum $\downarrow$ OVA-specific IgG1 in serum $\downarrow$ IL-5 in OVA-restimulated LN cells $\downarrow$ IL-10 in OVA-restimulated LN cells $\downarrow$	(19)
	Balb/c mice, HDM i.n.	Total cells in BAL $\downarrow$ Eosinophils in BAL $\downarrow$ Neutrophils in BAL $\downarrow$ CXCL1 in BAL $\downarrow$ Inflammation score in lung tissue $\downarrow$ Goblet cell hyperplasia in lung tissue $\downarrow$ Eotaxin-1 in lung homogenates $\downarrow$ IL-5, IL-9, IL-13 in HDM-restimulated LN cells $\downarrow$ IL-10 in HDM-restimulated LN cells $\downarrow$ IFN- γ , IL-17A in HDM-restimulated LN cells $\downarrow$	(19)

* Only statistically significant findings are reported except for results of histological lung tissue analyses.

^s Only results of treatment with *B. licheniformis* 467 vegetative cells are included in the table, *B. licheniformis* 467 spores were also analysed with partly differing results.

AHR: airway hyperreactivity; BAL: bronchoalveolar lavage fluid; HDM: house dust mite extract; IFN: interferon; IL: interleukin; i.n.: intranasal; i.p.: intraperitoneal; LN: lymph node; MHC: major histocompatibility complex; moDCs: monocyte-derived dendritic cells; OVA: ovalbumin; s.c.: subcutaneous; TNF: tumor necrosis factor

Mode-of-action analyses

As already mentioned, a number of cowshed bacteria have been isolated and successfully used in different animal model systems. All these bacterial strains have been proven to significantly reduce or inhibit allergic reactions. Thus, the question arises if the mode-of-action of the individual strains differs and, maybe even more important, if it would matter. The latter point seems to have been answered: it does. Ege et al. convincingly demonstrated in their 2011 landmark paper (11) that the diversity of the microbial encounter positively influences the protection of allergic diseases. One possible explanation for this diversity effect could be that engaging multiple innate pathways triggers an additive or even synergistic innate response which increases the immunomodulatory capability to avert allergic immune responses. Therefore, it is of great importance to dissect the individual immunological mechanisms and the structural components by which individual cowshed strains lead to allergy protection.

Acinetobacter lwoffii F78

The best-studied cowshed strain presumably is the Gram-negative bacterium *Acinetobacter lwoffii* F78 (15). First results (e.g., induced cytokine pattern as well as the upregulation of cell surface receptors) clearly supported the notion that polarization of T cells towards a Th1-response by dendritic cells (DC) is the mode-of-action. However, the structural component(s) actually responsible for this shift remained unclear at that point, since multiple innate pattern recognition receptors were engaged upon stimulation with *A. lwoffii* F78. Conrad et al. showed that prenatal exposure with *A. lwoffii* F78 also protects the progeny and that in order to achieve this protection maternal TLR signalling is required (24). Since the experiments were performed using quintuple TLR knockout animals, devoid of TLR2, 3, 4, 7, and 9, it was not known if one particular TLR was responsible. Further evaluation of different pathways that were engaged upon stimulation of human DCs with *A. lwoffii* F78 strongly pointed towards its LPS as the driving force for the allergy protection (23). Not only was the isolated LPS from *A. lwoffii* F78 (25) capable of inducing Th1-polarizing factors to a similar extent as the whole bacteria, but it could also be demonstrated that this depends on signalling through the LPS receptor TLR4. Furthermore, blocking TLR2 signalling did not reduce the upregulation of Th1-polarizing factors by whole bacteria and NOD2 signalling by itself is not sufficient.

Lactococcus lactis G121

Lactococcus lactis G121 was the second of the investigated cowshed strains in the original paper from 2007 by Debarry et al. (15). Despite being a Gram-positive bacterium, most of the immunologic responses that were analysed in the mouse model and upon stimulation of huDCs were remarkably similar to the responses towards *A. lwoffii* F78. Upregulation of costimulatory molecules, induction and release of IL-12 and downregulation of Th-2 promoting Notch ligand jagged-1 expression was nearly identical. However, already the delayed upregulation of the Th1-promoting Notch ligand delta-4 hinted towards different underlying molecular mechanisms. Furthermore, since Gram-positive bacteria do not contain LPS and thus do not signal via TLR4, other bacterial molecules and consequently other innate receptors must have been involved. The search for these molecules proved to be extremely laborious. Because of the delayed cellular response upon stimulation with *L. lactis* G121, we early on investigated if uptake of the bacteria was involved. Surprisingly, not only the uptake of *L. lactis* G121 was required in order to stimulate huDCs but also endosomal acidification was needed. Furthermore, these molecular requirements were also seen in a murine DC transfer model. In this model experimental sensitization is achieved by intranasal transfer of OVA-loaded BMDCs to naïve recipient mice. Thus, differences in the allergic outcome parameters in recipient mice upon allergen challenge can be directly attributed to the prior treatment of the transferred BMDCs. Based on these findings we started to screen endosomal innate receptors with respect to their relevance for *L. lactis* G121-driven activation. So far, we were able to demonstrate by comparison of BMDCs from wildtype and TLR13-deficient mice that signalling via TLR13 mainly contributes to the *L. lactis* G121 induced cytokine response in mouse BMDCs. Whether the TLR13 activating or other molecular patterns of *L. lactis* G121 are similarly operative in humans is a question currently studied.

Bacillus licheniformis 467

Within the microbial environment children are exposed to in cowsheds, the Gram-positive endospore-forming *Bacillus licheniformis* is very common. One particular strain, *B. licheniformis* 467, has been thoroughly investigated (16). As other investigated bacteria, also *B. licheniformis* 467 showed allergy-protective effects in a mouse model of allergic inflammation. However, the results exemplified clearly that not all bacterial strains are equally well suited for allergy protection. Surprisingly, although there was a clear difference between the induced polarization pattern of the spores vs. the vegetative bacteria (Th-1 vs. Th-2) and vegetative bacteria showed stronger TLR2- and NOD2-dependent activity, both prevented goblet cell hyperplasia but had only a marginal effect on airway hyperreactivity.

Staphylococcus sciuri W620 is another Gram-positive bacterium and has been characterized *in vivo* in OVA- and HDM- models (19). In both models, *S. sciuri* W620 prevented the hallmarks of allergic airway inflammation without inducing detrimental effects as it has been seen with *B. licheniformis* 467. Similar to the latter it utilized TLR2 and NOD2 for mediating cellular activation. However, although maturation of human DCs was achieved to a similar level as with the other bacteria reviewed here, a particular activation pattern was seen with *S. sciuri* W620: Whereas cytokines like TNF- α and IL-6 were induced, activation of human DCs with *S. sciuri* W620 did not lead to the release of IL-12, due to a lack of IL-12p35 mRNA upregulation. Rather, IL12p40 and IL-23p19 mRNAs were increased and hence, IL-23 was released. Interestingly, *S. sciuri* W620 exposure induced the IFN- γ production *in vitro* in human DC/T cell cocultures but no enhanced IFN- γ response in the mouse *in vivo* models. This result underpins that the transfer of results from *in vitro* experiments to the *in vivo* situation is not straightforward.

Bacterial mixes

Even from the characteristics of these four bacterial strains, it becomes evident that some activating features are shared among the strains (e.g., upregulation of costimulatory molecules, utilization of NOD2), whereas there are also a number of striking and possible important differences. There is differential use of TLRs and the pattern of the induced cytokines (as well as the upregulation of different polarizing factors and transcription factors, which are not discussed here) varies among the strains. In line with the fact that microbial diversity increases the protective effects, we hypothesized that this is reflected by the different stimulatory capabilities of the different strains (Fig.2). Thus, we are currently investigating if and how a combination of different bacterial strains changes the induced responses of the cells.

Clinical application

Given the lack of reliable preventive measures to protect children from developing allergic (airway) diseases it is well understood that the comprehensive epidemiological and preclinical findings on allergy protection by cowshed bacteria are also subject of considerations for clinical application. In fact, the idea of preventing the development of allergic diseases by administering bacteria is no completely new concept. Several clinical studies have been undertaken to examine the influence of oral administration of mostly probiotic bacteria on allergic outcomes in the last two decades. The results are inconsistent and two recent surveys on the overall findings including meta-analyses coincidentally conclude that a net benefit of probiotics could only be established for prevention of eczema with a low quality of evidence, but not for any other allergic disease (26, 27).

What will be the way to translate the exciting findings regarding allergy-protection by cowshed bacteria into a successful interventional approach? Probably, this question can only be answered retrospectively once a clinically proven safe and effective prophylaxis has been successfully developed. However, some essential points to consider become more and more apparent from the augmented knowledge in recent years.

First, the preclinical mode-of-action findings reviewed above which show the direct involvement of local dendritic cells in the protection from development of experimental allergic airway disease strongly support the concept that a different route of administration, namely intranasal administration of the beneficial bacteria, may be one of the factors or even the pivotal factor for a successful translation of the epidemiological observations into an effective interventional approach. Exposure of the local immunocompetent cells in the airways to the beneficial bacteria can directly be achieved by intranasal instead of oral administration. In line with this, our group found that the protective effect of

Lactococcus lactis G121 achieved in mice with intranasal administration (see table 1) was lost when the strain was administered intragastrically by a gavage or subcutaneously (Schermer O, Kauth M, unpublished data).

Second, the data on the differential effects of distinct bacterial strains and identified bacterial components involved on the overall allergy-protective outcome may point toward a mixture of those being the best approach for a pharmaceutical intervention to achieve the desired protective effect. However, the hypothetical 'best' mixture based on these results does not necessarily represent the most suitable approach for further clinical application due to the following reasons: i) all active pharmaceutical ingredients (API) of the mixture have to be producible in a constant quality in industrial scale, ii) analytical tools to control and to ensure both quality and stability of the API have to be developed and validated for every component and for the final mixture, iii) an increased risk of unwanted side effects which are intolerable for a prophylactic treatment in healthy children may occur due to interactions of the different components. This listing is by far not comprehensive but may exemplify some of the prerequisites for a successful translation beyond all necessary efficacy considerations.

Third, a whole non-pathogenic bacterial cell as API represents a natural mixture of molecules from various chemical classes compared to classical 'lead-target'-approaches in pharmaceutical development in which small chemical entities as the leads are tested for addressing one single cellular receptor/molecule as the target. As shown in the preclinical studies with whole bacterial cells and schematically summarized in fig.2, this well-defined mixture of molecules is able to intervene with the immune system on different levels, e.g., by addressing host cell surface receptors in a first step leading to internalization and cytokine induction without a pronounced inflammatory response. Subsequently, intracellular bacterial components are recognized by intracellular host receptors triggering further immunological mechanisms which finally prevent the induction of an allergic response upon allergen contact.

Taking together the current knowledge and regulatory constraints it seems reasonable to develop a primary prophylaxis to protect children from the development of allergic rhinitis and allergic asthma in form of nasal drops based on one or few of the Gram-positive cowshed bacterial strains for which their general suitability can be or already has been shown in thorough prequalification studies.

Future prospects

A lot of details concerning the allergy protection by cowshed bacteria were revealed in the last years starting with the first experimental report of Debarry *et al.* in 2007. The applied methods for detecting the relevant microbes in this specific environment of vast biological diversity have proven their suitability as they identified several bacterial species which showed strong allergy-protective properties in experimental *in vitro* and *in vivo* studies. Major advances have been achieved in deciphering the underlying mechanisms with sophisticated experimental techniques for some of them, e.g., *L. lactis* G121 and *S. sciuri* W620.

However, the variety of mechanisms with a protective impact on sensitization and subsequent allergic airway inflammation identified in preclinical studies with the different candidates is both fascinating and perplexing. Open questions remain as up to now it is still elusive which key steps are indispensable to induce an allergy-protective immune response. Further scientific research is needed to completely disentangle the mechanisms of allergic sensitization and its prevention. Nonetheless, the cumulative preclinical evidence which has been achieved so far leaves us with the strong impression that translation into a prophylactic medical intervention shall be possible. Early exploratory clinical studies, carefully designed based on the gathered knowledge with some of the thoroughly prequalified bacterial candidates, would be the appropriate next step towards this goal. Concomitant to the increasing recognition of the impact of the human microbiome on immune responses it has been

speculated that the farm effect might also originate from modulatory effects on this level (28). It is therefore desirable that the influence of intranasally administered bacteria on the airway microbiome would be assessed in upcoming studies.

The clear unmet medical need to have effective means for allergy prevention deserves all efforts to be undertaken and more resources to be allocated to foster the translation of the sound scientific findings summarized in this review into real functional medical interventions. The convincing scientific data pool gathered to date should be able to attract the resources necessary to examine the open questions and to explore - and hopefully confirm - the observed epidemiological and preclinical findings in a clinical setting of allergy protection with cowshed bacteria in the near future.

Acknowledgements

We would like to thank Prof. Albrecht Bufe for critical reading of the manuscript.

Figure legends

Fig. 1: Influence of intranasal treatment with different doses of *Lactococcus lactis* G121 in a mouse asthma model with ovalbumin (OVA)-induced sensitization and allergic airway inflammation. A) OVA-specific IgE titre in bronchoalveolar lavage (BAL) fluid, B) eosinophils in BAL fluid, C) IL-5, and D) IL-13 concentration in cell culture supernatant of OVA-restimulated spleen cells. Female Balb/c mice were sensitized by intraperitoneal injection of OVA on day 1 and day 14 and challenged with OVA aerosol on day 28 and 38. Mice were treated intranasally 14 times during the sensitization and early challenge phase with the indicated dose of *L. lactis* suspended in PBS or with PBS as control. Three days after the second challenge animals were sacrificed and BAL fluid, serum, splenocytes, and lung tissue were prepared for analyses. Detailed methods see Peters et al. (7).

Fig. 2: Known receptors and pathways involved in recognition and signaling of cowshed bacteria. The combination of different pathways, emanating from cell surface, endosomal and intracellular receptors and the strength by which these pathways are engaged lead to differential activation of dendritic cells.

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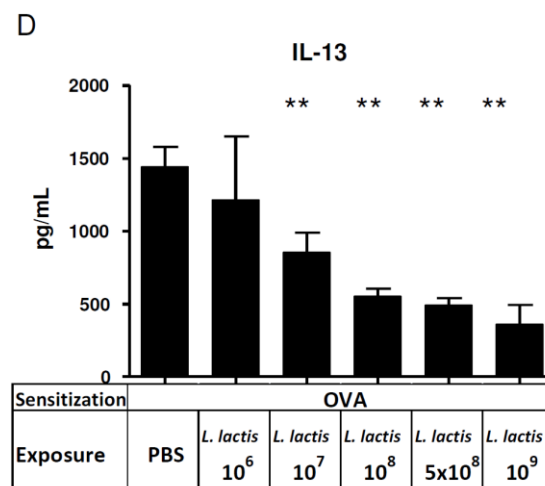
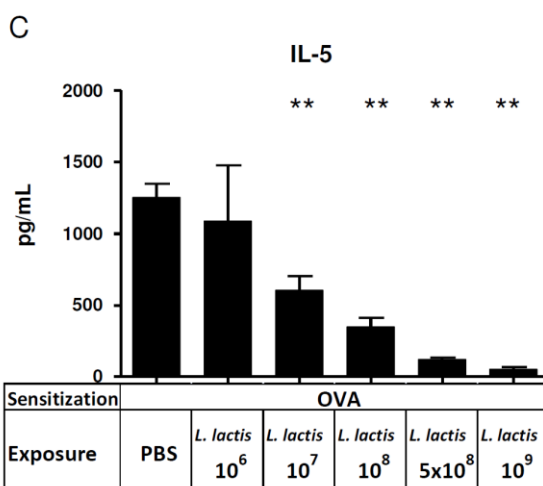
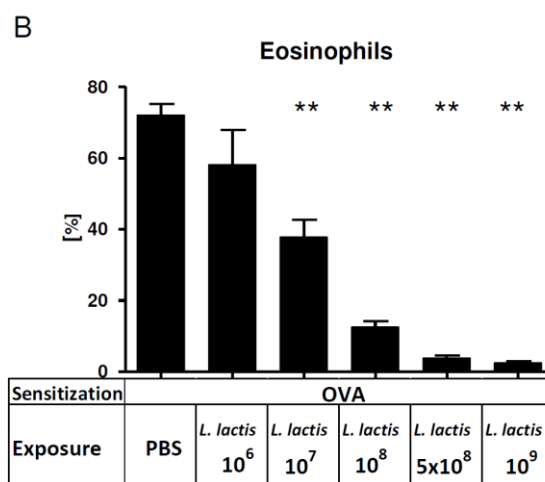
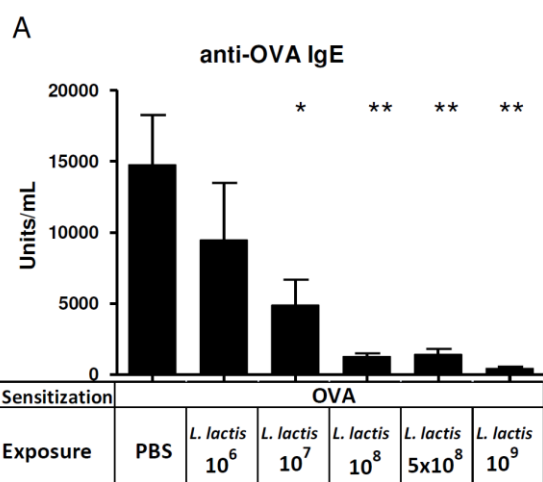
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● *A. lwoffii* F78

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