

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Farm-derived Gram-positive bacterium *Staphylococcus sciuri* W620 prevents asthma phenotype in HDM- and OVA-exposed mice

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Keywords

allergy; animal model; asthma; farming effect; prevention; proof-of-concept.

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Abstract

Background: Farm-derived dust samples have been screened for bacteria with potential allergeo-protective properties. Among those was *Staphylococcus sciuri* W620 (*S. sciuri* W620), which we tested with regard to its protective capacities in murine models of allergic airway inflammation.

Methods: We employed two protocols of acute airway inflammation in mice administering either ovalbumin (OVA) or house dust mite extract (HDM) for sensitization. Mechanistic studies on the activation of innate immune responses to *S. sciuri* W620 were carried out using human primary monocytic dendritic cells (moDC) and co-culture with autologous T cells.

Results: The allergeo-protective properties of *S. sciuri* W620 were proven in a T_H2-driven OVA model as well as in a mixed T_H1/T_H2 phenotype HDM model as demonstrated by abrogation of eosinophils and neutrophils in the airways after intranasal treatment. In the HDM model, lymph node cell T_H1/T_H2 signature cytokines were decreased in parallel. Studies on human moDC revealed an activation of TLR2 and NOD2 receptors and initiation of DC maturation following incubation with *S. sciuri* W620. Cytokine expression analyses after exposure to *S. sciuri* W620 showed a lack of IL-12 production in moDC due to missing transcription of the IL-12p35 mRNA. However, such DC selectively supported T_H1 cytokine release by co-cultured T cells.

Conclusion and clinical relevance: Our proof-of-concept experiments verify the screening system of farm-derived dust samples as suitable to elucidate new candidates for allergeo-protection. *S. sciuri* W620 was shown to possess preventive properties on airway inflammation providing the basis for further mechanistic studies and potential clinical implication.

Abbreviations

BAL, bronchoalveolar lavage; CFU, colony-forming unit; CI, confidence interval; DC, dendritic cells; GABRIELA, A Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community – Advanced Studies; HDM, house dust mite; IL, interleukin; LAB, lactic acid bacteria; moDC, monocyte-derived dendritic cells; NOD, nucleotide-binding oligomerization domain; OR, odds ratio; OVA, ovalbumin; PAS, periodic acid-Schiff; PBS, phosphate buffer saline; PCR, polymerase chain reaction; PRR, pattern-recognition receptor; SEM, standard error of the mean; SSCP, single-strand conformation polymorphism; TLR, Toll-like receptor.

Studies conducted in traditional farming sites identified dust collected in this environment to harbor a plethora of microorganisms, which might provide protection against allergies and asthma. In the context of exposure to environmental microorganisms, the GABRIELA study (A Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community – Advanced Studies) located several hot spots with a protective potential against allergic outcomes (1). In-depth analyses as presented by Ege et al. (2) identified a cluster of bacterial species to be inversely associated with atopy and asthma. Out of this cluster, we selected Gram-positive *Staphylococcus sciuri* W620 for an experimental proof-of-concept to demonstrate that the preceding analytical approach presented by Ege et al. (2) is suitable to identify microbial species with actual allergo-protective properties. Based on protocols initiating an allergic airway inflammation either with ovalbumin (OVA) or house dust mite (HDM), we employed *in vivo* models of murine airway inflammation to prove if *S. sciuri* W620 is capable to prevent experimental asthma under T_H2 - and T_H1/T_H2 -driven conditions. As a Gram-positive bacterium, it may provide protection by pathways different to those described previously for the farm-derived Gram-negative isolate *A. lwoffii* F78 (3, 4). We aimed to characterize cytokine patterns in response to *S. sciuri* W620 *in vivo* and *in vitro* to get first hints on the underlying mechanisms of immune cell activation and maturation.

Methods

Detection, isolation, and propagation of *S. sciuri* W620

For detection of *S. sciuri* W620, PCR-SSCP was performed as described previously (5). In addition, PCR targeting the *mecA* gene as a specific marker was performed according to Couto et al. (6). Further details on *S. sciuri* W620 detection, isolation, and propagation are given in the method section of the online supplement.

Animal experiments

Mouse models of allergic airway inflammation

Female Balb/c mice (6–8 weeks old) were housed in a pathogen-free facility with individually ventilated cages and received OVA- and HDM-free diet. All animal experiments were approved by the local authorities. At least 6–8 animals were used per group. In the OVA model, mice were sensitized to OVA on days 0, 7, and 14 by subcutaneous (s.c.) injections of 10 μ g OVA (grade VI; Sigma, Steinheim, Germany) in 200 μ l PBS. Sham-treated groups received 200 μ l PBS. The sensitization phase was followed on days 26, 27, and 28 by 20-min aerosol applications of 1% OVA or sham PBS, as described previously (7) (Fig. 1A).

In the HDM model, mice were exposed intranasally (i.n.) to 100 μ g *Dermatophagoides pteronyssinus* extract (Allergopharma, Hamburg, Germany) dissolved in 25 μ l PBS on days 0, 7, 14, and 21; control animals received 25 μ l PBS (Fig. 1B).

Treatment with *S. sciuri* W620

Anesthetized mice were treated i.n. with 10^8 colony-forming units (CFU) of lyophilized *S. sciuri* W620 suspended in 25 μ l

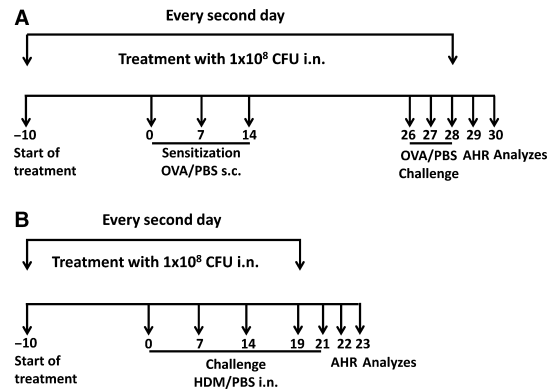


Figure 1 Sensitization and challenge protocols used for murine experimental asthma induction. (A) OVA model with subcutaneous sensitization and aerosol challenge. (B) HDM model with intranasal sensitization and challenge.

PBS in the HDM model and 50 μ l in the OVA model, respectively, or 25 μ l PBS 3 times per week, starting 10 days before the first sensitization and continuing over the whole sensitization and challenge process (Fig. 1A and B).

Characterization of the allergic phenotype

To characterize the allergic phenotype, the following parameters were analyzed: (i) differential cell counts in bronchoalveolar lavage (BAL), (ii) number of mucus-producing Goblet cells and inflammation score in lung histology, (iii) airway hyperresponsiveness to methacholine (Mch), (iv) serum immunoglobulins, (v) cytokines in BAL fluid and in restimulated lymph node cell supernatants after restimulation, and (vi) frequency of CD4⁺ Foxp3⁺ CD25⁺ T regulatory (Treg) cells in lung homogenates. All these methods are described in detail in the online supplement.

In vitro experiments in human cells

Transient transfection of HEK293 cells

HEK293 cells were transiently transfected with various pattern-recognition receptor (PRR) expression plasmids as described before (3). More details are given in the online supplement.

Generation and stimulation of *moDC*

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood of healthy donors as described previously (8). Subsequently, monocytes were isolated by counter-flow elutriation centrifugation (9); monocyte-derived DCs were then prepared as described by Sallusto and Lanzavecchia (10). For further details, see online supplement.

Expression of costimulatory molecules on human *moDC*

After 20 h of incubation with the indicated stimuli, cells were harvested and further analyses were carried out by using FACS analysis, real-time quantitative (RT) PCR, and ELISA. Further details are described in the online supplement.

Co-culture of moDCs with autologous naïve T cells

Co-cultures of moDCs with autologous naïve CD4⁺ T cells were performed as previously described (11). Cytokine protein and mRNA expression was measured as readout for T-cell activation. Further details are given in the online supplement.

Statistical analyses

Data analysis of dust samples was carried out using STATA 10.1 (StataCorp, College Station, TX, USA). Detection by PCR was used as a dichotomous variable split at the detection limit. The arbitrary density units of the SSCP analysis were used as log-transformed variables. Odds ratios (OR) with 95% confidence intervals (CI) were calculated by logistic regression for analyses of associations between SSCP and PCR products with asthma.

Data analyses of animal experiments and human dendritic cell experiments were performed with GraphPad Prism® (GraphPad Software Inc., La Jolla, CA, USA). All numeric data are expressed as means $\pm$ SEM and analyzed for significance by using one-way ANOVA and subsequent Tukey analysis. Differences were considered significant at $P < 0.05$.

Results

Exposure to *S. sciuri* W620 is significantly associated with asthma protection in farm children

In a population-based survey and using PCR-SSCP, we have previously identified several bacteria as candidates for asthma-protective agents (2). These included a cluster containing *Staphylococcaceae*, which was associated with a significantly lower asthma risk (2). This cluster contained among others the species *Staphylococcus sciuri*, which was confirmed by sequencing in triplets. Furthermore, a specific PCR targeting the *mecA* gene, which is considered specific for the species *S. sciuri*, revealed a similarly strong association with asthma protection (OR = 0.55 [0.22–1.34]) although barely missing statistical significance ($P = 0.188$), due to a reduced sample size ($n = 371$). The association between detection of *S. sciuri* by PCR-SSCP was significant (OR = 1.29 [1.03–1.61], $P = 0.025$) thereby cross-validating both methods. *S. sciuri* was detected in significantly higher amounts in mattress dust of farm children compared to nonfarm children (OR = 9.69 [6.04–15.54], $P < 0.001$).

Intranasal exposure to *S. sciuri* W620 in the OVA-induced mouse model of airway inflammation resulted in a decrease of hallmarks of a T_H2-driven asthmatic phenotype

To prove whether *S. sciuri* W620 is able to abolish a T_H2-driven airway inflammation in an experimental setup, we employed a well-established mouse model of experimental asthma based on a subcutaneous OVA-sensitization protocol (Fig. 1A). Mice were treated with *S. sciuri* W620 prior to and during sensitization and challenge. We found total BAL cell counts, eosinophils, and lymphocyte numbers as major hallmarks of airway inflammation to be significantly

decreased in *S. sciuri* W620-treated OVA-sensitized/challenged animals in comparison with the control group. Macrophage and neutrophil numbers were not affected by intranasal exposure to *S. sciuri* W620 (Fig. 2A).

Evaluation of PAS-stained lung sections revealed a significantly lower peribronchial/perivascular inflammation and reduced Goblet cell hyperplasia in animals treated with *S. sciuri* W620 compared to the OVA control group (Fig. 2B). As a functional parameter, airway hyperresponsiveness (AHR) was improved in the *S. sciuri* W620-treated group (Fig. 2C).

Intranasal *S. sciuri* W620 treatment was associated with significantly lower OVA IgE and OVA IgG1 serum levels compared to untreated animals while OVA IgG2a levels did not differ between the OVA and the *S. sciuri*/OVA groups (Fig. 2D).

Furthermore, we analyzed T-cell signature cytokine production in BAL and OVA re-stimulated lymph node cell supernatants. In BAL fluid, we found only IL-5, but not IL-10 and IFN- γ (Fig. 2E) to be significantly reduced following *S. sciuri* W620 exposure, whereas all these cytokine levels were ameliorated in lymph node cell supernatants (Fig. 2F).

Staphylococcus sciuri W620 prevents the asthma phenotype in a murine HDM-induced allergic airway inflammation model

Our data supported an abolishment of selectively T_H2-driven airway inflammation by i.n. treatment with *S. sciuri* W620 in the OVA model. To prove if *S. sciuri* W620 is also capable of preventing a mixed airway inflammation in mice, we employed a house dust mite (HDM) model, which in parallel displays neutrophilic and eosinophilic airway inflammation. According to the protocol given in Fig. 1B, mice were treated with *S. sciuri* W620 prior and during HDM extract application.

We observed a significantly reduced BAL cell numbers in *S. sciuri* W620-exposed allergen-challenged mice as compared to the control group (Fig. 3A, left panel). BAL differential cell counts indicated significantly lower numbers mainly of neutrophils (24 h after last challenge) and eosinophils (48 h after last challenge) (Fig. 3A).

Additionally, there were significantly lower numbers of PAS-positive mucus-producing cells in the airways of *S. sciuri* W620-treated allergen-challenged mice compared with HDM control animals (Fig. 3B).

Even though *S. sciuri* W620 treatment appeared to suppress HDM-induced airway inflammation, we could not observe any significant changes in HDM-specific IgG₁ and IgG_{2a} serum levels as shown in Fig. 3C. HDM-specific IgE serum levels were not detectable in any group.

Similar to the experiments within the OVA model, we analyzed T-cell cytokines in cell supernatants from the draining lymph nodes. To monitor a broad spectrum of T-cell-derived cytokines, we analyzed a panel of T_H1 (IFN- γ ; IL-17A), T_H2 (IL-4, IL-5, IL-9, IL-13), and Treg (IL-10) cytokines in re-stimulated T cells and observed an increase of these cytokines in the sham-treated HDM-administered animals, whereas mice treated with *S. sciuri* W620 were characterized by a significant reduction of all of these cytokines (Fig. 3D).

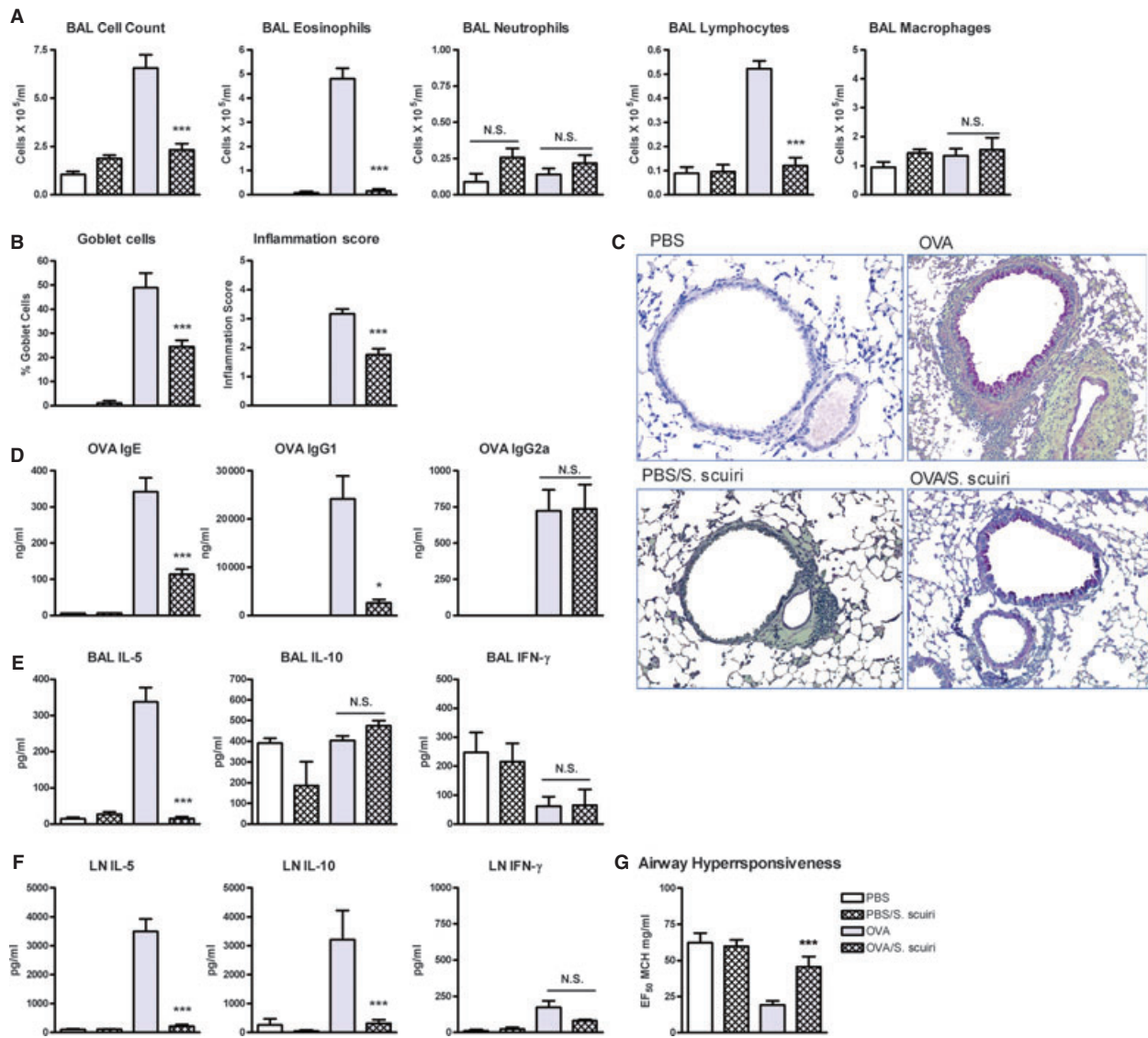


Figure 2 Intranasal application of *S. sciuri* W620 abolishes hallmarks of acute airway inflammation in a murine OVA-induced model of experimental asthma. (A) Total and differential cell counts in the BAL fluid of BALB/c mice subjected to an OVA s.c. adjuvant free (Protocol 1A); (B) Goblet cells and inflammation score in lung tissues; (C) Airway inflammation and mucus producing Goblet cells in PAS-stained histologic lung section. (D) Serum OVA-specific

immunoglobulins IgE, IgG1, and IgG2a; (E) Cytokine production in the BAL fluid measured by ELISA; (F) Cytokine production by lymph node cells after 72 h re-stimulation with OVA measured by ELISA; (G) Airway hyperresponsiveness measured by head-out body plethysmography. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Shown are means + SEM.

As eosinophils and neutrophils were found to be significantly reduced in the BAL fluid of *S. sciuri* W620-treated allergen-challenged mice, we also analyzed whether the chemokines CXCL1 (KC) and eotaxin-1 (CCL11) playing an important role in the recruitment of those cells into the lung are also affected. We observed markedly lower mRNA (data not shown) and protein levels of the two chemokines in *S. sciuri* W620-treated allergen-challenged mice compared to the sham-treated allergen-challenged group as depicted in Fig. 3F.

Microbial components have been proposed to drive suppression of allergic immune responses via induction of coun-

teracting T_H1 as well as Treg cell responses. Therefore, we also measured CD4⁺ Foxp3⁺ CD25⁺ Treg frequencies in lung homogenates. As shown in Fig. 3G, *S. sciuri* W620 treatment did not influence Treg frequencies in the lung in comparison with the sham-treated allergen-challenged group.

S. sciuri W620 activates TLR2 and NOD2 in transfected HEK293 cells

Studies on the activation of innate immune responses might further elucidate mechanisms preceding altered T-cell

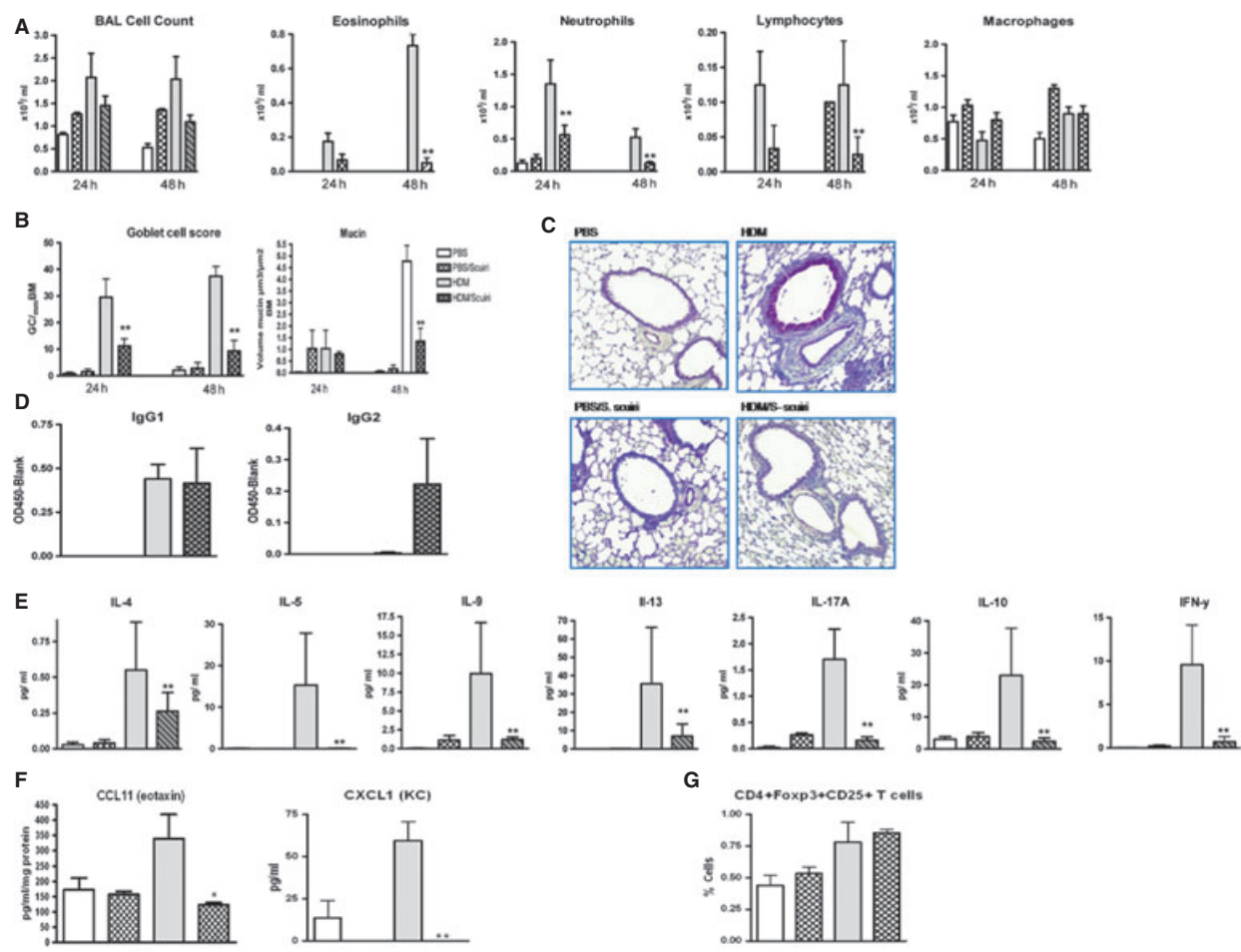


Figure 3 Intranasal application of *S. sciuri* W620 abolishes hallmarks of acute airway inflammation in a murine HDM model of experimental asthma. (A) Total and differential cell counts in the BAL of BALB/c mice subjected to a HDM model of experimental asthma, both 24 and 48 hours after the last challenge; (B) Goblet cells and inflammation score in lung tissues. (C) Airway inflammation and goblet cell producing cells in PAS-stained histologic lung section. (D) Serum HDM-specific immunoglobulins IgG1 and IgG2a.

(E) Cytokine production by lymph node cells after 72 h re-stimulation with HDM. (F) Protein levels of CCL11 (eotaxin) in lung homogenates (48 h after last challenge) and CXCL1 (KC) in BAL (24 h after last challenge). (G) Percentages of CD4 + CD25 + Foxp3 + regulatory T cells as determined by flow cytometry of homogenized lungs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Shown are means + SEM.

responses to *S. sciuri* W620 exposure. To explore *S. sciuri* W620 signaling through specific pattern-recognition receptors (PRR), we employed a well-established assay of transiently transfected HEK cells. Incubation of differentially transfected HEK cells with *S. sciuri* W620 resulted in a dose-dependent induction of Toll-like-receptor (TLR)2 activity comparable to that following TLR2 agonist Pam₃CysSK₄ exposure. In addition, intracellular NOD2 was activated by *S. sciuri* W620. There was no activation of HEK cells through TLR4 and NOD1 (Table 1 and Fig. S1).

Maturation of human moDC is initiated by *S. sciuri* W620

As *S. sciuri* W620 triggered signaling via innate immune receptors, it was next tested whether stimulation of moDC with the bacterium leads to maturation of these cells as indi-

cated by up-regulation of surface expression of co-stimulatory molecules. Incubation of moDC with *S. sciuri* W620 led to up-regulation of CD40, CD86, and CD80. In addition, moDC stimulated with the bacterium showed a higher expression of the antigen-presenting major histocompatibility complex (MHC) class II (Fig. 4).

S. sciuri W620 induces IL-23 but not IL-12p70 release by human dendritic cells due to lack of IL-12p35 mRNA induction

As *S. sciuri* W620 exposure to dendritic cells led to an activation and maturation of moDC, we aimed to analyze pro-inflammatory cytokine expression following bacterial activation by *S. sciuri* W620. We found IL-6, CXCL-8, and TNF- α production to be up-regulated after *S. sciuri* W620

Table 1 Innate immune receptors addressed by *S. sciuri* W620 in a HEK293 cell transfection system

PRR	TLR2	TLR4	NOD1	NOD2
Activation*	+	–	–	+

*CXCL-8-inducing capacity in HEK293 cells transiently transfected with indicated PRR (no activity has been detected with any other TLR). For detailed data, see Fig. S1.

treatment (data not shown). No release of IL-12p70 could be observed, whereas IL-23 was detected. As IL-12p70 is a heterodimeric cytokine consisting of the two subunits p35 and p40, mRNA expression of these subunits was analyzed separately by real-time PCR. Only the mRNA of IL-12p40 was detectable (Fig. 5). *S. sciuri* W620 induced transcription of IL-23p19 mRNA in moDC; hence, both subunits of IL-23, that is, IL23p19 and the IL12p40, were transcribed.

Co-culture of moDC with autologous T cells selectively induced T_H1 cytokine release

We finally investigated the influence of *S. sciuri* W620-exposed moDC on the activation of autologous naïve T cells. As depicted in Table 2, no induction of IL-4 and IL-10 was observed. However, *S. sciuri* W620-exposed moDC were able to induce the release of IFN- γ by T cells in comparable extend to LPS-stimulated moDC.

Discussion

Our proof-of-concept experiments clearly demonstrate that screening of farm-derived dust samples as conducted by Ege et al. (2) is a powerful method to identify bacterial species that harbor such properties. *S. sciuri* W620 is the first asthma-protective Gram-positive isolate from farm-derived dusts that could be identified by this structured approach.

Exemplary for other microorganisms that were found to be associated with asthma protection in these dust sample analyses, *S. sciuri* W620 was investigated in two murine models of acute allergic airway inflammation. Using the OVA model, we emphasized possible preventive effects of *S. sciuri* W620 on the development of a distinctly T_H2-driven allergic inflammation. The HDM model was set up to study asthma prevention under a mixed T_H1/T_H2 condition characterized by an airway inflammation with a paralleled neutrophil and

eosinophil influx into the lung. In both *S. sciuri* W620-exposed asthma phenotypes, a significantly diminished allergic airway inflammation characterized by a clear decrease of the respective inflammatory cells in the lung accompanied by a significant reduction of Goblet cells was achieved by treatment with *S. sciuri* W620 in comparison with sham-treated control animals. Additionally, in the HDM model, chemokines that attract eosinophils and neutrophils such as CCL11 (eotaxin-1) and CXCL1 (KC) were reduced in the lungs of *S. sciuri* W620-treated mice.

Previously, we have described *Acinetobacter lwoffii* F78 as a Gram-negative isolate from dust samples to protect against experimental asthma when applied intranasally in *in vivo* models with pre- and postnatal exposure (3, 4). *In vitro* experiments with *A. lwoffii* F78-exposed human moDCs revealed an activation of IL-12 production and a subsequent polarization of naïve T cells toward a T_H1 phenotype as shown by a strong IFN γ production in autologous naïve T cells co-cultured with *A. lwoffii* F78-exposed moDCs. These effects were mainly driven by *A. lwoffii* F78 LPS indicating that early exposure to Gram-negative bacteria might protect against retardation of immune maturation and persistence of the developing immune system within the T_H2-driven prenatal milieu (11). In analogy to these *A. lwoffii* F78 experiments, treatment of human moDCs with *S. sciuri* W620 also led to a maturation of dendritic cells in this case via TLR2/NOD2-mediated signaling processes. Contrary, no release of IL-12 was observed after incubation of moDCs with the Gram-positive bacterium. mRNA analyses revealed that the IL-12p35 gene was not transcribed in dendritic cells incubated with *S. sciuri* W620 while mRNA transcripts for IL-12p40 as well as IL23p19 were detectable. In consequence, IL-23, a heterodimer assembled of the IL-12p40 and IL-23p19 subunits, could be detected in supernatants from *S. sciuri* W620-exposed cells while production of IL-12 was absent. Actually, it was previously described that binding and signaling through TLR2 may inhibit IL-12 production while IL-23 release was increased (12). IL-12 plays a major role in mounting appropriate immune responses to pathogens and was characterized to have a central role in the development of T_H1 responses (13, 14). Hence, missing IL-12 production might explain incomplete T_H1 maturation and diminished T_H1 cytokine production in animal models. However, *in vitro* co-culture experiments revealed that naïve T cells are instructed to produce IFN- γ possibly due to IL-23 release by moDCs. Whereas it is known that *in vivo* IL-23 preferentially acts on memory

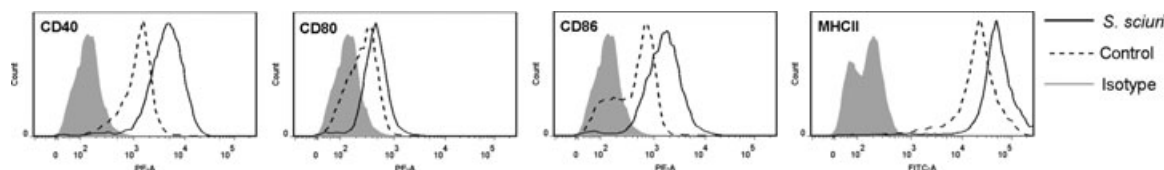


Figure 4 *S. sciuri* W620 induces maturation of moDCs. MoDCs were stimulated with 10^7 cfu/ml *S. sciuri* W620 or left untreated (control). After 20 h, surface expression of CD40, CD80, CD86, and MHCII was analyzed by flow cytometry. Gray filled histograms

show isotype control antibody binding, black dashed line surface expression on untreated control cells, and black lines expression after *S. sciuri* treatment. Representative FACS plots of $n \geq 8$ individual analyses are shown.

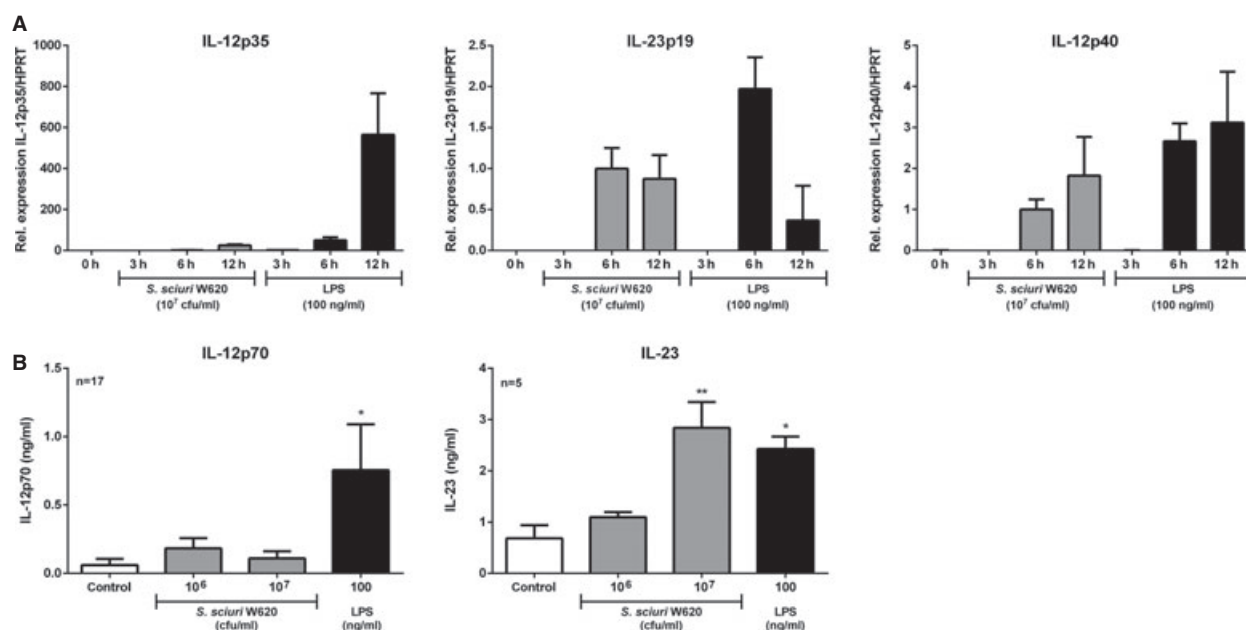


Figure 5 *S. sciuri* W620 induces IL-12p40 and IL-23p19 mRNA in MoDCs but not IL-12p35 and leads to release of IL-23 but not IL-12p70. (A) MoDCs were stimulated with LPS [100 ng/ml] or *S. sciuri* W620 [10^7 cfu/ml] for 0, 3, 6, and 12 h before mRNA was isolated. Expression of IL-12p35, p40, and IL-23p19 mRNA was calculated relative to HPRT expression by real-time PCR. Shown are

results of one of five experiments. (B) MoDCs were left untreated (control) or stimulated with LPS or *S. sciuri* W620 at indicated concentrations for 20 h. IL-12p70 and IL-23 content was measured in the supernatants by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, shown are means + SD.

Table 2 Cytokine production by human moDC/naïve T-cell (TC) co-cultures in absence and presence of *S. sciuri* W620 and LPS (as control)*

	moDCs			moDCs + TC		
	Medium	LPS [100 ng/ml]	<i>S. sciuri</i> W620 [10^7 cfu/ml]	Medium	LPS	<i>S. sciuri</i> W620 [10^7 cfu/ml]
IFN- γ	0.01 $\pm$ 0.01	0.05 $\pm$ 0.03	0.17 $\pm$ 0.11	0.40 $\pm$ 0.12	3.07 $\pm$ 1.45	2.91 $\pm$ 1.07
IL-10	0.02 $\pm$ 0.00	0.01 $\pm$ 0.00	0.00 $\pm$ 0.00	0.02 $\pm$ 0.01	0.16 $\pm$ 0.07	0.10 $\pm$ 0.04
IL-4	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.01 $\pm$ 0.01	0.00 $\pm$ 0.01	0.09 $\pm$ 0.05	0.06 $\pm$ 0.03

*All data are depicted as mean $\pm$ SEM in [ng/ml] of at least 5 independent experiments.

CD4⁺ T cells to produce IFN- γ (15), under *in vitro* conditions this might be different. In line with the *in vitro* studies, in both *in vivo* experiments, no IL-12 production was detectable in lungs and lymph nodes of *S. sciuri* W620-treated animals, whereas IL-23 production tended to be increased without reaching statistical significance (data not shown). Nevertheless, in both *in vivo* models, no increase of IFN- γ could be observed in the BAL and the lung-draining lymph nodes of *S. sciuri* W620-treated animals following local allergen challenge. This implies that *S. sciuri* W620 seems to act *in vivo* via a rather generalized suppression of T-cell activities at least during the effector phase, which is also supported by the observation of missing Treg induction.

Our experiments demonstrated that continuous *S. sciuri* W620 exposure is capable to abrogate inflammatory conditions in the lung. So far, out of Gram-positive bacteria, pro-

biotic lactic acid bacteria (LAB) were shown to provide anti-inflammatory effects mainly in gut (16) but also in the lung when orally given (17, 18). Recently, we demonstrated that intranasal application of *Lactococcus lactis* G121 isolated from farm-derived dust samples abolished the asthmatic phenotype in OVA-sensitized mice (3). Similar to treatment with *S. sciuri* W620, we observed a decrease of lymph node cell-derived cytokine production after application of *L. lactis* G121 to OVA-sensitized mice (H. Harb, H. Garn, P. I. Pfefferle, unpublished data).

Taken together, observations of this and our previous studies suggest that protection against airway inflammation by Gram-positive and Gram-negative bacteria is achieved by different modes of action. The farm effect is mainly based on the microbial diversity found in rural farming sites (1). Combination of different mechanisms of action

might enhance protective effects as signaling through multiple pathways may balance the immune response toward tolerance. The screening system presented by Ege et al. (2) may offer an array of candidates to be analyzed in single-strain experiments for mechanistic pathways and in mixed application for further clinical treatment of allergic diseases.

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Author contributions

SH, HHb, HHe, HR, HG, and PP were involved in the design of the study; ME, EvM, MM, JM, and JB were

involved in epidemiology; SH, HHb, ZM, PP, and HG performed animal experiments; KS and HHe carried out *in vitro* studies; all authors were involved in the preparation of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplement S1. Methods and results

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Online supplement

Farm-derived Gram positive bacterium *Staphylococcus sciuri* W620 prevents asthma phenotype in HDM and OVA-exposed mice

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METHODS

Detection of *S. sciuri* W620 by SSCP

PCR-SSCP was performed as follows: the variable regions 4 and 5 of the 16S ribosomal DNA gene were amplified using universal primers com1 5'-CAGCAGCCGCGGTAATAC-3' and com2Ph 5'-Ph-CCGTCAATTCCTTTGAGTTT-3' as first described by Schwieger and Tebbe ¹. PCR products were purified, digested to single stranded DNA, loaded on SSCP gels and the electrophoresis was done at 400 V for 16 h at 20 °C ². The bands were silver stained and dried at room temperature ³. The arbitrary density units of the SSCP analysis were used as logtransformed variables.

The PCR targeting the *mecA* gene was performed with primers SAMECA358 5'-ATCCATCAATATTGAACCA-3' and SAMECA1482 5'-TATATCTTCACCAACACC-3' ⁴. Each PCR reaction consisted of a total volume of 25 µl containing 0.5 µM of each primer 1.5 mM magnesium chloride, 0.2 mM dNTP and 2.5 Units Taq polymerase (Invitrogen). The PCR thermal cycling conditions were as follows: initial heating for 15 min at 95°C and 50 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C and a final extension for 4 min at 72°C. Specificity was checked by testing *Staphylococcus aureus* ATCC 25923, *Staphylococcus intermedius* DSM 20373, *Staphylococcus epidermidis* tetM1 (own culture collection), *Staphylococcus vitulinus* DSM 15615, *Micrococcus bovicus* DSM 15607, *Micrococcus caseolyticus* DSM 20597, *Jeotgalicoccus halotolerans* DSM 17274, *Jeotgalicoccus psychrophilus* DSM 19085, *Salinococcus roseus* (DSM 5351) and *Salinococcus jeotgali* (DSM 22324). Detection by PCR was used as a dichotomous variable split at the detection limit.

Isolation and propagation of *S. sciuri* W620

S. sciuri W620 was isolated from farm-derived dust by conventional culture techniques (Baird-Parker Agar Base supplemented with 5% egg yolk tellurite enrichment (BD); incubation 24 h at 37°C).

Preliminary identification of *S. sciuri* 620 was based upon microscopic characteristics, positive catalase reaction, positive oxidase test and negative coagulase test. Further identification was carried out by the use of api-Staph (bioMérieux, Germany). The identity was confirmed by PCR-SSCP (as described above). For reference, *S. sciuri* DSM 20345 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig) was analyzed in the same manner.

As the amplicons of the “test-“ and the reference-strain showed the same behavior in the SSCP gel, both bands were carefully excised, amplified with the primers com1 and com2Ph as previously described ², purified with the QIAquick PCR purification kit (Qiagen, Germany) and sequenced (Sequiserve, Germany). The sequence of the “test-organism” was compared with the reference organism by using the BLAST proGram (a genome database of GenBank in the National Center for Biotechnology Information, Bethesda, MD) and showed 100% conformity.

The bacterial strain *S. sciuri* W620 was grown in TSB medium containing 0.3% yeast extract for 5 h at 37°C. For *in vitro* studies, harvested bacteria were killed in PBS containing 1 mg/ml gentamicin (Invitrogen, Carlsbad, USA) for 30 min at 37°C.

Animal studies

Characterization of the allergic phenotype in murine experimental OVA and HDM models

Bronchoalveolar lavage (BAL) and differential cell counts

Mice were terminally anesthetized with ketamine plus rompun[®] for further analyses 48 h after the last challenge. Using a tracheal cannula, BAL was performed one time using 1 ml PBS containing 1x protease inhibitor cocktail (Roche). Total leukocytes were counted with an automated cell counter (Casy TT; Schaefer Systems; Reutlingen, Germany), and cell-free supernatant was stored at -20°C for cytokine measurements. Cytospin preparations were stained with Diff-Quick (Merz & Dade AG, Duding, Switzerland). Macrophages, lymphocytes, eosinophils and neutrophils were identified by standard morphologic criteria, and 100 cells were counted per cytospin.

Lung Histology

Sections of paraformaldehyde (4⁰% wt/vol)-fixed, agarose-embedded lung tissues were cut and stained with periodic acid/Schiff (PAS) as described previously ⁵. PAS-stained sections were viewed and random images collected under 20 × objective. The number of Goblet cells in the bronchial epithelium was quantified and expressed as cell number per mm basement membrane. Inflammation score was assessed as described previously ⁶.

Lung function analysis

Lung function analysis was carried out using the non-invasive method of head-out body-plethysmography. The mid-expiratory airflow (EF₅₀) was measured to assess bronchial responsiveness to increasing concentration of aerosolized β-methacholine (MCh) at 24 hours after the last OVA aerosol challenge as described previously ⁷.

Measurement of OVA-specific and HDM-specific immunoglobulins

Blood samples were taken from the axillary vessels 48h after the last challenge and serum levels of anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a were measured by ELISA, as previously described ⁷.

For detection of HDM-specific IgE, IgG1 and IgG2a, Maxi-Sorp plates (Nunc, Denmark) were coated with 50 µg/ml HDM extract in bicarbonate buffer (pH 9.6) overnight at 4°C. Subsequently, coated wells were blocked with 1% w/V BSA in PBS for 2 h at RT. After washing, serum samples (1:100) were incubated overnight at 4°C, washed and incubated with the corresponding biotin-labeled anti-Ig antibody (BD Pharmingen, San Diego, USA) overnight at 4°C. Plates were washed and incubated with streptavidin-peroxidase (Sigma, Taufkirchen, Germany) for 30 min at RT. After development with substrate (BM Blue POD Substrate Roche, Mannheim, Germany) the reaction was stopped using H₂SO₄ and ODs were read at 450 nm.

Restimulation of lymph node cells

Single-cell suspensions of mediastinal lymph node cells were generated by filtering through a 100 µm cell-strainer, washing and resuspending in RPMI medium with 10% FCS. After counting, lymph node cells were seeded in 96-wells at 2x10⁶/ml and stimulated with or without HDM extract or OVA (50 µg/ml) for 72 h at 37°C. Cell-free supernatants were collected and stored at -20°C until cytokine measurement.

Measurement of cytokines and chemokines

Cell-free supernatants from BAL or re-stimulated lymph node cells from the OVA model were analyzed for IL-5, IL-10 and IFN- γ as described previously ⁵. The ELISA detection limit of each ELISA was 10 pg/ml. The level of different T_H1, T_H2 and Treg cytokines in cell-free supernatants of restimulated lymph nodes from the HDM model were measured by Cytometric Bead Array using a FACSArray (BD Bioscience) as previously described ⁶. Levels of chemokines (CXCL1) and (CCL11) were measured by ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, USA).

Flow cytometry of regulatory T (Treg) cells

The percentages of Treg (CD4+CD25+Foxp3) cells in lung homogenates were determined by FACS analysis. Briefly, lungs were digested with collagenase/DNAse (Roche, Basel, Switzerland) and resulting cell populations were restimulated with PMA/ionomycin/Brefeldin A for 4 h and stained firstly with anti-CD4-V450 (BD, Heidelberg, Germany) and anti-CD25-PE-Cy7 (eBioscience, San Diego, USA). After T cell surface marker staining, cells were fixed with FoxP3-Fixation kit (eBioscience, San Diego, USA) and permeabilized with 0.3 % Saponin followed by intracellular staining with FoxP3-AlexaFluor 700 (Biolegend, San Diego, USA). Every staining included respective negative and isotype controls. Fluorescence signals were acquired by flow cytometry (FACS Aria III, BD, Heidelberg, Germany) and analyzed using FACSDivaTM software. Cells in the lymphocyte gate were used for analysis.

In vitro experiments on human cells

Generation and stimulation of moDC

Monocytes (>90 % purity) were cultured at 10⁶ cells/ml in RPMI (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10 % heat-inactivated fetal calf serum (Biochrom AG,

Berlin, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA), 500 U/ml GM-CSF (ImmunoTools GmbH, Friesoythe; Germany) and 500 U/ml IL-4 (ImmunoTools) exchanging half of the medium including cytokines every 2-3 days. At day 7 cells were harvested and re-cultured in complete medium in 24-well plates at a density of 10^6 cells/ml. Cells were either left untreated or stimulated with 100 ng/ml lipopolysaccharide (LPS) from *Salmonella enterica* sv. Friedenau and the indicated concentrations of *S. sciuri* W620 for 20 h.

Activation of transiently transfected HEK cells

HEK293 cells were plated at a density of 5×10^4 /ml in 96-well plates in DMEM (PAA Laboratories) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS . The following day, cells were transiently transfected using Lipofectamine according to manufacturer's instruction (Invitrogen, Karlsruhe, Germany). The expression plasmid coding for human CD14 was a kind gift of D.T. Golenbock (Worcester, MA, USA), and the Flag-tagged version of human TLR2 was a kind gift from P. Nelson (Seattle, WA, USA). Flag-tagged human TLR4 (P. Nelson) was been further subcloned into pREP9 (Invitrogen). The human MD-2 expression plasmid was a kind gift from K. Miyake (Tokyo, Japan). NOD1- and NOD2-coding pcDNA3 plasmids were a kind gift of Philip Rosenstiel (Kiel, Germany) and further subcloned into the V5 and His-tag containing Gateway-vector pcDNA-DEST40 (Invitrogen). Plasmids were used at 100 ng (10 ng for CD14 and MD-2) per transfection. After 24 h, cells were stimulated for another 18 h and CxCL-8 content of supernatants was quantified by ELISA (Invitrogen) according to the manufacturer's instruction.

Expression of costimulatory molecules on human moDC

The mAb CD40-PE and CD80-PE and IgG1-PE were purchased from Beckman Coulter (Krefeld, Germany), the mAb CD86-PE, MHC-II-FITC as well as IgG2a-FITC were obtained from BD (Becton Dickinson, Franklin Lakes, USA). Cells were analyzed by flow cytometry on a BD LSR II using the FACSDiva software (BD). Data analysis was performed with the FlowJo V10 program (Tree Star, Inc., Ashland, USA).

Co-culture of moDCs with autologous T-cells

Autologous naïve CD4⁺ T cells were isolated from elutriation derived lymphocyte fraction by negative selection using Naive CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, >95 % purity). DCs were washed and plated together with naïve T cells in 96-well plates for cytokine measurements or 6- well for mRNA at a ratio of moDCs to T cells = 1:10. 96-well co-culture was restimulated with 50 ng/ml Phorbol-12-myristate-13-acetate (Merck, Darmstadt, Germany) and 2 µg/ml Ionomycin (Invitrogen) after 5 days for 5 h. Cells for mRNA were harvested after 5 days of co-culture without restimulation. Measurement of IFN γ , IL-4 and IL-10 cytokine levels as well as mRNA isolation was carried out as described below.

Real-time quantitative RT-PCR

Total RNA was extracted from moDC after 0 h, 3 h, 6 h, and 12 h of incubation with the indicated stimuli using RNeasy Mini Kit from Qiagen (Hilden, Germany) and reverse-transcribed into cDNA using Oligo(dT)12-18 and Superscript III Reverse Transcriptase (Invitrogen). Transcripts were quantified by real-time quantitative PCR on Light Cycler 480 with the Light Cycler 480 SYBR Green I Master Mix (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. For each sample, ratio of specific mRNA

abundance was calculated to the amount of hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. Primer sequences are listed in the online supplement.

Measurement of cytokines

Supernatants of moDC were harvested after 20 h of incubation with the indicated stimuli. Cytokine concentrations were analyzed by ELISA specific for human IL-12p70, human IL-23 (both R&D Systems, Inc., Minneapolis, USA) and human CxCL-8 (Invitrogen) according to manufacturer's instructions.

Table S1. Primer sequences for human moDC mRNA expression RT-PCR analyses

	Primer	Primer sequence (5'-3')
1	IL-12p40-for	AAAGGAAGATGGA
2	IL-12p40-rev	TGCTGCTTTTGACA
3	IL-12p35-for	GCTCCAGAAGGCCA
4	IL-12p35-rev	AGGCCAGGCAACTC
5	IL-23p19-for	CAGTGTGGAGATGG
6	IL-23p19-rev	CCGATCCTAGCAGC
7	HPRT-for	GTCAGGCAGTATAA
8	HRPT-rev	GGGCATATCCTACA

RESULTS

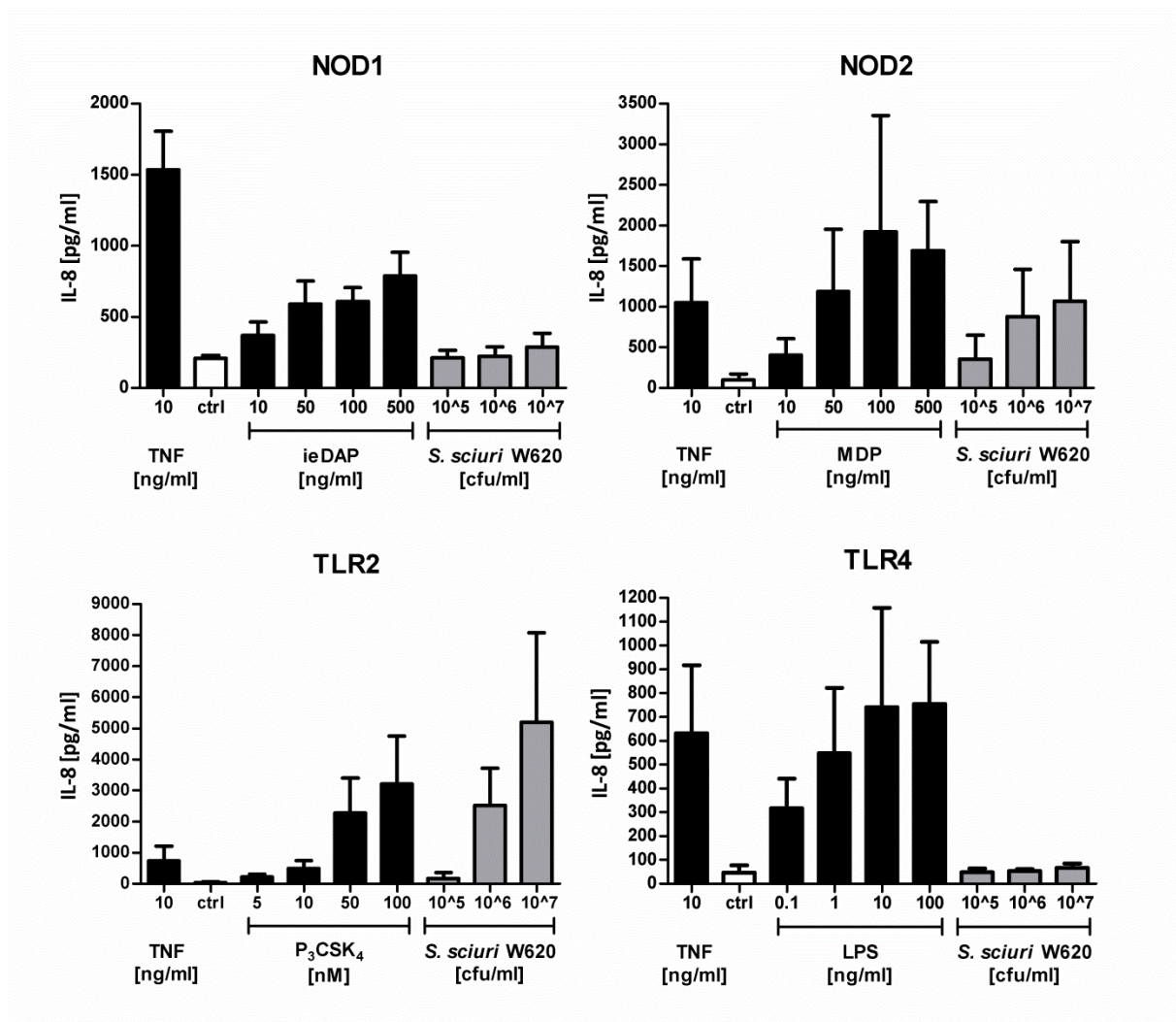


Fig. S1 Innate immune receptors addressed by *S. sciuri* W620 in a HEK293 cell transfection system. CXCL-8-inducing capacity in HEK293 cells transiently transfected with indicated PRR (no activity has been detected with any other TLR).

