# The hygiene hypothesis in autoimmunity: the role of pathogens and commensals

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Abstract | The incidence of autoimmune diseases has been steadily rising. Concomitantly, the incidence of most infectious diseases has declined. This observation gave rise to the hygiene hypothesis, which postulates that a reduction in the frequency of infections contributes directly to the increase in the frequency of autoimmune and allergic diseases. This hypothesis is supported by robust epidemiological data, but the underlying mechanisms are unclear. Pathogens are known to be important, as autoimmune disease is prevented in various experimental models by infection with different bacteria, viruses and parasites. Gut commensal bacteria also play an important role: dysbiosis of the gut flora is observed in patients with autoimmune diseases, although the causal relationship with the occurrence of autoimmune diseases has not been established.
Both pathogens and commensals act by stimulating immunoregulatory pathways. Here, I discuss the importance of innate immune receptors, in particular Toll-like receptors, in mediating the protective effect of pathogens and commensals on autoimmunity.

Atopy
A genetic predisposition to the cumulative development of common allergies, for example, atopic dermatitis and allergic asthma. Atopy involves phenomena of cutaneous or general hypersensitivity to allergens.

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The hypothesis that infections can protect against atopy was first formulated by Strachan in 1989. It was based on the finding that a higher frequency of allergic rhinitis and atopic dermatitis was observed in first-born children compared with siblings born later[1,2] and assumed that first-born children are less frequently exposed to common infections than their siblings. The hypothesis was confirmed by epidemiological studies and was extended to other allergic diseases[3]. The term hygiene hypothesis was coined in 2000 and is understood not as a problem of individual behavioural hygiene but rather of the broad environmental infectious burden. In parallel, various authors have shown in experimental models that the occurrence of autoimmune disease is prevented by infection with distinct pathogens[4–9] (BOX 1). Furthermore, it was shown that non-obese diabetic (NOD) mice developed spontaneous autoimmune diabetes at high incidence only if bred in a specific-pathogen-free (SPF) environment. The occurrence of various infections could prevent the onset of disease[10] (BOX 1). On the basis of these observations and other epidemiological and experimental data, it was proposed that the hygiene hypothesis for allergy should be extended to autoimmune diseases[10,11].

The hypothesis was initially based on the fact that the decrease in overall infection frequency was negatively correlated with the substantial increase in the frequency of allergic and autoimmune diseases observed in industrialized countries over the past 40 years. Robust epidemiological data supported the correlation, but the underlying mechanisms and the issue of causality remained unclear. Moreover, with the recent emergence of metagenomics, it has become possible to determine the composition of commensal gut-resident bacteria, which has led to studies on the role of the gut microbiota in the hygiene hypothesis.

In this Review, I present the epidemiological and experimental data that shed light on potential underlying mechanisms of the hygiene hypothesis. The focus is on animal models and clinical autoimmune diseases, mainly insulin-dependent diabetes mellitus (IDDM or type 1 diabetes), multiple sclerosis and systemic lupus erythematosus (SLE), for which a wealth of data is available. I also present experimental data gathered in allergic and other immune-mediated diseases that are of value in elucidating the mechanisms that underlie the hygiene hypothesis.

## Overview of the epidemiological data

Over the past few decades, the frequency of allergic and autoimmune diseases has increased considerably in industrialized countries, whereas the incidence of major infectious and parasitic diseases has decreased[10]. By contrast, the frequency of common seasonal childhood

**Box 1 | Infectious agents (and their derivatives) that protect NOD mice from IDDM**

Here, we mostly concentrate on the data concerning the NOD mouse, as this is the model for which a very large amount of data is available.

**Bacteria**
*Mycobacterium avium*[145]
*Mycobacterium bovis*[146]
*Mycobacterium bovis* (BCG)[85]
*Mycobacterium tuberculosis* (CFA)[84]
*Salmonella enterica* subsp. *enterica* serovar Typhimurium[147]
Gram-positive bacterial lysate (OM85)[39]

**Viruses**
Coxsackievirus B3 (REFS 148,149)
Coxsackievirus B4 (REFS 150,151)
Encephalomyocarditis virus[152]
Lymphocytic choriomeningitis virus[9]
Lactate dehydrogenase-elevating virus[153]
Mouse hepatitis virus[154]
Murine gammaherpesvirus 68 (REF. 155)

**Parasites**
*Fasciola hepatica*[109]
Filaria (*Litomosoides sigmodontis*)[107,108]
*Heligmosomoides polygyrus*[104,156,157]
*Schistosoma mansoni*[100,101]

BCG, Bacillus Calmette–Guérin; CFA, complete Freund's adjuvant; IDDM, insulin-dependent diabetes mellitus; NOD, non-obese diabetic.

infections remains unchanged. The frequency of allergic diseases (for example, asthma, atopic dermatitis and food allergy) continues to increase[12,13]. The frequency of IDDM is also steadily increasing[14] and affecting children at a younger age[15]. Though the number of studies that have focused on multiple sclerosis is more limited, the trend for this disease is similar when considering the examples of Norway and Japan[16–19].

Until recently, lower-income countries exhibited a high frequency of infectious diseases and a low frequency of autoimmune and allergic diseases. This pattern is evolving, and some rapidly developing countries (for example, China) are now starting to show the same trends as industrialized countries[20]. The parallel trend of the incidence of immune-related diseases is interesting, as it argues for a common epidemiological basis. However, aetiological factors unique to each disease (for example, a virus) cannot be excluded.

The existence of a north–south gradient in Europe, with a higher prevalence of autoimmune diseases in the north, was first reported in the early 2000s[10,11] and persists today. This gradient can be extended to the whole world, excluding some low-income countries with no available disease registries. The geographical distribution of IDDM and multiple sclerosis is the mirror image of that of tuberculosis, hepatitis A and traveller's diarrhoea (FIG. 1). This is clear when comparing Europe and Africa and even countries within Europe for certain diseases[10]. These differences may be explained by various factors, for example, climate and sun exposure, which may alter vitamin D levels[21], and importantly, the socio-economic differences between high-incidence and low-incidence areas[10] (FIG. 1), which have been well documented in allergic diseases[22] and IDDM[23]. These findings are relevant, as a low socio-economic level is often associated with poorer sanitary conditions.

Important studies were carried out in Karelia, a Russian republic located close to the Finnish–Russian border. The Finnish and Karelian populations belong to the same ethnic group and live in the same climate, yet have different social and economic environments[24] and show wide differences in the frequency of allergic and autoimmune diseases. Allergic asthma and IDDM are considerably less frequent in Karelian children than in Finnish children[25,26]. Concerning IDDM, the occurrence of anti-islet β-cell autoantibodies is similar in the two countries[27], but the frequency of overt disease (hyperglycaemia) differs. This observation suggests that the protective effective underlying the hygiene hypothesis exerts itself by preventing the progression of disease. These data also suggest that the role of climate is minimal, due to the similarities between the two countries.

Compelling results were also seen in studies of migrants that had moved from countries with low incidence of allergic and autoimmune diseases (Pakistan and Bangladesh) to countries with high incidence (the United Kingdom). The frequency of allergic asthma[28], IDDM[29,30] and multiple sclerosis[31,32] was as high in the descendants of these migrants as in the host country population. This finding suggests that genetic factors do not contribute to the difference in disease frequencies between countries. Interestingly, the protective effect of the original environment does not manifest itself below a certain age threshold. Moving after birth to a country with a high incidence of allergic asthma or multiple sclerosis only results in a high incidence of said disease in migrant children if they moved before reaching a specific age (5 years for asthma[28], 15 years for multiple sclerosis[31,32]). This threshold should be considered when discussing the role of the gut microbiota, which is fixed in composition in humans at age three[33]. These migrant studies were not initially undertaken to investigate the hygiene hypothesis. Rather, it was thought that young migrants might contract an infectious agent that caused the allergic and autoimmune diseases studied. Below, we discuss why this assumption is improbable.

A direct link between the total number of prior infections and the occurrence of allergic and autoimmune diseases has been hard to establish from epidemiological studies based on questionnaires, potentially because it is difficult for patients (or their parents) to recall past infections. This difficulty explains the interest in indirect markers of hygiene. For example, it has been shown that asthma is less frequent in children enrolled in day care, where they are exposed to infections, than in children kept at home[34]; thus, day care could be considered an indirect marker. However, this observation was not made for IDDM and multiple sclerosis. For asthma[2] and IDDM[35], more robust data have been gathered from other indirect indicators, such as birth order and social and economic indicators.

It is difficult to draw conclusions about the role of vaccinations in the development of autoimmune diseases. The prevention of infections by vaccination is associated with the overall reduction of infectious

Hygiene hypothesis
A hypothesis that postulates that an increased frequency of infections contributes to a decrease in autoimmune and allergic diseases.

Non-obese diabetic (NOD) mice
An inbred mouse line that spontaneously develops an autoimmune syndrome including insulin-dependent diabetes mellitus (IDDM or type 1 diabetes).

Traveller's diarrhoea
A digestive tract disorder provoked by eating contaminated food or drinking contaminated water. In the context of our discussion, it is a self-limited pathology that illustrates the presence of a basic health environment.

Anti-islet β-cell autoantibodies
Autoantibodies to various β-cell-specific autoantigens that are markers of the destruction of insulin-producing β-cells, which is the hallmark of insulin-dependent diabetes mellitus (IDDM or type 1 diabetes).

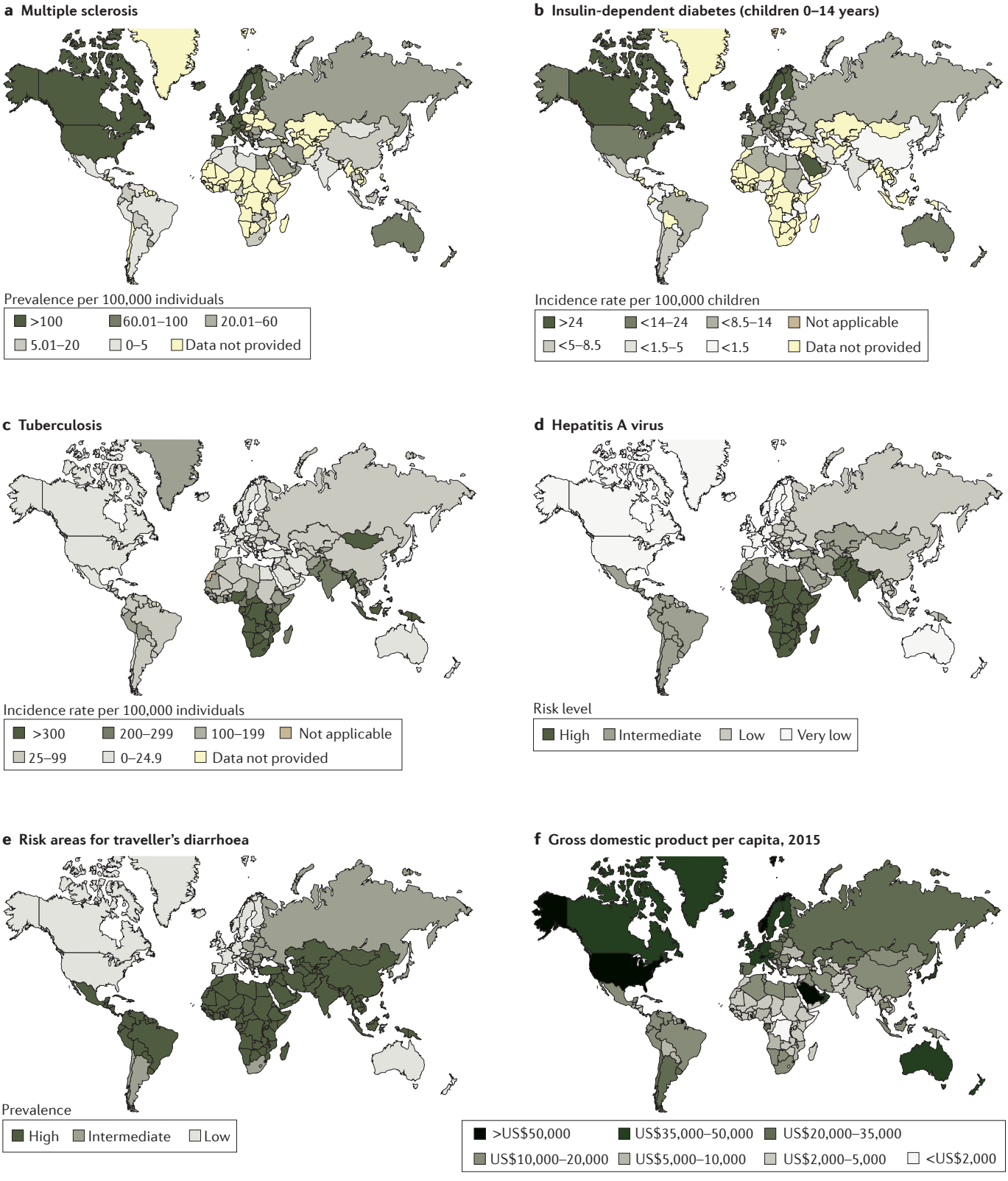


Figure 1 | **The geographical distribution of autoimmune disease, infectious disease and wealth.** This figure shows the geographical distribution of two prototypic autoimmune diseases (multiple sclerosis, panel **a** (data from REF. 198) and insulin-dependent diabetes, panel **b** (data from REF. 165)), three infectious diseases (tuberculosis, panel **c** (data from REF. 199); hepatitis A virus, panel **d** (data from REF. 200); and traveller's diarrhoea, panel **e** (data from REF. 201)) and wealth (gross domestic product, panel **f** (data from REF. 202)). The increased frequencies of autoimmune disease in industrialized countries that have lower frequencies of infectious disease provides evidence of the protective effect of infection. This supports the importance of the hygiene hypothesis in the development of autoimmune disease.

diseases and thus may contribute to the increase in frequency of autoimmune diseases. However, this potential contribution is difficult to prove because in most countries, many individuals in the population are vaccinated.

The considerable difference in the frequency of parasitic diseases between most countries in the north and south must also be stressed. Parasites have a strong capacity to inhibit the occurrence of allergic and autoimmune diseases in experimental models and in humans, which explains the negative effect of anti-parasitic treatments on the occurrence of atopy[36].

## Evidence for a causal relationship

To demonstrate a causal relationship between decreases in infections and increases in autoimmune diseases, experimental evidence is essential, as in experimental animal models, the effect of infection on the occurrence of disease can be directly assessed. This approach is very difficult in humans. There is one report observing that infestation with the porcine parasite *Trichuris suis* improved the clinical condition of patients with multiple sclerosis[37]. However, this observation awaits confirmation, and there are obvious ethical concerns regarding testing this effect experimentally.

Much more convincing is the well-documented demonstration that NOD mice (BOX 1), which spontaneously develop experimental IDDM, and F1 hybrid New Zealand mice (NZB x NZW) mice that spontaneously develop SLE are completely protected from spontaneous autoimmune disease development after they have been infected with various bacteria, viruses or parasites. These two well-studied models of spontaneous autoimmunity are prototypic of the two major categories of human autoimmune diseases: organ-specific (NOD mice) and non-organ-specific ((NZB x NZW) F1 mice).

Strikingly, >90% of female NOD mice and ~20–40% of male NOD mice develop spontaneous IDDM when raised in SPF sanitary conditions, but the frequency of diabetes decreases considerably when the mice are not raised under SPF conditions ('conventional' conditions). However, when the offspring of these mice are decontaminated (delivery by hysterectomy in isolators), the incidence of disease returns to 90% in female pups, starting from the first generation after decontamination[10]. When these 'clean' mice are then infected with a pathogen (BOX 1), IDDM development is completely prevented. Similarly, infection of (NZB x NZW) F1 mice with *Plasmodium berghei* prevents SLE-associated nephritis and prolongs survival[4]. The development of SLE in these mice is also prevented by infection with murine gammaherpesvirus 68 (REF. 38).

Interestingly, with the exception of some intestinal parasites, many of the infections caused by the pathogens listed in BOX 1 have no obvious relationship with the gut. In addition, the protective effect of certain bacteria[39] and parasites[40,41] is observed with pathogen-derived extracts or molecules. Thus, it appears that the protective effect of infection does not require a live infectious agent.

## The role of the gut microbiota

As mentioned above, the availability of metagenomics to analyse the diversity in the gut microbiome provided the incentive to encompass commensals within the framework of the hygiene hypothesis. Many studies have focused on analysing the composition of the gut microbiota in patients presenting with autoimmune disease. In patients with various autoimmune conditions, a reduction in gut microbiota diversity (dysbiosis) was observed (TABLE 1). In most studies, patients presented with an established disease, which did not allow researchers to explore the sequence of events in early disease and determine whether variations in the microbiota preceded disease manifestations. To answer this central question for IDDM, longitudinal studies were performed, in which the siblings of patients with diabetes were tested for anti-islet β-cell autoantibodies. The results show that the decline in gut microbiota diversity is first detected just before the advent of overt disease (hyperglycaemia) but well after the appearance of autoantibodies (seroconversion)[33].

The problem of proving a causal relationship therefore remains. Currently, it is not possible to tell if the observed changes in the microbiota are caused by the metabolic imbalance that is characteristic of clinical IDDM or by the inflammation within the target tissue. The metabolome related to the gut microbiota may also be important. At present, only a few studies have addressed the metabolome, and these are based on analysis of the microbiota in humans and mice[33,42].

Populations with different lifestyles have major differences in the composition of their gut microbiota, which could be associated with differences in hygiene and infectious burden. In support of this hypothesis, microbiota differences were observed when comparing the populations of industrialized and developing countries, for example, Northern Italy and Burkina Faso[43] or Bangladesh and the United States[44]. However, caution is needed in interpreting these data, as many factors in addition to hygiene differentiate these populations, particularly diet, which is well known to affect gut microbiota composition[45]. Important data were obtained in piglets reared in different sanitary environments. Piglets delivered and housed in a 'clean' environment showed a substantial difference in the composition of the gut microbiota compared with piglets reared under conventional conditions[46]. Relevant here is the case of Finland and Karelia, as important variations in the gut microbiota in populations from these countries mirror the differences in the frequency of autoimmune IDDM[47].

Experimental models allow us to directly investigate the potential causality between the diversity of the gut microbiota and autoimmune diseases. The elimination of the gut microbiome (achieved in germ-free mice or by treatment with broad-spectrum antibiotics) provided clearcut results in NOD mice. In both germ-free and antibiotic-treated mice, the frequency of IDDM increased considerably when compared with that of SPF mice[48–51]. However, it is important to recall, first, that a similar increase in disease frequency is obtained in SPF mice compared with mice housed under conventional

Dysbiosis
An imbalance of the microbial flora that most frequently affects the digestive tract. Dysbiosis can also be detected in other 'barrier' organs such as the skin, the lungs or the vagina.

Metabolome
The metabolome consists of all signalling molecules (for example, metabolites and hormones) detected in a biological sample. The metabolome thus defines a given physiological or pathological state and is therefore dynamic.

Germ-free mice
Mice born by hysterectomy under sterile conditions and raised in isolators to guarantee an environment totally devoid of pathogenic and commensal germs.

Table 1 | **The composition of the gut microbiota in human autoimmune diseases**

| Disease | Microbiota analyses | Presence of microbiota dysbiosis | Patients (*n*) | Clinical readout | Refs |
|---|---|---|---|---|---|
| Autoimmune diabetes | 16S rRNA | Yes | 4 | β-cell autoAbs* and clinical disease | 166 |
| | Shotgun metagenomic sequencing | Yes | 4 | Clinical disease | 167 |
| | 16S rRNA | Yes | 18 | β-cell autoAbs | 168 |
| | 16S rRNA | Yes | 16 | Clinical disease | 169 |
| | Quantitative cultures‡ | Yes | 35 | Clinical disease | 170 |
| | 16S rRNA | Yes | 29 | β-cell autoAbs | 171 |
| | 16S rRNA | No | 22 | β-cell autoAbs | 172 |
| | 16S rRNA | Yes | 21 | Clinical disease | 173 |
| | 16S rRNA | Yes | 35 | β-cell autoAbs and clinical disease | 174 |
| | 16S rRNA and Shotgun metagenomic sequencing | Yes | 11 | β-cell autoAbs and clinical disease | 33 |
| | 16S rRNA | Yes | 15 | Clinical disease | 175 |
| | 16S rRNA | Yes | 10 | β-cell autoAbs | 176 |
| | 16S rRNA and Shotgun metagenomic sequencing | Yes | 199 | β-cell autoAbs | 47 |
| | 16S rRNA | No | 10 | Long-standing clinical disease | 177 |
| Multiple sclerosis | 16S rRNA | Yes | 20 | Clinical disease | 178 |
| | 16S rRNA | Yes | 18 | Clinical disease | 179 |
| | 16S rRNA | Yes | 31 | Clinical disease | 180 |
| Systemic lupus erythematosus | 16S rRNA | Yes | 20 | Clinical disease | 181 |

*Presence of circulating autoantibodies (autoAbs) specific to β-cell antigens. ‡Faecal flora analysis was done using quantitative cultures on selective and non-selective media and under thermal and atmospheric conditions for bacterial and fungal growth. rRNA, ribosomal RNA.

conditions[10], and second, that the elimination of the gut microbiota is a substantial manipulation that may impact the immune system in an artificial way. Remarkably, despite the elimination of the gut microbiota, the immune system is still capable of mounting a vigorous autoimmune response, which leads to IDDM.

By contrast, experimental autoimmune encephalomyelitis (EAE) was more difficult to induce in germ-free mice[52,53]. These data are in keeping with the pioneering work of Goverman *et al.*, who showed that transgenic mice that express a T cell receptor specific for myelin basic protein (MBP) developed spontaneous EAE when housed in a conventional facility but not in an SPF facility[54]. This development was suggested to be the result of the requirement for colonization by commensals for the differentiation of T helper 17 ($T_H17$) cells, which have an established pathogenic role in EAE. In support of this hypothesis, gnotobiotic mice monocolonized with segmental filamentous bacteria (SFB), which promote differentiation of $T_H17$ cells in the gut lamina propria, developed EAE after disease induction to a similar extent as mice housed under conventional conditions[53,55,56]. Another interpretation has emerged, with recent data showing the importance of the inflammasome, which is differentially activated in EAE models depending on the adjuvant used[57,58] (BOX 2). Importantly, these results suggest a mechanism behind the apparently discrepant role of microorganisms in spontaneous experimental IDDM (adjuvant-free) compared with EAE (induced by administration of autoantigen and adjuvant). This may also explain why clinical multiple sclerosis follows the hygiene hypothesis trend (as does clinical IDDM) and does not replicate the results seen in EAE models. The presence of disease in these models depends on the degree of 'danger signal' (adjuvant) needed to activate the inflammasome.

Another approach to prove a causal relationship between hygiene and autoimmune diseases is to attempt to prevent the development of disease by administering commensal bacteria before disease onset. Convincing experimental results have been reported in NOD mice and in EAE models through the use of various probiotics[59–62]. In both cases, the administration of the probiotic mixtures before the onset of the disease almost completely prevented disease manifestations. This remarkable effect remains poorly understood. Probiotics may act by altering the composition of the gut microbiota, an effect that has not been clearly demonstrated so far, or alternatively by an intrinsic pharmacological effect.

More compelling evidence would be provided by the use of microbiota faecal transplant to transfer the predisposition to autoimmunity. However, this is difficult to achieve because it is hard to find suitable 'recipient' mouse strains with adequate controls. Interestingly, experiments have demonstrated that a degree of diabetes resistance can be conferred by transplanting the gut microbiota from NOD male mice into NOD female mice[63]. However, these data await full confirmation.

Experimental autoimmune encephalomyelitis
(EAE). A demyelinating allergic encephalomyelitis produced by the injection of brain tissue or purified proteins of the nervous system or their derived peptides in the presence of an adjuvant.

Gnotobiotic mice
Germ-free mice whose intestinal microflora is reconstituted by a single commensal bacterium (monocolonized mice).

Probiotics
Gut commensal bacteria available as single or combined species delivered orally and putatively endowed with a health benefit.

Box 2 | **Inflammasome modulation by pathogens and commensals to induce or promote immune regulation**

- Inflammasomes are a set of large multimolecular complexes that form in the cytosol and are composed of a sensor protein (NOD-, LRR- and pyrin domain-containing 1 (NLRP1), NLRP3, NLRC4, absent in melanoma 2 (AIM2) and pyrin) that is activated by pathogen-associated molecular patterns and damage-associated molecular patterns, an adaptor molecule (the adaptor protein ASC) and a catalytic protein (pro-caspase 1). Once the sensor protein is activated, the oligomer induces self-cleavage of pro-caspase 1 into active caspase 1, which in turn cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, which are released from the cytoplasm through pyroptosis (inflammatory cell death)[158].
- Inflammasomes were initially described in cells of the innate immune system, such as macrophages and dendritic cells, but are now also recognized in endothelial, glial and neuronal cells, which explains the growing interest in the field of neurodegenerative and autoimmune diseases of the central nervous system (CNS) (for example, multiple sclerosis and experimental autoimmune encephalomyelitis (EAE))[57]. Essential components of the inflammasome are critical for the development of EAE: mice deficient in NLRP3 are protected from disease, and only mild disease is observed in mice deficient in ASC and caspase 1 (REF. 57). It is well established that activation of NLRP3 drives the migration of inflammatory cells to the CNS[159,160]. It is also highly relevant that IFNβ, a first-line therapy in multiple sclerosis (also used in EAE) for which the mode of action had remained hitherto obscure, is an inhibitor of NLRP3 inflammasome activation[159,160]. Forms of EAE (induced using high doses of adjuvant or an acute infection) that are resistant to IFNβ have been observed: these forms are NLRP3 independent[57]. Recent observations also suggest that pertussis toxin, which is frequently used to enhance EAE, activates the pyrin inflammasome[58].
- A pathophysiological role for inflammasome activation is also proposed in rheumatoid arthritis and inflammatory bowel disease[161,162].
- The link between the hygiene hypothesis and inflammasomes becomes evident when one consults the impressive list of molecules produced by many bacteria or viruses that inhibit the assembly and/or signalling of the inflammasome (reviewed in REF. 163).
- A synthetic analogue of a derivative of the parasitic filarial nematode *Acanthocheilonema viteae* downmodulates the activity of the inflammasome and effectively protects against collagen-induced arthritis[164].
- IFNβ is cited above as an inhibitor of the inflammasome, but in general, type I and type II interferons possess this same inhibitory activity. This implies that inhibition of the inflammasome may result from stimulation with Toll-like receptor ligands (for example, poly I:C or CpG oligodeoxynucleotides) that induce the production of type I interferons.

Faecal transplant experiments have also been performed using immunocompromised NOD mice that were genetically deficient for MYD88, an adaptor protein downstream of Toll-like receptor (TLR) signalling. These mice developed IDDM when raised under germ-free conditions, but not when raised under SPF conditions[64]. The composition of the gut microbiota was substantially different in *Myd88*$^{-/-}$ mice compared with wild-type NOD mice raised under SPF conditions, and transplantation of the gut microbiota of *Myd88*$^{-/-}$ NOD mice protected wild-type NOD mice from disease[65]. These experiments provide the first robust evidence that the microbiota plays a role in controlling IDDM.

Several indirect observations indicate that the intestinal microbiota also plays a part in the genesis of human autoimmune diseases. First, children born by caesarian section, who are not exposed to vaginal commensal bacteria, have an increased incidence of IDDM and multiple sclerosis[66–68]. The increase is not large and is not present in all studies, but it is supported by a meta-analysis[66]. Second, the administration of broad-spectrum oral antibiotics in early childhood appears to moderately increase the incidence of IDDM and multiple sclerosis[69–74]. Last, preliminary data show that the administration of probiotics can prevent the appearance of anti-β-cell autoantibodies, but only in children with a high familial risk of autoimmune IDDM[75,76]. There are more data published on the clinical use of probiotics in allergy, including a recent meta-analysis that shows the beneficial effect of probiotic supplementation during pregnancy and infancy on atopic dermatitis[77].

Antigenic competition
The competition for recognition of the cognate antigen for soluble factors (cytokines) driving the proliferation and differentiation of antigen-specific lymphocytes.

## The hygiene hypothesis: mechanisms

According to the hygiene hypothesis, it is assumed that the reduction in the frequency of infections contributes directly to the increase in the frequency of autoimmune diseases. However, we must address the mechanisms whereby infections mediate this protective role. There are many different infectious diseases and commensal organisms. It is conceivable, therefore, that different mechanisms may underlie the relationship between infection and autoimmunity, as well as the role of intestinal commensal bacteria, in the development of autoimmunity.

Pathogens are associated with disease and effector immune responses, whereas commensals are not. Pathogens and commensals may therefore influence the development of autoimmune conditions in different ways, but there may also be common pathways. Here, I first discuss the role of anti-pathogen immune responses in the control of lymphocyte homeostasis. I then discuss the effect of microorganisms on immune regulation, which can apply to both pathogens (including parasites) and commensals. This includes the stimulation of TLRs, which may represent a common mechanism for the protective effect of pathogens and commensals on autoimmunity.

### *Homeostasis*

One potential mechanism that underlies the hygiene hypothesis is antigenic competition. Antigenic competition has been known to lead to reduced immune responses to a first antigen when a distinct second antigen is administered concomitantly or few days

after the first[78,79]. One mechanism that may explain antigenic competition is the variation in the capacity of antigen-presenting cells to display peptides derived from the processing of the first or the second (the 'competing') antigen[80,81]. Antigenic competition has been observed for immune responses towards weak antigens (for example, autoantigens) when these are administered concomitantly or shortly after a stronger antigen (for example, an antigen derived from an infectious agent), demonstrating the relevance for the hygiene hypothesis. Competition for homeostatic factors, namely, cytokines such as IL-2, IL-7 and IL-15, is currently the focus of much attention. The importance of cytokines in lymphocyte reconstitution after depletion is well known[82,83]. To extend these data to the hygiene hypothesis, it would be essential to know if the proliferation and differentiation of self-reactive lymphocyte clones is diminished by a strong concomitant anti-infectious response.

### *Immune regulation by pathogens*

Numerous studies have focused on the mechanisms that mediate the protective effect of infections, or of the derivatives of infectious agents, on experimental autoimmune diseases. The results are concordant in showing that the protective effect mainly involves immune regulatory pathways, which include specialized immune cell subsets and key cytokines (FIG. 2). The majority of these studies have been performed in the NOD mouse model of IDDM.

Experimental approaches to examine the immune regulatory cells and pathways that may be modulated by pathogens include measuring the frequency of adaptive or innate-like regulatory lymphocyte subsets (in particular, $CD4^+CD25^+FOXP3^+$ regulatory T ($T_{reg}$) cells, cytokine-producing natural killer (NK) cells and invariant NKT (iNKT) cells), assessing the functional capacity of these cells by adoptive transfer to uninfected recipients, and measuring the production of cytokines such as IL-4, IL-10 and transforming growth factor-β (TGFβ). The elimination of specific regulatory cell subsets or cytokines has also been carried out.

There is compelling evidence to show that specific pathogens exert their protective effect through unique modes of action. For example, complete Freund's adjuvant (CFA) or infection with Bacillus Calmette–Guérin (BCG) protects NOD mice from disease, even when administered at an advanced stage of IDDM (up to 10 weeks of age)[84,85]. It has been proposed that activation of NK cells[86] or the stimulation of $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells may underlie this effect[87]. Moreover, experiments have indicated a key role for IFNγ in the protective effect that CFA exerts on IDDM. For example, it has been shown that NOD mice deficient for IFNγ are resistant to the protective effects of CFA and BCG and develop disease at similar rates to wild-type mice, whereas NOD mice deficient for IL-4 or IL-10 production do not[88,89]. These results are counterintuitive, as IDDM is a $T_H1$ cell-mediated disease and IFNγ, which is a $T_H1$ cell-polarizing cytokine, would have been expected to worsen the disease.

Another important example is that of *Escherichia coli*-derived lipopolysaccharide (LPS), a TLR4 agonist, which exerts a protective effect in wild-type NOD mice but not in CD28-deficient NOD mice; these mice are deprived of thymus-derived $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells and are not protected from IDDM[59,90]. Furthermore, $CD4^+CD25^+$ $T_{reg}$ cells from LPS-treated NOD mice can adoptively transfer IDDM protection to untreated syngeneic recipients[91]. Notably, Kaufman and colleagues were the first to show that the adoptive transfer of LPS-activated B cells from NOD mice protected from IDDM *in vivo*[92]. Since then, the concept has emerged of regulatory B ($B_{reg}$) cells that produce IL-10 and effectively suppress autoimmune responses[93–95]. More refined phenotypic and functional characterization of $B_{reg}$ cells is rapidly progressing. Recent data have also described a regulatory role for a subset of plasmocytes that produce IL-10 and IL-35 (REFS 96,97). Importantly, the cytokine-producing ability of these cells, which is rapid and independent of B cell receptor stimulation, may be induced by many infectious agents, including *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Listeria monocytogenes* and *Schistosoma mansoni*[98]. All of these elements highlight the importance of analysing the involvement of $B_{reg}$ cells and regulatory plasmocytes in the context of the hygiene hypothesis.

The administration of the bacterial extract OM-85 (derived from Gram-positive bacteria such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus viridans* and *Moraxella catarrhalis*) prevents the development of IDDM in NOD mice but loses its effect when TGFβ, but not IL-10, is neutralized[39].

Virus infection can also have a protective effect. For example, newborn or young prediabetic NOD mice infected with lymphocytic choriomeningitis virus (LCMV) or with coxsackievirus B3 are fully protected from developing IDDM[8,99]. Virus infection induced the expression of programmed cell death 1 ligand 1 (PDL1) on lymphoid cells, which prevented the expansion of a set of diabetogenic $CD8^+$ T cells expressing programmed cell death protein 1 (PD1) and increased the frequency of TGFβ-producing $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells[99].

Regarding the protective effect of parasites, it has been shown that *S. mansoni* infection in NOD mice prevents IDDM and that this was associated with the production of IL-10 (REF. 100) and the stimulation of iNKT cells[101]. Furthermore, the helminth *Heligmosomoides polygyrus,* which also protects NOD mice from IDDM, increases the number of $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells as well as the production of TGFβ[102]. Interestingly, TGFβ production appeared to be essential for the protective effect of *H. polygyrus*[103], whereas the elimination of $T_{reg}$ cells had no effect on IDDM development[104]. However, *H. polygyrus* produces a molecule that mimics the biological effects of TGFβ, which complicates the interpretation of these findings[41]. In a mouse model of inflammatory bowel disease (IBD), the protective effect of *H. polygyrus* on disease development correlated with an altered composition of the gut microbiota, with a substantial increase in the frequency of members of the bacterial family Lactobacillaceae[105]. Another interesting study shows

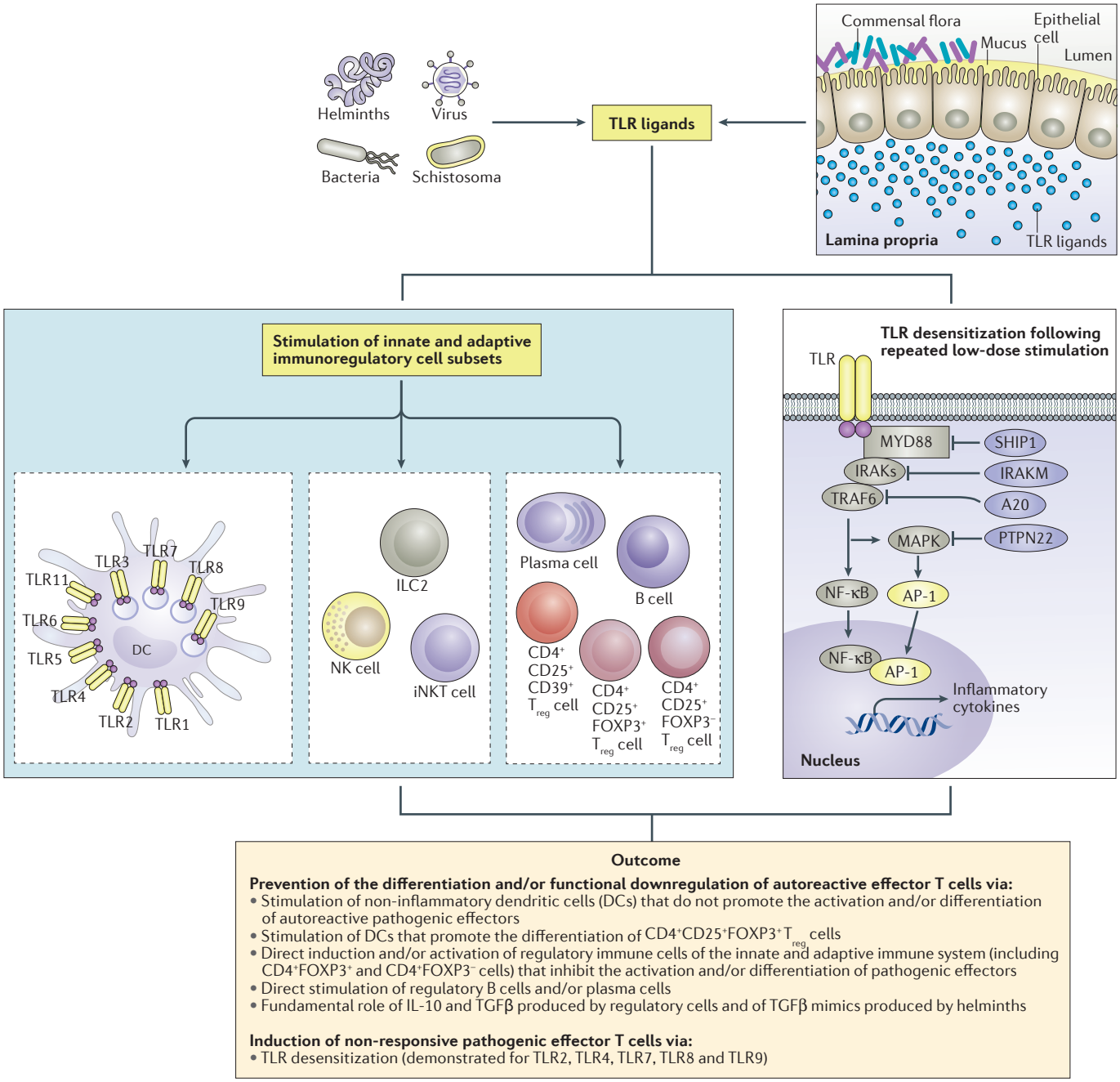


Figure 2 | **Stimulation of immune regulation by pathogens and commensals: the role of TLRs.** The protective effect of pathogens and commensals on autoimmune diseases is triggered by the presence of pathogen-associated molecular patterns (PAMPs), including Toll-like receptor (TLR) ligands. PAMPs can have indirect or direct effects on antigen-presenting cells (APCs) and on various cells at the interface of innate and adaptive immunity, such as natural killer (NK) cells, invariant NKT (iNKT) cells and innate lymphoid cells (ILCs), in particular group 2 ILCs (ILC2s). These cell populations, through their capacity (via cell–cell contact and/or cytokine production) to interact with the adaptive immune system efficiently control pathogenic autoimmune responses. In addition, $CD4^+CD25^+FOXP3^+$ or $FOXP3^-$ regulatory T ($T_{reg}$) cells are induced by pathogens and commensals and potentially regulatory B cells and plasma cells. Concerning cytokines, animal models outline the role of IL-10 and/or transforming growth factor-β (TGFβ). APCs and regulatory lymphocytes are the likely source of these cytokines. In addition, some parasites may produce mimics of TGFβ. The figure also addresses putative mechanisms that link TLR signalling and triggering of a 'non-response'. The interaction of TLRs with their ligands delivered at a low dose and repeatedly results in desensitization. As the response of TLRs to conventional stimulation is an inflammatory response, desensitization leads to a lack of inflammation, which may protect against autoimmunity. The desensitization effect has been shown for TLR2, TLR4, TLR7, TLR8 and TLR9. There is concordant data showing that, from a signalling point of view, desensitization involves phosphatases including SHIP1, IRAKM or PTPN22, but also molecules of unclear function, such as A20. Known for many years as an inhibitor of nuclear factor-κB (NF-κB) and apoptosis, A20 also has ubiquitin-regulatory properties and negatively regulates various inflammatory signalling cascades.

that infection with *Trichuris muris* protects mice deficient in the Crohn's disease susceptibility gene *Nod2* from IBD. This protection is associated with a polarized $T_H2$ immune response that favours a protective microbiota that is rich in Clostridiales at the expense of inflammatory Bacteroidales. Interestingly, the authors also showed that individuals from helminth-endemic regions harbour a similar protective microbiota[106].

Another parasite, the filaria *Litomosoides sigmodontis,* provided protection from IDDM in the NOD mouse model, which was associated with a $T_H2$-mediated immune response to the parasite[107]. However, NOD mice that lack the $T_H2$-polarizing cytokine IL-4 remained sensitive to the protective effect of the parasite, indicating that the $T_H2$-polarized immune response was dispensable. The parasite also promoted an increase in the frequency of peripheral $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells, but the removal of these cells also did not reverse the protective effect of *L. sigmodontis* infection[107]. Instead, the protective effect of the worm was shown to be mediated by TGFβ; however, it is unclear if the TGFβ is produced by host cells or if it is a parasite-produced TGFβ homologue[108].

The trematode *Fasciola hepatica* also protects against autoimmune IDDM in NOD mice, an effect that was associated with the M2 polarization of macrophages that have immune regulatory functions[109]. Moreover, it was shown that treatment with *F. hepatica* excretory–secretory products can also attenuate EAE. The treatment increased $T_H2$ cell responses and the frequency of $T_{reg}$ cells, yet protection against EAE was independent of these factors. Instead, the accumulation of eosinophils and the release of IL-5 (by $T_H2$ cells) and IL-33 (normally released by damaged barrier epithelial cells) were shown to be the key correlates of protection[110]. These data also suggest that subsets of innate lymphoid cells (ILCs)[111] other than NK and iNKT cells, in particular group 2 ILCs (ILC2s), whose activation and expansion are driven by IL-33, have important functions in mediating the protective effect of some pathogens on autoimmunity. Importantly, in all the presented examples, the protective effect of the parasites can be recapitulated by inoculating animals with parasite derivatives[40,41,101,109].

### *Immune regulation by commensals*

Like pathogens, commensal bacteria can induce immune regulatory pathways. This has been observed *in vivo* in models of autoimmune disease, by administrating probiotic preparations or specific commensal bacteria. Probiotics were shown to have a protective effect in models of IDDM[59,60,112] and EAE[62,113–115], and in both models, protection was dependent on IL-10 production by $CD4^+CD25^+$ cells located in the gut and lymph nodes draining the diseased tissue[62,113–115]. In a model of EAE, the protective effect of probiotics was observed even when disease-causing IL-17-producing cells were present in large numbers[113], and this protective effect correlated with an increased production of IL-10 by $T_{reg}$ cells. Similarly, it was shown that a single constituent, polysaccharide A (PSA), of the human commensal bacterium *Bacteroides fragilis* has a protective effect after oral administration in mouse models of EAE and experimental colitis[115–117]. Protection correlated with the presence of IL-10-producing $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells in the intestine.

It has been shown that subsets of commensal bacteria have a considerable effect on $T_H17$ and $T_{reg}$ cell differentiation[118]. In particular, SFB have been shown to induce the differentiation of $T_H17$ cells[55,56,119]. Commensal bacteria of the genus *Clostridium* induce $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells, and oral administration of a mixture of several *Clostridium* species to germ-free mice was shown to induce $T_{reg}$ cells in the colonic lamina propria[120,121].

A recent study showed that the protective effect of LPS on IDDM depends on its bacterial source[47]. *E. coli*-derived LPS isolated from the microbiota of individuals living in Karelia (who are at low risk of developing IDDM[47]) exerted a substantial protective effect in NOD mice. By contrast, *Bacteroides dorei*-derived LPS isolated from the gut microbiota of individuals living in Finland (who have a higher incidence of diabetes) had no effect when administered to NOD mice. This observation is all the more striking, as there are important differences in the chemical structure of these two LPS molecules[47].

The data I have described above for both pathogens and commensals point to the selective action of different environmental agents on specific immunoregulatory cell types. However, it will be important to further refine the characterization of the relevant immunoregulatory cells. Thus, if the role of $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells has been well studied, the role of $CD4^+FOXP3^-$ $T_{reg}$ cells must also be examined. These cells include T regulatory 1 lymphocytes that were described by Roncarolo and colleagues[122,123], which are IL-10-dependent and produce IL-10 and TGFβ, and $CD4^+FOXP3^-LAP^+$ T cells that were initially described by Weiner and colleagues in oral tolerance models[124], which produce TGFβ. Finally, it will be important to consider the function of mesenchymal stem cells[125,126] and myeloid-derived suppressor cells[127] as immune regulatory targets of pathogens and commensals.

### *Signalling through Toll-like receptors*

TLRs are one class of the innate immune system pattern recognition receptors. TLRs recognize distinct microbial components and directly activate immune cells. Thus, TLRs have a central role in the initiation and regulation of physiological and pathological immune responses. Considerable interest has focused on TLRs in recent years to dissect their signalling pathways and to find more effective adjuvants for vaccines. In almost all cases, TLRs were considered in their role as stimulators of immune responses.

A wealth of data indicates that signalling via TLRs may be the common denominator that underlies the protective effect of pathogens and commensals in autoimmune diseases. A large number of studies have used either TLR ligands or genetic approaches to

Table 2 | **Effect of *in vivo* TLR ligand administration on experimental autoimmune diseases**

| TLR ligand | Target TLR | Disease | Effect on disease | Target cell(s) and mechanism |
|---|---|---|---|---|
| Poly I:C | TLR3 | Spontaneous IDDM in NOD mice | Disease protection (the first report by Serreze *et al.*[130] was published in 1989, several years before the discovery of TLR3)[59] | • Protection is lost in NOD mice with *Cd1d1* deletion, which lack iNKT cells[59]<br>• Effect on marginal zone B cells, which acquire suppressive properties upon TLR3 ligation[182] |
| Zymosan | TLR2 | Spontaneous IDDM in NOD mice | Disease protection[128] | Increase in $CD4^+FOXP3^+$ $T_{reg}$ cells[128] |
| | | Experimental autoimmune encephalomyelitis | Prevention and treatment of established disease[139,184] | • Zymosan, through stimulation of TLR2 and dectin 1, triggered activation of β-catenin in DCs, which in turn expressed the retinoic acid metabolizing enzyme retinaldehyde dehydrogenase and IL-10. These DCs were then programmed to induce $CD4^+FOXP3^+$ $T_{reg}$ cells[184–186]<br>• Zymosan also activated F4/80 macrophages to produce TGFβ[187] |
| P40 protein (*Klebsiella pneumoniae*) | TLR2 | Spontaneous IDDM in NOD mice | Disease protection[59] | To be determined |
| Pam3Cys lipopeptide | TLR2 | Spontaneous IDDM in NOD mice | Disease protection[59] | To be determined |
| Pam3CSK4 | TLR2 | Spontaneous IDDM in NOD mice | Disease protection[134,138] | • Increase in $CD4^+CD25^+$ $T_{reg}$ cells[134]<br>• Increase in the production of IL-10 by antigen-presenting cells[134] |
| LPS | TLR4 | Spontaneous IDDM in NOD mice | Disease protection[59,92] | • $FOXP3^+$ $T_{reg}$ cells, as suggested by the fact that NOD mice with *Cd28* deletion, which lack thymic and peripheral $FOXP3^+$ $T_{reg}$ cells[90], resisted the protective effect[59]<br>• TLR4 agonists may also directly induce IL-10-producing B cells[92] |
| Resiquimod (R848) | TLR7 | Spontaneous IDDM in NOD mice | Disease protection[59] | To be determined |
| Empty plasmid DNA or CpG ODNs | TLR9 | Spontaneous IDDM in NOD mice | Disease protection[131] | To be determined |
| CpG ODNs | TLR9 | Diabetes in TCR-transgenic NOD mice (NOD 8.3)* | Rapid activation of IGRP-specific cytotoxic $CD8^+$ T cells and IDDM onset[183]. Further proving the TLR9-mediated effect, the CpG ODN-induced diabetogenic effect was abolished by coadministration of the TLR9 antagonist ODN 2088 (REF. 183) | IGRP-specific $CD8^+$ T cells[183] |
| | | SLE | Aggravation of disease[188] | DCs[188] |
| IRS 954 | TLR7 and TLR9 dual antagonists | SLE | Disease improvement[188,189] | DCs[188,189] |

*Derived from a diabetogenic $CD8^+$ T cell clone specific for IGRP and exhibiting an early and very aggressive disease. DC, dendritic cell; FOXP3, forkhead box P3; IDDM, insulin-dependent diabetes mellitus (hyperglycaemia); IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein (also known as G6Pase 2); iNKT cell, invariant natural killer T cell; IRS, immunoregulatory sequence; LPS, lipopolysaccharide; NOD, non-obese diabetic; ODN, oligodeoxynucleotide; SLE, systemic lupus erythematosus; TCR, T cell receptor; TLR, Toll-like receptor; $T_{reg}$ cells, regulatory T cells.

analyse the involvement of TLR signalling in the control of autoimmunity (TABLES 2,3). The first indications that TLRs may be protective came from experiments involving the parenteral administration of agonists for TLR2 (REFS 128,129), TLR3 (REFS 59,130), TLR4 (REF. 59), TLR7 (REF. 59) and TLR9 (REF. 131) to NOD mice, before IDDM onset, and showed that any of these TLR agonists could prevent disease development. This observation was counterintuitive because TLR agonists are expected to increase autoimmune responses, rather than reduce them.

Remarkably, the majority of the TLR ligands tested protect against autoimmunity, but through different mechanisms depending on the targeted TLR. Thus, the effect of the TLR3 ligand poly I:C is dependent on iNKT cells but not on $CD4^+CD25^+FOXP3^+$ $T_{reg}$ cells, whereas conversely, the protective effect of the TLR4 ligand LPS depends on $T_{reg}$ cells and not on iNKT cells[59] (TABLE 2). In addition to this direct action on different subsets of cells that are involved in immune regulation (TABLE 2), there is compelling evidence to show that TLR ligands may

Table 3 | **Effect of genetic ablation of TLR and TLR adaptor molecules on the development of experimental autoimmune diseases**

| Gene ablation | Effect on disease in wild-type mice | Effect on disease in germ-free mice |
|---|---|---|
| ***Spontaneous IDDM in NOD mice*** | | |
| *Myd88* | Complete disease protection* (REFS 59,64) | No effect on disease[64] |
| *Tlr2* | Disease attenuation[138] | No effect on disease[144] |
| *Tlr3* | No effect on disease[190] | ND |
| *Tlr4* | Disease acceleration[144,191] | No effect on disease[144] |
| *Tlr9* | Disease prevention[183,190] | ND |
| ***Experimental autoimmune encephalomyelitis*** | | |
| *Myd88* | Disease protection‡ (REFS 192–195) | ND |
| *Tlr2* | Disease attenuation[195] | ND |
| *Tlr2* in $CD4^+$ T cells | Disease attenuation[196] | ND |
| *Tlr4* | No effect on disease[195] or accelerated disease[194] | ND |
| *Tlr1* | No effect on disease[195] | ND |
| *Tlr6* | No effect on disease[195] | ND |
| *Tlr9* | Decreased[195] or accelerated disease§ (REF. 194) | ND |
| ***Systemic lupus erythematosus*** | | |
| *Tlr9* | Markedly exacerbated disease, associated with an increase in activation of pDCs and IFNα production[197] | ND |
| *Tlr7* | Disease protection[197] | ND |

*Transfer of the microbiota from *Myd88*$^{-/-}$ mice into wild-type mice protected from disease[65]. ‡*Myd88* inactivation of non-immunized recipients protected from disease induced by injection of encephalitogenic T cells from immunized wild-type mice, a model with no adjuvant. This protection from disease was mediated by IL-10, as disease transfer is re-established in mice lacking both MYD88 and IL-10[193]. §The contrasting findings are difficult to reconcile: a difference in the protocols used, notably concerning the adjuvant used, may be an explanation. In addition, a difference in the facility barriers cannot be excluded. IDDM, insulin-dependent diabetes mellitus (hyperglycaemia); *Myd88*, myeloid differentiation primary response gene 88; ND, not determined; NOD, non-obese diabetic; pDC, plasmacytoid DC; TLR, Toll-like receptor.

trigger intracellular negative signalling pathways, which is discussed below.

***TLRs as cellular targets of both pathogens and commensals.*** There are a number of cellular pathways linked to TLRs that are induced by commensals and pathogens (or their derivatives) that have a protective effect on autoimmune disease. For example, PSA derived from the commensal organism *B. fragilis* has been shown to induce a unique subset of IL-10-producing $CD4^+$ $T_{reg}$ cells in the intestine, and the protective effect was abolished in mice genetically deficient for IL-10 (REFS 115–117). Transcriptome analysis revealed that these $T_{reg}$ cells also expressed TGFβ2 (but not TGFβ1)[117]. Recent data suggested that the hallmark of PSA-induced $T_{reg}$ cells was the expression of CD39, the dominant cellular ectonucleotidase shown to be essential for the suppressor activity of $T_{reg}$ cells. Importantly, it was shown that TLR2 expression on T cells is necessary for PSA-mediated induction of IL-10 and CD39 expression, which, in turn, is instrumental for the protection of both EAE and colitis[117,132,133]. Another example of commensals mediating protection against autoimmunity through TLRs is the acceleration of IDDM in TLR4-deficient NOD mice raised in SPF conditions. The disease incidence in these mice is comparable to that observed in both TLR4-deficient and wild-type NOD mice raised in germ-free conditions (TABLE 3).

An example of pathogen-induced TLR-mediated protection is provided by LCMV infection in NOD mice, which protects the mice from developing IDDM. However, this effect was abrogated in mice deficient for *Tlr2* (REF. 134). In this context, it is interesting to note that TLR ligands can act on $T_{reg}$ cells indirectly, through an effect on dendritic cells (DCs) that stimulate the development of $T_{reg}$ cells ($FOXP3^+$ or $FOXP3^-$), or they can also directly act on $T_{reg}$ cells. Notably, the major Gram-negative bacterial virulence factor LPS, which protects against autoimmunity, is a prototypic TLR4 ligand.

***Receptor desensitization or 'TLR tolerance'.*** Desensitization occurs when a receptor decreases its signalling response after prolonged ligand or agonist exposure and can be a result of the inhibition of positive regulators or the induction of negative regulators of a particular signalling cascade[135]. In the case of TLRs, a short stimulation at optimal doses by TLR ligands elicits inflammation and may initiate an immune response, whereas repeated low-dose administration of the same ligands results in a non-response, which is reversible after discontinuation of the treatment. TLR tolerance was first demonstrated in 1946 (termed endotoxin tolerance), when it was shown that repeated injections of low doses of LPS (otherwise known as endotoxin) fully protected the treated mice against a lethal (high-dose) challenge with LPS, which induces a massive release of tumour necrosis factor (TNF) by macrophages[136].

It is now known that LPS signalling mechanisms that are inhibited in 'endotoxin-tolerized' macrophages involve both TLR4 and TLR2 and result from the impaired expression and/or functions of common intermediates that are involved in LPS-triggered TLR signalling[137]. More recent data indicate that LPS tolerance interferes with TLR4 signalling by inhibiting the downstream phosphorylation of the signalling intermediates LYN and SRC and their recruitment to TLR4 and by increasing the activity and expression of downstream phosphatases including PP2A, PTPN22, PTP1B and MKP1 (REF. 135).

Another example of desensitization is the prevention of IDDM in NOD mice, which is referred to as 'TLR2 tolerance' (REF. 138). Using an adoptive transfer model, the authors showed that repeated treatment of the NOD recipients of diabetogenic T cells with the TLR2 agonist Pam3CSK4 inhibited development of IDDM. Pam3CSK4 also prevented autoimmune diabetes recurrence otherwise observed on syngeneic islet grafts transplanted into overtly diabetic NOD mice[138]. Interestingly, peritoneal macrophages from NOD mice treated *in vitro* with Pam3CSK4 expressed lower levels of IRAK1 and IRAK4, which are positive transducers of TLR2 signalling, whereas expression of the inhibitory transducer IRAKM (also known as IRAK3) was increased[138], which further supports that TLR2 receptor desensitization plays a central role in this model[138].

TLR2 desensitization was also reported to have a role in the prevention of EAE. Administration of low doses of two different TLR2 ligands, Pam2CSK4 or Lipid 654 (L654), to naive recipients adoptively transferred with encephalitogenic (EAE-inducing) T cells showed down-regulation of TLR2 signalling and attenuation of EAE[139]. Interestingly, L654 is a microbiome-derived TLR2 ligand that is present in healthy human serum but substantially decreased in the serum of patients with multiple sclerosis[139]. The authors concluded that microbiome products that access the systemic circulation are not pro-inflammatory but could play an immune regulatory role by maintaining TLR tolerance or desensitization. Another interesting example was seen in a mouse model of EAE (induced following immunization with a proteolipid protein peptide and adjuvant), where repeated doses of a synthetic TLR7 ligand, 1V136, diminished the severity of disease and the expression of chemokines in the spinal cord[140].

The *in vivo* relevance of TLR desensitization in humans was observed in a report of children raised on dairy farms, an environment rich in LPS, who have a lower incidence of allergy[3]. It was also shown that chronic exposure to low-dose endotoxin or farm dust protected mice from asthma induced by house dust mites[141]. Lung epithelial cells showed a reduced production of cytokines that normally activate DCs to induce $T_H2$ cell responses. Interestingly, the LPS-induced TLR4 desensitization that may be responsible for this effect targets the lung epithelium and requires the ubiquitin-modifying enzyme A20 (REF. 141). Thus, the farming environment may protect against allergy by modifying the communication between barrier epithelial cells and DCs through A20 induction. Within the field of allergy, the concept has emerged that the addition of TLR ligands to allergen desensitization protocols markedly improved the effectiveness of the treatment. In a mouse model of birch-pollen-induced asthma, only epicutaneous immunotherapy using a recombinant allergen (Bet v 1) in combination with a TLR7 agonist (R848) substantially improved the signs of airway hyperreactivity[142]. Two allergy vaccines containing TLR agonists have been investigated in clinical trials: Pollinex Quattro (Allergy Therapeutics, UK) (containing the TLR4 agonist monophosphoryl lipid A) and AIC (containing TLR9-specific CpG motifs) have proved to be safe and showed efficacy in controlling allergic rhinitis. Various TLR ligands (CRX-675, a TLR4 agonist; AZD8848, a TLR7 agonist; VTX-1463 a TLR8 agonist; and 1018 ISS and QbG10, TLR9 agonists) are currently in clinical development for allergic rhinitis and asthma[143].

These results, although informative, are not sufficient to give a complete picture of the situation. However, they suggest that depending on the pharmacological properties of the TLR ligand (agonist or antagonist) and the kinetics of the TLR stimulation (acute or chronic), a stimulating or inhibitory signal will be delivered. It is therefore necessary to consider the global response to pathogens or commensals as the result of the integration of the positive and negative signals delivered to TLRs. This is in keeping with the 'balanced signal hypothesis' proposed by Chervonsky and colleagues based on their results with NOD mice genetically deficient for various TLR genes or their adaptor transducing molecules and raised under germ-free and SPF conditions[144]. These data indicate that both pathogens and commensals stimulate TLRs and that the consequence of stimulation for autoimmune disease depends on the specific TLR. This concept paves the way for the selective immunopharmacology of individual TLRs.

## Conclusions

In summary, the data presented here provide solid support for the hypothesis that pathogens, parasites and commensal microorganisms can protect against a variety of autoimmune conditions — an effect that so far appears to be nonspecific, as various microorganisms and parasites can induce protection against different autoimmune conditions. Thus, a reduction in infection rates is likely to be one of many factors that has led to an increase in the frequency of certain autoimmune diseases, as discussed here. Interestingly, this phenomenon does not apply to all autoimmune diseases. For example, there is no, or at best weak, evidence linking the hygiene hypothesis to rheumatoid arthritis. Not all infections are protective, or they may be so to various degrees, and it is still unknown which infections provide protection. Mycobacteria and helminths have a key role, but the question is still unresolved for a number of other infectious agents, though the epidemiological data on young children indicate that common infections might be important.

Syngeneic islet grafts
Islet transplants between syngeneic (genetically identical) donor and recipient individuals, which therefore does not give rise to allograft rejection. These grafts performed in diabetic non-obese diabetic mice provide a robust model to test for recurrence of the autoimmune disease.

Another issue that deserves further clarification is that of the respective role of pathogens and commensals. As discussed above, the working hypothesis that suggests the involvement of TLRs is supported by robust experimental data and has the additional merit of providing a common mechanism for both pathogens and commensals. An essential element will be to prospectively test the effect of new treatments to prevent autoimmune disease that are based on the hygiene hypothesis. This would provide both a confirmation of the proof of principle and potential therapeutic advances, particularly in the area of preventive medicine. The best candidates would be bacterial or parasite extracts or well-defined chemical TLR ligands. However, given that such products could be designed to be administered as a preventive approach to large populations of healthy individuals, their safety needs to be vigorously tested to rule out any possible side effects.


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*Tom van der Poll, Frank L. van de Veerdonk, Brendon P. Scicluna and Mihai G. Netea*

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**Author biography**

Jean-François Bach is an immunologist who contributed to the understanding of the mode of action of immunosuppressive agents in organ transplantation and autoimmune diseases as well as to the development of new strategies for the treatment of autoimmune insulin-dependent diabetes (for example, cyclosporin and CD3 monoclonal antibodies). More recently, he has focused on the mechanisms underlying the hygiene hypothesis, particularly in autoimmunity. He developed his career at the Hôpital Necker-Enfants Malades in Paris, France. He was Secrétaire Perpétuel of the French Academy of Sciences from 2006 to 2016.

**Key points**

- The initial application of the hygiene hypothesis for autoimmune diseases proposed in the early 2000s has been confirmed and consolidated by a wealth of published data in both animal models and human autoimmune conditions.
- The hygiene hypothesis probably explains the uneven geographical distribution of autoimmune diseases in the world. Individuals migrating from countries with low incidence of autoimmune diseases to countries with high incidence develop the disease with the frequency of the host country, provided that migration occurred at a young age and under a threshold that varies according to the disease.
- Pathogenic bacteria, viruses and parasites are often endowed with strong protective effects on autoimmunity even when infection occurs late after birth.
- Gut commensal bacteria may also have a protective role in autoimmunity when administered early in life.
- Pathogens, parasites and commensals essentially act by stimulating immune regulatory pathways, implicating the innate and the adaptive immune system. Importantly, the effect is seen with both living organisms and their derivatives or purified extracts.
- Both pathogens and commensals stimulate pattern recognition receptors, including Toll-like receptors (TLRs) to protect against autoimmunity. This effect may be mimicked by TLR agonists acting through pharmacological stimulation or desensitization of the target receptor.

**Figure permissions**

FIG.1: Original data sources

**Panel a** | Atlas of MS 2013. https://www.msif.org/wp-content/uploads/2014/09/Atlas-of-MS.pdf

**Panel b** | Patterson, C. *et al.* Diabetes in the young — a global view and worldwide estimates of numbers of children with type 1 diabetes. *Diabetes Res. Clin. Pract.* **103**, 161–175.

**Panel c** | Global tuberculosis report 2016. http://apps.who.int/iris/bitstream/10665/250441/1/9789241565394-eng.pdf?ua=1

**Panel d** | Screening for hepatitis during the domestic medical examination for newly arrived refugees. https://www.cdc.gov/immigrantrefugeehealth/pdf/domestic-hepatitis-screening-guidelines.pdf

**Panel e** | Caisse des Français de l'Étranger. https://www.cfe.fr/pages/votre-sante/guidespatho.php?id=126 [French]

**Panel f** | International Monetary Fund. World Economic Outlook Database. https://www.imf.org/external/pubs/ft/weo/2015/01/weodata/index.aspx

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**ToC blurb**

000 **The hygiene hypothesis in autoimmunity: role of pathogens and commensals**

*Jean-François Bach*

The hygiene hypothesis postulates that an increased frequency of infections contributes to a decrease in autoimmune and allergic diseases. Here, Bach summarizes the epidemiological and experimental evidence supporting this hypothesis and discusses the importance of innate immune receptors in mediating the protective effect of pathogens and commensals on autoimmunity.