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## OPINION

# Interactions between commensal intestinal bacteria and the immune system

Andrew J. Macpherson and Nicola L. Harris

Although we might shudder at the thought of billions of bacteria living in our lower intestine, we are colonized by these passengers shortly after birth. However, the relationship is mostly of mutual benefit, and they shape our immune system throughout life. Here, we describe our developing understanding of the far-reaching effects that the commensal flora have on mucosal and systemic immunity and their relevance to the effects of hygiene on human disease.

Most studies of the immune system aim to understand the way in which it responds to infectious pathogens. By analysing immune mechanisms in animal models of infectious disease, we learn how the immune system responds to biologically important challenges. Yet clinically apparent microbial infections are the exceptions in our harmonious coexistence with vast numbers of non-pathogenic microorganisms; these microorganisms enter our body from the environment shortly after birth and colonize the mucous membranes and skin epithelia. In the lower intestine alone, the density of commensal bacteria in the lumen reaches  $10^{12}$  organisms per gram of intestinal contents, with approximately 1,000 species present (BOX 1). So when the physiologist J.B.S. Haldane once commented that even the Archbishop of Canterbury is 65% water<sup>1</sup>, he omitted to mention that the head of the Church of England also consists of more bacterial cells than eukaryotic cells and

that the combined number of genes in these bacteria exceeds the total number of eukaryotic genes by a factor of at least 100.

Usually, we ‘peacefully’ coexist with our commensal microflora, in a good example of MUTUALISM, which has been established through

evolution on both sides. The bacteria benefit from the stable habitat, which is rich in energy sources from the food we ingest; and we salvage heat energy from compounds such as cellulose, which would otherwise be indigestible because we lack the necessary enzymes. Also, some bacterial compounds, including short-chain fatty acids and phylloquinone (vitamin K1), are used in host anabolic pathways. Moreover, the commensal microflora compete with incoming foreign microorganisms for space and resources, thereby making it more difficult for true pathogens to become established. However, this seemingly ideal balance is sometimes disturbed: the development of human INFLAMMATORY BOWEL DISEASE<sup>2</sup> (the incidence of which is 1 in 500 in Western populations) and possibly some autoimmune diseases are associated with immune responses to environmental microorganisms or mild pathogens.

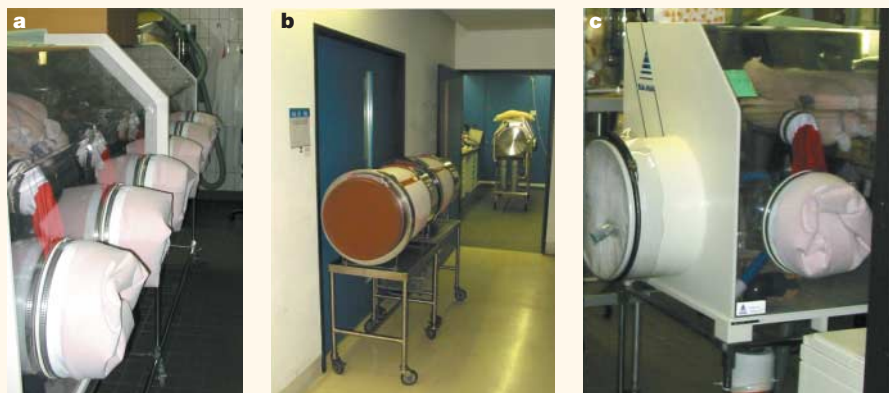
Here, we discuss the pervasive way in which the environmental flora shapes both the mucosal and systemic immune systems. It is clear that immune composition and lymphoid structures differ when this flora is present compared with when it is absent; however, we are only beginning to understand the mechanisms and functions of the adaptive changes that occur when environmental bacteria colonize the host. One of the consequences is to physically limit the live bacteria to the intestinal lumen. Yet, the functional consequences of the systemic

## Box 1 | Intestinal microorganisms

Bacteria are the main type of microorganism present in the mammalian intestine, although other types are also found, including protozoa and fungi. The stomach and small intestine have relatively low bacterial densities ( $10^3$ – $10^5$  organisms per gram or ml of luminal contents in mice, consisting mainly of acid-tolerant lactobacilli and streptococci). The distal portion of the small intestine, the ileum, is a transition zone with higher bacterial densities ( $10^8$  per gram) and species diversity, but the most dense colonization is in the colon ( $10^{10}$ – $10^{12}$  per gram), which hosts more than 400 bacterial species. In the lower intestine, anaerobes predominate, particularly the *Bacteroides*, bifidobacteria, fusobacteria and peptostreptococci (each group present at approximately  $10^9$  per gram); by contrast, aerobes and facultative aerobes, including enterobacteria and lactobacilli, are present at only moderate densities ( $10^6$ – $10^8$  per gram).

There are two main difficulties in understanding and measuring these complex flora. First, a comparison of two techniques used to assess faecal bacterial numbers — counting colonies of culturable bacteria and estimating numbers using smears — shows that less than 50% of intestinal bacteria can be cultured. This is because of the precise oxygen requirements of some species and their fastidious (and largely unknown) nutrient requirements. Second, although most measurements have been made using faecal bacteria, the intestine is not a homogeneous environment — groups of bacteria can also exist on the surface of the mucus layer or deep within it.

Fortunately, there are ways of overcoming the difficulties in culturing intestinal bacteria. The 1.5-kb gene encoding 16S ribosomal RNA is present in multiple copies in bacterial chromosomes, and it is highly polymorphic. Therefore, the nucleotide sequence of this gene (obtained after amplification by PCR) can be used to determine the species of each organism<sup>81</sup>, and the gene can serve as a target (in species-specific *in situ* hybridization) for studying the spatial arrangement of each bacterial group.



**Figure 1 | Keeping germ-free mice in an isolator.** It has been possible to keep experimental animals in entirely germ-free conditions for the past 50 years or more, by initially delivering the pups by sterile Caesarean section and hand rearing them aseptically. **a** | After this, it is less labour intensive to keep the mice free from colonization by environmental organisms by breeding them in an isolator, which is ventilated with sterile filtered air under positive pressure. Gnotobiotic (germ-free) animal husbandry uses sterile food, water and bedding. It is important to note that germ-free animals have no bacteria in the intestine or on other body surfaces, whereas specific-pathogen-free (SPF) mice are only devoid of known mouse pathogens and do contain intestinal bacteria. **b** | A germ-free isolator can be loaded with sterile supplies using a transport ring, which is small enough to fit in an animal-unit autoclave. **c** | The ring is connected to a port on the isolator using a flexible plastic sleeve that contains a glove. (The inside of the sleeve is sterilized by spraying it with peracetic acid immediately before it is connected to the isolator and the transport ring.) The internal door on the isolator and the external seal on the transport ring are then opened from the outside using the gloves, and sterile materials are brought in. Samples are taken routinely from the husbandry materials and the mice, to ensure that the colony remains germ free. Mice can either be experimentally manipulated in the isolator, or they can be transferred into a sterile lamina-flow hood, by connecting the isolator to a port on the side of the hood. Germ-free mice can easily be recolonized with bacteria by placing a single mouse that has normal flora into the cage.

immune alterations are also important, because there is evidence that the hygiene status of humans influences predisposition to allergy and/or autoimmunity. We believe that there are important differences between the observed improvements in human hygiene and the current animal models that are used to study the immunological consequences of these improvements, and we consider that these differences are crucial for understanding and modelling the effects of hygiene on systemic immunopathology.

### Mucosal immune adaptation

There is no question that the host is highly adapted to the presence of commensal intestinal bacteria. The evidence for this comes from comparing germ-free mice (FIG. 1), which have no commensal microflora, with specific-pathogen-free (SPF) animals of the same strain, which contain a simple flora (BOX 2). The mucosal immune system is undeveloped in germ-free animals: they have hypoplastic PEYER'S PATCHES that contain few germinal centres, as well as greatly reduced numbers of IgA-producing plasma cells and LAMINA PROPRIA CD4<sup>+</sup> T cells<sup>3,4</sup> (FIG. 2). The immunological abnormalities in germ-free animals are not confined to the mucosal immune system: the spleen and lymph nodes

are relatively structureless, with poorly formed B- and T-cell zones<sup>5</sup> (FIG. 2) and abnormal high endothelial venule morphology<sup>6</sup>. The mice also have hypogammaglobulinaemic serum, mainly because of reduced levels of IgG<sup>7</sup>. Furthermore, the gene-expression profile of the intestinal epithelial-cell layer is altered in the absence of commensal bacteria<sup>8</sup>. All of these abnormalities can be reversed within several weeks of colonizing germ-free animals with commensal bacteria, which can be achieved by placing an SPF mouse in a cage that contains germ-free animals (FIG. 1). Such experimental colonization of germ-free animals might seem artificial, but similar colonization occurs in every neonate within days of its birth<sup>9</sup>.

### Systemic immune ignorance

The physical barrier that separates the large numbers of commensal intestinal bacteria from the underlying tissues is only a simple (single cell) epithelial layer; although it is reinforced by a layer of mucus, the secretion of IgA and the production of antibacterial molecules (such as DEFENSINS) by the epithelium<sup>10</sup> (FIG. 3). Considering this, it is not surprising that in both humans and experimental animals some bacteria can penetrate this barrier to reach deeper tissues<sup>11–13</sup>. Challenge studies<sup>14</sup>

show that although commensal bacteria are killed within hours by macrophages, they can survive for several days inside dendritic cells (DCs). However, DCs that have been loaded with commensal bacteria in the Peyer's patches do not penetrate farther than the mesenteric lymph nodes, so DCs that are primed by live bacteria are usually restricted to the mucosal immune system. It is important to note that this *in vivo* priming effect is lost if the bacteria are killed by heat treatment, so it is not mediated by lipopolysaccharide and/or other bacteria-derived immunostimulatory molecules. (Although by internalizing live bacteria, DCs might concentrate these bacterial compounds.) So, IgA production, and probably intestinal T-cell responses, can be selectively induced by DCs loaded with commensal bacteria, and this increased local secretion of IgA limits the penetration of commensal bacteria<sup>14</sup>. It has long been established that both B and T cells are activated in the Peyer's patches, after which they recirculate through the intestinal lymphatics, enter the bloodstream at the level of the thoracic duct and home back to the intestinal lamina propria. Because DCs containing live commensal bacteria are confined to the mucosal immune system, the induction of T- and B-cell responses is focused at the mucosa where it is required.

Although there is a strong locally induced immune response, because commensal bacteria do not usually penetrate beyond the mesenteric lymph nodes, we believe that the systemic immune system is largely ignorant of these organisms. An example of this is that responses that prime the production of IgG specific for *Enterobacter cloacae* (the main aerobe in our SPF mouse colony) are not seen in unmanipulated mice colonized with these bacteria<sup>15</sup>. This occurs as a result of IGNORANCE, rather than tolerance, because intravenous injection of a small dose of *E. cloacae* causes a highly reproducible priming response<sup>15</sup>. The priming of systemic immunity to commensal bacteria is largely unnecessary, because the innate immune system can destroy the few organisms that do penetrate the intestinal barrier — mice only become unable to coexist with their intestinal flora when there are serious deficiencies in the phagocytic biocidal mechanisms that generate reactive oxygen and nitric oxide radicals<sup>16</sup>.

So, on the whole, we believe that mice are systemically ignorant of particulate (live) commensal intestinal bacteria; however, soluble bacterial degradation products that reach the systemic circulation are probably responsible for the differences in the organization of secondary-lymphoid structures<sup>17</sup> and the different concentrations

## Box 2 | The 'Schaedler flora' in experimental animals

As shown in FIG. 1, experimental animals can be bred and maintained in a sterile environment. These germ-free or gnotobiotic animals have no microorganisms in the gut or on other body surfaces. Starting with germ-free mice, Russell Schaedler and colleagues<sup>82</sup> at the Rockefeller Institute for Medical Research, New York, USA, carried out experiments in the 1960s in which they reintroduced simple mixtures of defined intestinal bacteria. They showed that inoculation with coliform bacteria alone led to high stable levels of these bacteria, which are only a minor component of the gut flora in a normal mouse. To attempt to introduce a simple, balanced flora that would colonize the gut without compromising the health of the mice, a cocktail of eight species eventually became popular: *Escherichia coli* var. *mutabilis*, *Streptococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, group N *Streptococcus*, *Bacteroides distasonis*, a *Clostridium* species and a species of extremely oxygen-sensitive (EOS) spiral-shaped (fusiform) bacteria. In 1987, the National Cancer Institute of the United States revised this 'Schaedler flora' to include a *Flexistipes* fusiform bacterium and three further EOS fusiform species<sup>53,83</sup>.

Specific-pathogen-free (SPF) mice are defined on the basis of a negative screen for specific known pathogens. They are usually derived from a commercial breeding colony, which might or might not have been rederived (by Caesarean section or embryo transfer) in a germ-free environment. The most rigorous derivation of SPF mice is to be derived germ free and then colonized with modified Schaedler flora.

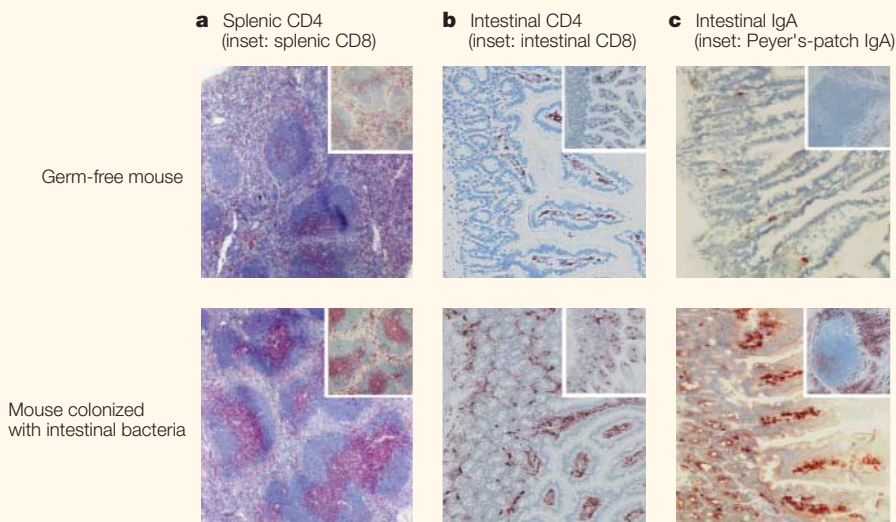
It is clear that the flora of experimental animals generated in this way is far simpler than in humans (approximately 1,000 species) or in conventional experimental animals. Although this defined gut population should remove much of the background variability observed in experiments, it is often assumed that the flora remains stable while subsequent generations of animals are bred (in SPF facilities). However, the composition of the flora (and particularly the anaerobes) is not often checked, and it is relatively easy for new species to be introduced, either from human handlers or from animals sourced from other SPF suppliers. Scientists are only aware of the mouse pathogens that are routinely tested for, and the immunological community is generally ignorant of the environmental flora of the experimental animals that they use.

and diversity of serum immunoglobulins in germ-free and SPF animals<sup>18,19</sup>. Indirect evidence is provided by studies<sup>20,21</sup> showing that the hypogammaglobulinaemia of germ-free animals is more pronounced when they

are fed an elemental diet (containing hydrolysed amino acids and purified lipids and carbohydrates) rather than autoclaved food, which contains (dead) bacterial material. Although these elemental diets are often

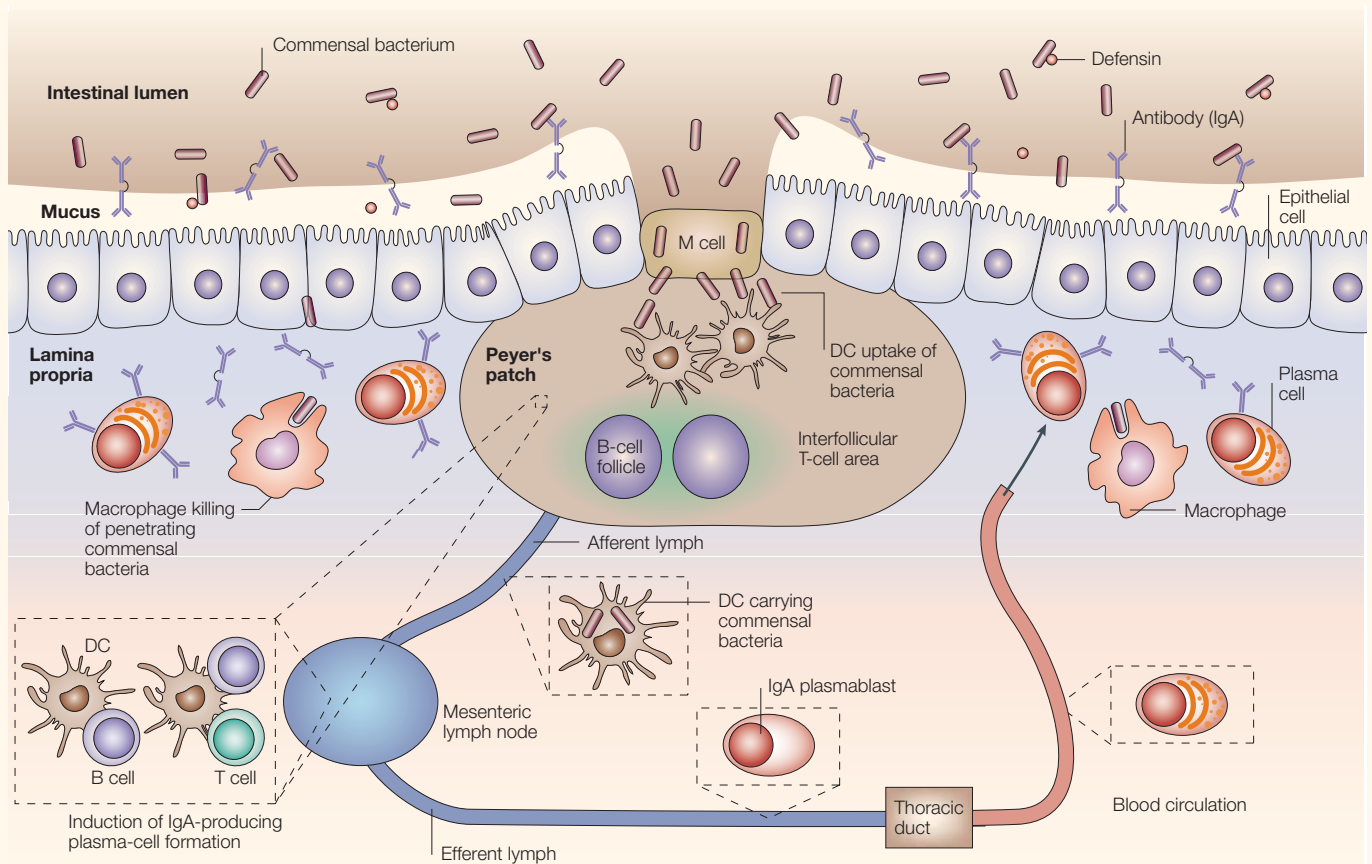
referred to as antigen free (because they lack full-length proteins), the animals still consume milk (during lactation in the neonatal period) and groom themselves. We argue that the important difference between the background immune stimulation of germ-free animals fed a sterile (autoclaved) natural-ingredient diet and those fed an elemental diet probably results from the microbial material contaminating the autoclaved food (rather than the presence of full-length proteins), because soluble proteins introduced into the intestine are generally thought to be tolerogenic rather than immunogenic<sup>22</sup>. The relative impact on immune reactivity of reducing the levels of contaminating microbial molecules or full-length proteins could easily be tested by selective experimental supplementation.

Compared with germ-free mice that are fed autoclaved food, germ-free mice fed an elemental diet have highly reduced levels of serum IgG and IgA, reduced numbers of splenic IgG-producing cells and fewer circulating lymphocytes<sup>19</sup>. Despite this, serum IgM concentrations and the diversity of the IgM REPERTOIRE are preserved<sup>19</sup>. When these mice were challenged with a model antigen together with adjuvant, they mounted a strong specific IgG response, but the polyclonal response that leads to increased levels of non-antigen-specific IgG was considerably diminished. This reduction in the polyclonal response was also seen in germ-free animals that were fed sterile food (in comparison with SPF mice), but it was more marked in germ-free mice that were fed the elemental diet<sup>23</sup>. In addition, the usual increase in the frequency of somatically hypermutated immunoglobulins (of the IgG isotype) that occurs during ageing also depends on exposure to environmental antigens (from commensal gut microorganisms), because this has been shown to occur to a lesser extent in germ-free mice<sup>24</sup> than in SPF mice. So, bacteria-driven alterations that result from 'bathing' the immune system in immunostimulatory bacterial molecules cause a baseline level of immune activation<sup>25</sup>; this increases the degree of polyclonal stimulation to protein antigens administered with adjuvants and might also improve the immune response against invasive pathogens. It is well known that patients with agammaglobulinaemia are protected against infections with encapsulated bacteria by treatment with pooled gammaglobulin preparations from healthy donors. Furthermore, experiments on rats have shown that crossreactive antibodies can sufficiently bind the repetitive capsular polysaccharides of bacteria to



**Figure 2 | The presence of intestinal bacteria has a large impact on lymphoid structures of both the intestine and systemic tissues.** Histological sections of the spleen and intestine are shown for a germ-free wild-type C57BL/6 mouse and for a C57BL/6 mouse colonized with intestinal bacteria. In the absence of intestinal bacteria, the spleens have relatively few germinal centres and poorly formed T-cell (pink) and B-cell zones (a). The intestines of germ-free mice have low numbers of lamina propria CD4<sup>+</sup> cells (brown) (b), greatly reduced numbers of IgA-producing cells (brown) (c) and hypoplastic Peyer's patches. The histological abnormalities of germ-free mice reverse within weeks of colonization with intestinal bacteria, which can be achieved, for example, by placing a normal colonized mouse in the same cage. These images are reproduced with permission from REF. 3 © (2001) Elsevier.





**Figure 3 | Immune defences against commensal intestinal bacteria.** Commensal bacteria are present at a high density in the intestinal lumen (up to  $10^{12}$  bacteria per gram of luminal contents). Most commensal bacteria reside outside the layer of mucus that covers the intestinal epithelial cells. Some bacteria can be killed by antibacterial molecules, such as defensins, which are produced by the epithelial cells. Bacteria that penetrate the enterocyte epithelial layer are rapidly killed by the macrophages in the lamina propria. Commensal bacteria can also penetrate the specialized follicle-associated epithelium, containing M cells, which lies over the Peyer's patches. These bacteria are also rapidly killed by macrophages, but small numbers can survive for several days in dendritic cells (DCs). This enables the interaction of DCs with T and B cells in the Peyer's patches and/or the migration of DCs to the draining mesenteric lymph nodes. (DCs that contain live bacteria induce IgA-producing plasma cells more effectively than heat-killed bacteria.) Although DCs loaded with commensal bacteria can traffic to the mesenteric lymph nodes, the lymph nodes function as a barrier, and the loaded DCs cannot penetrate farther to reach the systemic secondary-lymphoid tissues. The result is that the induction of immune responses by live bacteria is confined to the mucosa itself. Following activation, B- and T-cell blasts can leave the mesenteric lymph nodes through the efferent lymph, enter the bloodstream at the thoracic duct and home back to the intestinal mucosa.

induce the fixation of complement and the opsonization of the bacteria<sup>26</sup>. One disadvantage is that the peripheral self-tolerance that is usually generated by the presentation of proteins on immature DCs<sup>27</sup> might be compromised by bystander activation of the DCs in the presence of bacterial degradation products.

The presence of commensal intestinal bacteria, therefore, has clear structural and functional consequences for the systemic immune system. Because even high doses of commensal bacteria do not penetrate to systemic secondary-lymphoid structures<sup>14</sup>, and immune changes in germ-free animals are accentuated when they are fed a purified diet of hydrolysed amino acids, we propose that the baseline setting of the immune system is a response to contamination of the immune environment with soluble breakdown products from microorganisms.

Interactions between the systemic and mucosal immune systems have traditionally been studied by examining oral tolerance. Such studies involve the administration of a T-CELL-DEPENDENT ANTIGEN, either by ingestion or mucosal application, then the measurement of suppression of the response to later systemic immunization. Although oral tolerance can be induced in germ-free animals<sup>22</sup>, the kinetics of the systemic response to immunization are delayed<sup>28</sup>, probably because of the immaturity of systemic secondary-lymphoid organization under germ-free conditions. This makes the interpretation of oral-tolerance results in germ-free animals too complicated to allow direct comparison with SPF animals.

#### Improving human hygiene

We now turn from examining the effects of experimentally manipulating the density and diversity of environmental antigens on mice

to assessing whether experiments with 'ultra-clean' mice (that is, germ-free mice or SPF mice with a restricted flora) can help us to understand the patterns of human disease.

Hygiene is even more complex to investigate in the human population, because poor hygiene usually occurs together with several confounding factors: poor nutrition, lifestyles with lower technology, and less access to medical care (for registering the incidence of disease). The importance of these variables needs to be considered in epidemiological studies.

Autoimmunity and allergy, which result from inappropriate and overactive immune responses, are disadvantages arising from our ability to combat infectious disease. The 'hygiene hypothesis' states that as we improve hygiene, there are fewer infectious challenges, and the subsequent response of the immune system leads to allergy (BOX 3).

## Box 3 | The 'hygiene hypothesis'

In 1989, Strachan<sup>29</sup> coined the term 'hygiene hypothesis'. This hypothesis states that a leading cause of the increased incidence of allergy in today's population is the decrease in exposure to common infections during early life, which occurs as a result of smaller family size and improved hygienic conditions. Considerable epidemiological and experimental evidence supports the hypothesis, including studies examining airborne viruses, mycobacteria, orofaecal microorganisms and helminths<sup>84,85</sup>. Two popular theories that offer explanations for the hygiene hypothesis are immune deviation and counter regulation.

It is well known that the T helper 1 (T<sub>H</sub>1)-cell cytokine interferon- $\gamma$  can suppress the differentiation of T<sub>H</sub>2 cells<sup>86</sup> and the production of IgE<sup>87</sup>, which are associated with atopy. Because the neonatal immune system has been described as showing a T<sub>H</sub>2-cell bias<sup>88</sup>, proponents of immune deviation argue that exposure to microorganisms that induce T<sub>H</sub>1-cell responses is required to prevent the development of atopic (T<sub>H</sub>2-cell) responses<sup>89</sup>.

The counter-regulation model states that the production of immunoregulatory factors after exposure to microorganisms limits the development of unrelated immune-mediated disease<sup>84</sup>. Indeed, experimental infection of mice with *Mycobacterium vaccae* can elicit a population of CD4<sup>+</sup>CD45RB<sup>low</sup> regulatory T cells that attenuate ovalbumin-induced airway inflammation through the production of interleukin-10 (IL-10) and transforming growth factor- $\beta$ 1<sup>90</sup>. IL-10-producing cells that are induced by infection with enteric helminths have also been shown to protect mice from immunopathology associated with the subsequent ingestion of a food allergen<sup>91</sup>.

We argue here that neither explanation is likely to account entirely for the long-term consequences of altered hygiene conditions and that alterations in T- and B-cell repertoires after pathogenic infections probably contribute more to the differences in incidence of immunopathology.

There is reasonable clinical epidemiological support for this theory. Children from families of lower socio-economic status or with more siblings have decreased incidence of atopy, presumably because of exposure to more infectious agents<sup>29</sup>. There is also an inverse correlation between previous infection with mycobacteria or viruses, including hepatitis A virus (HAV), and the subsequent development of asthma<sup>30–32</sup>, and other studies<sup>33–36</sup> show that children brought up on farms are protected from the development of asthma. However, it is usually unclear which of these infections can actually induce a protective effect and which are surrogate markers of poor hygiene in a complex environment.

For those exposed to HAV, protection against asthma development is more pronounced in individuals carrying a six-amino-acid insertion at position 157 of the *TIMI* (T-cell immunoglobulin domain and mucin domain 1) gene, which encodes the cell-surface receptor through which HAV infects human cells<sup>37</sup>. This receptor is also expressed by activated CD4<sup>+</sup> T helper 2 (T<sub>H</sub>2) cells<sup>38</sup>. It remains unclear whether the polymorphism in *TIMI* alters HAV infectivity or alters the immune response against the virus. However, the results indicate that, in this case, HAV itself is responsible for protection against allergy, rather than just being a surrogate for another protective infection.

One suggested explanation for the observed protection against allergy is that interaction with 'unhygienic' microorganisms causes

immune deviation, thereby skewing immune responses away from the neonatal T<sub>H</sub>2-cell bias towards T<sub>H</sub>1-cell responses<sup>39</sup> (BOX 3). However, it has been difficult to show this phenomenon directly, and it does not explain three other epidemiological observations: first, there is a similar negative association between atopy and infection with helminths (which are known to induce T<sub>H</sub>2-cell cytokines)<sup>40</sup>; second, humans with an immunodeficiency resulting from genetic lesions that affect T<sub>H</sub>1-cell cytokine pathways do not have an increased incidence of allergic disease<sup>41</sup>; and third, the increase in the incidence of allergy in recent decades has been accompanied by similar increases in autoimmune diabetes<sup>42</sup> and coeliac disease<sup>43</sup> — conditions that are usually considered to be T<sub>H</sub>1-cell-biased diseases. Because the demographics of autoimmunity are similar to those of allergy, they need to be considered together when modelling the effects of hygiene.

Another possible explanation for the effects of altered hygiene on allergy and/or autoimmunity is that there are differences in the induction of regulatory T cells (BOX 3). Certain infectious agents, such as *Bordetella pertussis*, have been reported to induce the development of T regulatory 1 (TR1) cells as a method of evading host responses<sup>44</sup>. A similar induction of regulatory T cells has also been described following infection with hepatitis C virus<sup>45</sup>, *Helicobacter hepatis*<sup>46</sup> and helminths<sup>47</sup>. In addition, a strong bias in CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T-cell subsets

during thymic selection can result in autoimmunity<sup>48,49</sup>. Patients with mutations in the forkhead box P3 (*FOXP3*) gene (required for the development of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells<sup>50</sup>) also show systemic autoimmunity, eczema and increased serum IgE levels<sup>51</sup>, so a global lack of regulatory T cells does affect immune-system homeostasis. Yet, in a lymphocyte-replete human or experimental animal, the ability of infections to alter regulatory T-cell populations is probably weak, considering the poor antigen-specific proliferative expansion of regulatory T cells<sup>52</sup>. Therefore, we think that it is improbable that functional alterations in regulatory T cells explain the relationship between improved hygiene and increasing incidence of allergy and autoimmunity in the population.

**Hygiene in experimental animals**

Because we can manipulate the population of environmentally derived microflora by controlling the husbandry of rodents, we should also be able to investigate the underlying basis of the hygiene effect. It is crucial to appreciate that whereas germ-free mice have no microorganisms colonizing their body surfaces, neither germ-free nor SPF animals have been exposed to any known pathogen. Hygienic humans have a diverse intestinal and body-surface flora, but they take measures to reduce their exposure to pathogens. The environmental conditions that we manipulate for experimental animals (when comparing mice with a restricted commensal flora and germ-free mice, neither of which are exposed to pathogens) are therefore quite different from improvements in human hygiene (in which early exposure to pathogens is reduced). Humans have a much more diverse (approximately 1,000 species) and dense (approximately 10<sup>12</sup> bacteria per gram of luminal contents) commensal bacterial flora than SPF mice<sup>53</sup> (BOX 2). In fact, in contrast to humans, most rodent models of autoimmunity actually show reduced incidence in conditions of improved hygiene<sup>54–59</sup>. Similarly, spontaneous and induced models of inflammatory bowel disease are abrogated in SPF or germ-free conditions<sup>60–63</sup>. There are rare exceptions to this: notably, autoimmune gastritis following neonatal thymectomy, the incidence of which is unchanged in germ-free conditions<sup>64</sup>; and diabetes in non-obese diabetic mice, in which the incidence is increased<sup>65,66</sup>.

There are potentially three ways in which microorganisms interact with the immune system: an infection in which live microorganisms can proliferate systemically; the penetration through the mucosa

## Glossary

## DEFENSINS

A family of proteins exhibiting bactericidal properties. They are secreted by immune cells (particularly neutrophils), intestinal Paneth cells and epithelial cells.

## EXHAUSTION

Non-responsiveness of the immune system resulting from the deletion of specific thymocytes (central tolerance) and the deletion or functional inactivation of specific T cells in the periphery (peripheral tolerance) in the presence of large quantities of antigen.

## IGNORANCE

Non-responsiveness of the immune system in the presence of a given antigen, despite the existence of specific T and B cells capable of mounting a functional response.

## INFLAMMATORY BOWEL DISEASE

Immune-mediated inflammation of the bowel. There are two main forms: Crohn's disease, which is a granulomatous segmental inflammation affecting any part of the intestine, and ulcerative colitis, which is a mucosal inflammation involving the rectum and extending for a variable distance along the colon. In developed countries, the incidence of inflammatory bowel disease is approximately 1 in 50,000. It usually starts in early adult life and continues afterwards with a relapsing, remitting course.

## LAMINA PROPRIA

The layer of the intestine between the epithelial cells and the most superficial smooth-muscle layer.

## MIMICRY

Resemblance between epitopes contained within microbial and host proteins, leading to crossreactivity of T cells in the host.

## MUTUALISM

The relationship between two different species that live in close proximity and benefit from one another.

## PEYER'S PATCHES

Collections of lymphoid tissue located in the mucosa of the small intestine, with an outer epithelial layer containing specialized epithelial cells, called M cells.

## REPertoire

The spectrum of B or T cells. Defined according to the specificities of the B-cell- or T-cell-receptors that are present immediately before onset of a clinically important infection.

## T-CELL-DEPENDENT ANTIGEN

To generate an antibody response to a T-cell-dependent protein antigen requires recognition of the antigen (in the context of MHC molecules) by helper T cells and cooperation between those antigen-specific T cells and B cells that recognize the same antigen.

## TOLL-LIKE RECEPTORS

Cell-associated pattern-recognition receptors that recognize molecules unique to microorganisms, resulting in immune-cell activation and production of pro-inflammatory molecules.

lymph nodes were smaller and nephritis was reduced compared with animals on the natural-ingredient diet, thereby demonstrating the possible impact of microbial products on the resulting immunopathology.

## Modelling human hygiene effects

Instead of the current method of comparing germ-free and colonized animals, in which large differences in lymphoid structure are apparent, we think that deliberate, defined experimental infections will be required to model the way in which improvements in human hygiene during early life lead to a greater incidence of allergy and autoimmunity. Indeed, it is possible to show short-term alterations in susceptibility to induced allergic responses following defined experimental infections. For example, previous infection with *Mycoplasma* or *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) can attenuate airway inflammation that is induced experimentally using ovalbumin, at least when mice are challenged within 1–2 weeks of clearing the infection<sup>68,69</sup>. Previous pulmonary infection with influenza virus can also provide protection against bronchial hyperresponsiveness in mice<sup>70</sup>. This effect is dependent on **interferon- $\gamma$**  production by lung-resident memory CD8<sup>+</sup> T cells, which can be re-activated by nonspecific stimuli encountered during allergen challenge<sup>70</sup>. These studies provide evidence for the ability of pathogenic infections to alter unrelated immune responses through immune deviation mechanisms, but they do not model the long-term effects of early exposure to pathogenic agents and how this prevents the development of allergy and/or autoimmunity.

Infections result in an immune response that is partly specific (T-cell clones specific for peptides derived from pathogens and high-affinity neutralizing antibodies specific for surface epitopes)<sup>71,72</sup> and partly nonspecific (class-switch recombination of natural antibody specificities, resulting mainly from bystander help provided by specific T-cell clones)<sup>67,73</sup>. We propose that infection causes an alteration of the T-cell repertoire that could also account for the hygiene effect, without necessarily involving T<sub>H</sub>1–T<sub>H</sub>2 immune deviation or T-cell-mediated regulation. The presence of a large quantity of antigen can eliminate or inactivate T-cell clones; this functional exhaustion has well-recognized effects that abrogate antiviral<sup>74,75</sup> or anti-tumour<sup>76</sup> immune responses. Exhaustion of virus-specific T cells can also clearly occur in wild-type immunocompetent mice that are infected with lymphocytic choriomeningitis virus<sup>77</sup>. Because T cells that are specific for

of small numbers of commensal environmental organisms that cannot proliferate efficiently; and systemic penetration of soluble microbial molecules that activate TOLL-LIKE RECEPTORS on immune cells. The biggest difference between humans living under hygienic conditions and those under 'primitive' (non-hygienic) conditions is the decreased exposure of the former to systemic infections, whereas SPF mice are not exposed to pathogens and even their intestinal bacteria do not usually reach the systemic immune system. We think that reduced exposure to clinically obvious infections is therefore probably crucial for the 'hygiene effect' in humans, whereas the decreased nonspecific immune activation in germ-free animals (because of the absence of soluble microbial products) is the key to the beneficial effect in many autoimmune models<sup>67</sup>.

Evidence of the potential importance of microbial products (and the possibility of confusion with specific infections) is demonstrated by the induction of autoimmune haemolytic anaemia in mice transgenic for erythrocyte-specific autoantibodies<sup>57</sup>. The pathogenic autoantibodies in this strain are produced by B1 cells derived from the pleuro-peritoneal lineage. In germ-free conditions, there are few B1 cells, and no autoimmune disease develops. In SPF conditions, the animals

have B1 cells but still show no haemolytic anaemia. The immunopathology is only seen when the strain is maintained in conventional conditions; however, defined infections of SPF animals have not yet revealed the causative pathogen. In this case, it is clear that a pathogen can trigger autoimmunity; yet, because injection of lipopolysaccharide has the same effect, the consequences of pathogenic infection probably result from increased exposure to microbial products.

A second example is provided by studying the role of environmental antigens in the spontaneous development of autoimmunity in MRL/*lpr* mice, which have a mutation in the *Fas* (CD95) gene and therefore have defective lymphocyte apoptosis<sup>59</sup>. Under conventional conditions, these animals spontaneously develop an autoimmune syndrome that is characterized by lymphoproliferation (mainly of a CD4<sup>+</sup>CD8<sup>-</sup> T-cell subset), high serum immunoglobulin levels (including multiple autoantibodies), vasculitis and nephritis. When the MRL/*lpr* strain was bred in a germ-free environment but fed an autoclaved natural-ingredient diet, there was no difference in lymphoproliferation or autoimmune pathology compared with animals bred in conventional conditions. However, when the germ-free MRL/*lpr* animals were fed a sterile elemental diet, the



particular microbial antigens are selectively deleted or inactivated during exhaustion, the consequence is unresponsiveness to particular peptide antigens, rather than generalized immunosuppression. Moreover, because there is a hierarchy of the dominance of T-cell epitopes in complex pathogens<sup>78</sup>, such repertoire attrition would probably result in the dominance of alternative epitopes, rather than increasing susceptibility to the organism; however, the immune response to more simple crossreactive proteins (such as allergens or autoantigens) would be lost. Therefore, specific infections can shape the T-cell repertoire and possibly limit the ability of B cells to generate high-affinity antibodies that are capable of causing immunopathology. An alternative model is that an increased density of intestinal commensal flora<sup>79</sup>, resulting in higher penetration of soluble microbial molecules, might modify the T-cell repertoire by facilitating tolerance through activation and clonal deletion of specific T cells that would be repeatedly exposed to non-replicating antigen. Moreover, it has been shown that functional exhaustion can explain the tolerization against experimental allergic encephalomyelitis or renal tubular interstitial nephritis that can be induced following administration of autoantigen in incomplete Freund's adjuvant<sup>80</sup>. Clearly, the specific exhaustion or tolerance of cross-reactive T cells that is required to model this repertoire-dependent version of the hygiene hypothesis has not yet been studied in detail, and this will require sophisticated sequential studies of the CD4<sup>+</sup> T-cell repertoire in various infections.

## Conclusions

In this perspective, we discuss the fact that live commensal bacteria are confined to the mucosal immune compartment because they are readily killed by macrophages and can only survive in dendritic cells; therefore, the systemic immune system is essentially ignorant of these organisms. In humans, epidemiological associations have been made between improved conditions of hygiene and increased incidence of allergy and autoimmunity. We argue that although most animal models of autoimmunity are ameliorated under SPF or germ-free conditions, it is not reasonable to compare this with improved conditions of human hygiene, because the experimental conditions only affect organisms that are not usually seen in large numbers by the systemic immune system. The reduced systemic immune responsiveness observed when the number of colonizing environmental microorganisms is low (in ultraclean animals)

probably reflects poor secondary-lymphoid tissue organization and low baseline activation, resulting from reduced penetration of soluble microbial products throughout the whole body.

The epidemiological evidence for the hygiene hypothesis is persuasive, but to dissect the mechanisms using *in vivo* models requires studying long-term effects of experimental pathogenic infection on the later induction of allergic and/or autoimmune disease. Pathogens can certainly leave their imprint on the composition and structure of the immune system. The converse of the hygiene hypothesis, MIMICRY — which is the triggering of immunopathology by a previous infection — also provides a clinically persuasive explanation for the development of rheumatic fever and Guillain-Barré syndrome, but it is not easy to model satisfactorily. Understanding the long-term pathogenic and environmental influences on immune-system function is yet another frontier for immunologists to cross.

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Competing interests statement  
The authors declare that they have no competing financial interests.

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