# The old friends hypothesis: evolution, immunoregulation and essential microbial inputs

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In wealthy urbanised societies there have been striking increases in chronic inflammatory disorders such as allergies, autoimmunity and inflammatory bowel diseases. There has also been an increase in the prevalence of individuals with systemically raised levels of inflammatory biomarkers correlating with increased risk of metabolic, cardiovascular and psychiatric problems. These changing disease patterns indicate a broad failure of the mechanisms that should stop the immune system from attacking harmless allergens, components of self or gut contents, and that should terminate inappropriate inflammation. The Old Friends Hypothesis postulates that this broad failure of immunoregulation is due to inadequate exposures to the microorganisms that drive development of the immune system, and drive the expansion of components such as regulatory T cells (Treg) that mediate immunoregulatory mechanisms. An evolutionary approach helps us to identify the organisms on which we are in a state of evolved dependence for this function (Old Friends). The bottom line is that most of the organisms that drive the regulatory arm of the immune system come from our mothers and family and from the natural environment (including animals) and many of these organisms are symbiotic components of a healthy microbiota. Lifestyle changes that are interrupting our exposure to these organisms can now be identified, and many are closely associated with low socioeconomic status (SES) in wealthy countries. These insights will facilitate the development of education, diets and urban planning that can correct the immunoregulatory deficit, while simultaneously reducing other contributory factors such as epithelial damage.

## 1. Introduction

It was noted as early as 1873 that the prevalence of hay fever was increasing amongst wealthy urban populations but not amongst farmers (1). Increases in allergic disorders accelerated during the 20th century (2, 3), and this focused attention on Th2 responses. However it was soon realised that the prevalences of autoimmune disorders and inflammatory bowel diseases (IBD) that involve different effector pathways of the immune system were often rising in parallel at the same time and in the same places (2, 4, 5). This suggested a broad failure of immunoregulation allowing multiple branches of the immune system to target harmless allergens, autoantigens and gut contents. Moreover even in individuals with no obvious inflamed target organ, systemically raised levels of inflammatory biomarkers were increasingly being observed in wealthy developed countries

(6), and correlated with an increased risk of metabolic, cardiovascular (7) and psychiatric disorders (8–10).

These observations point to a broad failure of regulation of the immune system. Therefore the recent increases in chronic inflammatory disorders are likely to be attributable to recent changes in the stimuli that drive the development of immunoregulatory mechanisms. Expansion of the repertoire of effector lymphocytes of the immune system is driven by microbial inputs, but so is expansion of the regulatory anti-inflammatory arm that censors inappropriate immune responses and terminates redundant inflammation (11). This is true in the gut which has received most attention (12, 13), but it is equally true for the skin (14, 15) and the lungs (11). The mechanisms involved are outlined in a later section.

The "Old Friends Hypothesis" therefore uses an evolutionary framework to identify the microbial inputs that drive immunoregulatory circuits (16, 17). These are likely to be found amongst organisms with which humans co-evolved as hunter-gatherer omnivores and on which we may be in a state of evolved dependence (18). This paper outlines the evolution of our relationship to microorganisms, and the evolution of the immune system, and then considers many broad categories of microorganism, with particular attention to their role in driving immunoregulation and in allergic disorders. But no hypothesis in biology is ever the whole answer. The Old Friends Hypothesis should be considered together with the need for biodiversity in the microbial input (19), and the increased risks of allergic disorders when epithelia are exposed to substances driving "danger signals" and increased permeability (20–22) and **Figure 1**.

## 2. Evolution from and with microorganisms

Cellular life forms came into existence about 3.8 billion years ago. Approximately 1.5 billion years later an endosymbiotic event led to an organism similar to an alpha-proteobacterium living inside another organism, where it evolved to become the mitochondrion (23). This appears to have happened only once and led to the evolution of all eukaryotic life forms. About 65% of human genes have their origins in Bacteria, Archaea and eukaryotic microbes (24), including genes responsible for synthesizing neurotransmitters in the brain (25).

So we evolved from microorganisms but we also evolved in a world dominated by them. Calculating the biomass of major life kingdoms in terms of carbon content reveals that bacteria rank second after plants in total biomass, comprising approximately 7 gigatons of carbon compared to humans who only constitute 0.06 gigatons. Additionally, there are around $10^{30}$ bacteria, archaea, and fungi present on earth, outnumbering humans by a ratio of $10^{20}$ to 1. The symbiotic microbiota in our guts is at least as abundant as our own human cells, and they produce more than ~30% of the small molecules present in our peripheral blood, affecting our physiology in largely unexplored ways (26). These gut organisms were initially separated from host tissues by a chitin barrier, which persisted in arthropods and annelids. In chordate invertebrates such as tunicates, the barrier persists and is embedded in a mucin gel. In the most primitive vertebrates, a more substantial mucus layer is produced, and in mammals, the chitin layer is absent permitting the complex mucus layers to interact with microorganisms. Some organisms
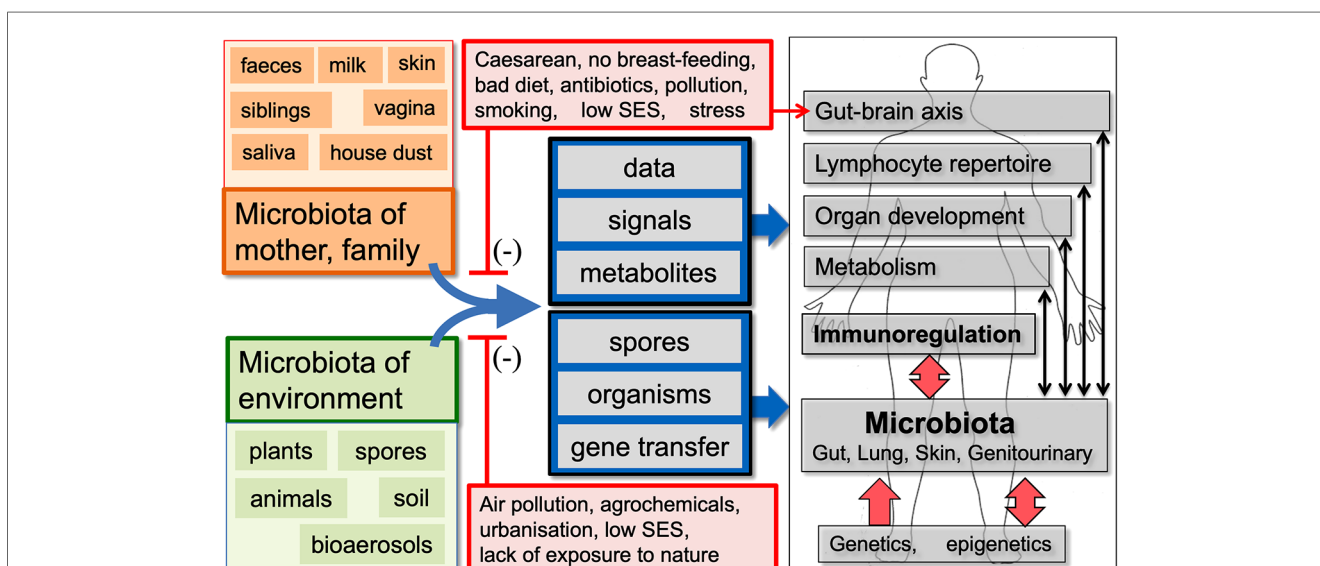


FIGURE 1
A summary of the old friends hypothesis. We co-evolved with the microbiota of mother, family and the natural environment, which are involved in multiple physiological functions and provide essential data and signals that drive development of the effector and regulatory arms of the immune system. Factors that distort these microbiota and/or decrease our exposure to the microbiota-derived signals and metabolites that drive immunoregulation may be contributing to the increase in chronic inflammatory disorders. In some human communities many, but not all, of the factors that distort microbial inputs are associated with low socioeconomic status (SES). (Infections and vaccines that enhance "Trained Immunity" rather than immunoregulation are not shown, but are discussed in the text.)

adhere to the mucus, are nourished by it, and regulate the function of underlying cells (27). This is reminiscent of the situation in plants, where molecules secreted by the roots attract and nourish microorganisms that engage in symbiotic nutrient exchange and signaling (28).

The gut microbiota has co-evolved with humans. Comparing human and ape microbiota to calculate the date when humans and the great apes diverged from a common ancestor yields a date similar to that indicated by comparisons of their chromosomal or mitochondrial DNA (29). Moreover many bacterial and at least one archaeal strain have co-diversified and spread across the planet with their human hosts. These organisms are becoming "obligate symbionts", losing many genes they would need to survive outside the human gut (30). They are co-evolved components of our physiology.

## 2.1. Co-evolution of the immune system and microorganisms

How did the immune system cope with this massive co-evolving community of diverse microbial species in close contact with the host tissues? The innate immune system uses inherited pattern recognition receptors (PRR) to recognise conserved microbial components. However, pathogens can evolve quickly and produce structures that are not recognized by existing PRRs. The innate immune system can try to adapt by duplicating and modifying PRR genes, but this process is slow and results in a cluttered genome. The adaptive immune system in vertebrates solves this problem by using somatic hypermutation to create a large variety of receptors with little increase in genetic complexity. This involves mutating the genes that encode B and T lymphocyte receptors to produce a diverse array of clones. While this reduces the genetic load, it also creates new problems such as the production of useless or autoreactive lymphocytes. However, since each clone expresses only one receptor, any cell line that recognises nothing or that recognises the host's own tissues can be eliminated. Most autoreactive cells are eliminated in the thymus which expresses self-antigens. However in order to select and retain lymphocyte clones that recognise a diversity of microorganisms, and that can manage and tolerate a diverse microbiota while eliminating pathogens, the adaptive immune system must obtain data from microbial inputs, acquired mostly from mother, family and the environment (31). Thus the immune repertoire of each new individual is matched to the microbial environment into which that individual was born.

## 3. What do microbial exposures do for the immune system

It is evident from the previous paragraph that the developing immune system requires data from microbial inputs. However this is only one of many essential functions of microbial inputs, many of which have profound effects on the regulatory arm of

the immune system. These are listed in Table 1 and in the following sections.

## 3.1. Data

An input of microbial antigens and epitopes is needed in early life to select the mutated lymphocyte clones that need to be retained as memory cells. All life forms are constructed at least in part from variants of the same building blocks that evolved long ago in early microorganisms (24, 25). This is important because it means that if individual humans or animals are exposed to a sufficiently diverse range of microbial epitopes, the lymphocyte clones that are retained and expanded will, by chance, contain some that recognise viruses or other pathogens to which the individual was never previously exposed such as HIV or COVID-19 (31–33). This point emphasises the need for exposure to microbial biodiversity (19). Interestingly, biodiversity of the gut microbiota has a strong correlation with health (64). Even declining health in old age is associated with a decrease in gut microbiota biodiversity (35, 65). However other possible functions of biodiversity are suggested in the next paragraph.

## 3.2. Biodiversity

It may not be only the diversity itself that is important but also the increased likelihood of having essential species that drive functions such as immunoregulation (discussed later) (36,37), or other undiscovered necessary functions. For example, a single protein synthesised by a single organism is required for the expansion of the pancreatic β cells in the zebra fish (66). We do not know if there are similar hidden requirements in the human microbiota because we cannot recolonise germ-free humans with one species at a time in order to identify such dependencies.

Another possibility is that complex ecosystems are more stable. Species diversity can protect ecosystems from excessive damage caused by environmental change because having many species increases the chance that some can quickly adapt to the new conditions (67).

Biodiversity also prevents dangerous biofilm formation. An organism's physiology changes when it forms biofilm. Some organisms become more pathogenic, resistant to the immune system and even resistant to antimicrobials. Much of the pathology caused by

*Candida albicans* occurs when it switches from yeast to hyphal forms during biofilm formation (68). In patients with IBD, gut microbiota can penetrate the mucus barrier and form biofilm that adheres to the epithelial surface. Bacteria from the microbiota of healthy donors do not cross human intestinal epithelial cell monolayers *in vitro* but organisms from the biofilm can do so (69). High biodiversity of the gut microbiota may affect quorum sensing signals and prevent the switch to biofilm.

TABLE 1 Some microbial inputs and their functions.

| Input | Effect on immune system | Refs. |
|---|---|---|
| **Data** | | |
| Diverse microbial epitopes | Select diverse repertoire of useful lymphocyte clones. Expand lymphocyte repertoire to recognise novel pathogens | (31–33) |
| Microbial epitopes from microbiota transported to thymus | Select lymphocyte clones that recognise gut microbiota<br>Tolerate symbiotic partner organisms | (34) |
| **Biodiversity, organisms** | | |
| Diverse organisms | Populate microbiota. Drive organ development. Set up regulation of metabolic and immune systems | (35–37) |
| Spores | Trapped by mucus and cilia, swallowed. Expand or restore the gut microbiota. Treg-inducing strains. | (38) |
| Low dose pathogens | Disarm in airways, swallow.<br>Immunity to common pathogens. "Trained Immunity" | (39, 40) |
| **Metabolites** | | |
| Short chain fatty acids from fermentation of fibre, polysaccharides | Inhibition of histone deacetylases (HDACs) and activation of G-protein-coupled receptors (GPCRs).<br>Anti-inflammatory effects. Increased Treg activity | (41, 42) |
| Bacterial tryptophan catabolites | Indoles and indolepropionic acid promote differentiation of Treg via the aryl hydrocarbon receptor (AhR) | (43) |
| Secondary bile acids | Less Th17 cells and enhanced production of RORγ + Treg<br>Anti-inflammatory effects. Increased Treg activity. | (12, 44) |
| Branched chain amino acids | Maintain Treg. Enhance immunoregulation | (45) |
| **Signals** | | |
| Microbial components (e.g. some LPS & muramic acid derivatives) | Signals via Pattern Recognition Receptors (PRR)<br>Tolerance, immunoregulation, innate immune system regulation. Drive release of *TNFAIP3* (A20) | (46–49) |
| DNA | Horizontal gene transfer. Adapt strains to gut ecosystem and adapt metabolic repertoire to diet | (50–52) |
| **Extracellular vesicles and microRNA** | | |
| Microbial extracellular vesicles and miRNA | Influence promotion by dendritic cells of T cell differentiation into various effector types or Treg | (53) |
| **Pathogens and vaccines** | | |
| Infection; crowd infections of childhood or other pathogens | Death or immunity to the pathogen. "Trained Immunity" after some infections or during persistent infection. Epigenetic modulation of adaptive immune system | (54–57) |
| Vaccines | Immunity to the pathogen. Some live vaccines also provide non-specific survival benefit via "Trained Immunity" | (58–60) |
| **Other inputs** | | |
| Bacteriophages from people or environment | Regulate composition of gut microbiota. Release of inflammatory components by lysis. Depletion of anti-inflammatory strains when phage imbalance? | (61–63) |

## 3.3. Metabolites that modulate immunoregulation

The roles of the major gut microbiota-derived metabolites in the regulation of immune function were reviewed recently (12) and will be outlined very briefly here and in Table 1. Short chain fatty acids (SCFA) such as acetate, propionate and butyrate, derived from the microbial fermentation of dietary fibre, can enhance production of IL-10, TGF-β and Treg (12, 41). Secondary bile acids generated by the microbiota, notably derivatives of lithocholic acid, can downregulate Th17 cells and enhance production of RORγ + Treg (12, 44). Several bacterial tryptophan catabolites including various indoles and indolepropionic acid promote Treg differentiation via the aryl hydrocarbon receptor (AhR) (12, 43). Branched-chain amino-acids such as valine, leucine and isoleucine are present in the diet, but are also generated by the microbiota, and maintain Treg (45).

The Treg populations in the skin are attracted and expanded by signals from skin microbiota (15). Lipases from components of this microbiota (*Corynebacterium, Staphylococcus,* and *Micrococcus*) act on lipids secreted by sebaceous glands to generate butyrate and other SCFA (70), which have immunoregulatory properties as outlined above. Moreover, some authors suggest that ammonia-oxidising bacteria (AOB) and archaea that colonised human skin in the past converted the high levels of ammonia and nitrate in human sweat into nitrite and nitric oxide which entered through the skin and exerted immunoregulatory functions (71), notably, downregulation of Th2 responses (72). Because these organisms are exquisitely sensitive to alkylbenzene sulfonate detergents this may no longer occur, though clinical trials applying AOB to the skin are being performed (73).

## 3.4. Microbial components and signals that modulate immunoregulation

Exposures to microbial components that activate PRRs such as TLR4, TLR2, TLR9 or AhR or PI3K/Akt/mTORC1 signaling systems drive the establishment of immunoregulatory mechanisms. These microbial components transiently trigger inflammation, but repeated low dose exposures may prime anti-inflammatory mechanisms due to the release of IL-1β which can induce tolerance to itself and to endotoxin (LPS) (74). Therefore exposure to these microbial signals informs the immune system about the nature of the microbial environment and triggers immunoregulatory epigenetic adjustments. For example, LPS induced Treg via tolerogenic dendritic cells and TGF-β in an animal model (75). LPS in dust in the farming environment may protect against allergic responses by inducing A20 in lung epithelial cells (76). A20 is a potent inhibitor of the NF-κB signaling pathway and its expression is increased in Amish farmers using traditional farming methods (46, 47).

In mice, administering a TLR2 agonist resulted in a decrease in Th17 cells and an increase in type 1 regulatory T cells in the spleen. This led to a reduction in the severity of Experimental Autoimmune Encephalomyelitis (EAE) (77). The same researchers discovered that patients with Multiple Sclerosis (MS) had significantly lower levels of a TLR2 agonist derived from bacteria in their blood compared to healthy individuals (77).

TLR9 is an intracellular PRR that recognizes CpG motifs that are not methylated. These motifs, common in microbes, typically trigger an inflammatory response. However, some variants of CpG motifs and other microbial DNA sequences may have anti-inflammatory properties (78, 79). This appears to be the case for many species of *Lactobacillus* (80) which could explain why the probiotic effects of lactobacilli depend on the presence of TLR9 in the gut (81).

DNA is exchanged by horizontal gene transfer (HGT) both between different gut-resident microorganisms, and between gut microbiota and organisms in the environment (50–52). This exchange can occur between species that diverged in an evolutionary sense in the distant past, and constitutes a global network of gene exchange that can help strains to adapt rapidly to new diets and metabolic needs (51, 52). It is not clear whether HGT modulates the organisms that drive development of immunoregulatory mechanisms.

## 3.5. Extracellular vesicles and microRNA: a 2-way dialogue

Host-derived microRNAs (miRNA) in membrane-bound extracellular vesicles (EV) can modify microbial gene expression. Mice that lack the miRNA-processing enzyme, Dicer, cannot form miRNAs. Such mice developed abnormal gut microbiota and exacerbated colitis, but administrating faecal miRNA corrected these abnormalities (82). So the host regulates gene expression in the gut microbiota. But this type of communication is 2-way. Recent work has shown that microbiota-derived EV have profound physiological effects on host metabolism, on the regulation of the immune system and on Treg numbers (53).

## 3.6. Bioaerosols

The microbial diversity of air is comparable to that of seawater, soil, and the human gut (83). Moreover, the lungs and airways constitute a sense organ with cellular sensors that can detect biogenic aerosols in inhaled air whether derived from microorganisms or from other sources such as plants. Plant polyphenols like quercetin, resveratrol and curcumin can reduce inflammation through the AhR (84). Microbial pigments such as phenazines and naphthoquinones can also regulate inflammation and anti-bacterial responses (48). All of these molecules and others from algae and higher plants can inhibit protein kinases of the PI3K/Akt/mTORC1 signaling system, which is believed to have an anti-inflammatory effect (49). This is likely to be relevant to the immunoregulatory benefits of exposure to the natural environment discussed later.

## 4. Which are the microbial exposures that matter

With this evolutionary, developmental and pharmacological background we can begin to ask which microbial exposures are the ones that drive the immunoregulatory mechanisms that seem to be deficient in modern developed countries.

## 4.1. Crowd infections of childhood

The seminal observation that hay fever was less prevalent in children with older siblings (85) led initially to speculation that modern domestic hygiene was reducing exposure to the common infections of childhood leading to imbalances within the immune system. However this hypothesis was unlikely because humans cannot be in a state of evolved dependence on these infections. They are mostly "crowd infections" that could not have persisted in isolated ancestral hunter-gatherer groups. Measles, for example, probably did not hit human populations until late in the Roman empire when appropriately large populations existed (86–88).

The debate was rapidly resolved when epidemiological studies revealed that these crowd infections of childhood do not protect from allergic disorders (89–91), and often trigger or exacerbate them (89, 92, 93).

We now understand that the protective effect of older siblings, while unquestionably correct, is likely to be due to increased transmission of the microbiota of mother and of the natural environment, as discussed later (94, 95). This does not mean that the common infections of childhood have no effect on the immune system but their effect, apart from inducing immunity to themselves, is non-specific activation of the innate immune system ("Trained Immunity") rather than amplification of down-regulatory anti-inflammatory pathways, as briefly explained in the next section.

### 4.1.1. "trained immunity"

In the 1930s, Pullinger observed that infecting cattle or guinea pigs with *M. tuberculosis* conferred resistance to *Brucella abortus* (96). Pullinger and subsequent authors attributed this to nonspecific activation of monocytes (96, 97). Further studies showed cross-protection between unrelated parasite species, and between *Listeria monocytogenes* and influenza virus (98). This work was largely forgotten until it was reported in the 1980s that live vaccines such as measles, polio, smallpox, and the Bacillus of Calmette and Guérin (BCG) could enhance resistance to unrelated infections (58, 59). These effects are mediated by epigenetic modulation of several components of the innate immune system, including natural killer cells and monocytes as Pullinger and Elberg had suggested (60, 99). "Trained Immunity" is not known to enhance the immunoregulatory pathways that are deficient in rich urban communities, but in view of the interconnected nature of all aspects of the immune system it is likely that changing patterns of Trained Immunity will be found to be relevant (100). This constitutes a major gap in our knowledge.

## 4.2. Mother and other people

The transfer of the co-evolved human microbiota (29, 30) from mother (and siblings) to infant is critical for the development of the infant's microbiota, as well as the immune and metabolic systems (94). Certain lifestyle factors, such as Caesarean deliveries, lack of breastfeeding, poor diet (discussed in greater detail below and in Table 2), antibiotic use, and insufficient mother-infant intimacy, can reduce this transfer, and are associated with an increased risk of immunoregulatory disorders (94, 95, 101). Some organisms in the child's microbiota appear later and continue to accumulate until 5 years of age (131). These organisms are probably acquired from other family members and at day-care centres, as well as from the natural environment. Microbial strains can be transmitted person-to-person through normal social and mother-infant interactions both within and outside the home (132, 133). Such transfers may be diminished by modern lifestyles.

## 4.3. The home

Can exposure to the microbiota of modern homes be considered essential? If we think in terms of evolution the response must be that it depends on the home. In the past, humans lived in natural shelters such as caves, or constructed homes from natural materials like stones, mud, branches, and leaves. Later more sophisticated homes were constructed by rearranging natural materials such as timber, stone, straw, soil, clay, animal dung, thatch or turf. The microbial makeup of such homes would have been similar to that of the surrounding natural environment. Even when damp and decaying, the organisms present would have been those with which humans co-evolved. However, modern homes made from synthetic materials, biocide-treated timber, plywood, and synthetic gypsum board harbour a microbiota that is different from that of the natural environment (134, 135). This difference is more pronounced in urban homes that are distant from nature (136). Furthermore, when a modern home is damp and deteriorating, as is often the case in households of low Socioeconomic Status (SES), the bacterial and fungal microbiota can produce secondary metabolites that are hazardous to human health, leading to varying degrees of "Sick Building Syndrome" (104–137) and increased likelihood of children being hospitalized for respiratory infections (107). Therefore, it is improbable that the unnatural microbiota of modern homes, especially when the home is of low SES, provides necessary or desirable microbial exposure for infants. However, when the microbiota of homes is similar to that of farms and the natural environment, it can be beneficial, particularly for disorders related to faulty immunoregulation like asthma, as discussed later (138–140).

TABLE 2 Low socioeconomic status (SES) and distortion of microbial exposures.

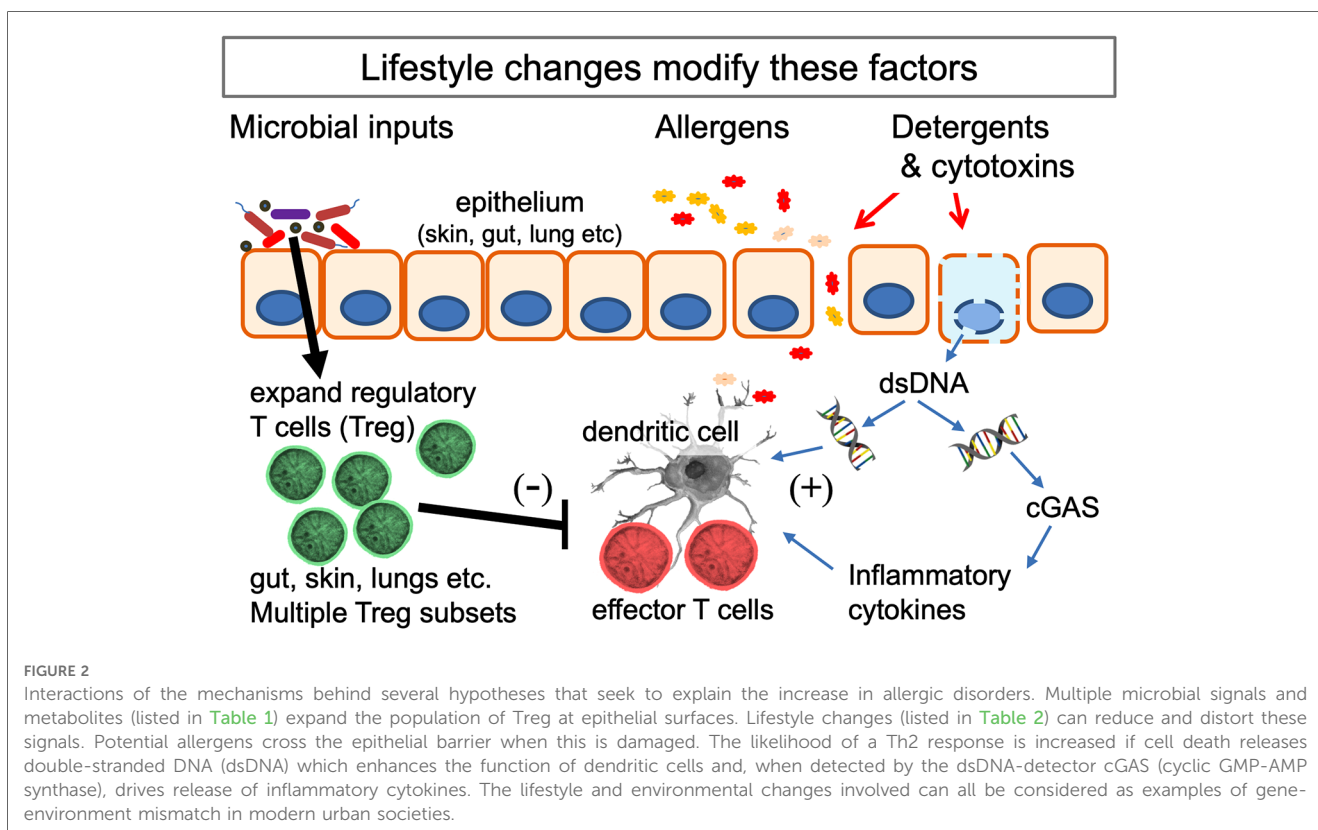| Disruptors of required microbial exposures | | Effects | References |
|---|---|---|---|
| Modern delivery and neonatal care | Caesarean deliveries | Delayed development of mature microbiota in neonate. | (94, 95, 101) |
| | Lack of breast-feeding | Reduced milk-derived prebiotics | |
| Pollution | Traffic, air pollution | Direct effects on microbiota, and indirect effects via host immune system and damaged epithelia | (28, 102, 103) |
| | Agrochemicals | | |
| | Damp, sick-building | Toxic microbial secondary metabolites | (104–107) |
| | Exposure to cleaning and hygiene products | Epithelial damage. Th2 adjuvant effects | (20–22) |
| Lack of green space | Little exposure to strains and spores from nature | Low biodiversity of microbiota Less immunoregulatory strains Increased psychiatric disorders Metabolic/cardiovascular disorders | (108–111) |
| | Less sunlight, vitamin D | Defective immunoregulation, altered microbiota | (112, 113) |
| Stressors | Drug abuse, violence, heat, noise, sleep disorders | Changes to microbiota and reduced biodiversity via signals within the gut-brain axis | (114–116) |
| Poor diet | Unvaried | Low biodiversity of microbiota | (64, 117) |
| | Ultraprocessed: emulsifiers, excitotoxins, | Leaky gut, neuronal damage. Deficient micronutrients | (118, 119) |
| | Low fibre | Low short chain fatty acids (SCFA) | (12, 41, 120) |
| | Low vitamins | Potential deficiencies | (121) |
| | Obesity | Metabolic problems Cardiovascular problems | (42, 122) |
| | Sugars, artificial sweeteners | Distorted microbiota. Raised glycaemic response | (123) |
| Education | Smoking | Switch from aerobes to anaerobes, biofilm and *Clostridioides difficile* | (124) |
| | Antibiotic misuse | Exposure *in utero* or in early life correlates with metabolic and immunoregulatory problems | (125–127) |
| | | Reduced deconjugation of sex hormones, reduced re-uptake, distorted sexual maturation | (128) [Discussed and referenced in (129)] |
| | Vaccine hesitancy and refusal | Infection risk and lack of beneficial non-specific vaccine effects | (130) |

### 4.3.1. Cleaning agents: are our homes too clean?

Another relevant variable is the use of cleaning agents. The media have publicised the notion that the increase in allergic disorders might be attributable to reduced microbial exposures caused by excessive personal or domestic cleanliness (141). However epidemiological studies seeking correlations between the use of cleaning agents and allergic disorders have produced wildly discordant results (142, 143). We have suggested elsewhere that this might be due to a failure to take into account a probable effect of exposing infants to aerosols of cleaning agents (22). Cleaning agents, especially when used as sprays, have been thought to have harmful effects on the lungs of adult cleaning personnel who are exposed to them every working day (144). Detergents cause increased epithelial permeability and cytotoxins cause local cell damage that provides "danger signals" and activates allergic defence mechanisms in the airways or gut (20, 21, 145, 146). For instance, food antigens usually cause tolerance, but if the antigen is detected in the gut in the presence of cell death an allergic Th2 response may be generated (20). This antigen then becomes a proxy for the cytotoxic molecule (which is often not itself immunogenic), so subsequent exposures will trigger an allergic reaction, even if the cytotoxin is absent. Exposure to detergents and cytotoxins is most likely in homes of low SES where infants are crawling in confined spaces while their mothers use trigger sprays containing potentially toxic cleaning products. In a UK cohort, where use of cleaning agents correlated with wheeze and atopic eczema (142), it was noted that the most intense use of chemical household products correlated with low educational level, smoking, and poor, crowded housing (147). The infant airway in which immunoregulatory balance is being established, is likely to be very sensitive to these exposures. In animal models successful Th2 adjuvants cause some cytotoxicity and release of double stranded DNA (dsDNA) (146) which enhances antigen presentation by MHC Class II (148). Moreover dsDNA activates the local immune system via the airway dsDNA sensor (cGAS; cyclic GMP-AMP synthase) pathway (149), which is essential for the induction of airway allergy (150). Further details of this pathway can be found here (151) and in Figure 2.

## 4.4. Natural environment

In addition to the microbiota of mother and family, our evolving hunter-gatherer omnivore ancestors were inevitably exposed to the microbiota of the natural environment and of animals that were hunted, eaten or domesticated. During the late 19th century Blackley noted that farmers were less prone to hay fever than were people residing in urban areas (1). Since then, numerous studies have supported the notion that early-life exposure to a farming environment can reduce the prevalence of allergic disorders (46, 138). Some of the protective effect appears to come from early life exposure to farm animals (152) or dogs (139, 153) which cause measurable changes to the gut microbiota (154). Furthermore, living close to green spaces can also decrease the risk of allergic sensitization (155). Some of these studies have revealed immunological evidence that strongly suggests a cause-and-effect relationship, rather than a mere coincidental



FIGURE 2

Interactions of the mechanisms behind several hypotheses that seek to explain the increase in allergic disorders. Multiple microbial signals and metabolites (listed in Table 1) expand the population of Treg at epithelial surfaces. Lifestyle changes (listed in Table 2) can reduce and distort these signals. Potential allergens cross the epithelial barrier when this is damaged. The likelihood of a Th2 response is increased if cell death releases double-stranded DNA (dsDNA) which enhances the function of dendritic cells and, when detected by the dsDNA-detector cGAS (cyclic GMP-AMP synthase), drives release of inflammatory cytokines. The lifestyle and environmental changes involved can all be considered as examples of gene-environment mismatch in modern urban societies.

association (46, 47, 155). For example, Amish farmers who use traditional farming techniques have a very low incidence of allergic disorders and were found to have increased biomarkers of immunoregulation, compared to industrialized Hutterite farmers (46, 47). Similarly, exposing children to natural biodiversity in their school playgrounds in a controlled clinical trial resulted in an increase in peripheral blood biomarkers of immunoregulation (109). The evidence supporting the protective effect of exposure to the microbiota of the natural environment is less complete for other chronic inflammatory disorders, but there is suggestive evidence for IBD (156), autoimmune diseases (38) and psychiatric disorders (110) and metabolic and cardiovascular diseases (111).

### 4.4.1. Soil

Which organisms from the natural environment are important in addition to those from animals? Although there is no direct evidence linking soil consumption to health benefits, it is evident that soil is a significant source of microbial exposure in natural settings. Soil microorganisms become airborne in dust during dry conditions, but also when raindrops impact the soil, because tiny explosions of soil organisms occur, releasing them into the air (157). Additionally, soil organisms settle on food, particularly in farmers' markets where washing and packaging are minimal. Moreover geophagy (consumption of soil) is an evolved behaviour. It is probable that all vertebrates, especially in early life, engage in it, and the green iguana is a well-studied example of this (158). Many primate species, including gorillas, orangutans, and chimpanzees have been observed eating soil (159, 160). Geophagy is a common practice in many cultures (160, 161), and is frequently observed during pregnancy, not only in underdeveloped rural societies but also in Western cultures where it is often considered a pathological manifestation of pica. Many of the Treg-inducing and immunoregulation-enhancing strains of bacteria are found in soil (13, 162).

#### 4.4.1.1. Bacteriophages

There about $10^9$ phages/gm of soil so vast quantities are taken in every day in food and drinking water. Bacteriophages are the most numerous biological entities in the gut and constitute about 95% of the gut virome. They influence the composition of the microbiota and the turnover of susceptible species, and therefore regulate the release of pharmacologically active microbial components and metabolites (61, 63). The overall effect on immunoregulation is poorly documented but there is a report of low diversity of gut bacteriophages in children who develop the autoantibodies implicated in type 1 diabetes (T1D), or the clinical disease (163). It is possible, therefore, that contact with appropriate bacteriophages from mother, other people and the natural environment influences immunoregulation.

#### 4.4.1.2. Spores

Soil is also a major source of spores which are particularly relevant to immunoregulation. Spores are resilient and can survive in the environment for centuries [reviewed in (164)]. It appears that around 60% of bacterial genera in the gut can produce spores,

including some that were not previously known to do so (165, 166). Spores play a vital role in transmitting strictly anaerobic organisms essential to human health from one person to another through the environment, since the spores are not killed by oxygen (165, 166). Therefore spore-forming anaerobic organisms are probably among the components of a child's microbiota that appear later in infancy and continue to accumulate until the age of 5 (131, 166). Many of these organisms are critical because they make SCFA which have numerous essential physiological roles (12, 41), and promote expansion of the Treg population (13, 38) as outlined in Table 1.

## 4.5. Low dose pathogens

Inevitably there are sometimes pathogens in respired air, but very low doses typically lead to protective immune responses rather than disease. When bacteria attach to the nasal mucosa, released exosomes may transfer inducible nitric oxide synthase to neighbouring epithelial cells and increase release of nitric oxide (167). Pathogens also trigger the release of cathelicidin, and other human antimicrobial peptides (AMP) of which there are more than 100 (39). When bacteria and cathelicidin enter the cell the NLRP3 inflammasome is activated and a cascade of events is initiated, including the activation of caspase 1, the death of infected cells, and the release of pro-inflammatory cytokines IL-1β and IL-18. These events enhance inflammation and recruit neutrophils which are induced to form networks of extracellular fibres consisting mainly of DNA called Neutrophil Extracellular Traps (NET). These NETs contribute to inactivation of microorganisms (39).

Ultimately, these mechanisms in the airways kill or disarm the respired organisms, which are then taken up by the lymphoid tissue of Waldeyer's ring or exposed to acid in the stomach before being sampled by the dendritic cells in the small bowel (40). In this way, inspired low doses of pathogens may provide useful data to the immune system and prime immunity to potential pathogens, but they do not appear to be crucial for setting up immunoregulation.

## 4.6. Sequence of exposures

The order in which live or killed vaccines are given can determine the nature of their non-specific effects (59), indicating that the order in which vaccines are given determines the epigenetic changes that they induce. This may also apply to the sequence in which infections are experienced (168). For example it has been suggested that acute lymphocytic leukaemia (ALL) may result from delayed exposure to an agent that, during human evolution, would have been encountered in very early life (169). Interestingly ALL is more common when early life exposure to microorganisms is reduced, such as after Caesarean delivery, lack of breastfeeding and lack of older siblings (169). This is similar to risk factors for immunoregulatory disorders like allergies (94, 95).

A similar concept has been proposed to explain the increase in T1D that seems to have appeared in parallel with the development of

the modern Western lifestyle. The prevalence of T1D fell after the introduction of rotavirus vaccines administered at 2 months (170).

## 4.7. Other infections

It was reported 20 years ago that hay fever and asthma were less frequent in subjects seropositive for hepatitis A virus (HAV), *Toxoplasma gondii*, and herpes simplex virus 1 (171). It was not clear whether these infections were markers of plentiful food-borne and orofaecal microbial exposure to family and environment, or whether the infections were themselves enhancing immunoregulation. Some of these are persistent infections which deserve to be considered because they may drive immunoregulation in order to limit immunopathological damage to the host. A few particularly obvious examples of infections that exert non-specific effects on the immune system are considered briefly below.

### 4.7.1. Helicobacter pylori

*Helicobacter pylori* was carried by humans for much of our evolutionary history. However, the use of antibiotics has reduced the seroprevalence of *H. pylori* to less than 10% among native-born citizens in Western urbanized countries. This could be relevant because epidemiological surveys have shown an inverse relationship between *H. pylori* seropositivity and childhood asthma (56), and experiments in mice indicate that *H. pylori* drives expansion of Treg subsets expressing CXCR3 or RORγt and demethylation at the FOXP3 locus (172).

### 4.7.2. Herpes viruses

More than 90% of adults have been infected with at least one of the five most common species of Herpes virus (HSV-1, HSV-2, varicella zoster, Epstein–Barr virus, cytomegalovirus). These viruses tend to remain latent but periodic reactivation can influence the state of the immune system. Mice that were latently infected with either murine gammaherpesvirus 68 or murine cytomegalovirus were found to be resistant to bacterial pathogens such as *Listeria monocytogenes* and *Yersinia pestis* (55). This resistance is attributed to intermittent reactivation followed by cytokine-mediated activation of macrophages. We are unaware of evidence that these viruses protect significantly from allergic disorders.

### 4.7.3. Tuberculosis

Latent tuberculosis infection (LTBI) is very common in developing countries and the persistent presence of these organisms has been demonstrated in multiple tissues (173). There is a small risk of developing clinical tuberculosis. However, treating LTBI in non-HIV-infected individuals does not provide a survival benefit because it increases mortality from other causes (57). Similarly BCG, a live vaccine derived from a mycobacterium, appears to protect the elderly from respiratory virus infections (174). In other words LTBI and BCG vaccine both induce "Trained Immunity" outlined earlier. But do they induce immunoregulation? Some early studies suggested an inverse correlation between tuberculin test positivity and atopic disorders

(175) but this did not prove cause and effect. Many studies have subsequently investigated whether BCG vaccination protects from allergies (176). The conclusion is that there can be a weak protective effect if the BCG is given to neonates, particularly in babies at greater risk because of atopic disorders in the parents (177), but the effects are small.

### 4.7.4. Helminths

The rise in inflammatory disorders has been attributed to the decline in helminth infections because helminths drive immunoregulatory mechanisms to protect the host from fatal immunopathology (178). Some authors propose that we have evolved to rely on this background immunoregulation so that the absence of helminths results in an excessively inflammatory immune response (179). It was logical to postulate evolved dependence on helminths and this author has endorsed it in the past but it now seems more likely that adaptation of the developing immune system to the presence of helminths was largely epigenetic and is lost after a few generations without helminths. Various helminth species reside in distinct sites, such as blood, tissues, bladder, or gut, and each species damps down inflammation through a unique mechanism (178). Additionally, helminth burdens can vary widely between individuals, even when they live in comparable geographical areas. As a result, there is no "inevitable" helminth-related factor that would have driven the evolution of a permanent germ-line-encoded adaptation leading to evolved dependence [discussed in (180)]. Intermittent environmental factors or infectious stresses are managed through reversible epigenetic adaptations, not via germline encoded mutations which would lead to frequent gene-environment mismatch. These epigenetic mechanisms explain occasional reports of lower prevalence of allergic symptoms in children who were infected with geohelminths in early life [discussed in (178)] but meta-analyses suggest that overall, helminthiases are associated with increased allergic manifestations probably driven by the powerful Th2 responses that they evoke (181).

This probable role of epigenetics also helps us to understand the conflicting results of helminth therapy trials in MS. It is reported that when Argentinian MS patients become naturally infected with helminths they would have encountered during childhood, disease progression can be halted (182). In early life their immune systems developed in the presence of these helminths and consequent epigenetic adjustments necessitate their continued presence. However, in regions where helminths have not been endemic for multiple generations, trials of helminth therapy for MS or other autoimmune disorders have been disappointing (183–185). While evolution turns the inevitable into a necessity, it allows the intermittent or temporary to become an option through epigenetic adjustments. In the absence of helminths in Europe and the USA the need for them has faded.

#### 4.7.4.1. Anecdotal evidence of efficacy may be valid

There is a wealth of anecdotal evidence for the efficacy of self-administered therapy with a variety of helminths. However helminth therapy in wealthy developed countries is probably only

effective in individual patients with specific genetic backgrounds and immunoregulatory deficits for which a particular helminth product happens to be relevant. This is how helminth products may be utilized in the future. However, until we know how to identify the appropriate combination of patient, genetics, disease, and helminth product, clinical trials may not yield useful results, and it is difficult to justify attempts to reconstruct the human biome with a helminth component.

# 5. Causes of failed microbial exposures; links to diet, SES and epidemiology

Table 2 lists some of the ways in which modern life-styles are reducing or distorting essential immunoregulatory exposures to the microbiota of mother, family and the natural environment. Many of them have been mentioned earlier in the text and will not be discussed in detail here. All the factors in the list alter microbial exposures and the microbiota, but clearly some of them are also detrimental to health in ways that are independent of effects on immunoregulation. For example, pollution alters the microbiota of the environment (186) and of the exposed public (103), but it is also directly toxic to humans. Smoking, while closely linked to low SES in many countries has profound effects on the microbiota (124), but is also directly toxic and carcinogenic. We need to know how much of the health deficit caused by smoking and pollution is due to distorted microbiota.

Similarly, diet has a major effect on the gut microbiota but it is increasingly suspected that some ingredients of ultraprocessed foods such as detergent-like emulsifiers and excitotoxins are also inherently toxic, causing a leaky gut and neuronal damage (118, 119). But poor diets can also fail to support immunoregulatory microbiota. For example an unvaried diet can reduce biodiversity of the microbiota (64, 117). Some modern diets associated with low SES can be frankly proinflammatory. There has been a disturbing increase in the incidence of cancers, notably breast and colorectal, appearing in people less than 50 years old (187). Recent studies suggest a role for exposures in early life (187), and the modern low SES Western diet is an obvious candidate. This diet is likely to be low in fibre (41) and SCFA (120) which are necessary for the establishment of immunoregulation as outlined in Section 3.3. Similarly, excessive consumption of sugars (including fructose) and artificial sweeteners leads to distortion of the microbiota, raised glycaemic responses, obesity and metabolic and cardiovascular problems (123–188). Such diets may also be deficient in micronutrients and vitamins, leading to potential deficiencies (12, 121).

These dietary factors, and others listed in Table 2, are closely associated with low SES in wealthy urban settings. In a classic study the difference in life expectancy in some parts of the British isles between wealthy and deprived areas was as high as 27 years (189). We and others have argued recently that much of this SES-associated health deficit might be secondary to inappropriate microbial exposures and inappropriate microbiota (190–192). We need to find out how much of the health deficit

is mediated in this way because this might inspire new ways to combat the SES-linked health deficit.

## 5.1. SES and inconsistent epidemiology

Epidemiologists seeking links between low SES and allergic disorders should perhaps take note of the items in this list which can perhaps explain discordant results in this literature. In developing countries, low SES is often associated with subsistence agriculture in rural settings with abundant exposure to the natural environment. On the other hand, in wealthy urbanised countries low SES is associated with living in polluted urban slums with little access to green space while the wealthy have gardens and rural holiday homes. Similarly in developing countries access to Caesarean deliveries and antibiotics may be difficult for people of low SES, whereas in some rich countries, where these things are readily available, misuse of Caesarean deliveries and antibiotics can be more frequent amongst people of low SES, perhaps because they lack awareness of the disadvantages (193). These points can explain the enormous discrepancies between different epidemiological studies of allergic disorders which, for example, may or may not find links with low SES (194).

## 5.2. Epidemiology and multiple Treg subtypes

Treg biology provides another explanation for discordant epidemiology. There are several different subsets of Treg, which act at different stages in the development of an allergic response, including the initial expansion of T cell clones, polarisation towards Th2, regulation of IgE vs. IgG4 production and late effector pathways such as control of mast cell activation (195). Thus some patterns of Treg activation will block sensitisation, while other patterns of activation will fail to block sensitisation, but may still block clinical manifestations. This explains some instances of a lack of correlation between IgE or skin-prick test positivity and clinical manifestations of allergic disease (196). Different Treg subpopulations, located in different sites, might also explain the very variable correlation between skin, airway and gut manifestations of allergy (197, 198). We need more understanding of the role of different microbial exposures in expanding Treg cells that operate at each stage of the allergic response (195), and at different epithelial surfaces (198).

# 6. Conclusions

The Old Friends hypothesis emphasises the role of exposures to microorganisms with which humans co-evolved as essential drivers of the regulatory and anti-inflammatory arm of the immune system. Any hypothesis that seeks to explain the increases in allergic disorders must include an immunoregulatory component. Exposure to allergens and weakening of epithelial barriers certainly

contribute (21), but they are unlikely to cause allergic responses unless there is also cell death providing the Th2-adjuvant effect of dsDNA (149–151), and unless defective immunoregulatory mechanisms permit it (17). Moreover, we need to explain the often simultaneous increases in other chronic inflammatory disorders such as autoimmunity and IBD (2, 4, 5) and systemic inflammatory states that predispose to cardiovascular, metabolic and psychiatric problems (6, 7, 199). Malfunctioning immunoregulation is likely to be fundamental to all these states, but most phenomena in biology are multifactorial. Things are most likely to happen when there are multiple reasons for them to do so. Indeed all the major hypotheses, whether they involve hygiene, "Old Friends", biodiversity, novel allergens or leaky epithelium (Figure 2), can be regarded as different manifestations of a set of interacting gene-environment mismatches caused by modern lifestyles (200, 201).

We argue that mother, family and the natural environment (including the animals within it) provide the major microbial exposures that are needed to populate the gut microbiota and set up the immunoregulatory pathways that are defective in rich urban societies. The implication is that this mechanism is fundamental to all the current hypotheses. We now know that humans have indeed co-evolved with the microbiota, some members of which are becoming obligate symbionts (29, 30). We have minimal understanding of the physiological roles of these organisms, but we do know that at all body surfaces the microbiota drives development of both the effector and regulatory arms of the immune system (11–15). The biology of the multiple subtypes of Treg and the functional differences of Treg in different body sites can explain many of the wildly variable epidemiological findings on issues such as links to SES, correlations between different allergic disorders, and between sensitization and clinical manifestations (195, 198).

Infections also impact the immune system but mostly they provide data, specific immunity and non-specific activation of the innate immune system ("Trained Immunity") rather than immunoregulation. Germ-line-encoded evolved dependence on infections is unlikely unless the infection was effectively inevitable throughout much of our evolution.

Finally, the Old Friends hypothesis points to many ways in which we can optimize exposures to the necessary microbial inputs. Almost all the detrimental factors listed in Table 2 can be offset by education, improving diets, minimizing use of antibiotics, reducing pollution, improving living conditions, designing better housing and providing access to green space.

## Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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