DEVELOPING ANIMAL MODELS FOR POLYMICROBIAL DISEASES

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Polymicrobial diseases involve two or more microorganisms that act synergistically, or in succession, to mediate complex disease processes. Although polymicrobial diseases in animals and humans can be caused by similar organisms, these disease are often also caused by organisms from different kingdoms, genera, species, strains, substrains and even by phenotypic variants of a single species. Animal models are often required to understand the mechanisms of pathogenesis, and to develop therapies and prevention regimes. However, reproducing polymicrobial diseases of humans in animal hosts presents significant challenges.

There is now compelling evidence that many infectious diseases of humans (FIG. 1) and animals (TABLE 1) are caused by more than one microorganism. The mixed microbial nature of these diseases has been recognized since the early 1920s but there has been renewed interest in this topic since the 1980s[1], signalled by the publication of four important reviews (REFS 2–5) from 1982 to the present date. Polymicrobial diseases (see BOX 1 for nomenclature) can be caused by the synergistic or sequential action of infectious agents from either the same or different kingdoms, genera, species, strains or substrains, or by different phenotypic variants of a single species[6]. Polymicrobial diseases share underlying mechanisms of pathogenesis, such as common predisposing factors (BOX 2), but each disease has unique aspects. Although the molecular mechanisms of some polymicrobial infections are known, other polymicrobial diseases are not well understood. Owing to their complexity, the study of polymicrobial infections requires a multidisciplinary approach and specific *in vitro* methodologies and animal models. The development of assay systems and treatment and prevention regimes is needed.

Multiple diverse *in vitro* systems have been used to study polymicrobial diseases (BOX 3). Although *in vitro* methods are crucial for understanding polymicrobial diseases, rigorous, reproducible and relevant animal models of human diseases are essential for the prevention and treatment of these co-infections[7–10]. All animal models of human diseases have inherent limitations but they also have important advantages over *in vitro* methods, including the presence of organized organ systems, an intact immune system and, in inbred mice, specific genetic backgrounds, and the availability of many reagents for characterizing the immune response to sequential or co-infecting microorganisms. The availability of mice with specific genetic backgrounds can have a pivotal role in understanding the mechanisms of pathogenesis of polymicrobial diseases, as exemplified by studies on septic peritonitis[11–17], periodontal disease[18] and Lyme arthritis[19,20]. Understanding the molecular mechanisms underlying polymicrobial diseases of veterinary importance has also been facilitated by the use of animal models. These veterinary systems are useful examples for those researchers attempting to develop animal models of complex human diseases.

So far, most animal models for human polymicrobial diseases are rodents, usually mice, but also rats, gerbils, cotton rats and chinchillas. Other animal models include non-human primates, which are useful for modelling diseases that are caused by microorganisms with a restricted host range. For most human viral co-infections of clinical importance, good animal models and culture systems are lacking and are urgently required.

This review provides an overview of the pathogenesis of selected polymicrobial diseases, the molecular basis for some of these co-infections and describes animal models that have been developed to mimic these diseases.

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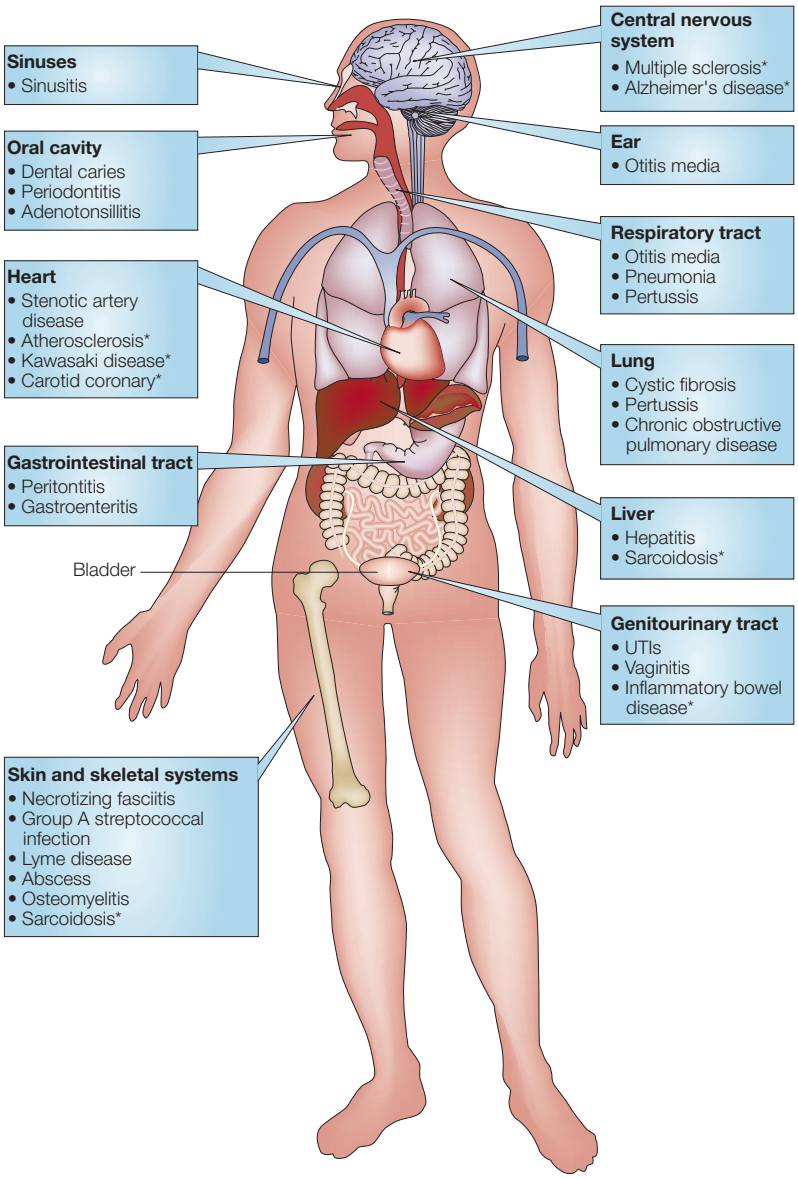

Figure 1 | **Human polymicrobial diseases.** Polymicrobial diseases and suspected polymicrobial diseases (indicated by an asterisk) are listed in the anatomical niche in which the disease pathology is mainly observed.

Table 1 | **Examples of polymicrobial diseases**

Host	Infection or disease
Cattle	Bovine respiratory disease complex (BRDC), bovine gastroenteritis
Sheep	Ovine conjunctivitis, ovine foot rot, chronic non-progressive pneumonia
Pigs	Porcine atrophic rhinitis, porcine gastroenteritis, porcine respiratory disease complex (PRDC)
Poultry	Poult enteritis mortality syndrome, infectious coryza

Viral co-infections

Infections involving bovine viral diarrhoea virus. In cattle, infections with bovine viral diarrhoea virus (BVDV) can be clinically asymptomatic or can cause severe symptoms. The outcome depends on whether the primary infection occurred *in utero* or after birth[21] and whether the primary infection was with a cytopathic or non-cytopathic biotype of BVDV. Milder, congenital persistent infection follows foetal infection with non-cytopathic BVDV. Death, or culling from the herd within 1 year of birth after failure to thrive, is common; however some persistently infected calves seem healthy at birth and survive for several years. Mucosal disease follows congenital persistent infection and is due to co-infection with cytopathic and non-cytopathic BVDV *in utero*. Conversely, acute bovine diarrhoeal disease is induced by primary post-natal infection with either of the BVDV biotypes and can result in severe respiratory, enteric or reproductive disease. The severity of the disease depends on the relative virulence of the viral strain, physical and environmental stresses, and co-infection with another pathogen.

Porcine co-viral diseases. Porcine reproductive and respiratory syndrome (PRRS) results from infection with the PRRS virus (PRRSV) followed by infection with a bacterial or viral co-pathogen. Co-infecting viruses include porcine coronavirus, swine influenza virus[22–24] and pseudorabies virus[25]. Different plaque variants of PRRSV can also co-infect a porcine host[26]. Porcine post-weaning multisystemic wasting syndrome (PMWS) is due to co-infection of pigs with porcine circovirus 2 (PCV-2) and porcine parvovirus (PPV)[27,28]. PCV-2 and PPV are thought to enter the host through tonsillar macrophages and viraemia results within 3 days of infection. PCV-2 and PPV can replicate in circulating peripheral monocytes and contribute to both cell-associated viraemia and viral distribution throughout lymphoid tissues[28].

Hepatitis virus co-infection. Human co-infections with multiple hepatotropic viruses from the hepatitis virus group are well documented. Co-infection with multiple hepatitis viruses is possible owing to their similar routes of transmission and ability to chronically infect the host. Hepatitis A virus (HAV) co-infection of individuals that are chronically infected with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) results in a disease of increased severity and risk of death. Moreover, HBV–HCV co-infection occurs in 10–15% of HBV patients, and hepatitis G virus (HGV)–HCV co-infection occurs in 10–20% of individuals with chronic HCV infection; however, HBV–hepatitis D virus (HDV) co-infection occurs only in the setting of co-infecting HBV. VIRAL INTERFERENCE, in which replication of one virus is suppressed by another virus, is an intriguing aspect of triple HBV–HCV–HDV infection — HDV can suppress both HBV and HCV replication[29]. In a retrospective study of patients with hepatitis virus co-infections, HDV was dominant by RT-PCR detection of HDV RNA in triple co-infections, but in dual co-infections there were alternating dominant roles for either HBV or HCV. Multiple hepatotropic viral infections are associated with reduced HCV replication but increased pathology. Patients with dual or triple co-infections have more severe liver disease pathologies than patients that are

VIRAL INTERFERENCE
One virus suppresses the replication of another.

Box 1 | **Nomenclature of polymicrobial diseases**

For diseases and infections that involve two or more microorganisms, several terms are used in the literature, often interchangeably, and include: polymicrobial diseases, complex diseases, complicated infections, co-infections, concurrent infection, polybacterial diseases, dual infections, mixed infections, synergistic infections, superinfections and secondary infections. In this review, co-infection will be used throughout for clarity, to distinguish infection with more than one microorganism. The term polymicrobial disease is used to describe diseases that result from co-infections.

infected with HCV alone, and HAV co-infection has been implicated in the deterioration of patients that are chronically infected with other hepatitis viruses[30].

Co-infection with HIV and hepatitis virus or HTLV. Owing to shared routes of infection and the ability to induce chronic infections, co-infection with hepatitis viruses and HIV is common[30,31]. HIV infection is often associated with chronic HBV and HCV infections[32]. This association is particularly strong in cases where there is a history of acquisition of HIV by a parenteral route — predominantly in injection drug users. Similarly, co-infection with HIV and human T-lymphotropic virus (HTLV) — two families of retroviruses that share common mechanisms of transmission and tropism for immune system cells, particularly T lymphocytes — occurs in a significant number of individuals worldwide[33]. It is unclear if co-infection alters the pathogenicity of either virus, or results in unique clinical features, but there are now data that indicate that HTLV-2 might modulate the progression and outcome of HIV-1 infection[33].

Co-infection with HIV and herpes simplex virus. In 1994, Kaposi's sarcoma-associated herpesvirus (KSAV), also known as human herpesvirus type 8 (HHSV-8), was isolated. KSAV is found in all clinical variants of Kaposi's sarcoma and is also associated with primary effusion lymphoma and a plasma-cell variant of multicentric Castleman's disease, both of which are AIDS-related lymphoproliferative diseases[34,35]. Infection with herpes simplex virus type 2 (HSV-2) increases the risk of acquiring and transmitting HIV[36,37]. Recent estimates indicate that in HSV-2-positive individuals, 52% of the sexually transmitted risk of HIV infection can be attributed to infection with HSV-2. The increased risk might be due to HSV-2 reactivation, which disrupts the epithelial barrier and recruits activated CD4 cells — host cells for HIV — into the herpes lesion[38]. Or, the role of HSV-2 in HIV transmission might be due to the recruitment of HIV-infected CD4 cells to HSV-infected lesions. In addition, HSV regulatory proteins could upregulate HIV replication and promote viral shedding at the mucosal surface. Taken together, these observations indicate that prevention of HSV-2 infection could reduce the risk of HIV infection and transmission[39].

Animal models for viral co-infections

Modelling human viral diseases in an animal host is challenging owing to the host-range restriction of most viruses. Although greater primates such as chimpanzees and gibbon apes are often susceptible to

Box 2 | **Conditions that predispose animals and humans to polymicrobial diseases**

Stress, lifestyle and metabolic diseases
Stress can be caused by living conditions that are overcrowded — for example, military barracks and college dormitories. Metabolic diseases that are known to predispose humans to polymicrobial diseases include diabetes, cancer and stress-induced immunosuppression. Lifestyle choices that predispose humans to polymicrobial diseases include smoking and diet. All of these factors can underlie polymicrobial diseases such as respiratory tract illnesses, necrotizing ulcerative gingivitis, oral and periodontal diseases and acute interstitial pneumonia of cattle.

Alterations in mucosal surfaces owing to microbial activity
Cell-surface changes at the mucosae might include changes in the expression of receptors for adherence and uptake of microorganisms, or changes in mucosal secretions induced by microbial activity. Examples of polymicrobial diseases that can occur after mucosal alterations include influenza A virus neuraminidase enzyme activity, which facilitates *Streptococcus pneumoniae* adherence, and bovine rhinotracheitis virus, which can cause an increase in elastase activity that promotes *Mannheimia haemolytica* mucosal colonization.

Pro-inflammatory cytokine induction
Induction of cytokines including tumour-necrosis factor (TNF)-α and interleukin (IL)-1 can increase adherence of bacteria including *S. pneumoniae* and *Haemophilus influenzae*. Cytokine induction can increase the severity of diseases such as periodontitis. Latent HIV-1 is reactivated by cytokine induction through the action of microbial lipopolysaccharides.

Microbial virulence determinants
In some instances production of a virulence factor by one microorganism can increase the risk of infection or colonization by a second microorganism. This might include sharing virulence factors, such as adhesins; for example, *H. influenzae* shows enhanced adherence when pretreated with *Bordetella pertussis* adhesins.

Impaired innate or acquired immunity
Infection with a microorganism that results in an impaired immune system predisposes the affected individual to infection with other microorganisms, or can allow infection of a niche that is usually protected in the body. Co-infections that occur in HIV-1 infected individuals, such as thrush caused by *Candida albicans*, are good examples of polymicrobial diseases that result from this underlying condition.

Box 3 | New *in vitro* methods used to study polymicrobial diseases

There are several new *in vitro* methods, including:

- Genomic sequencing of individual microorganisms and mixed microbial ecosystems and the use of meta-genomics to study the genomes of uncultured microbial communities.
- Molecular phylogenetic studies, such as genotyping or 16S rRNA analyses, to determine the genetic relatedness or diversity of microbial community members.
- Genome-wide transcription profiling using microarrays to assess the rates of transcription during polymicrobial infection.
- Fluorescence-based imaging and detection methods such as laser confocal microscopy using fluorescent probes, fluorescent *in situ* hybridization (FISH) using species-specific 16S rRNA-directed oligonucleotide probes and the use of transcription and translation reporter gene constructs.
- Analyses of inter-genera bacterial signalling such as quorum sensing.
- Use of biofilm chambers and continuous culture flow cell reactors to study polymicrobial diseases.
- Co-infections of cell lines, tissue and organ cultures and extracted teeth.
- Laser capture microdissection of colonized infected tissues.

human viruses, in contrast with rodent hosts, the use of greater primates for modelling human viral disease is limited by differences in the clinical presentation of disease — some diseases are asymptomatic in primates — and the expense of using primate models in research[40]. Given the difficulties of modelling diseases caused by individual viruses, it is not surprising that models of virus co-infections, such as HIV and HCV, have not been established.

A variety of small animal and lower-order non-human primate model systems have been developed to model human viral co-infections. Mice and ferrets have been used to study interference between influenza A virus (IAV) strains, as well as interference between cold-adapted influenza A and B vaccine reassortants and wild-type viruses[41–43]. Murine hosts have been used to study how one retrovirus can block infection by a second retrovirus[44], and to define the role of the tissue tropisms of HELPER VIRUSES on the disease specificity of a co-infecting oncogene-containing retrovirus such as the type of tumour that is induced[45]. BALB/c and NIH Swiss mice have been used as models to analyse a putative pathogenic interaction between a murine leukaemia virus and a polyomavirus[46]. Rabbits have been used to produce models of mixed HTLV-1 and HIV-1 co-infection[47] and co-infection with HTLV types I and II[48].

Rhesus and pig-tailed macaque monkeys have been used to model co-infection with simian immuno deficiency virus (SIV) and simian acquired immunodeficiency syndrome retrovirus type 1 (SRV-1)[49]. More recently, macaques have been used to define the susceptibility to co-infection with two human HIV-2 isolates[50]. In this model, co-infections were established in macaques that were simultaneously exposed to both viruses, whereas in macaques that were sequentially CHALLENGED, co-infections were only observed if challenge with the second HIV-2 isolate occurred early after challenge with the first HIV-2 isolate and before full SEROCONVERSION. Chimpanzees have also been used to study HIV-1 subtype B strain co-infections[51] and interference between hepatitis viruses[52,53].

HELPER VIRUS
A virus in a mixed infection that provides a complementing function so that a co-infecting defective virus can replicate.

CHALLENGED
Inoculated with an infectious agent.

SEROCONVERSION
The development of antibodies in serum as a result of infection or immunization.

Viral–bacterial co-infections

Bovine respiratory disease complex. Pneumonic pasteurellosis, or 'shipping fever', in cattle is due to bacterial co-infection of a virus-compromised host and is known as bovine respiratory disease complex (BRDC). Co-infection with one of a selection of bovine viruses and members of the *Pasteurellaceae* family, including *Mannheimia haemolytica, Pasteurella multocida* and *Haemophilus somnus* — commensal bacteria of the ruminant upper respiratory tract — cause BRDC. Although these bacteria are non-pathogenic commensals, disease can be caused when a viral infection compromises the respiratory tract. Intranasal delivery of bacteria into animal models does not result in disease, which indicates that the role of the viral co-pathogen is crucial in BRDC[54]. In the absence of a viral infection, large infectious doses of bacteria must be inoculated directly into the lung to cause disease in the animal model, and the pathology is different from that of BRDC. Bovine herpesvirus 1 infection increases bacterial colonization, decreases ciliary activity in the respiratory tract, increases the frequency of adherence and invasion by *P. multocida* and *M. haemolytica*[55], delays recruitment of polymorphonuclear neutrophils (PMNs) to the lungs, causes apoptosis of PMNs and infected $CD4^+$ T cells, downregulates major histocompatibility complex (MHC) class I synthesis and surface expression, and increases expression of the *M. haemolytica* leukotoxin receptor Cd11-α/CD18 on PMNs. Similarly, bovine respiratory syncytial virus disrupts host respiratory tract defences by destroying ciliated respiratory epithelial cells and blocking interferon (IFN)-α and -β activities[56–61].

BRDC pathology results from the effects of pathogen and host virulence factors. *M. haemolytica* produces multiple virulence factors, including a leukotoxin of the repeat in toxin (RTX) family that activates PMNs, induces production of inflammatory cytokines, results in cytoskeletal changes and causes apoptosis. Leukotoxin-activated PMNs are crucial to pathogenesis and inflammatory mediators released by neutrophils are thought to be essential because inflammation and most of the pathology in BRDC is absent

in neutrophil-depleted animals. In pigs, porcine respiratory disease complex (PRDC) is a similar disease complex that is caused by co-infection with one of several porcine respiratory tract viruses and members of the *Pasteurellaceae* family[62–66].

Porcine reproductive and respiratory syndrome. PRRS is caused by PRRSV co-infection with multiple bacterial pathogens including *Streptococcus suis* type II[67], *Bordetella bronchiseptica*[68], *Mycoplasma hyopneumoniae*[69] and *Actinobacillus pleuropneumoniae*[70].

Poult enteritis and mortality syndrome (PEMS). In turkeys, PEMS is caused by turkey coronavirus, avian pneumovirus or Newcastle disease virus co-infection with enteropathogenic *Escherichia coli*[71,72].

Lower respiratory tract polymicrobial diseases. In humans, respiratory tract viruses predispose individuals to bacterial infections of the lower respiratory tract and during the influenza pandemics of 1918 and 1957 bacterial pneumonia significantly contributed to mortality. There are multiple examples of human diseases that are viral–bacterial co-infections, including invasive group A streptococcal infection after infection with varicella-zoster virus (the causative agent of chickenpox)[73–75], otitis media (OM), gastroenteritis, exacerbations of chronic obstructive pulmonary disease (COPD), a severe and aggressive form of periodontitis, sinusitis and bronchopneumonia (FIG. 1). Despite the diverse spectrum of diseases and anatomical niches, there are common underlying mechanisms involved in these co-infections. Often, viral disruption of host defences has a role in the development of bacterial co-infections.

Middle-ear polymicrobial diseases. In otitis media, which is a middle ear infection, a synergistic interaction that results in disease owing to co-infection with an upper respiratory tract virus and three bacterial species — *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHI) and *Moraxella catarrhalis* — is well documented. However, certain viruses such as respiratory syncytial virus (RSV) and rhinovirus seem to predispose affected individuals more often to bacterial OM. The saying that children "get a cold and a week later develop OM" is substantiated by epidemiological data that indicate a seasonal influence on the coincidence of 'colds' and OM, as well as evidence for a peak incidence of virus isolation that is coincident with, or immediately preceding, peak incidence of OM (FIG. 2). In the recent Finnish OM Cohort Study and Finnish OM Vaccine Trial, the relationship between viruses and OM was supported by data that showed the presence of a virus in either nasopharyngeal aspirates or middle-ear fluid specimens in 54% or 67% of OM cases in these studies, respectively[76]. Rhinovirus was the most commonly isolated virus, followed by enterovirus and RSV. A specific virus was detected in two-thirds of all cases of acute OM in young children, but only those viruses that are tested for can be detected, so this figure is likely to underestimate the proportion of acute OM events with viral co-infection.

The mechanisms of synergy between pathogens in OM have been analysed using *in vitro* methods and animal models (reviewed in REF. 7). Briefly, viral infection compromises the protective functions of the Eustachian tube, alters respiratory-tract secretions, damages the mucosal epithelial lining, interferes with antibiotic efficacy, modulates the immune response and enhances bacterial adherence[77] and colonization[78] to predispose the host to bacterial OM. Influenza and parainfluenza viruses have neuraminidases that remove sialic acids from host-cell glycoproteins, which results in the exposure of receptors for pneumococci. The activity of neuraminidases allows the adherence of and/or colonization by *S. pneumoniae*[79], which is one of the primary aetiological agents of acute OM.

Although all upper respiratory tract viruses can disrupt the host respiratory tract defences, each virus has a specific pathology. Not surprisingly, there are specific partnerships between viruses and bacteria in OM. In the chinchilla model (FIG. 2) IAV predisposes the host middle ear to *S. pneumoniae*-induced or pneumococcal OM and adenovirus infection predisposes the host middle ear to NTHI OM. IAV does not predispose the chinchilla host to NTHI-induced OM, nor does adenovirus predispose the host to either *M. catarrhalis*-induced OM[80] or to pneumococcal OM[78,81]. Virus and bacteria synergy seems to be maintained in adults and children. The oropharynges of 15% of adults with experimental IAV infection were heavily colonized with *S. pneumoniae* six days after viral challenge[82], whereas isolation rates for other middle-ear pathogens were unaffected. In children, *S. pneumoniae* is cultured more often from middle-ear fluids that contain IAV than from those that are culture-positive for either RSV or parainfluenza virus[83].

Cystic fibrosis polymicrobial diseases. Upper respiratory viruses predispose the host to bacterial invasion of the lower respiratory tract and are often detected in patients with COPD[84] or cystic fibrosis (CF). In addition to bacterial factors, host determinants also have a role in co-infections of the CF lung. CF patients do not have a higher incidence of viral disease compared with non-CF individuals, but viral disease produces more significant pathology. It has been proposed that CF patients have impaired innate immunity, which allows increased virus replication and upregulated cytokine production. In turn, this results in increased bacterial colonization of the lung. Zheng and co-workers[85] showed that increased virus replication in CF patients is due to the absence of the antiviral nitric oxide synthesis pathway. This was attributed to impaired activation of signal transducer and activator of transcription (STAT1), which is an important component of the antiviral defences of the host. Compromising innate immunity provides a mechanism for the severity of viral disease in CF and the establishment of bacterial co-infections.

Expression of virulence determinants by *Pseudomonas aeruginosa*, a pathogen of CF patients, can depend on signals produced by other bacteria. Transcriptional profiling *in vitro* coupled with research in an animal model showed that adding exogenous signalling molecules,

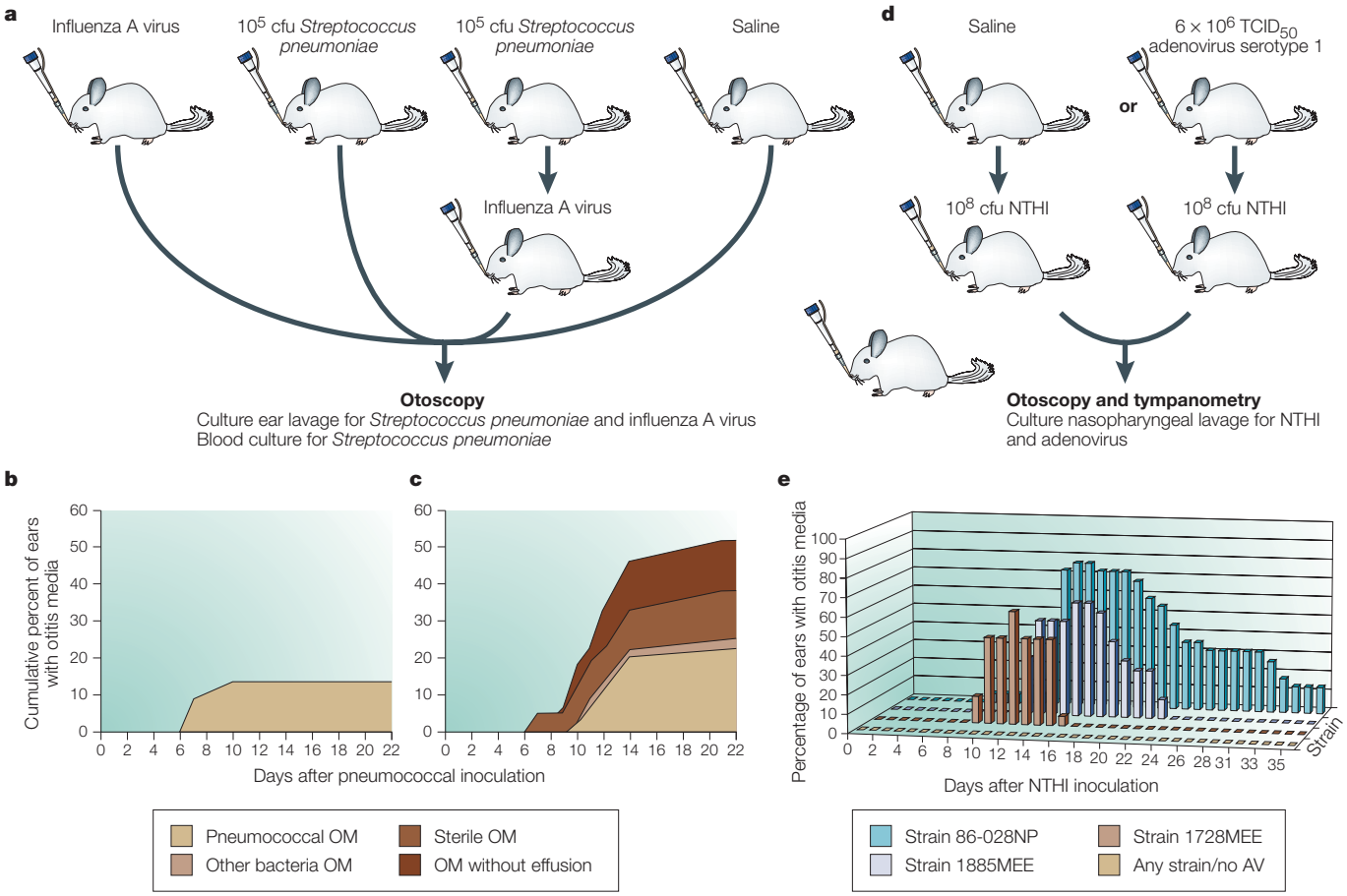

Figure 2 | **A chinchilla experimental model for otitis media. a** | Chinchillas were infected intranasally using a pipettor with influenza A virus, 10^5 colony-forming units (cfu) of *Streptococcus pneumoniae* or sterile saline. After 2 days, one set of animals inoculated with *S. pneumoniae* was inoculated intranasally with influenza A virus. All animals were assessed for 22 days by nasopharyngeal lavage every 2–3 days to detect cultures of *S. pneumoniae* or influenza A virus, by otoscopy to detect symptoms of otitis media, by aspirates from middle-ear effusions to culture *S. pneumoniae* and influenza A virus and by cardiac puncture to culture *S. pneumoniae*. The graphs in panels **b** and **c** show the incidence of ears with otitis media (OM) that were observed following each infection regime. Panels b and c are reproduced with permission from REF. 93 © (1980) American Society for Micobiology. **d** | Chinchillas were infected intranasally using a standard pipettor with either saline (control) or 6 x 10^6 $TCID_{50}$ adenovirus serotype I. Seven days later, the same animals were inoculated intranasally with 10^8 cfu of one of three clinical isolates of nontypeable *Haemophilus influenzae* (NTHI, strains #86-028NP, #1885MEE or #1728MEE). **e** | All animals were assessed for 35 days by otoscopy and tympanometry (every 2 days after adenovirus inoculation) for OM and by nasopharyngeal lavage on days 1,4,7,10,14,18,21,28 and 35 to assess NTHI by culturing. $TCID_{50}$, tissue culture infectious dose 50%, where 50% of aliquots initiate infecton.

such as autoinducer-2, or the production of this molecule by the oropharyngeal bacterial flora upregulated the expression of genes that encode virulence factors[87]. Modulation of gene expression by interspecies communication between normal flora and pathogenic bacteria could therefore have a role in polymicrobial diseases.

Periodontitis. Some herpesviruses, including human cytomegalovirus (HCMV), Epstein–Barr virus type 1 (EBV-1) and HSV, have been implicated in the pathogenesis of a severe and highly aggressive form of periodontitis through co-infection with *Porphyromonas gingivalis*[87,88]. HCMV and HSV were detected at significant levels using PCR in periodontal disease and were shown to be good predictors of the presence of *P. gingivalis*. EBV-1 was not linked to isolation of *P. gingivalis* but was also predictive of active disease[87].

Animal models for viral–bacterial co-infection

Pneumococcal pneumonia after influenza virus infection. A sequential inoculation model has been developed in mice to probe the mechanisms of the interaction between *S. pneumoniae* and IAV. Mice infected simultaneously with *S. pneumoniae* and IAV displayed gradual weight loss and increased mortality, commensurate with an additive effect. Conversely, mice infected with *S. pneumoniae* seven days after IAV infection uniformly died within 24 hours and had significant bacteraemia — lethality was due to overwhelming pneumococcal septicaemia[89]. This model is being used to define the molecular mechanisms of the lethal synergy of IAV with *S. pneumoniae*[90]. The activity of viral neuraminidase was found to be crucial to this synergistic relationship[91] and, in common with OM, has an important role in predisposing both the upper and lower respiratory tracts to invasion by *S. pneumoniae*.

Meningococcal bacteraemia after influenza virus infection. A murine model has been developed to reproduce the pathogenesis of human meningococcaemia, which often results in serious symptoms or death[92]. In this model, adult BALB/c mice are infected intranasally with a mouse-adapted IAV, then seven or ten days later, are co-infected with *Neisseria meningitidis*. Fatal meningococcal pneumonia and bacteraemia occurred in mice challenged at seven, but not ten, days after IAV infection. Meningococcal pneumonia and bacteraemia did not develop in mice that were not co-infected with IAV. Susceptibility to lethal infections correlated with peak interferon-γ production in the lungs and decreased IAV load and production of IL-10, which indicates that transient IAV-induced modulation of host immunity has a role in susceptibility to *N. meningitidis* co-infection.

Otitis media. The only viral–bacterial co-infection model for OM is the chinchilla. Before 1980, most OM studies in the chinchilla used inoculation of pathogens directly into the middle ear. Although this induces disease in almost all of the animals inoculated and is therefore extremely useful for studies of therapeutics and surgical intervention strategies, it bypasses all of the early steps in the development of the pathogenesis of the disease, including colonization of the nasopharynx, ascension of the Eustachian tube and initiation of infection in the middle ear. Giebink and co-workers[93], developed a clinically relevant model in which chinchillas were challenged intranasally with both *S. pneumoniae* and IAV. This study showed that 4% of the chinchillas that were infected with IAV alone, and 21% of those inoculated with *S. pneumoniae* alone, developed OM, but of the animals that were co-infected with both microorganisms, ~67% developed OM (FIG. 2). This model has been useful in defining the molecular mechanisms of IAV predisposition to pneumococcal OM[94–96] and to investigate the role of pneumococcal virulence determinants in OM[81].

To study the pathogenesis of OM mediated by NTHI, a chinchilla model that uses a co-challenge method was developed. In this model, adenovirus infection can predispose the chinchilla to NTHI invasion of the middle ear[97] (FIG. 2); however, IAV infection has no effect. This adenovirus–NTHI co-infection model has been used to study the mechanisms of adenovirus predisposition to NTHI-induced OM[98,99], to identify new NTHI virulence determinants[100] and to assay the relative efficacies of different NTHI-derived vaccine candidates for OM[101–103]. A cotton rat model of RSV and NTHI co-infection has also been developed to study co-infections of the respiratory tract[104]. In the cotton rat, colonization of the respiratory tract with NTHI increased to a maximum level four days after infection with RSV and colonization was increased compared with rats that had not been infected with RSV. NTHI colonization of the respiratory tract was increased by RSV co-infection and, although the mechanisms underlying this relationship are not understood, this model might be useful to determine the mechanisms of RSV predisposition to bacterial OM.

A common theme has emerged from these models that upper respiratory tract viruses of both animal and human hosts can predispose the respiratory tract to infection by pasteurellaceae in BRDC and PRDC in animals and periodontitis, sinusitis, COPD and OM in humans.

Pulmonary infections of humans. A mouse model has been developed to evaluate the role of respiratory dendritic cells (RDCs) in viral–bacterial co-infections[105]. RDC migration from the lungs to the secondary lymph nodes after infection with pulmonary virus is monitored by the use of a fluorescent dye. After inoculation with influenza virus, the rate of RDC migration to the draining peribronchial lymph nodes increased, but this only occurred during the first 24 hours after virus infection. After 24 hours, RDCs did not migrate, despite virus replication and pulmonary inflammation. Moreover, viral infection suppressed additional RDC migration in response to either a second pulmonary virus infection or administration of BACTERIAL CPG DNA. In addition to suppressed RDC migration, there was also suppression of an antiviral pulmonary $CD8^+$ T-cell response. It seems likely that the transient suppression of RDC migration and the delayed development of an effective adaptive immune response to a second infection might be another mechanism by which influenza virus predisposes the host to bacterial co-infection.

Bacterial co-infections

Atrophic rhinitis. Infection with more than one bacterial species is common in animals and man. In pigs, atrophic rhinitis (AR), which is characterized by severe atrophy of the nasal TURBINATES, is caused by co-infection with strains of *B. bronchiseptica* and heat-labile toxin-producing strains of *P. multocida*[106–108]. *P. multocida* can adhere to respiratory tissues, but co-infection with *B. bronchiseptica* allows more efficient colonization by *P. multocida*. *P. multocida* produces a dermonecrotic toxin called PMT (for *P. multocida* toxin), which interferes with normal bone modelling in both the nasal turbinates and long bones in swine, and is distinct from the *B. bronchiseptica* dermonecrotic toxin (DNT). In porcine models, PMT causes a more serious form of AR known as progressive AR, whereas *B. bronchiseptica* infection alone induces a milder, or non-progressive, form of the disease.

Bacterial co-infections of humans include orofacial infections[109], adenotonsillitis[110], persistent osteomyelitis[111], peritonitis[112], chronic sinusitis[113], abscesses[114,115], necrotizing fasciitis and approximately one-third of urinary tract infections (UTIs) in the elderly[116] and in renal transplant patients[117]. Two important bacterial co-infections are periodontitis and vaginosis.

Periodontitis. Periodontal disease causes tooth loss and is associated with systemic vascular diseases such as atherosclerosis and carotid coronary stenotic artery disease[118]. Periodontitis in an expectant mother can contribute to both low birth weight and pre-term labour[119]. Periodontal disease is initiated by the formation of a

BACTERIAL CPG DNA
Immunostimulatory bacterial DNA that is enriched in unmethylated CPG dinucleotides.

TURBINATE
A small curved bone along the lateral wall of the nasal passage.

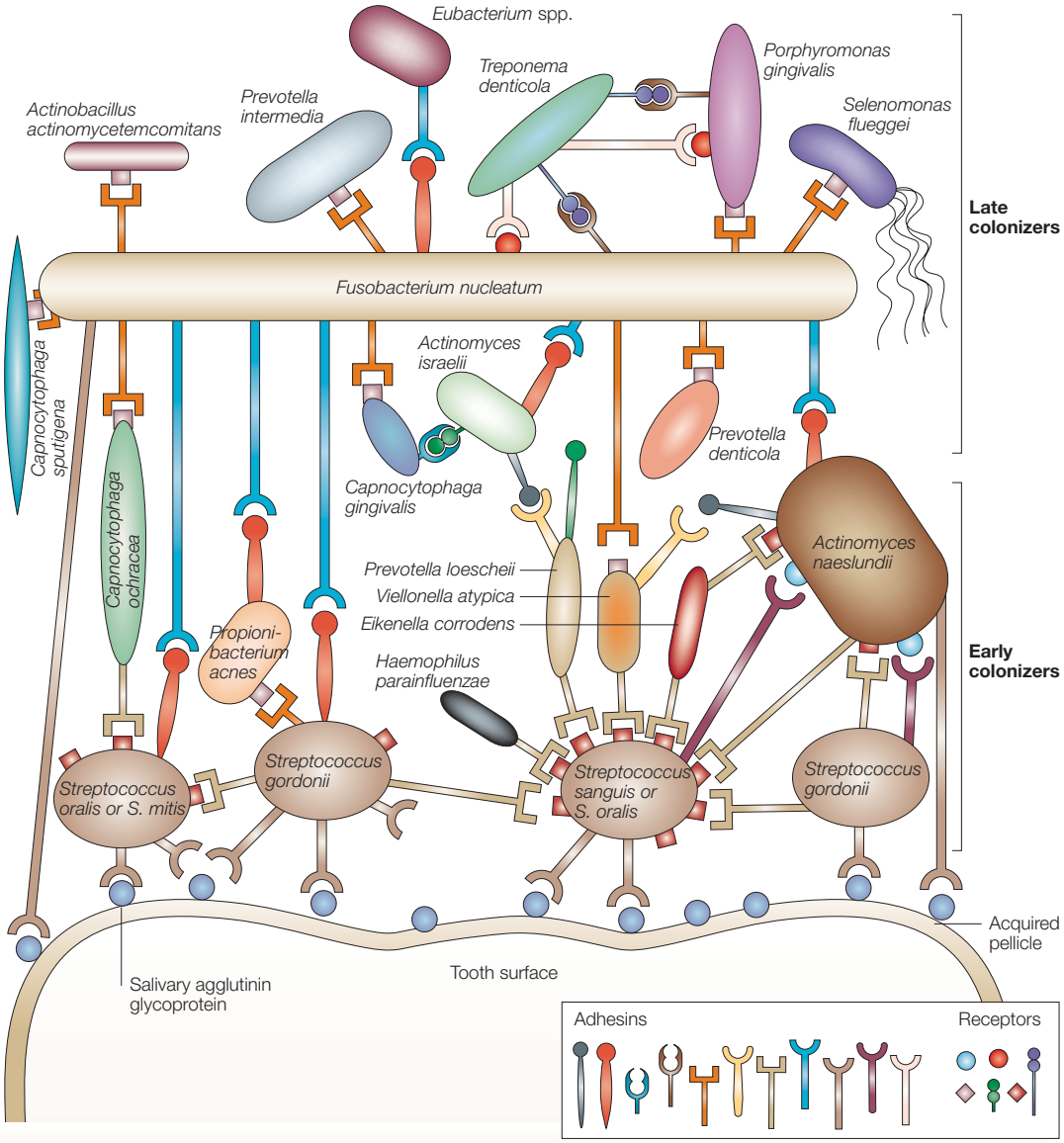

Figure 3 | **A model of oral biofilm formation.** The tooth surface is covered by an acquired pellicle comprised of lipids and proteins, including salivary agglutinin glycoprotein. The pellicle is recognized by primary colonizing bacteria (*Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus gordonii* and *Streptococcus sanguis*) that express receptors for salivary agglutinin glycoprotein. Other bacteria then colonize in a spatial and temporal manner as shown, using receptors and adhesins to eventually form dental plaque. Reproduced with permission from REF. 194. © (2002) American Society for Microbiology.

biofilm on the tooth surface followed by bacterial invasion of gingival tissues[119]. Teeth have a non-shedding surface and are located in a warm, moist environment, so are a particularly suitable niche for biofilm formation by the oral microbial flora. In periodontitis, coaggregation — a process in which genetically distinct bacteria become interconnected by specific adhesins — is central to the formation of complex multispecies biofilms[120] (FIG. 3). In dental plaque, primary colonizers such as *Streptococcus gordonii*, and other oral streptococci that express adhesins, provide a film on which other bacterial colonizers assemble the biofilm. It was thought that the abundance of plaque that formed was responsible for the induction of periodontitis but, at present, the favoured hypothesis is that the quality of the plaque formed, in terms of microbial constituents, is the main predictor for periodontal disease.

Actinobacillus actinomycetemcomitans, P. gingivalis and *Bacteroides forsythus* are the main periodontal pathogens. Periodontal disease covers a range of clinical symptoms and there are multiple forms of periodontitis in children and adults. In individuals under 20 years of age, *A. actinomycetemcomitans* is the main bacterial pathogen, whereas in adults aged 35 years or older, periodontitis has been linked to *P. gingivalis* and *B. forsythus*. Spirochaetes, especially *Treponema denticola,* have been implicated in periodontitis despite the fact that most oral spirochaetes have not been successfully cultured. Recent studies using molecular phylogenetic techniques have implicated new bacterial

species or phylotypes — including members of the uncultivated bacterial division TM7 — in periodontitis, dental caries and halitosis[121–127]. In many of these studies, not only were new species and phylotypes identified, but bacteria that are known to be oral pathogens were found to be numerically minor, which was expected because ~50% of oral flora have not been cultivated[123].

In localized juvenile periodontitis (LJP), the leukotoxin of *A. actinomycetemcomitans*, like that of *M. haemolytica*, is the best-studied virulence factor. This leukotoxin selectively kills PMNs and macrophages *in vitro*, and PMNs and macrophages are important components of the host defence *in vivo*. Expression of this leukotoxin is variable among *A. actinomycetemcomitans* isolates and the leukotoxin-expression phenotype correlates with differences in the promoter region of the leukotoxin gene operon[128]. A subset of leukotoxin-overproducing strains are more virulent and are associated with LJP in humans.

Neutrophil abnormalities seem to be an important predisposing condition for periodontal disease. In addition, loss of tooth attachment and bone resorption, which are important events in periodontal disease, occur together with increased IL-1 and tumour-necrosis factor (TNF) activities. The production of IL-1 and TNF (both of which are pro-inflammatory cytokines) has been correlated with the spread of inflammatory cells to connective tissues, the loss of connective tissue attachment, osteoclast formation and the loss of alveolar bone. An overzealous host response to periodontal pathogens, resulting in excessive production of IL-1 and TNF, is hypothesized to be responsible for much of the damage that occurs in periodontal disease[129].

Vaginosis. Bacterial vaginosis (BV) is a human bacterial co-infection. The mucosal environment of the vagina is influenced by developmental and hormonal changes[131]. The most common bacterial constituents of the vaginal microflora are lactobacilli, including *Lactobacillus crispatus* and *Lactobacillus jensenii*[131]. When these hydrogen peroxide (H_2O_2)-producing lactobacilli are outcompeted by anaerobic and facultatively anaerobic members of the vaginal flora, BV develops with a concomitant rise in vaginal pH, which further promotes the growth of the resident lactobacilli. BV is common, occurring in 5–51% of the global female population[130] and the role of lactobacilli in the maintenance of vaginal homeostasis has been well studied. Women with stable bacterial colonization have a reduced risk of developing BV[132]. Normal vaginal flora has a role in defence against the acquisition of other pathogenic microorganisms, including those that are responsible for sexually transmissible diseases (STDs), and BV is a strong predictor of STD acquisition[133]. Compared with subjects with normal vaginal flora, subjects that have BV are more likely to test positive for *Neisseria gonorrhoea* and *Chlamydia trachomatis*. Recently, BV has also been found to be associated with an increased risk of HSV-2 infection[134].

Animal models for bacterial co-infections

Periodontal disease. In most individuals, periodontal pathogens trigger an inflammatory response that effectively prevents microbial colonization and invasion of adjacent gingival tissues. However, individuals that have specific IL-1 polymorphisms that result in increased levels of IL-1 expression are predisposed to periodontal disease. Using this criterion, a mouse model of polymicrobial-induced osteoclastogenesis, bacterial penetration, leukocyte recruitment and soft-tissue necrosis has been developed to clarify the role of cytokines in periodontal disease. In this model, the dental pulp of the first mandibular molars is exposed by surgically clipping the mesial cusps and then a mixture of putative oral pathogens is inoculated into the dental pulp (FIG. 4a). By monitoring the size of osseous lesions, tissue necrosis, osteoclastogenesis, osteoclastic activity, inflammatory cell recruitment and bacterial penetration into tissue periodontal disease, pathogenic mechanisms can be investigated[135]. IL-1 or TNF receptor signalling does not seem to be required for bacteria-induced osteoclastogenesis and bone loss in this model, but does have a crucial role in protecting the host against anaerobic co-infections.

A rat model of periodontitis was developed to test adherent (rough) and non-adherent (smooth) variants of *A. actinomycetemcomitans* for virulence, as well as to assess phenotypic reversion *in vivo*[136]. In this model, the normal flora of the oral cavity of Sprague–Dawley rats is reduced by antibiotic treatment, after which rats are inoculated with *A. actinomycetemcomitans* by either normal ingestion of food layered with bacterial cultures, oral swabbing or gastric lavage (FIG. 4b). When clinical isolates of *A. actinomycetemcomitans* were compared with laboratory-adapted variants, Fine *et al.*[136] found that the clinical strains were more efficient at colonization and persisted longer in the rat oral cavity than laboratory strains. Rough variants were more efficient colonizers of the rat oral cavity than smooth variants, regardless of the method of inoculation, although feeding was the preferred method owing to the similarity with human disease. Importantly, rats that were orally infected with *A. actinomycetemcomitans* by feeding developed immunoglobulin G (IgG) antibodies to the bacteria and had bone loss that was typical of periodontitis. This model has not been used to study the process of bacterial co-infection in periodontitis, but has been used to identify a gene locus that is important in virulence and which mediates tight adherence by *A. actinomycetemcomitans*[137].

A primate model (*Macaca fascicularis*) of periodontal disease uses silk ligatures tied around the posterior teeth to induce plaque accumulation and the initiation of periodontitis[138]. So far, this model has only been used for single pathogen studies, but is considered to be a relevant animal model of periodontal disease owing to the similarity of clinical and histological features with those of periodontal disease of humans, and because, in this model, periodontal destruction is clearly triggered by bacterial infection[139].

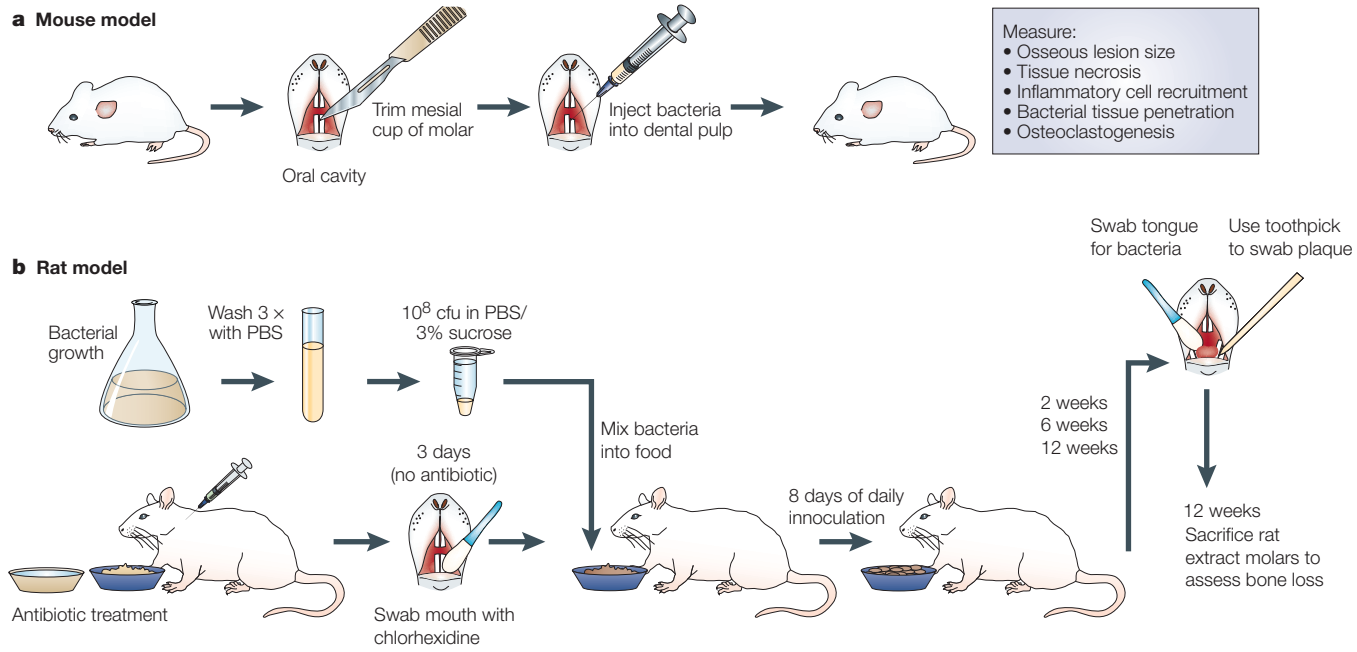

Figure 4 | **Animal models for periodontal disease. a** | In the mouse model the molar is trimmed to expose the dental pulp. Bacterial suspensions that are being tested for the ability to cause periodontal disease are injected into the dental pulp and the mouse is monitored for signs of periodontal disease by methods that include examination of osseous lesions, tissue necrosis, inflammatory cell recruitment, bacterial tissue penetration and osteoclastogenesis. **b** | In the rat model bacteria are grown using standard laboratory procedures and washed 3 times with phosphate-buffered saline (PBS) supplemented with 3% sucrose. The rats are pretreated with antibiotics and the mouth is swabbed with chlorhexidine to deplete the oral flora. The bacterial suspension is mixed into the rat's food so that this animal model replicates, as far as possible, the natural route of infection for periodontal disease. After daily inoculation the rats are assessed for bacterial colonization and bone loss. Using this model, different strains of bacteria and the contribution of different virulence loci can be tested. cfu, colony-forming units.

Peritonitis and sepsis. Although the role of pathogenic enterococci and their role in peritonitis is not understood, many putative virulence factors have been identified using animal models. Available animal models include systemic infection in mice and compartmentalized infection in rats, and the bacterial virulence factors that have been identified using each model differ[140]. This indicates that both host and pathogen factors contribute to peritonitis and, perhaps, that the animal models are quite different. Nevertheless, these models have identified a role for cytokines in septic shock, a protective role for IL-10 against lethal shock[141], a role for STAT4 in the mortality seen in bacterial co-infection sepsis[142] and helped to define the role of the classical pathway of complement activation in defence against polymicrobial peritonitis[143]. Animal models of bacterial co-infection peritonitis and/or sepsis can involve any of the following methods for induction of infection: peritoneal implantation of microbe-filled gelatin capsules[140,141]; intraperitoneal injection of faecal suspensions[17] or caecal ligation and puncture[112,142–151].

Mycotic co-infections

Candida and mixed infections. As defined by Soll and his colleagues[6], mycotic co-infections with the fungal pathogen *Candida* spp. can be due to co-infection with *Candida* and bacteria, or co-infection with multiple *Candida* species. Co-infection with multiple *Candida* strains and SUBSTRAINS are also found. Regardless of the co-pathogens, mycotic co-infections of the oral and vaginal cavities, on indwelling prosthetic devices, or systemic infection of the blood can present significant therapeutic challenges. Difficulty in treating some of these infections is partly attributed to the formation of biofilms by *Candida* spp.[152] Biofilm formation on devices such as prosthetic heart valves and catheters has been studied *in vitro*[95]. When cultured on a variety of catheter materials, *Candida* spp. form biofilms comprising a matrix of microcolonies of both the yeast and the filamentous hyphal forms. In studies of mixed microbial populations, *Candida* spp. form biofilms with several bacterial species, including *Staphylococcus epidermidis* and oral streptococcal species. The receptor for *Candida albicans* co-aggregation with *S. gordonii* is a complex cell surface polysaccharide that is expressed on the surface of the bacterium. The interaction between yeast cells and oral streptococci or other bacteria has important implications for the mechanisms of yeast infections of the oral cavity, in addition to promoting biofilm formation on a variety of surfaces. In the oral cavity, *Candida*–bacterial interactions are responsible for denture stomatitis, angular cheilitis and gingivitis, and also have a role in periodontitis[153].

SUBSTRAINS
Genetic variants of a single strain as determined by techniques such as multilocus enzyme electrophoresis or random amplified polymorphic DNA analysis.

***Candida* mixed-phenotype co-infections.** A new category of polymicrobial diseases has been proposed for *Candida* spp. in which the infection is due to phenotypic heterogeneity[154]. In addition to the hypha–bud transition, *C. albicans* has a reversible, high-frequency phenotype switch that can be identified by differences in colony morphology. *C. albicans* cells of two phenotypic phases have different virulence characteristics. The ability of this human pathogen to rapidly switch between phenotypes could be a higher-order pathogenic trait. Support for this hypothesis comes from studies in which strains that cause deep tissue mycoses were shown to switch at higher frequencies than those that cause superficial infections. Furthermore, pathogenic *C. albicans* strains that were isolated from the oral cavity switch at higher rates than commensal strains that were isolated from the same site. A clinically relevant example of the role of both phenotype and mating-type switching in disease was characterized by Brockert *et al.*[155], who investigated oral cavity and vaginal isolates of *C. glabrata* in three patients with vaginitis. The results of this study showed that switching occurs at sites of infection, that different switch phenotypes of the same strain can dominate in different anatomical locations in the same host and that mating-type switching occurs *in vivo*.

***Candida*–bacterial co-infections.** These co-infections can cause disease in the lower respiratory tract. In CF patients, clinical specimens that also harbour *C. albicans* contain nine times the amount of *P. aeruginosa* compared with patients that do not harbour *C. albicans*[156]. Moreover, sputum samples of 6–70% of CF patients contain *C. albicans* in addition to *P. aeruginosa*. Hogan and Kolter[157] showed that *P. aeruginosa* forms a dense biofilm on *C. albicans* filaments *in vitro* and, in doing so, kills the fungus. *P. aeruginosa* fails to bind to, or kill, the yeast form of *C. albicans*. It is unclear if a similar relationship between these two pathogens functions *in vivo* but, as several *P. aeruginosa* virulence factors that are important in human disease are also involved in killing the fungal filaments, this co-culture system could prove useful for the study of the pathogenesis of *P. aeruginosa*-induced disease.

Animal models for mycotic co-infections

***Candida* infections.** Owing to the ability of *Candida* spp. to switch between bud and hypha (or hypha-like) forms as well as to switch phenotype, all animal models of *Candida* infection are likely to represent one or another of the multiple polymicrobial states that have been proposed for this microorganism. A rat model of oral colonization has been used to compare the relative pathogenicity of different *Candida* strains as well as to determine the effect of chemotherapeutic immunosuppression on the ability of *Candida* spp. to switch from a commensal to an invasive phenotype[158]. A rat model of oral candidiasis has also been developed and used to assay isogenic derivatives of a virulent *C. albicans* strain for the biological consequences of these genetic manipulations[159]. A murine host has been used as a model of systemic candidiasis[160] and there is also a murine model for *C. glabrata*-induced vaginitis[161]. In the *C. glabrata* model, the increased susceptibility of non-obese diabetic mice to *C. glabrata*-induced vaginitis compared with their non-diabetic counterparts indicates a link between susceptibility to diabetes and infection with *C. glabrata*. In addition to studies of *Candida* genetics and pathogenicity[162], this model is useful for the evaluation of the relative efficacy of antimycotic agents and probiotics for the prevention of vaginitis.

An animal model of haematogenously disseminated candidiasis has recently been developed[163] that can investigate the role of phenotype switching in candidiasis. In this model (FIG. 5) mice were injected with engineered *C. albicans* strains in which the transition between yeast and filamentous forms is under the control of a doxycycline-regulated promoter. Mice that were infected with strains that switched to the filamentous form died, whereas those infected with strains that could not switch from the yeast to the filamentous form survived, despite the fact that the fungal burdens in both groups were nearly identical. These data indicate that the filamentous form is important for mortality but that the yeast form of *C. albicans* is important for dissemination to deeper tissues.

Parasitic co-infections

Parasite–parasite co-infections. Several human diseases have mixed parasitic aetiologies, including co-infections with *Plasmodium* spp. and nematodes. In some studies, co-infection with a helminth seemed to confer protection against severe complications of malaria[164], but this is not always the case. When infected controls with a low helminth burden were compared with those with circulating helminth schizonts, co-infection with *Ascaris lumbricoides* was found to be associated with protection from cerebral malaria. In addition, a later study showed a significant association between *Ascaris* infection and the risk of co-infection with *Plasmodium falciparum* and *Plasmodium vivax,* indicating that pre-existing *Ascaris* infection might increase host tolerance to co-existing *Plasmodium* spp.[165] Subsequently, helminth-infected patients were found to be more likely to develop falciparum malaria compared with those that were not co-infected[166]. Moreover, the risk of developing falciparum malaria increased with the number of co-infecting helminth species. Collectively, these findings indicate that a helminth-mediated helper T cell 2 (T_H2) shift (an immune response that is biased towards that which is characteristic of a T_H2-mediated response) might have a complex impact on malaria co-infection — decreasing antisporozoite immunity but inducing a protective outcome against severe complications of malaria. Although the underlying mechanism is less clear, Mwatha *et al.*[169] showed that exposure of *Schistosoma mansoni*-infected children to *P. falciparum* had a significant influence on the severity of hepatosplenomegaly (enlargement of the liver and spleen) that was observed in co-infected children.

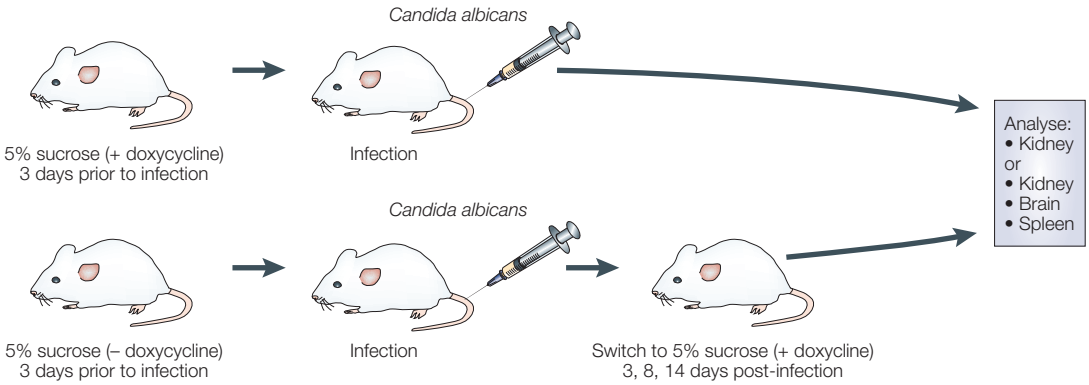

Figure 5 | **A mouse model for candidiasis.** Using an engineered strain of *Candida albicans* the switch from the yeast to the filamentous forms can be modulated by growth on doxycycline[163]. After pretreatment with this antibiotic, the mice are infected by injection into the tail vein with *C. albicans* and the mice can be analysed to determine the virulence of the yeast and filamentous forms — under the strict control of antibiotic treatment. In this model, candidiasis can be assessed by measuring the fungal burden in different parts of the anatomy and through histopathological examination.

Virus–parasite co-infections. Schistosomiasis is a chronic helminth infection that is caused by *S.mansoni*. In HCV and *S. mansoni* co-infection, there is a higher incidence of viral persistence and accelerated damage to the liver than when the patient is infected with either infectious agent alone. In a recent study, stimulation of $CD4^+$ T cells with HCV antigens produced a type 1 cytokine profile in patients infected with HCV, whereas in patients that were co-infected with HCV and *S. mansoni*, a type 2 cytokine predominance was evident despite the fact that T cells that were recovered from both patient populations responded in the same manner to stimulation with schistosomal antigens[168]. The helminth-induced inability to generate an HCV-specific $CD4^+/T_H1$ T-cell response has been shown to have a role in the persistence and severity of HCV infection, which indicates that the induction of a strong cellular immune response through new therapeutic approaches might limit subsequent liver damage in those individuals with chronic HCV infection[169].

Parasite–bacteria co-infections. One example of co-infection with a parasite and bacterium in humans is that of *Borrelia burgdorferi* (the causative agent of Lyme disease) and the intra-erythrocytic parasite *Babesia microti*. Both of these pathogens are transmitted by the tick vector *Ixodes scapularis*. Co-infection can occur by a bite from a single tick carrying multiple pathogens, or from multiple tick bites. The first cases of Lyme borreliosis and babesiosis co-infection were reported in the mid 1980s, with parasite–bacteria co-infection rates of up to 33% among those with confirmed tick-borne infection in certain populations. Although ticks can also harbour the human pathogen *Anaplasma phagocytophilum*, Lyme borreliosis and babesiosis co-infection accounts for ~80% of polymicrobial disease in the eastern United States. Consistent with the theme for other co-infections involving a parasite, patients that harbour both of these pathogens had more severe and longer-lasting symptoms than those with Lyme borreliosis alone[170,171].

BYSTANDER ANTIGENS
Factors that can activate T cells without their specific antigen — without triggering through their T-cell receptor.

Animal models for parasitic co-infections

Co-infection with *Schistosoma* species and *Plasmodium* species has been modelled in field voles and mice since 1956 (REF. 172), with conflicting observations concerning the ability of one parasite to suppress the capacity of the other to infect the host[173,174]. Results obtained seem to depend on the *Plasmodium* species used as well as the immune status of the host. *S. mansoni* is, however, a potent inducer of a T_H2 dominant response, not only to itself but also to other BYSTANDER ANTIGENS that are present in a host, so it does have an influence on the clinical outcome in these co-infections[175,176]. Synergistic interactions between specific protozoans and helminths are often ascribed to the immunosuppression that is characteristic of protozoan infections[177] and which is observed in the mouse, which is the main model for these infections

A mouse model has been used to model the arthritis and carditis that can occur in co-infections with *B. microti* and *B. burgdorferi*[19]. Co-infection resulted in a significant increase in symptoms of arthritis. This increase was correlated with a reduction in concentrations of the cytokines IL-10 and IL-13. A mouse model for tick-borne Lyme arthritis mediated by co-infection with *B. burgdorferi* and a causative agent of human granulocytic ehrlichiosis (HGE) has been developed[178,179] (FIG. 6). Co-infection results in increased titres of both pathogens and more severe arthritis than does infection with *B. burgdorferi* alone. Co-infection resulted in reduced concentrations of IL-12, IFN-γ and TNF-α and increased concentrations of IL-6. IFN-γ expression in macrophages was suppressed, which might indicate a reduction in phagocytic activity in co-infection. These models will allow us to define the modulation of host immune responsiveness that occurs in those individuals that are simultaneously or sequentially infected with multiple tick-borne pathogens[179].

Co-infection following viral immunosuppression

Co-infections can arise as a result of the virus-induced immunosuppression that is characteristic of a subset of human viral pathogens, the best characterized of which is HIV.

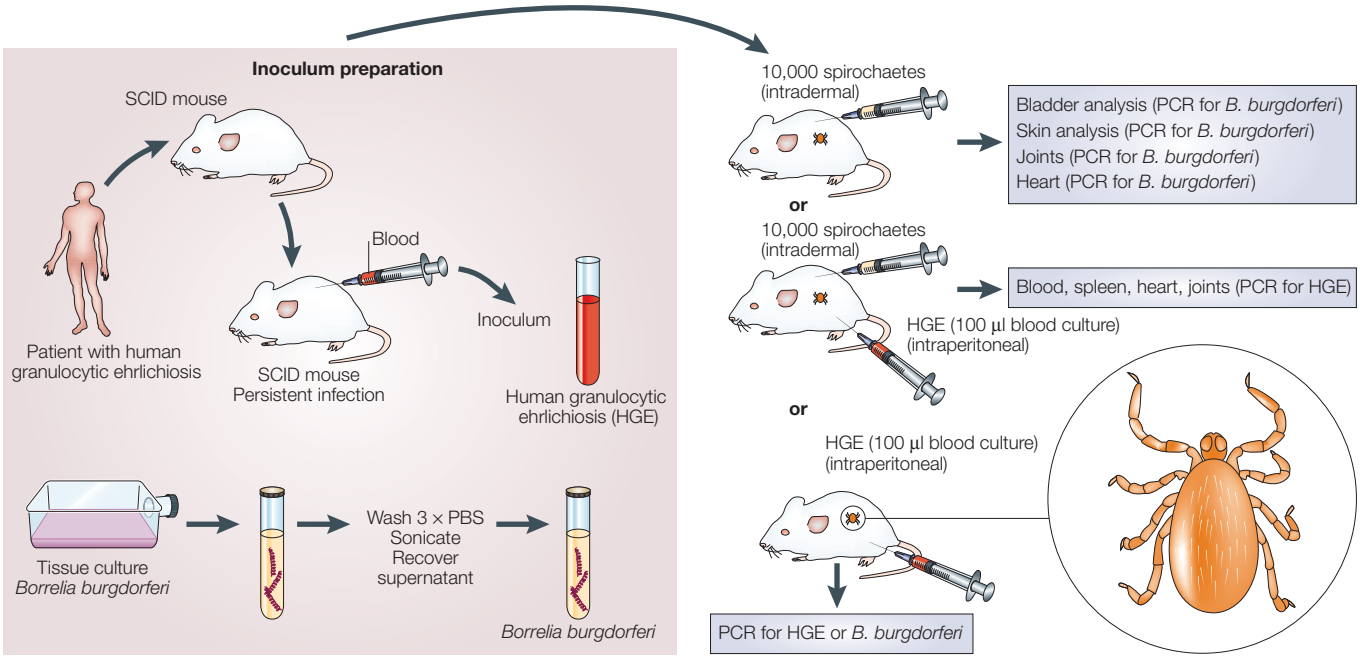

Figure 6 | **Animal model for Lyme disease and human granulocytic ehrlichiosis (HGE) co-infection.** These diseases share a tick vector, *Ixodes scapularis*, and to analyse whether *Ehrlichia* sp. and *Borrelia burgorferi* (the causative agents of HGE and Lyme disease, respectively) co-infection leads to increased severity of spirochaete-induced Lyme arthritis a mouse model has been developed. Mice are infected intradermally with either spirochaetes (*B. burgdorferi* cultured *in vitro*) or HGE (blood culture from a SCID mouse, see inset panel)[178]. Arthritis and presence of the two pathogens can then be determined through histopathology, PCR to detect bacterial DNA and by assessing immune responses. Ticks were allowed to feed on all groups of mice to assess transmission of the pathogens. After feeding, PCR (HGE) and immunofluorescence (*B. burgdorferi*) were used for pathogen detection.

HIV co-infections. Owing to a reduction in the number and/or activity of $CD4^+$ T cells, natural killer (NK) cells, dendritic cells and macrophages, HIV-infected individuals have multiple defects in immune responses. These defects lead to increased susceptibility to so-called opportunistic infections that, in turn, augment the developmental course of AIDS. Co-infections are diverse and include: *Pneumocystis carinii*, a ubiquitous fungus; *Toxoplasma gondii*, a coccidian zoonotic infectious agent; *Mycobacterium tuberculosis*, a bacterium; and *Leishmania* spp., which are protozoa[32,180].

In addition to systemic diseases, localized infections with *Candida* spp., such as thrush in the oral cavity, are common co-infections in HIV-infected individuals[181]. The commensal oral flora acquires an invasive phenotype in the HIV-infected host, and *C. albicans* is indicative of a defect in host T-cell immunity in HIV infection[182]. Oropharyngeal candidiasis develops in ~20–50% of HIV-infected patients and often precedes the development of a more invasive *Candida* infection, oesophageal candidiasis. The progressive immunosuppression that is characteristic of HIV infection provides a mechanism for the development of oesophageal candidiasis, which is a reportable AIDS-defining opportunistic illness.

Another disease of the oral cavity in HIV-seropositive patients is necrotizing ulcerative periodontitis (NUP), which is a disease that is characterized by ulcerated gingival papillae[122]. HIV-seropositive NUP is similar to HIV-seronegative necrotizing ulcerative gingivitis (NUG) and is characterized by a surface biofilm of mixed microbial flora overlying a subsurface flora comprising dense aggregates of spirochaetes. In contrast to NUG, high levels of yeast and herpes-like viruses were observed using transmission electron microscopy examination of tissues recovered from the former patient group. Herpes-like particles were observed in 56.5% of biopsies obtained from HIV-infected patients with NUP. These findings correlate well with those of Contreras and co-workers[183,184] in which co-infection with herpesvirus was associated with high levels of periodontopathic bacteria. The role of viruses in the pathogenesis of NUP or periodontitis is not known but, in addition to inducing immunosuppression, it has been suggested that viruses might promote the overgrowth of bacterial pathogens and/or induce the release of tissue-destroying cytokines by host cells[185].

Measles virus co-infections. Bacterial infection after measles virus infection is common. Measles virus is not usually lethal, but measles virus-induced immunosuppression results in increased susceptibility to infection with *S. pneumoniae*, *H. influenzae*, *S. aureus*, *P. aeruginosa*, *S. pyogenes* and *C. trachomatis* and to infection with other viruses such as adenovirus and HSV. These co-infections are responsible for the morbidity and mortality that are associated with measles virus infection. Measles virus infection of B cells results in secretion of a soluble factor that inhibits proliferation of cells of the lymphoid lineage. B cells that are infected with measles

virus cannot present antigen to T cells and have a diminished capacity to secrete Ig or proliferate. Susceptibility to bacterial co-infection is likely owing to these underlying immune defects that result in the hallmark of measles virus infection — inhibition of the proliferation of $CD4^+$ and $CD8^+$ T cells[186–189].

Animal models for co-infections

A primate model of HIV-induced immunosuppression that developes cutaneous leishmaniasis has been developed in rhesus macaques. In this model, macaques are chronically infected with SIV, and then co-infected with *Leishmania major* metacyclic promastigotes by intradermal injection. Lesion size, parasite load and SIV viraemia are measured weekly. This model has been used to assay both the synergistic relationship between these two pathogens and the responsiveness to, and relative protective efficacy of, CpG oligodeoxynucleotides delivered to co- and mono-infected macaques[190]. Recently, a rhesus monkey model for SIV predisposition to *Mycobacterium leprae* co-infection has been developed, which showed that co-infection increases the susceptibility to leprosy regardless of the timing between the two infections[191].

As mentioned earlier, measles virus-induced immunosuppression often leads to bacterial co-infection. To understand the mechanisms of co-infection, a murine model of combined measles virus and *Listeria monocytogenes* infection was developed[192]. In this model system, transgenic mice expressing the human measles virus receptor CD46 are co-infected with measles virus and *L. monocytogenes*, or are challenged with the bacterial pathogen alone. Mice co-infected with measles virus were more susceptible to infection with *L. monocytogenes* and this susceptibility corresponded with a reduction in the macrophage and PMN populations in the spleen, as well as a reduction in IFN-γ production by $CD4^+$ T cells. A reduction in $CD11b^+$ macrophages and IFN-γ producing T cells was found to be due to reduced proliferative expansion and not due to either increased apoptosis or altered distribution of these cells between the spleen, blood or lymphatics. The ability of measles virus to suppress both innate and adaptive immune responses is thought to be responsible for the increased susceptibility to bacterial co-infection.

The future of polymicrobial disease research

Molecular methods are now being used together with conventional culture techniques to determine the identity of the full complement of microorganisms that are involved in co-infections and to determine the interactions between these microorganisms. As a result, additional diseases of polymicrobial origin will be identified. This will necessitate the development of new animal models and new *in vitro* methods for the study of polymicrobial diseases. Uncovering the molecular mechanisms that are involved in the pathogenesis of complex diseases might show that changes in lifestyle, such as smoking cessation or dietary changes, could prevent co-infections. Developing methods to disrupt biofilms are one target for researchers. New antimicrobials and vaccine candidates for both the predisposing and the co-infecting microorganisms will be sought. Therapeutic approaches for polymicrobial diseases might include the use of probiotics for the treatment or prevention of vaginal infections, gastroenteritis, inflammatory bowel disease, UTIs and periodontitis. Moreover, advances in nanotechnology and biomedical engineering will allow the development of new ways to deliver these therapeutic or preventative agents in a disease- or site-specific manner such as the design and use of 'intelligent implants'[193]. These indwelling devices might be embedded with sensors to detect the biofilm-forming microorganisms and signal the release of antimicrobial agents stored in an internal reservoir. As the organizers of the first satellite conference on diseases of mixed microbial aetiology (see the online links box) stated — polymicrobial diseases are "a concept whose time has come"[1].

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Competing interests statement

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