

MULTIPLE CONSEQUENCES OF THE CHANGING EDUCATIONAL INPUT TO OUR IMMUNE SYSTEMS

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SUMMARY

The idea that diminishing exposure to microorganisms in the environment might play a role in the rising incidence of allergies was put forward nearly 30 years ago. Soon after the same idea was suggested for autoimmune disease. We would now add inflammatory bowel disease (IBD; Crohn's disease and ulcerative colitis) to this list, and place all three groups of disorder (allergies, autoimmunity, IBD) under a common umbrella title of "diseases of immunodysregulation", all attributable to a failure of maturation of immunoregulatory cell populations and networks. Meanwhile it has become clear that some behavioural and mood disorders are also increasing at a worrying rate in the rich, developed countries, and some of these are epidemiologically associated with the immunoregulatory disorders, and accompanied by cytokine changes that permit a tentative unifying hypothesis involving regulatory roles of interleukin 10 (IL-10) in the immune system and central nervous system.

Recent laboratory and clinical studies suggest that microorganisms can influence the "diseases of immunodysregulation" without causing overt infections. Changes in commensal flora (such as gut flora) or exposure to immunogenic but harmless saprophytes or vaccines, or alteration of our microbial exposure by antibiotics and hygiene can all exert significant effects. This information provides us with some extraordinary challenges, because although hygiene, vaccines and antibiotics are the three most useful and cost-effective achievements of medicine, the next decade will see us trying to discover how to improve vaccines so that they more closely mimic the "educational" input that the immune system requires, and how to supplement the effects of conventional vaccines with probiotics, and how to devise novel vaccines that are used, not to combat a specific infection, but rather to exploit their non-specific immunoregulatory properties.

INTRODUCTION: THE HYGIENE HYPOTHESIS AND ALLERGIC DISORDERS

In 1976 Gerrard and his colleagues, higher levels of IgE than their white
noted that North American Indians had neighbours, but a much lower incidence

of allergic symptoms (Gerrard et al., 1976). They suggested explicitly that “atopic disease is the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths” (Gerrard et al., 1976). This constitutes one of the earliest statements of the hygiene hypothesis.

Numerous subsequent epidemiological studies have confirmed that there is a steadily rising incidence of allergic symptoms in the rich developed, hygienic countries (Strachan et al., 1997) and several aspects of the epidemiology are compatible with the view that the relevant environmental change is the decreasing exposure to microorganisms. These include the protective effects of large family size, and of being low down in the birth order, especially if the individual has “dirty older brothers” (Matricardi et al., 1998; Strachan et al., 1997). A trend towards increases in allergic manifestations is also now being seen in urban Africans, when compared to the corresponding rural population (Yemaneberhan et al., 1997).

Further support came from a number of studies showing a negative correlation between allergic manifestations and evidence of response to organisms transmitted by the oro-faecal route (Matricardi et al., 2000), the incidence of tuberculosis (von Mutius et al., 2000), BCG vaccination (Aaby et al., 2000), and tuberculin skin test positivity (Shirakawa et al., 1996). While other

studies failed to confirm the latter observations in other environments (Alm et al., 1997; Strannegard et al., 1998), the interpretation of tuberculin test data, and the immunological effects of exposure to mycobacteria are complex matters and the negative results are not convincing (Rook and Stanford, 1999). At that time there was widespread belief that there is a reciprocal “see-saw” downregulation of Th1 responses by Th2, and of Th2 responses by Th1. Therefore several authors expanded the view that the rising incidence of allergies might be due to a failure of our hygienic modern environments to drive sufficient Th1 activity (Holt, 1995; Hopkin, 1997; Rook and Stanford, 1998; von Mutius, 1998). With hindsight this was clearly never a viable hypothesis. First, it entirely ignored the possibility that Th2-inducing infections such as helminths might also protect (Gerrard et al., 1976). Secondly the Th1/Th2 seesaw hypothesis ignored the fact that the incidence of Th1-mediated autoimmune diseases is rising in parallel with allergic diseases, with remarkably tight correlation between the two (Stene and Nafstad, 2001).

The major themes of this article are therefore the nature of the diseases that might be increasing because of altered inputs to our immune systems, the nature of the altered input, and the prospects for using this knowledge for novel immunotherapies.

THE HYGIENE HYPOTHESIS AND AUTOIMMUNE DISEASE

With hindsight it should have been obvious that the “hygiene hypothesis” could not be explained only by changes in the Th1/Th2 cytokine balance. It was already known that microbial exposure can influence autoimmunity in animal models, whether that autoimmunity is

mediated by Th2 or Th1 cells. Secondly, human Th1-mediated autoimmunity, such as type 1 diabetes (Stene and Nafstad, 2001) and multiple sclerosis (Celius and Vandvik, 2001; Pugliatti et al., 2001; Sumelahti et al., 2001) have been increasing at a rate similar to the

increase in allergies (*Stene and Nafstad, 2001*). Taken together these facts suggest that the hygiene hypothesis applies equally to these Th1-mediated disorders, and cannot therefore be a simple Th1/Th2 imbalance (*Rook, 2000*).

For instance, 25 years ago *Kohashi and colleagues (1985)* studied the induction of adjuvant arthritis in germ-free mice, and compared the incidence and severity of disease with that seen after re-colonisation of the gut with single species. The gut flora could either enhance or inhibit the disease depending on the bacterial species used. Similarly bacterial exposure profoundly affects rodent models of arthritis in HLA B27 rats (*Taurog et al., 1994*), pristane-induced Lupus (*Hamilton et al., 1998*), and autoimmune thyroiditis (*Penhale and Young, 1988*). Moreover a single dose of Freund's complete adjuvant protected non-obese diabetic (NOD) mice from a Th1-mediated autoimmune diabetes (*Sadelain et al., 1990*), and striking differences in the manifestations of diabetes in NOD mice in different animal houses have been attributed to

differing microbial exposure (*Todd, 1991*).

The most striking evidence comes from the astonishingly close correlations between the incidences of allergic symptoms and type 1 diabetes whether the analysis is confined to Europe, or extended to countries outside Europe (*Stene and Nafstad, 2001*). Therefore Th2-mediated and Th1-mediated pathologies are increasing in parallel (*Stene and Nafstad, 2001*).

Does this mean that Th1/Th2 balance has no role at all? It is interesting that one study has suggested that despite the parallel increase in allergies and type 1 diabetes *within communities*, these diseases tend *not* to occur in the same individuals. Children with type 1 diabetes, and their siblings, may be partially protected from allergic disorders (*Douek et al., 1999*). This implies that there might be an underlying environmental trend that is increasing the risk of both types of disorder, but that which disorder develops is determined by the individual's genetic background and immunological experience.

THE HYGIENE HYPOTHESIS AND INFLAMMATORY BOWEL DISEASE

The picture becomes clearer when a third group of diseases is considered. There have been definite increases in inflammatory bowel disease (IBD) over the last 10 years. In Iceland, the incidence of ulcerative colitis has doubled, and Crohn's disease has increased 3-fold (*Bjornsson et al., 1998*). IBD is commoner in the North of Europe than the South, though the gap may be closing (*Shivananda et al., 1996*), and there have been similar increases in Italy over a similar period (*Trallori et al., 1996*). Any doubts about these conclusions, based on changing diagnostic criteria, for example, have been dispelled by

rigorous recent studies in Scandinavia and Scotland (*Lindberg et al., 2000; Sawczenko et al., 2001*).

Once again there are animal models of IBD that suggest the possibility that microbial exposure can influence the incidence and severity of the disease. Interleukin-2 knockout (IL-2(-/-)) mice get colitis and autoimmunity unless germ-free. In germfree IL-2(-/-) mice the disease can be induced by antigen in Freund's complete adjuvant (*Ehrhardt et al., 1997*), and is associated with IL-12-driven Th1 without a Th2 component. The same is true of IL-10 knockout mice (*Kuhn et al., 1993*), and it is the

more recent work in which the role of IL-10 and regulatory T-cells has been defined in murine models of IBD that has consolidated the view that IBD belongs within the group of diseases that

falls under the “hygiene hypothesis” umbrella, and cast light on the mechanisms involved. This is discussed in the next section.

THE HYGIENE HYPOTHESIS AND REGULATORY T-CELLS; DISEASES OF “IMMUNODYSREGULATION”

Thus we would now place all three groups of disorder (allergy, autoimmunity, and inflammatory bowel disease) under a common umbrella title of “diseases of immunodysregulation”, all attributable to a failure of maturation of immunoregulatory cell populations and networks.

Regulatory cells and inflammatory bowel disease

Current evidence suggests that inflammatory bowel diseases (Crohn's disease and ulcerative colitis; IBD) are disorders in which the physiological mechanisms that inhibit immune responses to gut content (food or microbes) are failing. For instance IBD occurs in mice that lack IL-10 (gene knockout) and also in mice with severe combined immunodeficiency (SCID) that receive effector T-cells (CD4⁺CD45RB^{high}) without the appropriate regulatory cells (reviewed in *Asseman and Powrie, 1998*). As in the models of autoimmunity discussed below the regulatory cells are characteristically CD25⁺ and their function involves CTLA4 (*Read et al., 2000*). There is good evidence that the regulatory cells that can stop the inflammatory process are associated with abundant IL-10, and they have been denoted Tr1 cells (T-regulatory 1) (*Groux et al., 1997*).

Regulatory cells and autoimmunity

Regulatory cells that can suppress in-

flammation mediated by autoimmunity, have also been characterised. It is not clear whether these constitute several different cell types or whether they are variants of a single regulatory cell lineage. A wide variety of organ-specific autoimmune disorders can be shown to be controlled by CD25⁺ T-cells that also express mRNA for IL-4, TGF- β and IL-10 (*Seddon and Mason, 2000*). They are likely to be related to the IL-10-secreting Tr1 cells that can downregulate the Th1-mediated inflammation in models of inflammatory bowel disease (*Groux et al., 1997*). These cells are found in man, and for instance they can inhibit the nickel-specific Th1 responses of nickel-reactive individuals (*Cavani et al., 2000*). They may also be related to Th3 cells that secrete TGF- β and IL-10 (*Fukaura et al., 1996*). These are readily derived from human peripheral blood (*Kitani et al., 2000*). They can inhibit both Th1-mediated (*Cavani et al., 2000; Fukaura et al., 1996*) and Th2-mediated autoimmunity (*Bridoux et al., 1997*). Similarly T-cells engineered to secrete TGF- β will also downregulate both Th2-mediated and Th1-mediated responses (*Thorbecke et al., 2000*).

Regulatory T-cells and allergy

A variety of different types of cell have been implicated in the regulation of allergic responses. These include CD8-expressing cells (*MacAry et al., 1997*), and γ/δ T-cells. The latter have been implicated in the control of airway hyperresponsiveness (*Lahn et al., 2001*), and

in the development of tolerance and IL-10 production in response to low dose oral antigen (Fujihashi et al., 1999). It has been suggested very tentatively that γ/δ T-cells downregulate airway responsiveness to allergen challenge by controlling the 'repair' response of the airway epithelium to damage mediated by α/β T-cells (Holt and Sly, 1999). A different subset of T-cells, that may be identical to the Tr1 cells implicated in suppression of IBD, and perhaps also to Th3 cells, can inhibit the Th2-mediated response to allergen *in vivo* (Cottrez et al., 2000). These cells also release IL-10 which is already known to specifically decrease IgE production by IL-4-stimulated peripheral blood mononuclear cells *in vitro* (Punnonen et al., 1993). It can also downregulate IL-5 production by T-cells (Zuany-Amorim et al., 1996).

These findings do appear to relate to man, because the diameter of a house-dust mite (HDM)-induced wheal was inversely related to the quantity of mRNA for IL-10 induced by HDM *in vitro* (Macaubas et al., 1999). Similarly there is evidence for deficient release of IL-10 into the airways in human asthmatics (Borish et al., 1996).

Parasites, regulatory T-cells and allergy

Further support has come from the recent claim that decreased atopy in children infected with *Schistosoma haematobium* correlates with increased release of IL-10 from peripheral blood mononuclear cells in response to worm antigens *in vitro* (van den Biggelaar et al., 2000). This interpretation helps to explain the work of Gerrard and of Lynch outlined earlier (Gerrard et al., 1976; Lynch et al., 1998), and is acceptable if we assume a biphasic relationship between parasite load and the effect on atopy. This proviso is necessary because in studies of European

children, those who were *Ascaris*-IgE seropositive had 10-fold higher levels of total IgE, higher prevalence rates of allergen-specific IgE seropositivity and higher prevalence of allergic rhinitis and asthma (Dold et al., 1998). Similarly in a separate study the means of total serum IgE and blood eosinophils were significantly higher in *Toxocara*-seropositive than in the seronegative group, and allergic asthma was associated with *Toxocara* seroprevalence (Buijs et al., 1997). Thus low level infection with Th2-inducing parasites clearly exerts a non-specific adjuvant effect on the Th2 response to allergens. It might however be true that chronic exposure to very high parasite loads induces sufficient anti-inflammatory IL-10 to non-specifically downregulate inflammation due to other causes. Thus the observations in children with Schistosomiasis, although greeted with much enthusiasm, pose a number of puzzles that are highlighted by the studies on *Toxocara* (Buijs et al., 1997) and *Ascaris* (Dold et al., 1998). It is extremely difficult to explain the increase in the incidence of allergic disorders in the developed countries in recent decades on the basis of changing exposure to parasites. Moreover in Estonia, where the prevalence of allergy is low, parasites are very infrequent, so the relevant changes in microbial exposure must involve other types of organism (Julge et al., 1997).

Symptomless atopy, or atopy leading to allergic disorders?

A further difficulty is that the Schistosomiasis study looked at total and specific IgE and at the prevalence of positive skin prick reactions to HDM. It did not study allergic symptoms (van den Biggelaar et al., 2000). In a separate study in Ethiopia the rural population had significantly higher levels of skin prick test sensitivity to HDM than the urban population, but a lower

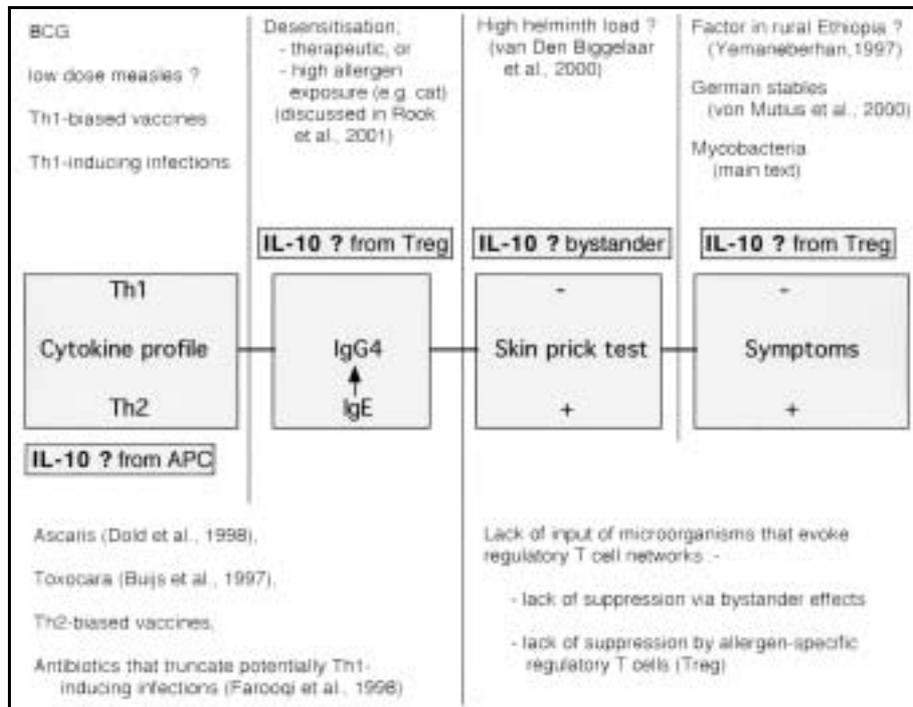


Figure 1: Regulatory cells may act at multiple levels to inhibit the inflammatory process. This figure focuses on the effects of IL-10. If antigen-presenting cells release IL-10 during the initiation of the response there may be bias towards Th2. In contrast, at later stages in the pathway, IL-10 from regulatory T-cells can switch from IgE to IgG4, downregulate skin prick test positivity, or attenuate allergic symptoms in the airways. Different subsets of regulatory cells might be involved at each stage. Moreover the regulation can be a non-specific bystander effect of anti-inflammatory cytokines, or due to *allergen-specific* regulatory cells. Similar figures could be devised for autoimmunity and inflammatory bowel disease.

prevalence of wheeze and asthma (Yemaneberhan et al., 1997). Figure 1 therefore points out that downregulation

of allergic manifestations can occur at several different levels, and via non-specific and allergen-specific pathways.

THE HYGIENE HYPOTHESIS AND PSYCHIATRIC DISORDERS

At this point we wish to introduce a hypothesis of such potential importance that although still tentative, it deserves to be thought about. It has become clear that some behavioural and mood disorders are also increasing at a worrying rate in the rich, developed countries, and some of these are epidemiologically associated with the immunoregulatory disorders already discussed. These psychiatric problems are also accompanied

by cytokine changes that parallel those seen in allergic disorders, and that can account for behavioural changes. There are two major types of evidence to support this hypothesis.

1) Associations with inflammatory disorders that are themselves increasing

The incidence of autism is increasing at the same rate as allergies etc. (Kaye et

al., 2001). Moreover about 50% of autistic children have gastrointestinal symptoms, and there is evidence for a mild mucosal inflammation resembling mild IBD (Furlano et al., 2001). Chronic fatigue syndrome is also associated with allergies (Borish et al., 1998). Attention deficit hyperactivity disorder (ADHD) is somewhat controversially associated with allergic manifestations (Egger, 1997; Roth et al., 1991), and many cases probably represent an infantile form of depression. There also appears to be a more general association between allergies and depression. College students with a history of clinical depression were more likely to be allergic, and allergic subjects were more likely to have mood worsening after influenza (Bell et al., 1991). This association seems not to be merely attributable to the stress of the allergic symptoms, because first and second degree relatives of allergic individuals have higher levels of psychiatric disorder and vice versa. Relatives of asthmatic adolescents have a higher incidence of affective disorder, antisocial personality disorder and substance abuse (Wamboldt et al., 1996). This association was then confirmed in a much larger twin study of depression and allergy (Wamboldt et al., 2000).

2) Cytokines and mood disorders

It has been noted that major depres-

sion is accompanied by indications of systemic inflammation; leukocytosis, increased acute phase proteins, increased production of IL-1 β , IL-6, IFN γ , IL-2 (reviewed in Maes et al., 1999), and there is some evidence that antidepressant drugs (including SSRI's and TCA's, HCA's and lithium), can reverse these cytokine changes. The recent vogue for treating hepatitis and certain cancers with IL-2 and Interferon alpha (IFN- α) has highlighted the role of cytokines in mood. In many subjects IL-2 and IFN- α rapidly induce severe clinical depression (Capuron et al., 2000, 2001; Dantzer et al., 1999; Musselman et al., 2001). In others there is mania after withdrawal. Interestingly IFN- α production is high in individuals with allergy or chronic fatigue syndrome while IL-10 production is low (Borish et al., 1998). This may be important because IL-10 is not only able to suppress the inflammatory disorders discussed above, but also able to suppress the sickness behaviour caused by signals passing to the brain from inflammatory sites. Moreover IL-10 can do this even when injected directly into the brain (Bluthé et al., 1999; Nava et al., 1997). Thus potentially these disorders may be influenced by the same defective control of inflammation, and changing cytokine balance with IL-10 deficiency, as the overtly inflammatory disorders discussed earlier.

THE UNIFYING HYPOTHESIS AND ITS CONSEQUENCES

Thus various regulatory cell types secreting IL-10, if deficient in inhabitants of the clean developed countries, can provide an unifying hypothesis to explain simultaneously the increase in allergy, autoimmunity and IBD (Rook, 2000; Rook et al., 2000; Yazdanbakhsh et al., 2001), and possibly changing patterns of psychiatric disorder. Indeed IL-10-secreting cells such as certain

NKT-cells can also be involved in the induction of regulatory T-cells (Sonoda et al., 2001), as can IL-10 secretion by antigen-presenting cells. We have recently been surprised by the huge range of background cytokine expression even in normal people. Quantitative RT-PCR using mRNA from unstimulated peripheral blood mononuclear cells from normal tuberculin-positive donors,

revealed a 10,000x range of ratios of IL-4 mRNA to IFN- γ mRNA (*Seah and Rook, 2000; Seah et al., 2000*). We are not aware of a strictly quantitative analysis of IL-10 mRNA expression in unstimulated blood cells.

One consequence of the realisation that the balance of pro-inflammatory cytokines (whether Th1 or Th2) to regulatory cytokines may be the crucial factor, is a resolution of the bizarre compartmentalisation of immunology that led workers in the field of Th1-mediated autoimmunity to seek treatments that would deviate the Th1 response towards Th2, while workers in the field of allergy were trying to deviate the Th2 response towards Th1. It now appears probable that neither approach was either safe or desirable, and that to treat both types of disorder we should be trying to evoke regulatory T-cells.

For instance, in the experimental allergic encephalitis (EAE) model and in the induction of diabetes in non-obese diabetic (NOD) mice, both of which are primarily Th1-mediated, a superimposed Th2 response can make the situation worse (*Lafaille et al., 1997; Pakala et al., 1997*), as it does in models of infection (reviewed in (*Rook et al., 2000*)). Moreover if the immune response is incorrectly regulated, switching the incorrectly regulated Th1 response to Th2 may merely result in an incorrectly regulated Th2 response that causes a different but equally dangerous disease (*Baxter et al., 1994; Genain et al., 1996*). This appears to be what happened in a treatment trial in human multiple sclerosis, where the disease changed in nature without improvement

from the patients' point of view, and autoimmune thyroid disease also appeared, superimposed upon the modified MS (*Coles et al., 1999a; Coles et al., 1999b*). These issues were discussed in detail elsewhere (*Rook et al., 2000*). The solution to this dilemma is to distinguish between Th2 effector cells, which are potentially dangerous when superimposed upon a Th1 response and Th2-like regulatory cells secreting IL-10 and/or TGF- β , which are beneficial.

The same argument holds for attempts to treat allergy by deviating the response to Th1. Allergen-specific Th1 clones can entirely fail to downregulate a Th2 response (*Cottrez et al., 2000*) or airway hyper-reactivity (*Hansen et al., 1999*). Indeed they can contribute additional immunopathology despite their ability to downregulate aspects of the Th2 response such as eosinophil infiltration (*Hansen et al., 1999*). Similarly in a recent human clinical trial, administration of IL-12 to asthmatics caused some evidence of a switch towards Th1, and some fall in eosinophil count, but failed to decrease the late asthmatic response to allergen exposure (*Bryan et al., 2000*). Moreover a monoclonal antibody to IL-5, a key Th2 cytokine, greatly reduced eosinophil levels in blood and bronchoalveolar lavage, but also failed to affect the late asthmatic response to allergen exposure (*Leckie et al., 2000*).

An alternative approach is the induction of the physiological regulatory cells that can control both excessive Th1 and Th2 activity.

HOW DO MICROORGANISMS TRIGGER REGULATORY T-CELLS?

There is no definitive answer to this question yet. Figure 2 is a crude cartoon indicating that the precursors of effector

cells, (which are inevitably weakly anti-self because of positive selection in the thymus), and regulatory cells that block

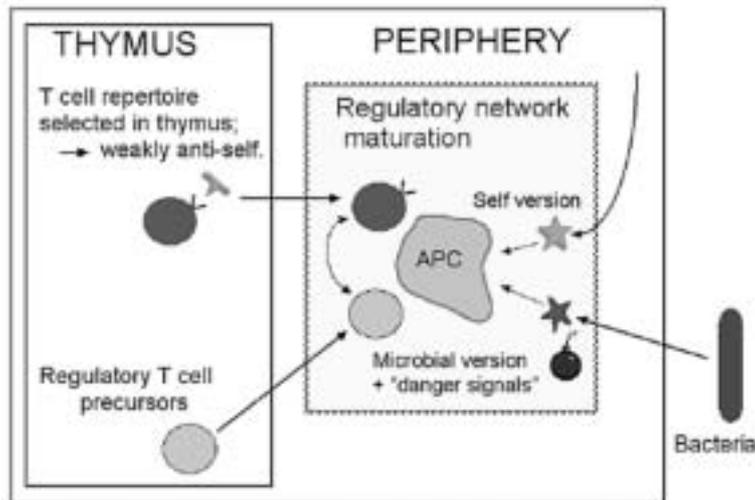


Figure 2: The induction of regulatory T-cells, and the requirement for bacterial or other microbial products is not understood in detail. Regulatory T-cell precursors need to encounter the antigen in the periphery, and the simultaneous presence of microbial homologues, presented in the context of microbe-derived “danger signals”, may be essential for the correct setting up of regulatory networks and self/non-self discrimination.

autoimmunity, may be generated together in the thymus (*Seddon and Mason, 2000*). Then for full function the regulatory cells need to encounter the self antigens in the periphery (*Seddon and Mason, 2000*). It may be at this point that cross-reactive microbial epitopes, presented in the context of microbial “danger signals” (*Matzinger, 1994*), exert a crucial influence on the appropriate development of regulatory networks. Much tolerance to allergens probably develops via mucosal surfaces, and indeed Th3 cells are preferentially induced following oral administration of antigen. It is interesting that oral tolerance is difficult to evoke in germ-free mice. This ability is restored by bowel flora or bacterial products

(*Sudo et al., 1997; Wannemuehler et al., 1982*). Effectively therefore, certain microbial components can act as “regulatory cell adjuvants”. One hypothesis to explain the development of regulatory responses involves the Notch-Notch ligand system. These molecules take part in cell lineage decisions in many organs, and may be involved in the effector/regulator decision in the immune system. High-dose peptide delivered intranasally to mice induces transient expression of Delta 1 (a Notch ligand) on inhibitory CD4+ T-cells. Ligation of the Notch1 receptor on neighbouring T-cells by Delta1+ regulatory T-cells inhibits clonal expansion of effector cells and leads to suppression (*Hoyne et al., 1999*).

THE MICROORGANISMS INVOLVED

If deficient exposure to certain microorganisms that act as regulatory T-cell adjuvants is behind the steady in-

crease in diseases of immunodysregulation in the developed countries, it becomes important to identify the organ-

isms involved. As far as recent trends in the developed countries are concerned the helminths and other parasites can probably be disregarded, as discussed earlier. In fact it is extremely unlikely that any single species or genus holds the key, but identification of any genera with the appropriate properties is clearly useful since it can lead to therapeutic trials.

Gram-negative bacteria

Recently some attention has been focused on Gram-negative bacteria, because allergic manifestations are less common in children living in an environment contaminated with high levels of endotoxin (lipopolysaccharide; LPS) (Gereda et al., 2000). This was confirmed by studies of farming families in Germany, where high levels of LPS are found in the homes and bedding of a population with a low incidence of allergies (von Mutius et al., 2000). LPS is a readily assayed and very robust component of Gram-negative bacteria, but this work does not in any way prove that these bacteria are the important ones. They might be, but we must remember that the LPS is an indicator of a lifestyle that will allow contamination with all types of organism, even if there is no way to screen easily for the others. It is however interesting that a polymorphism of CD14, which is involved in recognition of LPS by macrophages, is associated with some aspects of atopy (Baldini et al., 1999), but if the Notch-Notch ligand story is correct, LPS is an unlikely candidate as inducer of regulatory cells since its known interactions with Toll-like receptors will drive effector rather than regulatory cell proliferation.

Mycobacteria

The possible importance of the mycobacteria is suggested by several epidemiological approaches already dis-

cussed (Aaby et al., 2000; Shirakawa et al., 1996; von Mutius et al., 2000). Mycobacteria are not part of the normal commensal flora of man, but they are immensely common in mud and untreated water. There are more than 80 saprophytic species, and untreated water in developing countries can contain as many as 10^9 (approximately 1mg) per litre. Thus exposure to mycobacteria depends on lifestyle to an extent that is not true for any species that is part of the commensal gut flora, and so can help to explain the difference between developing and rich countries, and the urban-rural differences seen in both environments. The inhabitants of inner cities in the USA encounter mycobacteria very little, whereas inhabitants of developing countries usually have delayed hypersensitivity reactions to multiple environmental species as well as to tuberculosis. In fact they must have been very common in the environment throughout mammalian and human evolution, and it is possible that exposure to them is as much an evolutionary necessity as is exposure to a background level of LPS. It is important to note that children with defective receptors for IFN- γ , or IL-12 die from infections with saprophytic environmental mycobacteria (de Jong et al., 1998; Newport et al., 1996), which implies that in normal children, who frequently have such species associated with their tonsils (Stewart et al., 1970), the immune system is constantly and actively involved in their destruction. Conversely, the mycobacteria, when present in the environment, must be constantly modulating the immune system.

In mouse models BCG or *M. vaccae* can limit Th2 responses, even when given after the start of immunisation (Erb et al., 1998; Wang and Rook, 1998). This effect includes downregulation of airway hyper-reactivity (Zuany-Amorim et al., 2001). The

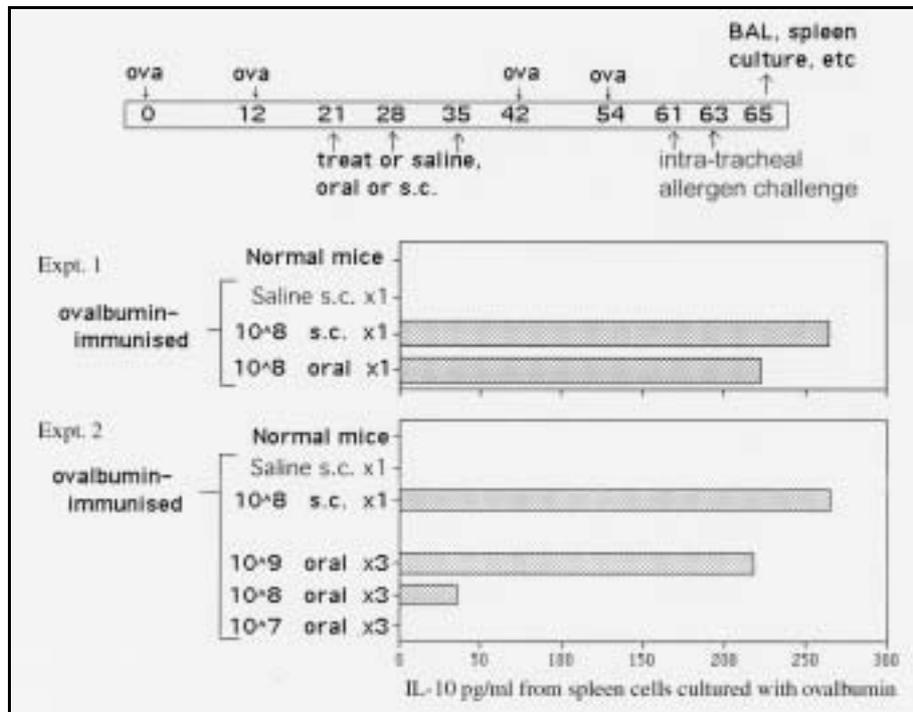


Figure 3: Spleen cells from Balb/c mice rendered allergic to ovalbumin did not release IL-10 in response to ovalbumin *in vitro*. However 0.1mg killed *M. vaccae* given s.c. at the times indicated, resulted in the presence in the spleen of a robust IL-10 response. Manifestations of the allergic response to intratracheal challenge were also suppressed, correlating with the presence of allergen-specific regulatory cells demonstrable in a cell transfer system. Orally administered *M. vaccae* (by gavage) was similarly active.

downregulation is mediated by IL-10-secreting allergen-specific CD4⁺ T-cells that can be transferred into allergic recipients (discussed in Rook et al., 2001) and Zuany-Amorim et al., submitted for publication). In view of the probable involvement of the gut in tolerance induction, it is interesting that mycobacteria, whether alive or killed, are subject to very rapid and efficient uptake via M cells (Fujimura, 1986; Lugton, 1999; Momotani et al., 1988). It has emerged recently that tiny doses of SRL172 or SRP299 (derivatives of heat-killed *M. vaccae* NCTC11659) given by the oral route are active in the Balb/c model of allergy to ovalbumin. A full account of this work will be published elsewhere (Rosa Brunet, L., Hunt, J., and Rook,

G.A.W., in preparation). Figure 3 shows, for example, that one dose of SRL172 given after the first 2 of 4 immunisations, caused the appearance in the spleen of cells that released IL-10 in response to ovalbumin *in vitro*. Again, allergen-specific regulatory cells are induced.

Clinical studies with mycobacterial derivatives in allergic disorders

Derivatives of *M. vaccae* administered by the intradermal route have been tested in several clinical studies in human asthma (Camporota et al., 2000; Hopkin et al., 1998; Shirtcliffe et al., 2001) and eczema (Arkwright and David, 2001). The results are encour-

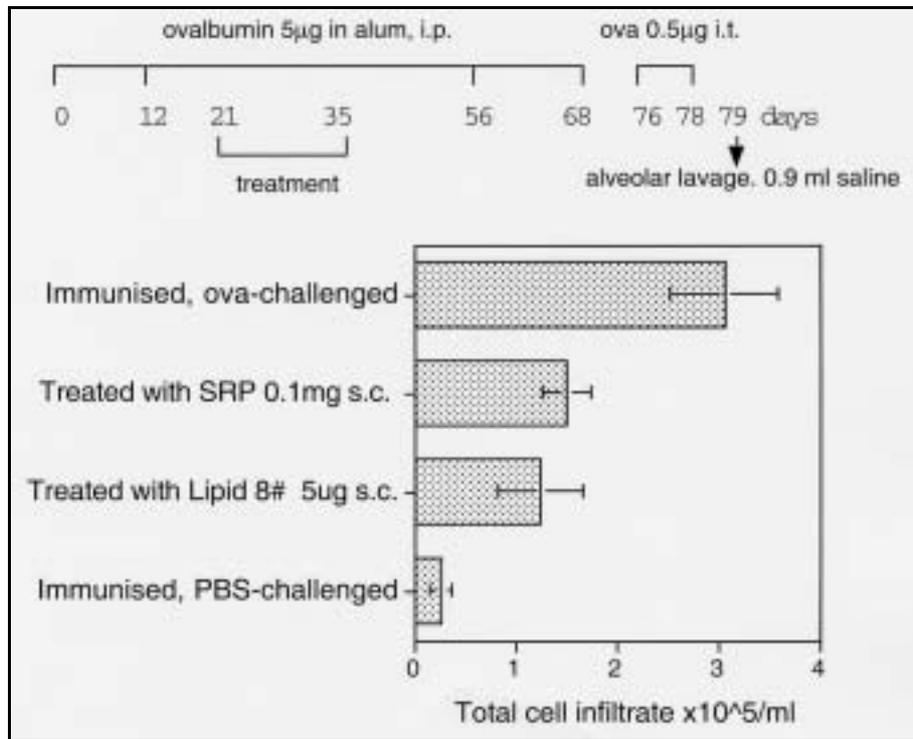


Figure 4: Using a protocol similar to that shown in Figure 3, two s.c. injections of lipid fraction 8 from *M. vaccae* NCTC11659 caused suppression of cellular infiltration into the lungs following intratracheal (i.t.) challenge with ovalbumin. This fraction can also lead to the presence of IL-10 secreting cells, and to suppression of eosinophilia, without any switch to Th1 (not shown). We speculate that some mycobacterial lipids might be essential to the immune system, and act as regulatory T-cell adjuvants.

aging, and a Phase II study is now in progress. Interestingly a delipidated preparation appeared to be inactive (Shirtcliffe et al., 2001), and recent work suggests that certain lipid fractions may be important active components of the organism (Figure 4).

Clinical studies with mycobacterial derivatives in autoimmune disease

M. vaccae derivatives have also shown promise in several clinical studies in psoriasis (Balagon et al., 2000; Lehrer et al., 1998), and BCG vaccine was recently tried in patients with multiple sclerosis (MS) (Ristori et al., 1999). Since BCG is predominantly a

Th1-inducing vaccine, and MS is a predominantly Th1-mediated disease, this trial represented a striking conceptual advance in which the physicians were thinking in terms of regulatory T-cells rather than Th1/Th2 balance (Rook et al., 2000). The results of this small preliminary study were most encouraging (Ristori et al., 1999).

Lactobacilli and the hygiene hypothesis

Lactobacilli were a common part of the diet of primitive man, but now occur mostly as a minor additive in some foods. Levels of gut *Lactobacilli* are lower in populations with high rates of allergy (Sepp et al., 1997). Therefore

this genus is also generating interest, and since it colonises the human gut there are exciting possibilities, and several small clinical studies have taken

place. These are covered by other chapters within this volume and will not be considered further here.

CONCLUDING REMARKS

As we live in increasingly hygienic and infection-free environments, so vaccination schedules become an increasingly large proportion of the educational input to our immune systems. If the immune system has evolved in the anticipation of a particular type and sequence of inputs, the change represented by modern vaccination schedules may be inappropriate. Vaccines will inevitably modify the priming of regulatory T-cells. First, they provide exposure to organisms in a sequence that is different from the evolutionarily experienced/programmed sequence. Peter Aaby has evidence that the *sequence of vaccination* has effects on subsequent overall health (personal communication). Secondly, the vaccines used prime an unbalanced response, mostly Th2-orientated, particularly in the USA where BCG is not used and hepatitis B vaccination at birth is routine. It is not clear that priming antibody responses drives adequate maturation of regulatory T-cells. Thirdly, most current vaccines *replace* the educational input previously provided by recovery from the infection, with a quite different type of immunological response (for instance antibody that blocks infection rather than a cell-mediated response that cures it). These changes are taking place against a background of antibiotic use, already

shown to affect susceptibility to allergies (*Farooqi and Hopkin, 1998*), that will also alter bowel flora and the education of the immune system.

Against this background there are now several studies indicating that vaccinations can affect the overall survival of children from diseases not directly related to the target infection for which the vaccine was intended. Thus measles vaccine and BCG were beneficial for overall survival, whereas Diphtheria-Pertussis-Tetanus (DPT) opposed this beneficial effect (*Kristensen et al., 2000*). Thus while many groups, including ours, are seeking to use vaccines as immunomodulators to correct inappropriate priming of immunoregulation in the developed countries, others are already showing that vaccines do indeed exert non-specific effects on health, even against the greater background of exposure to infection in a developing country (*Kristensen et al., 2000*). It is to be hoped that novel vaccine design and novel vaccination schedules with greater emphasis on the activation of regulatory cells, can work together with probiotics (or indeed with orally administered vaccines) to retain the huge benefits of vaccination, while simultaneously reducing the diseases of immunodysregulation.

LITERATURE

- Aaby, P., Shaheen, S.O., Heyes, C.B., Goudiaby, A., Hall, A.J., Shiell, A.W., Jensen, H., and Marchant, A.: Early BCG vaccination and reduction in atopy in Guinea-Bissau. *Clin. Exp. Allergy* 30, 644-650 (2000).
- Alm, J.S., Lilja, G., Pershagen, G., and Scheynius, A.: Early BCG vaccination and

- development of atopy. *Lancet* 350, 400-403 (1997).
- Arkwright, P.D. and David, T.J.: Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease. *J. Allergy. Clin. Immunol.* 107, 531-534 (2001).
- Asseman, C. and Powrie, F.: Interleukin 10 is a growth factor for a population of regulatory T-cells. *Gut* 42, 157-158 (1998).
- Balagon, M.V., Walsh, D.S., Tan, P.L., Cellona, R.V., Abalos, R.M., Tan, E.V., Fajardo, T.T., Watson, J.D., and Walsh, G.P.: Improvement in psoriasis after intradermal administration of heat-killed *Mycobacterium vaccae*. *Int. J. Dermatol.* 39, 51-58 (2000).
- Baldini, M., Lohman, I.C., Halonen, M., Erikson, R.P., Holt, P.G., and Martinez, F.D.: A polymorphism in the 5'-flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum IgE. *Am. J. Respir. Cell. Mol. Biol.* 20, 976-983 (1999).
- Baxter, A.G., Horsfall, A.C., Healey, D., Ozegebe, P., Day, S., Williams, D.G., and Cooke, A.: *Mycobacteria precipitate* an SLE-like syndrome in diabetes-prone NOD mice. *Immunology* 83, 227-231 (1994).
- Bell, I.R., Jasnoski, M.L., Kagan, J., and King, D.S.: Depression and allergies: Survey of a nonclinical population. *Psychother. Psychosom.* 55, 24-31 (1991).
- Bjornsson, S., Johannsson, J.H., and Oddsson, E.: Inflammatory bowel disease in Iceland, 1980-89. A retrospective nationwide epidemiologic study. *Scand. J. Gastroenterol.* 33, 71-77 (1998).
- Bluthé, R.M., Castanon, N., Pousset, F., Bristow, A., Ball, C., Lestage, J., Michaud, B., Kelley, K.W., and Dantzer, R.: Central injection of IL-10 antagonizes the behavioural effects of lipopolysaccharide in rats. *Psychoneuroendocrinology* 24, 301-311 (1999).
- Borish, L., Aarons, A., Rumbyrt, J., Cvietusa, P., Negri, J., and Wenzel, S.: Interleukin-10 regulation in normal subjects and patients with asthma. *J. Allergy Clin. Immunol.* 97, 1288-1296 (1996).
- Borish, L., Schmalig, K., DiClementi, J.D., Streib, J., Negri, J., and Jones, J.F.: Chronic fatigue syndrome: identification of distinct subgroups on the basis of allergy and psychologic variables. *J. Allergy Clin. Immunol.* 102, 222-230 (1998).
- Bridoux, F., Badou, A., Saoudi, A., Bernard, I., Druet, E., Pasquier, R., Druet, P., and Pelletier, L.: Transforming growth factor beta (TGF- β)-dependent inhibition of T-helper cell 2 (Th2)-induced autoimmunity by self-major histocompatibility complex (MHC) class II-specific, regulatory CD4+ T-cell lines. *J. Exp. Med.* 185, 1769-1775 (1997).
- Bryan, S.A., O'Connor, B.J., Matti, S., Leckie, M.J., Kanabar, V., Khan, J., Warrington, S.J., Renzetti, L., Rames, A., Bock, J.A., Boyce, M.J., Hansel, T.T., Holgate, S.T., and Barnes, P.J.: Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356, 2149-2153 (2000).
- Buijs, J., Borsboom, G., Renting, M., Hilgersom, W.J., van Wieringen, J.C., Jansen, G., and Neijens, J.: Relationship between allergic manifestations and *Toxocara* seropositivity: a cross-sectional study among elementary school children. *Eur. Respir. J.* 10, 1467-1475 (1997).
- Camporota, L., Corkhill, A., Long, H., Lau, L., Lordan, J., Stanford, J., Rook, G., Holgate, S., and Djukanovic, R.: Effects of intradermal *Mycobacterium vaccae* on allergen-induced airway responses and IL-5 generation by PBMC in mild to moderate asthma. *Am. J. Respir. Crit. Care. Med.* 161, 477 (2000).
- Capuron, L., Ravaut, A., and Dantzer, R.: Early depressive symptoms in cancer patients receiving interleukin 2 and/or interferon alfa-2b therapy. *J. Clin. Oncol.* 18, 2143-2151 (2000).
- Capuron, L., Ravaut, A., and Dantzer, R.: Timing and specificity of the cognitive changes induced by interleukin-2 and interferon-alpha treatments in cancer patients. *Psychosom. Med.* 63, 376-386 (2001).
- Cavani, A., Nasorri, F., Prezzi, C., Sebastiani, S., Albanesi, C., and Girolomoni, G.: Human CD4+ T-lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. *J. Invest. Dermatol.* 114, 295-302 (2000).
- Celius, E.G. and Vandvik, B.: Multiple sclerosis

- sis in Oslo, Norway: prevalence on 1 January 1995 and incidence over a 25-year period. *Eur. J. Neurol.* 8, 463-469 (2001).
- Coles, A.J., Wing, M., Smith, S., Corradu, F., Greer, S., Taylor, C., Weetman, A., Hale, G., Chatterjee, V.K., Waldmann, H., and Compston, A.: Pulsed monoclonal antibody treatment and autoimmune thyroid disease in multiple sclerosis. *Lancet* 354, 1691-1695 (1999a).
- Coles, A.J., Wing, M.G., Molyneux, P., Paolillo, A., Davie, C.M., Hale, G., Miller, D., Waldmann, H., and Compston, A.: Monoclonal antibody treatment exposes three mechanisms underlying the clinical course of multiple sclerosis. *Ann. Neurol.* 46, 296-304 (1999b).
- Cottrez, F., Hurst, S.D., Coffman, R.L., and Groux, H.: T-regulatory cells 1 inhibit Th2-specific response in vivo. *J. Immunol.* 165, 4848-4853 (2000).
- Dantzer, R., Wollman, E., Vitkovic, L., and Yirmiya, R.: Cytokines and depression: fortuitous or causative association? *Mol. Psychiatry* 4, 328-332 (1999).
- de Jong, R., Altare, F., Haagen, I.A., Elferink, D.G., Boer, T., van Breda Vriesman, P.J., Kabel, P.J., Draaisma, J.M., van Dissel, J.T., Kroon, F.P., Casanova, J.L., and Ottenhoff, T.H.: Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science* 280, 1435-1438 (1998).
- Dold, S., Heinrich, J., Wichmann, H.E., and Wjst, M.: Ascaris-specific IgE and allergic sensitization in a cohort of school children in the former East Germany. *J. Allergy Clin. Immunol.* 102, 414-420 (1998).
- Douek, I.F., Leech, N.J., Gillmor, H.A., Bingley, P.J., and Gale, E.A.: Children with type-1 diabetes and their unaffected siblings have fewer symptoms of asthma. *Lancet* 353, 1850 (1999).
- Egger, J.: Hyperkinetic syndrome. *J. Nutr. Env. Med.* 7, 353-357 (1997).
- Ehrhardt, R.O., Ludviksson, B.R., Gray, B., Neurath, M., and Strober, W.: Induction and prevention of colonic inflammation in IL-2-deficient mice. *J. Immunol.* 158, 566-573 (1997).
- Erb, K.J., Holloway, J.W., Soback, A., Moll, H., and Le Gros, G.: Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guérin (BCG) suppresses allergen-induced airway eosinophilia. *J. Exp. Med.* 187, 561-569 (1998).
- Farooqi, I.S. and Hopkin, J.M.: Early childhood infection and atopic disorder. *Thorax* 53, 927-32 (1998).
- Fujihashi, K., Dohi, T., Kweon, M.N., McGhee, J.R., Koga, T., Cooper, M.D., Tonegawa, S., and Kiyono, H.: γ/δ T-cells regulate mucosally induced tolerance in a dose-dependent fashion. *Int. Immunol.* 11, 1907-1916 (1999).
- Fujimura, Y.: Functional morphology of microfold cells (M cells) in Peyer's patches - Phagocytosis and transport of BCG by M cells into rabbit Peyer's patches. *Gastroenterol. Jpn.* 21, 325-335 (1986).
- Fukaura, H., Kent, S.C., Pietrusewicz, M.J., Khoury, S.J., Weiner, H.L., and Hafler, D.A.: Induction of circulating myelin basic protein proteolipid protein specific transforming growth factor beta (TGF- β)-secreting Th3 T-cells by oral administration of myelin in multiple sclerosis patients. *J. Clin. Invest.* 98, 70-77 (1996).
- Furlano, R.I., Anthony, A., Day, R., Brown, A., McGarvey, L., Thomson, M.A., Davies, S.E., Berelowitz, M., Forbes, A., Wakefield, A.J., Walker-Smith, J.A., and Murch, S.H.: Colonic CD8 and gamma delta T-cell infiltration with epithelial damage in children with autism. *J. Pediatr.* 138, 366-372 (2001).
- Genain, C.P., Abel, K., Belmar, N., Villinger, F., Rosenberg, D.P., Linington, C., Raine, C.S., and Hauser, S.L.: Late complications of immune deviation therapy in a nonhuman primate. *Science* 274, 2054-2057 (1996).
- Gereda, J.E., Leung, D.Y.M., Thatayatikom, A., Streib, J.E., Price, M.R., Klinnert, M.D., and Liu, A.H.: Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet* 355, 1680-1683 (2000).
- Gerrard, J.W., Geddes, C.A., Reggin, P.L., Gerrard, C.D., and Horne, S.: Serum IgE levels in white and metis communities in Saskatchewan. *Ann. Allergy* 37, 91-100 (1976).
- Groux, H., O'Garra, A., Bigier, M., Rouleau, M., Antonenko, A., de Vries, J.E., and Roncarolo, M.G.: A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses

- and prevents colitis. *Nature* 389, 737-742 (1997).
- Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., and Roncarolo, M.G.: A CD4⁺ subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, 737-742 (1997).
- Hamilton, K.J., Satoh, M., Swartz, J., Richards, H.B., and Reeves, W.H.: Influence of microbial stimulation on hypergammaglobulinemia and autoantibody production in pristane-induced lupus. *Clin. Immunol. Immunopathol.* 86, 271-279 (1998).
- Hansen, G., Berry, G., DeKruyff, R.H., and Umetsu, D.T.: Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103, 175-183 (1999).
- Holt, P.G.: Environmental factors and primary T-cell sensitisation to inhalant allergens in infancy: reappraisal of the role of infections and air pollution. *Paediatr. Allergy Immunol.* 6, 1-10 (1995).
- Holt, P.G. and Sly, P.D.: γ/δ T-cells provide a breath of fresh air for asthma research. *Nat. Med.* 5, 1127-1128 (1999).
- Hopkin, J.M.: Mechanisms of enhanced prevalence of asthma and atopy in developed countries. *Curr. Opin. Immunol.* 9, 788-792 (1997).
- Hopkin, J.M., Shaldon, S., Ferry, B., Coull, P., Antrobus, P., Enomoto, T., Yamashita, T., Kurimoto, F., Stanford, J.L., Shirakawa, T., and Rook, G.A.W.: Mycobacterial Immunisation in grass pollen asthma and rhinitis. *Thorax* 53 (Suppl. 4), Abstract S63 (1998).
- Hoyne, G.F., Dallman, M.J., and Lamb, J.R.: Linked suppression in peripheral T-cell tolerance to the house dust mite derived allergen Der p 1. *Int. Arch. Allergy Immunol.* 118, 122-124 (1999).
- Julge, K., Vasar, M., and Bjorksten, B.: The development of atopic sensitisation in Estonian infants. *Acta Paediatr.* 86, 1188-1194 (1997).
- Kaye, J.A., del Mar Melero-Montes, M., and Jick, H.: Mumps, measles, and rubella vaccine and the incidence of autism recorded by general practitioners: a time trend analysis. *Brit. Med. J.* 322, 460-463 (2001).
- Kitani, A., Chua, K., Nakamura, K., and Strober, W.: Activated self-MHC-reactive T-cells have the cytokine phenotype of Th3/T-regulatory cell 1 T-cells. *J. Immunol.* 165, 691-702 (2000).
- Kohashi, O., Kohashi, Y., Takahashi, T., Ozawa, A., and Shigematsu, N.: Reverse effect of gram-positive bacteria vs. gram-negative bacteria on adjuvant-induced arthritis in germfree rats. *Microbiol. Immunol.* 29, 487-497 (1985).
- Kristensen, I., Aaby, P., and Jensen, H.: Routine vaccinations and child survival: follow up study in Guinea-Bissau, West Africa. *Brit. Med. J.* 321, 1435-1438 (2000).
- Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W.: Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263-274 (1993).
- Lafaille, J.J., Keere, F.V., Hsu, A.L., Baron, J.L., Haas, W., Raine, C.S., and Tonegawa, S.: Myelin basic protein-specific T-helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J. Exp. Med.* 186, 307-312 (1997).
- Lahn, M., Kanehiro, A., Takeda, K., Konowal, A., O'Brien, R.L., Gelfand, E.W., and Born, W.K.: γ/δ T-cells as regulators of airway hyperresponsiveness. *Int. Arch. Allergy Immunol.* 125, 203-210 (2001).
- Leckie, M.J., ten Brinke, A., Bryan, S.A., Khan, J., Diamant, Z., O'Connor, B.J., Walls, C.M., Mathur, A.K., Cowley, H.C., Chung, K.F., Djukanovic, R., Hansel, T.T., Holgate, S.T., Sterk, P.J., and Barnes, P.J.: Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness and the late asthmatic response. *Lancet* 356, 2144-2148 (2000).
- Lehrer, A., Bressanelli, A., Wachsmann, V., Bottasso, O., Bay, M.L., Singh, M., Stanford, C., and Stanford, J.: Immunotherapy with *Mycobacterium vaccae* in the treatment of psoriasis. *FEMS Immunol. Med. Microbiol.* 21, 71-77 (1998).
- Lindberg, E., Lindquist, B., Holmquist, L., and Hildebrand, H.: Inflammatory bowel disease in children and adolescents in Sweden, 1984-1995. *J. Pediatr. Gastroenterol. Nutr.* 30, 259-264 (2000).
- Lugton, I.: Mucosa-associated lymphoid tissues as sites for uptake, carriage and excretion of tubercle bacilli and other pathogenic

- mycobacteria. *Immunol. Cell. Biol.* 77, 364-72 (1999).
- Lynch, N.R., Hagel, I.A., Palenque, M.E., Di Prisco, M.C., Escudero, J.E., Corao, L.A., Sandia, J.A., Ferreira, L.J., Botto, C., Perez, M., and Le Souef, P.N.: Relationship between helminthic infection and IgE response in atopic and nonatopic children in a tropical environment. *J. Allergy. Clin. Immunol.* 101, 217-221 (1998).
- MacAry, P.A., Holmes, B.J., and Kemeny, D.M.: Generation of rat MHC class-I-restricted ovalbumin-specific IgE inhibitory CD8+ T-cell clones. *Int. Arch. Allergy Immunol.* 113, 279-280 (1997).
- Macaubas, C., Sly, P.D., Burton, P., Tiller, K., Yabuhara, A., Holt, B.J., Smallacombe, T.B., Kendall, G., Jenmalm, M.C., and Holt, P.G.: Regulation of T-helper cell responses to inhaled allergen during early childhood. *Clin. Exp. Allergy* 29, 1223-1231 (1999).
- Maes, M., Song, C., Lin, A.H., Pioli, R., Kenis, G., Kubera, M., and Bosmans, E.: *In vitro* immunoregulatory effects of lithium in healthy volunteers. *Psychopharmacology (Berl.)* 143, 401-7. (1999).
- Matricardi, P.M., Franzinelli, F., Franco, A., Caprio, G., Murru, F., Cioffi, D., Ferrigno, L., Palermo, A., Ciccarelli, N., and Rosmini, F.: Sibship size, birth order, and atopy in 11,371 Italian young men. *J. Allergy Clin. Immunol.* 439-444 (1998).
- Matricardi, P.M., Rosmini, F., Riondino, S., Fortini, M., Ferrigno, L., Rapicetta, M., and Bonini, S.: Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma; epidemiological study. *Brit. Med. J.* 320, 412-417 (2000).
- Matzinger, P.: Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12, 991-1045 (1994).
- Momotani, E., Whipple, D.L., Thiermann, A.B., and Chevillat, N.F.: The role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal patches in calves. *Vet. Pathol.* 25, 131-137 (1988).
- Musselman, D.L., Lawson, D.H., Gumnick, J.F., Manatunga, A.K., Penna, S., Goodkin, R.S., Greiner, K., Nemeroff, C.B., and Miller, A.H.: Paroxetine for the prevention of depression induced by high-dose interferon alfa. *N. Engl. J. Med.* 344, 961-966 (2001).
- Nava, F., Calapai, G., Facciola, G., Cuzzocrea, S., Marciano, M.C., De Sarro, A., and Caputi, A.P.: Effects of interleukin-10 on water intake, locomotory activity, and rectal temperature in rat treated with endotoxin. *Int. J. Immunopharmacol.* 19, 31-38 (1997).
- Newport, M., Huxley, C.M., Huston, S., Hawrylowicz, C.M., Oostra, B.A., Williamson, R., and Levin, M.: A mutation in the interferon γ receptor gene and susceptibility to mycobacterial infection. *New. Engl. J. Med.* 335, 1941-1949 (1996).
- Pakala, S.V., Kurrer, M.O., and Katz, J.D.: T-helper 2 (Th2) T-cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. *J. Exp. Med.* 186, 299-306 (1997).
- Penhale, W.J. and Young, P.R.: The influence of the normal microbial flora on the susceptibility of rats to experimental autoimmune thyroiditis. *Clin. Exp. Immunol.* 72, 288-292 (1988).
- Pugliatti, M., Sotgiu, S., Solinas, G., Castiglia, P., Pirastru, M.I., Murgia, B., Mannu, L., Sanna, G., and Rosati, G.: Multiple sclerosis epidemiology in Sardinia: evidence for a true increasing risk. *Acta Neurol. Scand.* 103, 20-26 (2001).
- Punnonen, J.R., de Waal Malefyt, R., van Vlasselaer, P., Gauchat, J.F., and de Vries, J.E.: IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting accessory cell function in monocytes. *J. Immunol.* 151, 1280 (1993).
- Read, S., Malmstrom, V., and Powrie, F.: Cytotoxic T-lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192, 295-302 (2000).
- Ristori, G., Buzzi, M.G., Sabatini, U., Gignani, E., Bastianello, S., Viselli, F., Buttinelli, C., Ruggieri, S., Colonnese, C., Pozzilli, C., and Salvetti, M.: Use of Bacille Calmette-Guerin (BCG) in multiple sclerosis. *Neurology* 53, 1588-1589 (1999).
- Rook, G.A. and Stanford, J.L.: Skin-test responses to mycobacteria in atopy and asthma. *Allergy* 54, 285-286 (1999).
- Rook, G.A.W.: Clean living increases more than atopic disease. *Immunol. Today* 21,

- 249 (2000).
- Rook, G.A.W., Ristori, G., Salvetti, M., Giovannoni, G., Thompson, E.J., and Stanford, J.L.: Bacterial vaccines for the treatment of multiple sclerosis and other autoimmune diseases. *Immunol. Today* 21, 503-508 (2000).
- Rook, G.A.W., Rosa Brunet, L., and Hunt, J.: Cat allergen and sensitisation. *Lancet* 357, 2137 (2001).
- Rook, G.A.W. and Stanford, J.L.: Give us this day our daily germs. *Immunol. Today* 19, 113-116 (1998).
- Roth, N., Beyreiss, J., Schlenzka, K., and Beyer, H.: Coincidence of attention deficit disorder and atopic disorders in children: empirical findings and hypothetical background. *J. Abnorm. Child Psychol.* 19, 1-13 (1991).
- Sadelain, M.W., Qin, H.Y., Lauzon, J., and Singh, B.: Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes*. 39, 100583-101179 (1990).
- Sawczenko, A., Sandhu, B.K., Logan, R.F., Jenkins, H., Taylor, C.J., Mian, S., and Lynn, R.: Prospective survey of childhood inflammatory bowel disease in the British Isles. *Lancet* 357, 1093-1094 (2001).
- Seah, G.T. and Rook, G.A.W.: High levels of mRNA encoding IL-4 in unstimulated peripheral blood mononuclear cells from tuberculosis patients revealed by quantitative nested RT-PCR; correlations with serum IgE levels. *Scand. J. Infect. Dis.* 33, 106-109 (2000).
- Seah, G.T., Scott, G.M., and Rook, G.A.: Type 2 Cytokine Gene Activation and Its Relationship to Extent of Disease in Patients with Tuberculosis. *J. Infect. Dis.* 181, 385-389 (2000).
- Seddon, B. and Mason, D.: The third function of the thymus. *Immunol. Today* 21, 95-99 (2000).
- Sepp, E., Julge, K., Vasar, M., Naaber, P., Bjorksten, B., and Mikelsaar, M.: Intestinal microflora of Estonian and Swedish infants. *Acta Paediatr.* 86, 956-961 (1997).
- Shirakawa, T., Enomoto, T., Shimazu, S., and Hopkin, J.M.: The inverse association between tuberculin responses and atopic disorder. *Science* 275, 77-79 (1996).
- Shirtcliffe, P.M., Easthope, S.E., Soo, C., Weatherall, M., Tan, P.L.J., Le Gros, G., and Beasley, R.: The effect of delipidated, deglycolipidated (DDMV) and heat killed *M. vaccae* in asthma. *Am. J. Resp. Crit. Care Med.* 163, 1410-1414 (2001).
- Shivananda, S., J., L.-J., Logan, R., Fear, N., Price, A., Carpenter, L., and van Blankenstein, M.: Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 39, 690-697 (1996).
- Sonoda, K.H., Faunce, D.E., Taniguchi, M., Exley, M., Balk, S., and Stein-Streilein, J.: NK T-cell-derived IL-10 is essential for the differentiation of antigen-specific T-regulatory cells in systemic tolerance. *J. Immunol.* 166, 42-50 (2001).
- Stene, L.C. and Nafstad, P.: Relation between occurrence of type 1 diabetes and asthma. *Lancet* 357, 607 (2001).
- Stewart, C.J., Dixon, J.M., and Curtis, B.A.: Isolation of mycobacteria from tonsils, naso-pharyngeal secretions and lymph nodes in East Anglia. *Tubercle* 51, 178-183 (1970).
- Strachan, D., Sibbald, B., Weiland, S., Ait-Khaled, N., Anabwani, G., Anderson, H.R., Asher, M.I., Beasley, R., Bjorksten, B., Burr, M., Clayton, T., Crane, J., Ellwood, P., Keil, U., Lai, C., Mallol, J., Martinez, F., Mitchell, E., Montefort, S., Pearce, N., Robertson, C.F., Shah, J.R., Stewart, A.W., von Mutius, E., and Williams, H.C.: Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC). *Pediatr. Allergy Immunol.* 8, 161-176 (1997).
- Strachan, D.P., Harkins, L.S., and Golding, J.: Sibship size and self-reported inhalant allergy among adult women. *ALSPAC Study Team. Clin. Exp. Allergy* 27, 151-155 (1997).
- Strannegard, I.L., Larsson, L.O., Wennergren, G., and Strannegard, O.: Prevalence of allergy in children in relation to prior BCG vaccination and infection with atypical mycobacteria. *Allergy* 53, 249-254 (1998).
- Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C., and Koga, Y.: The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction.

- J. Immunol. 159, 1739-1754 (1997).
- Sumelahti, M.L., Tienari, P.J., Wikstrom, J., Palo, J., and Hakama, M.: Increasing prevalence of multiple sclerosis in Finland. *Acta Neurol. Scand.* 103, 153-158 (2001).
- Taurog, J.D., Richardson, J.A., Croft, J.T., Simmons, W.A., Zhou, M., Fernandez-Sueiro, J.L., Balish, E., and Hammer, R.E.: The germfree state [reverts development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J. Exp. Med.* 180, 2359-2364 (1994).
- Thorbecke, G.J., Umetsu, D.T., deKruyff, R.H., Hansen, G., Chen, L.Z., and Hochwald, G.M.: When engineered to produce latent TGF-beta1, antigen specific T-cells down regulate Th1 cell-mediated auto-immune and Th2 cell-mediated allergic inflammatory processes. *Cytokine Growth Factor Rev.* 11, 89-96 (2000).
- Todd, J.A.: A protective role of the environment in the development of Type 1 diabetes. *Diabetic Medicine* 8, 906-910 (1991).
- Trallori, G., Palli, D., Saieva, C., Bardazzi, G., Bonanomi, A.G., d'Albasio, G., Galli, M., Vannozzi, G., Milla, M., Tarantino, O., Renai, F., Messori, A., Amorosi, A., Pacini, F., and Morettini, A.: A population-based study of inflammatory bowel disease in Florence over 15 years (1978-92). *Scand. J. Gastroenterol.* 31, 892-899 (1996).
- van den Biggelaar, A.H., van Ree, R., Rodrigues, L.C., Lell, B., Deelder, A.M., Kremsner, P.G., and Yazdanbakhsh, M.: Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. *Lancet* 356, 1723-1727 (2000).
- von Mutius, E.: The environmental predictors of allergic disease. *J. Allergy Clin. Immunol.* 9-19 (1998).
- von Mutius, E., Braun, F.C., Schierl, R., Riedler, J., Ehlermann, S., Maisch, S., Waser, M., and Nowak, D.: Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin. Exp. Allergy.* 30, 1230-1234 (2000).
- von Mutius, E., Pearce, N., Beasley, R., Cheng, S., von Ehrenstein, O., Bjorksten, B., and Weiland, S.: International patterns of tuberculosis and the prevalence of symptoms of asthma, rhinitis, and eczema. *Thorax* 55, 449-453 (2000).
- Wamboldt, M.Z., Hewitt, J.K., Schmitz, S., Wamboldt, F.S., Rasanen, M., Koskenvuo, M., Romanov, K., Varjonen, J., and Kaprio, J.: Familial association between allergic disorders and depression in adult Finnish twins. *Am. J. Med. Genet.* 96, 146-153 (2000).
- Wamboldt, M.Z., Weintraub, P., Krafchick, D., and Wamboldt, F.S.: Psychiatric family history in adolescents with severe asthma. *J. Am. Acad. Child Adolesc. Psychiatry* 35, 1042-1049. (1996).
- Wang, C.C. and Rook, G.A.W.: Inhibition of an established allergic response to ovalbumin in Balb/c mice by killed *Mycobacterium vaccae*. *Immunology* 93, 307-313 (1998).
- Wannemuehler, M.J., Kiyono, H., Babb, J.L., Michalek, S.M., and McGhee, J.R.: Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. *J. Immunol.* 129, 959-965 (1982).
- Yazdanbakhsh, M., van den Biggelaar, A., and Maizels, R.M.: Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. *Trends Immunol.* 22, 372-377 (2001).
- Yemaneberhan, H., Bekele, Z., Venn, A., Lewis, S., Parry, E., and Britton, J.: Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. *Lancet* 350, 85-90 (1997).
- Zuany-Amorim, C., Creminon, C., Nevers, M.C., Nahori, M.A., Vargaftig, B.B., and Pretolani, M.: Modulation by IL-10 of antigen-induced IL-5 generation and CD4+ T-lymphocyte and eosinophil infiltration into the mouse peritoneal cavity. *J. Immunol.* 157, 377 (1996).
- Zuany-Amorim, C., Freschard, C., Trifilieff, A., Pay, G., Bowen, G., and Walker, C.: Long-term protective Effect of heat-killed mycobacterium vaccae on the allergic pulmonary inflammation in immunised mice. AAAAI Conference, March, Abstract, (2001).