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READDRESSING THE BALANCE: OLD FRIENDS, MICROFLORA AND IMMMUNOREGULATION

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SUMMARY

As a result of modern day living conditions, it has become critical to readdress the balance of stimuli of the immune system. To this end, a balance between effector T cells and regulatory T cell-mediated mechanisms is crucial. It is here proposed that renewed exposure to Old Friends and an appropriate intestinal microflora in the host is essential for immunoregulation. The efficient induction of an immunoregulatory network may prevent the development of diseases of immunodysregulation which are characterized by exuberant and inappropriate effector responses to harmless antigens. Vaccinations or oral supplements based on Old Friends may provide a novel therapeutic approach for the treatment of these diseases.

DISEASES OF IMMUNODYSREGULATION

Epidemiological data suggest that the incidence of diseases of immunodysregulation has increased dramatically in the last century in developed countries (*Wills-Karp*, 2001; *Bach*, 2002). These disorders are characterized by inappropriate immune responses leading to chronic inflammation. Example of immunodysregulatory diseases include allergic conditions such as allergic rhinitis and asthma, which results from inappropriate responses to harmless ubiquitous allergens; autoimmune diseases such as type I diabetes and multiple sclerosis which results from inappropriate responses to self antigens, and inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease, which are caused by inappropriate responses to the contents of the intestine. Interestingly, a rise in these conditions has yet to be reported in developing countries.

The escalation in some diseases of immunodysregulation in developed countries has been dramatic. A poignant example is asthma, which is now a common condition among children and young adults with prevalence rate reaching 17-30% in the UK, New Zealand and Australia. In contrast, prevalence remains relatively low (1-7%) in less developed countries such as in some Eastern European states, China and Indonesia (*ISAAC study*, 1998). Asthma is a multifaceted disease in which allergen exposure leads to both early and late phase airway inflammatory responses culminating in severe bronchoconstriction and airway remodelling. This disease has become of major public health importance, placing a heavy burden on both affected families and on society and adding further strain on the healthcare system.

The causes responsible for these observed increases in diseases of immunodysregulation remain unclear. It has been suggested that genetic factors alone cannot be blamed for the observed rise. It is unlikely that the number of people with a strong genetic predisposition to develop these conditions have increased over the last 50 years to the extent required to explain such rise in the incidence of these disorders. Hence, environmental factors may be playing a role (*von Mutius*, 2000; *Rook* and *Rosa Brunet*, 2002; *Guarner* et al., 2006). The change in living conditions which has occurred over the last century may favour the development of these diseases in a segment of the population which are predisposed to them (*Rook* and *Rosa Brunet*, 2002). Indeed, there have been a number of changes in our living environment which have resulted in a severe reduction in the stimuli needed to correctly educate the immune system. These include a reduced exposure to helminths and to saprophytic mycobacteria, once common encounters (*Wills-Karp*, 2001; *Yazdanbakhsh* et al., 2002; *Rook* and *Rosa Brunet*, 2005a). In addition, the inappropriate use of antibiotics and changes in diets has been shown to alter the intestinal microflora and significantly affect immune responses in the host (*Noverr* and *Huffnagle*, 2004; *Rook* et al., 2004).

THE HOST IMMUNE SYSTEM

The role of the immune system is to distinguish between self, to which no response is necessary, and foreign antigens, to which a response is required and an effective defence mechanism needs to be developed. Antigen presenting cells such as dendritic cells (DC), pick up antigens, process them into immunogenic peptides and present them to naïve CD4+ T cells. Depending on the immunostimulatory molecules on the DC and the cytokine milieu at the time of activation, the naïve CD4+ T cells develop into either T-helper 1 (Th1) or T-helper 2 (Th2) cells. The Th1 and Th2 phenotypes have long been considered mutually exclusive and regulating each other (*Coffman* and *Mossman*, 1991). It is the knowledge of this reciprocal regulation which has influenced the interpretation of the epidemiological data on diseases of immunodysregulation until very recently.

It was originally thought that a lack of Th1 stimulation was responsible for the increased incidence in asthma, a Th2 cell-mediated immunopathology. This hypothesis, named the Hygiene Hypothesis was based on the observation of an inverse relation between the development of hay fever and family size. Children in large families, exposed early and more frequently to childhood infections, were less likely to develop allergic diseases (*Strachan*, 1988). This hypothesis was further supported by a number of observations which suggested lower risk of developing allergies if one was exposed to conditions favouring Th1 stimulation as it may happen when children have older siblings or attend nursery schools and are therefore at increased risk of childhood infections, when they live with a dog, when they contract infections by the oral-faecal route etc. (*von Mutius*, 2000; *Wills-Karp*, 2001). Unfortunately, this hypothesis failed to explain the increase in concomitantly rising diseases of immunodysregulation. It was the lack of Th2 stimulation which was blamed for the increase prevalence in IBD (*Elliot* et al., 2005).

To add further confusion, it was a lack of both Th1 and Th2 stimulation which was thought to play a role in the development of autoimmune conditions (*Christen* and *Herrath*, 2005).

Hence, an altered balance between Th1 and Th2 cells fails to provide an adequate explanation for the rise in diseases of immunodysregulation in developed countries. The unifying hypothesis that explains this rise is not a reduced Th1 or Th2 stimulation but rather the lack of stimuli required for the maturation of regulatory T cells (Tregs).

REGULATORY T CELLS

Tregs comprise a number of distinct CD4+ T cell populations, which are all characterized by the preferential production of the immunoregulatory cytokines IL-10 and TGF-β. These cells effectively control both Th1 and Th2 effector populations and downregulate their responses. Two major categories of CD4+ Tregs have been described so far (*Hawroylowicz*, 2005; *Stock* et al., 2006). The first are naturally occurring, are characterized by the expression of CD25 on their cell surface and are derived from the thymus. They express high levels of the transcription factor Foxp3, which is essential for their development and function. The second are called adaptive Tregs, they are antigen-specific and are induced in the periphery under particular immunological conditions.

In the context of diseases of immunodysregulation rather than a Th1/Th2 balance, the crucial factor is the balance between effector and Treg cells. In the absence of optimal levels of immunoregulation, one may develop a specific immunodysregulatory disorder based on one's Th1/Th2 bias, one's genetic background and immunological history. The critical role Tregs have in preventing the development of immunodysregulation is given additional support by the observation that a genetic defect in the transcription factor Foxp3 induces a syndrome that includes symptoms of allergic diseases, autoimmunity and IBD. Indeed, Scurfy mice and patients with IPEX/XLAAD, who have a disruption in the Foxp3 gene, develop pathologies which encompass all three diseases of immunodysregulation (*Brunkow* et al., 2001; *Wildin* et al., 2001).

So what can be done to readdress the balance between effector and Treg cells. It has been hypothesized that because of the long evolutionary association with a number of relatively harmless organisms, these are recognized by the immune system as innocuous. Hence, rather than promoting effector immune responses which not only would be useless but potentially tissue damaging for the host, they preferentially induce immune responses towards regulatory modulation. Examples of these organisms include gastrointestinal helminths, the saprophytic mycobacterium, *Mycobacterium vaccae*, and lactobacilli such as *Lactobacillus casei* and *L. reuteri*. Reports suggest that exposure to such organisms may induce unusual maturation patters in DC facilitating their ability to induce Tregs or they may induce Tregs directly (*Maizels* and *Yazdanbakhsh*, 2003; *Rook* et al., 2004, *Guarner* et al., 2006).

THE HELMINTHS

Helminths were once common parasites of man leading to chronic infection of the host. The majority of human helminthiases, barring the cases of super-infections, are relatively benign with minimal deleterious consequences. Interestingly, infected host may even be protected from subsequent challenges by the same parasite species by a mechanism referred to as concomitant immunity.

Helminth infections are associated with Th2 responses and are characterized by high levels of IL-4, IgE isotype switching, eosinophilia, goblet cells hyperplasia and mastocytosis. Although allergic diseases and helminth infection are associated with similar immunological responses, they do not appear to overlap in patients. Indeed, a number of epidemiological studies have shown that when infected with helminths, the host has a reduce risk of developing clinical symptoms of allergic diseases such as airway hyperresponsiveness, wheeze and asthma (*Yazdanbakhsh* et al., 2002). Moreover, there is a strong inverse relationship between infection with *Schistosoma spp.* and *Ascaris lumbricoides* and skin reactivity to environmental allergens (*Yazdanbakhsh* et al., 2002).

There have been a number of hypothesis put forward to explain the protective effect of helminths. It was proposed that the high polyclonal IgE levels produced during helminth infections saturate IgE receptors, FcεRI, on mast cells and blocks the binding of specific IgE to environmental allergens. This prevents degranulation and immediate hypersensitivity reactions to allergens (*Lynch* et al., 1993). Alternatively, the production of IgG4 antibodies, a Th2 dependent isotype that is characteristic of helminth infection, can inhibit IgE mediated degranulation. The hypothesis for a role for the blocking antibody IgG4 has received particular attention as patient receiving allergen immunotherapy have been shown to switch to an IgG4 phenotype (*Akdis* et al., 2006).

A hallmark feature of helminthiasis is the hyporesponsiveness that develop during chronic infections (*Pearce* and *MacDonald*, 2002). For example, hosts chronically infected with *S. mansoni* have poor antigen specific T cell responses and reduced cytokine production. The production of IL-10, TGF-β and nitric oxide has been associated with this immunosuppression, which is not limited to parasite-specific antigens but includes unrelated antigens. Indeed, the immunological responses to tetanus toxoid after tetanus vaccination in schistosome-infected patients are reduced compared to healthy controls (*Sabin* et al., 1996). It is believed that during infections, helminths induce an immunoregulatory network characterized by the induction of Tregs and the modulation of dendritic cells favouring the maturation of Tregs (*van der Kleij* et al., 2002; *Maizels* and *Yazdanbakhsh*, 2003; *Hesse* et al., 2004; *McKee* et al., 2004).

Evidence is accumulating from experimental models and from epidemiological studies confirming an immunoregulatory circuit developed during infections with helminths (*Yazdanbakhsh* et al., 2002; *Maizels* and *Yazdanbakhsh*, 2003). The ability of these parasites to downregulate host immunity thereby preventing their elimination from the host and minimizing tissue pathology can be harness to prevent diseases of immunodysregulation. The hypothesis that helminth infections downregulate allergic reactions through the action of Tregs has found experimental confir-

mation recently. In a well executed study, *Wilson* and colleagues (2005) showed that infection with the murine helminth *Heligmosomoides polygyrus* significantly reduced the inflammatory cellular infiltrate in the lungs of allergen sensitized and challenged mice. Suppression was passively transferred from infected mice to uninfected sensitized mice by a cell population containing elevated numbers of CD4+ CD25+Foxp3+ T cells. These cells were characterized by high TGF-β expression and by IL-10 production. These data support the hypothesis that helminth infections elicit Tregs able to downregulate allergen induced lung pathology *in vivo*.

THE INTESTINAL MICROFLORA

The intestinal microflora may play a critical part in the regulation of immune responses in the host.

Alteration to the microflora has been associated with a number of immunological effects (*Noverr* and *Huffnagle*, 2005; *Guarner* et al., 2006). For example, germ-free mice have few intra-epithelial T and B cells, have smaller Peyer's patches, lower levels of IgA and most significantly are unable to develop oral tolerance. In spite of these effects, until recently the role of the microflora in diseases of immunodysregulation was neglected by investigators. There is now an increasing amount of epidemiologic and clinical data to suggest that alterations to the intestinal microflora predisposes to the development of these disorders. For example, the development of allergic diseases has been shown to correlate with the use of antibiotic early in life, altered faecal microflora and dietary changes (*Noverr* and *Huffnagle*, 2004, 2005).

Bacterial colonization of the intestine starts soon upon delivery and involves a succession of microbial populations which change with the infant's diet changes and development. By adulthood, the microflora which normally comprises for the most part anaerobes (97%) and only a small percentage of aerobic/facultative anaerobes (3%) is generally stable and is composed of both permanent residents and transient colonizers, introduced with food. Whereas approximately 30-40 species predominate, 400 to 1000 may be present in the intestine including among the anaerobes *Bacteroides, Bifidobacterium, Eubacterium, Fusobacterium, Clostridium* and *Lactobacillus*, among the facultative anaerobes *Escherichia coli*, *Salmonella* spp., *Enterococcus*, *Staphylococcus* and *Streptococcus* and even some fungi such as *Candida albicans*.

Epidemiological studies have reported alterations to the microflora of allergic individuals compared to healthy controls (*Kalliomaki* and *Isolauri*, 2003). Allergic states are associated with increased levels of aerobic and lower levels of anaerobic microbes. In particular, decreased levels of lactobacilli and bifidobacteria have been reported in children with allergies and atopic eczema (*Noverr* and *Huffnagle*, 2005). In a mouse model of ovalbumin-induced allergic pulmonary inflammation, alteration to the microflora though treatment with antibiotic exacerbated the development of allergic airway responses following allergen challenge (*Novarr* et al., 2005). Treatment with antibiotics and a single oral gavage of *Candida albicans*, altered the microflora with an increased fungal component persisting for 3 weeks. Treated mice were then chal-

lenged intranasally with ovalbumin. In the absence of systemic priming, mice with altered microflora developed vigorous airway allergic responses to ovalbumin. Pulmonary eosinophilia, goblet cells metaplasia, and levels of IgE, IL-5 and IL-13 were all significantly increased. In contrast, mice with unaltered microflora did not develop an allergic response following intranasal challenge. These studies suggest that changes to the intestinal microflora can break airway tolerance to an aeroallergen (*Noverr* et al., 2005).

It has been hypothesized that the microflora plays a role in promoting the development of Tregs that can downregulate Th2 responses developed in the airways. The gut mucosa has long been considered an ideal environment for the development and maintenance of Tregs (*Coombes* et al., 2005). Antigens acquired by DC in an anti-inflammatory environment such as in a microflora-balanced healthy intestine preferentially stimulate the generation of Tregs. Indeed, exposure to specific members of the intestinal microflora such as *L. casei* and *L. reuteri* has been shown to prime DC to promote the development of Tregs. The effect of these lactobacilli on the Th-cell polarizing capacity of DC was investigated *in vitro* by stimulating naïve T cells with the superantigen *S. aureus* enterotoxin B, in the presence of DC matured with these microbes. It was shown that *L. casei* and *L. reuteri* modulate DC function and prime for induction of IL-10- producing Tregs which are able to suppress T cell proliferation (*Smits* et al., 2005). Hence, a disruption to the intestinal microflora may lead to the development of inflammatory signals so that when inhaled/swallowed allergens are acquired by DC, these cells fail to induce Tregs because of altered balance in immune stimuli (*Noverr* and *Huffnagle*, 2004, 2005).

THE SAPROPHYTIC MYCOBACTERIA: *MYCOBACTERIUM VACCAE*

Saprophytic mycobacteria comprise at least 80 species, which are ubiquitous in mud and untreated waters. Exposure to mycobacteria has been a common occurrence throughout human evolution. Evidence for this shared evolutionary past is clearly indicated by the presence of a subset of T-cell which are CD1-restricted and appear to recognize only mycobacterial lipid and glycolipid (*Dutronc* and *Porcelli*, 2002). Exposure to mycobacteria has been significantly limited in developed countries where concrete and chlorinated waters have limited bacterial contact. Indeed, whereas the majority of individuals in developing countries respond positively to skin test to soluble antigens from environmental mycobacteria, only few individuals in developed countries do (*Rook* and *Rosa Brunet*, 2002).

Because of the long evolutionary association with saprophytic mycobacteria, it has been hypothesized that they are recognized by the innate immune system of the host as harmless. Therefore rather than mounting aggressive immune responses to organisms that are either non-pathogenic or only lead to self-limiting infections, the host responds to immunostimulatory clues from these organisms by developing immunoregulation. This hypothesis has been called the "The Old Friend hypothesis" and encompasses also helminthes and lactobacilli (*Rook* et al., 2003). Triggering mechanisms that induce immunoregulation through the exposure to Old Friends may be ex-

ploited therapeutically to prevent diseases of immunodysregulation. Evidences to support this hypothesis have been collected from a number of studies. Treatment with either live or dead mycobacteria inhibits allergen induced lung pathology in experimental models of allergic inflammation (*Walker* et al., 2003).

M. vaccae, a saprophytic mycobacteria isolated from mud of lake Kyoga in Uganda, has shown particular therapeutic promise. In experiments using heat killed *M. vaccae*, subcutaneous treatment was able to induce Tregs which suppressed allergen-mediated inflammatory responses. Treatment reduced the severity of pulmonary inflammation in mice sensitized and subsequently challenged with ovalbumin. Protection was long-lasting and associated with the development of CD4+ Tregs rather than with a Th1/Th2 phenotype switch (*Zuany Amorim* et al., 2002a,b; *Adams* et al., 2004). Transfer of *M. vaccae*-induced Tregs into allergen-sensitized mice prior to allergen challenge reduced subsequent pulmonary eosinophilia in an allergen-specific manner. This inhibition was shown to reside within the $CD45RB^{low}$ CD4+ T cell subset and was dependent on IL-10 and TGF-β. Treatment with neutralizing anti-IL-10 and anti-TGF-β antibody reversed the inhibitory effect of the $CD4+CD45RB^{low}$ population.

The effects of *M. vaccae* are not limited to the induction of Tregs. *M. vaccae* was shown to act also on pulmonary APC such as CD11c+ DC. These cells have received renewed attention as they have been shown to produce IL-10, stimulate development of Tregs, mediate airway tolerance and prevent the development of airway reactivity and asthma (*Akbari* et al., 2001). Treatment with *M. vaccae* correlated with the development of CD11c+ cells which express high levels of the immunoregulatory cytokines IL-10, TGF-β and IFN-α (*Adams* et al., 2004). It is hypothesized that because the development of Tregs is likely to be dependent on the maturation state of DC, influencing development of these cells is an additional strategy for immunoregulation.

Initial clinical studies investigating the therapeutic benefits of *M. vaccae* have been promising. A single intradermal injection of *M. vaccae* was administered to atopic individuals with asthma. Whereas treatment is usually well tolerated, a small scar may develop at the site of injections in some individuals. Patients were evaluated 3 weeks after treatment. Treatment with *M. vaccae* reduced the late phase response to inhaled allergens as well as serum levels of IgE and *in vitro* production of IL-5. However, none of these effects reached statistical significance (*Camporota* et al., 2003). A larger multicentre, phase II, randomized placebo-controlled study was conducted but was severely hampered by a large placebo effect. Treatment failed to improve mean weekly asthma symptoms as recorded in the patients' diary card (primary efficacy analysis), even though there was a trend towards significance. In spite of these initial disappointing findings, exploratory analysis did reveal a significant beneficial effect of treatment. When the data were adjusted for variation between treatment groups at baseline, patients receiving two doses of *M. vaccae* 6 weeks apart showed a significant reduction in asthma symptom scores and a significant decrease in asthma exacerbations. Additional studies to ascertain the therapeutic benefit of *M. vaccae* are required.

During evolution, the most likely route of exposure to *M. vaccae* was oral. A study to determine whether oral delivery of *M. vaccae* was effective in

reducing symptoms of pulmonary allergic inflammation revealed that the therapeutic effects were retained regardless of the delivery route. The intestinal epithelium is easily penetrated by mycobacteria whose size and hydrophobicity are ideal for uptake by M cells and Peyer's patches, leading to an interaction with the mucosal immune system. Once again the beneficial effects of *M. vaccae* are not associated with the development of Th1 responses but rather with the preferential induction of immunoregulatory cytokines such as IL-10 (*Hunt* et al., 2005). It is anticipated that exposure of the gut mucosa to *M. vaccae* may further facilitate the induction of Tregs as this site as long been considered an ideal environment for the development and maintenance of Tregs. These observations raise exciting new clinical possibilities. The ease of administration and the lack of scarring may be a preferred alternative to intradermal injection of *M. vaccae*.

CONCLUSIONS

It is here proposed that the reduced exposure to Old Friends is to blame for the significant increase in prevalence of diseases of immunodysregulation that has been observed in the last few decades. Old Friends are defined as those organisms that in virtue of their long standing evolutionary association with the human host, rather than inducing effector immune responses promote the development of immunoregulation. Among these, intestinal helminths, lactobacilli and *M. vaccae* feature prominently. Experimental evidence and clinical data is accumulating in support of the hypothesis that exposure to these organisms may facilitate the induction of Tregs by influencing the maturation of DC (*Maizel* and *Yazdanbakhsh*, 2003; *Rook* et al., 2004, *Guarner* et al., 2006).

It is critical that we learn to harness the immunoregulatory potential of these Old Friends (*Rook* et al., 2003; *Rook* and *Rosa Brunet*, 2005b). In order to readdress the balance of the immune system in terms of effector and Treg cell, the reintroduction of Old Friends in our modern living conditions is advisable. Whether as vaccinations to evoke specific mechanisms of immunoregulation or as oral supplements, this novel approach may offer therapeutic potential for the treatment of diseases of immunodysregulation.

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