Ramp-ing up allergies: Nramp1 (Slc11a1), macrophages and the hygiene hypothesis

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The 'hygiene hypothesis' suggests that a lack of infections favours the development of allergic disease. Mycobacteria and helminths are important in the hygiene hypothesis as infections caused by these agents generate regulatory mechanisms that can restore the immune balance. The hygiene hypothesis may be viewed as a complex multifactorial interaction between the environment and the genetic background of the individual, both of which contribute to the development of allergic disease. For instance, the Nramp1 gene (recently designated Slc11a1), which encodes bacterial resistance and determines the level of macrophage activation, affects treatment of allergic asthma with mycobacteria. Thus, Nramp1, and other innate immunity genes, might provide a link between the genetic background, the bacterial environment and the development of allergic diseases. It is likely that the macrophage has a crucial role in this link, connecting innate immunity with adaptive immunity in allergic disease.

An epidemic increase in allergic diseases has occurred during the past two decades. Studies in the 1970s suggested that 'allergic disorders may represent the continuing activity of an immune system made redundant by a clean, Western, lifestyle' [1]. In 1989, the possible relationship between infection and allergic disease re-emerged and was denominated as the 'hygiene hypothesis' [2]. It was demonstrated that a large family size or early placement in day care settings, which possibly results in a higher exposure to infectious agents, protects against the development of asthma. In addition, exposure to a farming environment early in life reduces the likelihood of developing atopic sensitization or asthma, probably due to exposure to higher levels of bacterial endotoxin [3]. A possible inverse relationship between exposure to specific common childhood infections and the development of allergic asthma has been addressed in several studies [4,5], but remains controversial. It is possible that the hygiene hypothesis might not be explained by a specific single infection. Rather, multiple and repetitive infectious factors during certain periods in the development of the immune system influence the development of allergy and asthma.

Recently, together with the revival of the concept of T regulatory (Treg) cells, a new consideration for the hygiene hypothesis has emerged. Allergic and autoimmune diseases are united by the fact that they both represent poorly regulated and exaggerated immune responses. Thus, a balance between regulated and deregulated immune responses, rather than the involvement of a T helper 1 (Th1)–Th2 balance, might be important [6]. Clearly, Treg cells control immune reactions to commensal and pathogenic bacteria. Therefore, exposure to microbes could have a huge impact on the development of Treg cells and hence on the development of allergic or autoimmune disease. This was elegantly illustrated in experiments using germfree mice in which it was impossible to elicit tolerance to an allergen [7]. Similarly, germfree rats were highly sensitive to induction of arthritis compared with specified-pathogen-free or conventional rats [8].

Infectious agents and the hygiene hypothesis

In general, three infectious candidates have received attention in the hygiene hypothesis (reviewed in Refs [9,10]): oro-faecal infections or gut commensals, helminths and mycobacteria. The candidature of mycobacteria was derived from experiments in animal models, in which bacillus Calmette–Guérin (BCG) or heat-killed *Mycobacterium vaccae* significantly reduced allergic and asthmatic manifestations [11,12]. By contrast, studies investigating the possible correlation between mycobacterial vaccination during early infancy or maturity and the subsequent development of atopy or asthma in humans are conflicting (reviewed in Ref. [13]). The frequency of administration of mycobacteria, the mycobacterial preparation used, the age at vaccination, and the varying natural exposure to environmental bacteria (including *Mycobacterium tuberculosis*) might have influenced these contradictory results. However, most likely, the genetic background of the individual contributes to the possible inverse association between infection and the development of allergy and asthma. The ability to mount a response to (myco)bacterial antigens is highly influenced

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by the genotype of the individual [14]. Host genetics are also an important factor in influencing the immune response in helminthic infections [15].

Nramp1 and the hygiene hypothesis

It is clear that mere changes in gene frequencies cannot explain the recent increase in the prevalence of allergic disease. A much more plausible alternative is that certain genetic factors and frequencies intermingle with changes in the environment (a 'gene-by-environment interaction'), which causes the increase in allergic disease in the fraction of the population that is susceptible to allergic disease. As the ability to mount a response to infectious agents is highly heritable, a genetic contribution to the inverse relationship between mycobacterial infection and the development of allergy and asthma is very plausible. In mice, resistance to intracellular bacteria, such as myco-bacteria, is under the control of the gene natural-resistance-associated macrophage protein 1 (*Nramp1*, recently designated *Slc11a1*) [16]. In humans, there is also an association or linkage of *NRAMP1* with suscepti-bility to infectious diseases [17].

The Nramp1 protein is a divalent cation transporter in the lysosomes and phagolysosomes of phagocytotic cells such as macrophages and neutrophils. The exact direction of the cation current is still a matter of debate, but it was demonstrated that the *Nramp1* gene is crucial in the metabolism and clearance of iron acquired by phagocytosis [18]. It is likely that intracellular bacteria and Nramp1 are competing for iron within the phagosome. The outcome of this competition might determine whether bacteria are able to proliferate. As a consequence, high levels of cyto-plasmic iron in $Nramp1^s$ (susceptible) macrophages could cause mRNA instability for a range of activation markers, contributing to the low capacity of $Nramp1^s$ macrophages to become activated when compared with $Nramp1^r$ (resistant) macrophages. This low level of macrophage activation is reflected in reduced secretion of several inflammatory mediators such as nitric oxide (NO), inter-feron (IFN)-γ, interleukin (IL)-6 and tumour necrosis factor (TNF)-α. By contrast, it was demonstrated that the *Nramp1* gene does not affect the function of other antigen-presenting cells (APCs), such as dendritic cells, in mice [19].

Interestingly in light of the hygiene hypothesis, the level of Th2-type responses is affected by polymorphisms in the *Nramp1* gene. The IgE and IL-4 response was lower in $Nramp1^r$ mice compared with $Nramp1^s$ mice after infection with an attenuated strain of *Salmonella typhi-murium* [20]. More relevant, the level of Th2 cytokines (IL-4, IL-5, IL-13), IgE and mast cell degranulation was much lower in $Nramp1^r$ mice compared with $Nramp1^s$ mice after allergen sensitization and aerosol challenge. However, the development of features of asthma (e.g. airway hyperreactivity) was not affected by *Nramp1* in this model [21]. In addition, the efficacy of a mycobacterial vaccine in lowering both allergic and asthmatic manifestations in the same mouse model for allergic asthma was much lower in $Nramp1^r$ mice compared with $Nramp1^s$ mice [22]. Perhaps polymorphisms in the human *NRAMP1* gene, and other infection-related genes, could contribute to the inverse correlation between bacterial infection and

allergic diseases. Polymorphisms in the genomic region of human *NRAMP1* are associated with the risk of atopy in BCG-vaccinated children [23]. Moreover, the *Nramp1* gene is involved in several other diseases of deregulated immunity: a polymorphism in the promoter region of *NRAMP1* has been associated with inflammatory bowel disease [24], rheumatoid arthritis [25], multiple sclerosis [26] and type 1 diabetes mellitus [27].

Analogous with *Nramp1*, a genetic variation in the gene encoding Toll-like receptor (TLR) 2 was recently shown to influence the protective effect of a farming environment. Farmers' children carrying a T-allele in the gene were less likely to develop atopic and asthmatic manifestations when compared either with farmers' children carrying an A-allele or nonfarmers' children. [28]. In the same study, a variation in the gene encoding TLR4 was inversely associated with allergen-specific IgE in the farmers' children only.

Together, these results point to a new concept in the hygiene hypothesis. *Nramp1*, *TLR2* and other genes involved in innate immunity provide a link between genes, the infectious environment and the development of disorders of deregulated immunity such as allergy and autoimmune disease (Box 1). The way infections affect predisposition to allergy and asthma is not universal, but can be modulated according to polymorphisms in genes involved in innate immunity.

The macrophage: a regulatory cell?

The role of molecules involved in both innate immunity and allergic diseases (such as *Nramp1*) points towards the macrophage as being an important immune regulatory cell. Macrophages are one of the first host cells encountered by both microorganisms and particulate antigens, such as allergens. Macrophages are the predominant immune cells within conducting airways and alveoli. This prominence strongly implicates macrophages in asthma, either as promoters or inhibitors of inflammatory responses in the lung. Macrophages are recruited to the airways of allergic subjects following allergen challenge and are important accessory and effector cells in inflammation in the airways. By contrast, alveolar macrophages might regulate and inhibit airway inflammation through multiple mechan-isms; although, unlike dendritic cells, they are poor APCs. This regulatory role of macrophages in immune responses, and antibody responses in particular, was first shown in experiments in Biozzi mice [29]. Macrophages of Biozzi high-antibody-responder mice displayed decreased consti-tutive and bactericidal activity and deceased antigen degradation as compared with macrophages of Biozzi low-antibody-responder mice. Subsequent studies showed that macrophages are suppressive in the lung, because depletion of alveolar macrophages increased pulmonary immune responses [30]. This suppressive effect was medi-ated directly, by the secretion of NO, IL-10 and/or prosta-glandin E_2 (PGE_2) (reviewed in Ref. [31]), or indirectly, by inhibition of pulmonary dendritic cell maturation by secretion of NO and TNF-α [32].

Recently, significant progress has been made in the definition of the heterogeneity of macrophages. In addition to classical activation by pro-inflammatory cytokines,

macrophages can be activated by type 2 cytokines such as IL-4 and IL-13 (alternatively activated macrophages) or ligation of Fcγ receptors (type II activated macrophages). These cells are not as well characterized as classically activated macrophages but they appear to be anti-inflammatory and suppress T-cell proliferation through IL-10 [33]. Unfortunately, it is not known whether *Nramp1* is expressed or whether it affects the function of these alternatively activated macrophages. Alternative activation of macrophages might be an initial attempt of the immune system to dampen the strong Th2

environment as seen in allergy and asthma. To act in this way, macrophages secrete several mediators, as discussed below.

NO
The secretion of NO is directly related to the degree of macrophage activation and is strongly influenced by the *Nramp1* gene. Murine *Nramp1*[r] macrophages showed a much higher level of NO in response to IFN-γ and infection with BCG compared with *Nramp1*[s] macrophages, which resulted in a higher bacteriostatic activity *in vitro* [34]. NO might also be of importance in the influence of macrophages on immune responses in the lung. The debate about the possible beneficial and/or detrimental effect of NO still continues [35]. Initially, on the basis of the observation that asthmatics show elevated levels of exhaled NO, it was believed that an increased production of NO was considered to contribute to allergic disease in the lung. By contrast, NO can directly inhibit the proliferation of both Th1 and Th2 cells [31]. Therefore, depending on the moment in time and the level of concentration, NO can be both anti- and pro-inflammatory. Human monocytes and macrophages produce NO under very restricted conditions and, therefore, the beneficial or detrimental effect of NO on allergic disease is still difficult to assess.

PGE_2
PGE_2 can be produced by macrophages in large amounts. Like NO, it has pleiotropic effects on the immune system. PGE_2 has stimulatory effects on the production of IL-4, IL-5 and IL-10 by Th2 cells [36]. By contrast, PGE_2 is generally viewed as an immunosuppressant mediator as it inhibits lymphocyte proliferation and production of IFN-γ and IL-2 by T cells [36], suppresses B-cell activity [37], and inhibits macrophage production of IL-1 and TNF-α [38]. Moreover, in asthma, PGE_2 is considered to be beneficial by inhibiting smooth muscle contraction and airway hyperresponsiveness [39]. However, as yet, nothing is known about the role of *Nramp1* in PGE_2 synthesis.

IL-10
The most interesting link between *Nramp1*, macrophages and the hygiene hypothesis might be IL-10. Interestingly, after stimulation, macrophages from susceptible *Nramp1*[s] mice have a lower TNF-α production, but a surprisingly higher IL-10 production, compared with macrophages from resistant *Nramp1*[r] mice [40]. In humans, a polymorphism in *Nramp1* that decreases macrophage activation (as measured by TNF-α production) was not only associated with tuberculosis, but also with a higher endotoxin-induced production of IL-10 [41]. Induction of IL-10 is probably used by *M. tuberculosis* to silence the immune system. In addition, IL-10 inhibits antigen-dependent activation of most of the cell types primarily involved in allergic reactions and prevents eosinophils from homing to the lung in experimental models of asthma. Moreover, some observations suggest that IL-10 production is defective in patients with asthma [42]. Interestingly, the level of production of IL-10 by macrophages and other APCs is important for the induction of Treg cells. A strong inhibitory effect of allergen-pulsed
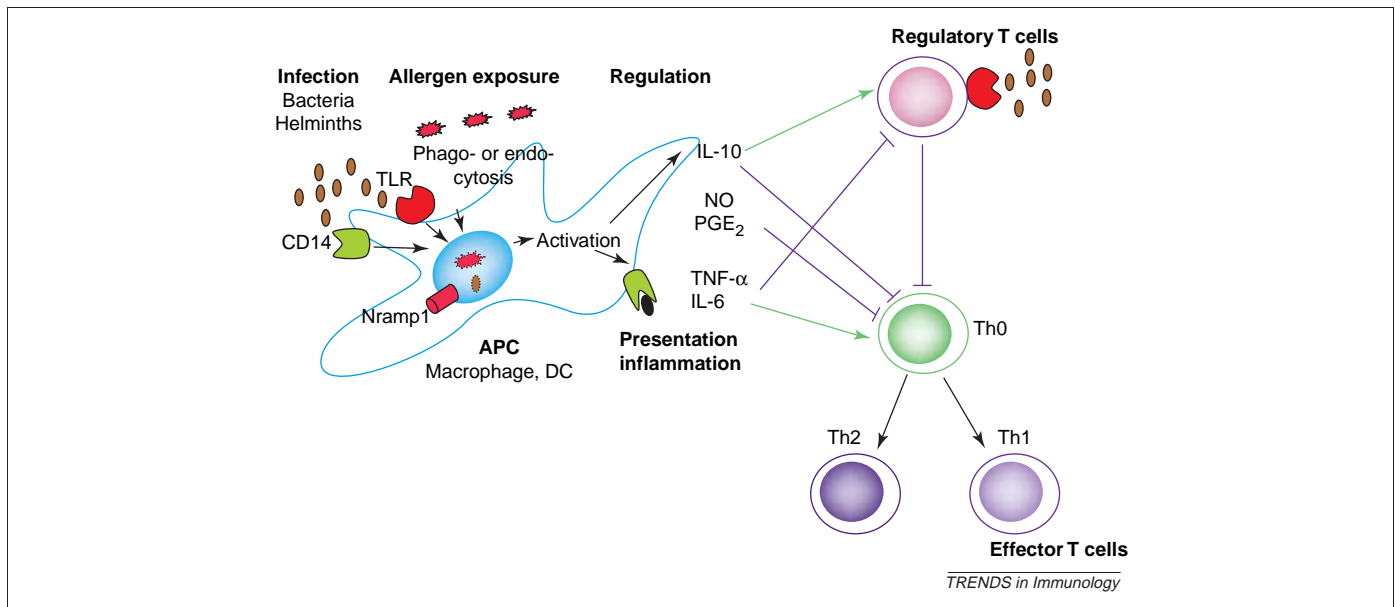
Figure 1. The APC and its central role in the activation and/or regulation of T-cell responses. Infectious agents and/or allergens can trigger APCs through receptors such as CD14 and TLRs or through phago- or endocytosis. In macrophages, the Nramp1 protein, present in phagosomes and lysosomes, mediates the outcome of this trigger. This outcome can be activation of the APC, secretion of IL-6 and TNF-α, and presentation of antigens, leading to an inflammatory response and the generation or stimulation of effector T cells. However, NO and PGE$_2$ can result in negative feedback, resulting in inhibition of the effector T-cell response. In addition, the APC can be initiated to produce IL-10, leading to inhibition of T-cell proliferation and to generation of Treg cells. Interestingly, TLRs are also expressed on Treg cells. This might provide a more direct pathway of inhibition of effector T-cell responses by infections [59]. Abbreviations: APC, antigen-presenting cell; IL-6, interleukin 6; NO, nitric oxide; Nramp1, natural-resistance-associated macrophage protein 1; PGE$_2$, prostaglandin E$_2$; TLR, Toll-like receptor; TNF-α, tumour necrosis factor α; Treg, T regulatory cell.

macrophages in a murine asthma model was dependent on the production of IL-10 by these cells, which possibly induced Treg cells [43]. Likewise, the reduction of allergen-specific Th2 responses in successful conventional allergen immunotherapy in humans was initiated and maintained by IL-10 produced by allergen-specific T cells and monocytes.

Importantly, a recent study showed that treatment with mycobacteria induced an increase in CD11c$^+$ cells in the lungs after allergen sensitization and challenge. This population of cells, which in the lung consist of DCs and macrophages, secreted increased levels of IL-10 and transforming growth factor (TGF)-β compared with the same cells from nontreated allergic mice [44]. During infection and/or allergen encounter, the outcome for both effector and suppressor T-cell activation probably depends on the ratio between regulatory mediators, such as IL-10 and PGE$_2$, and pro-inflammatory cytokines, such as IL-6 and TNF-α, secreted by APCs [45] (Figure 1).

Conclusions

'*Nature strives for the opposite and from it generates consonance*' (*Herakleitos*).

The hygiene hypothesis can probably best be explained by a mechanism involving regulation of the immune response. Deprival of infections might lead to a deregulation of the immune system and a subsequent shift to allergic and autoimmune responses. Microbial stimuli, the candidature of which has not clearly been defined so far, might act through the innate immune system and subsequently through key players in the process of activation and regulation of adaptive immune responses, including dendritic cells and Treg cells. However, in our

opinion, macrophages, which form the first line of defence in many tissues and are initiators of many immune responses, might also be important regulatory cells. The role of the macrophage in the regulation of allergic and autoimmune diseases is illustrated by the role of the *Nramp1* gene in these disorders. *Nramp1* and other genes involved in innate immunity provide a link between genetic background, the bacterial environment and the development of allergic diseases. Evolutionary pressure, which differs significantly between continents and ethnic groups, has caused differences in the frequencies of genes involved in the innate response to infectious agents. In our opinion, this different genetic makeup might underlie the worldwide difference in the prevalence of allergic disease. Certain gene polymorphisms that have been valuable in the past might not have been able to follow the drastic change in the environment observed in recent decades.

The current controversy about the efficacy of mycobacteria and their components in the treatment of allergic asthma in humans might be explained by the strong influence of genetic factors. To date, the use of microbial products in the prevention or therapy of allergic disease has not lead to the discovery of the expected 'asthma-vaccine' [46]. Future research and clinical trials should take the gene-by-environment interactions into account, even though this will make the interaction between allergic disease even more complex and extensive. In addition, as they play a crucial role in the hygiene hypothesis, it may be tempting to speculate that macrophages in the lung are a possible therapeutic target in the treatment of allergic asthma. Macrophages might need (artificial) infectious stimuli to exert their potent regulatory role in immune disorders.

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