The success of anti-TNF agents, as well as the more recent outburst of highly effective biologics targeting the IL-23/IL-17 axis, should not overshadow the fact that there is still a sizable number of nonresponder patients who might benefit by an alternative strategy.

The mouse model described by Gunderson *et al.* (2013) uncovers an important aspect of CD8 T-cell pathogenicity in skin inflammation mediated by IFN-γ, while posing new critical questions. Answering them will enhance our understanding of the pathological mechanisms underlying psoriasis and could ultimately lead to novel effective therapeutic strategies.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Toll-Like Receptors Link Atopic March to the Hygiene Hypothesis

Lajos Kemény[1,2] and Kornélia Szabó[1]

In recent decades, the prevalence of atopic diseases has increased substantially worldwide, but their molecular pathologies are now being elucidated. The report by Haapakoski *et al.* in this issue suggests that the manner in which the immune system encounters an allergen is key to the subsequent polarization of its responses, and the presence of microbial ligands appears to be important in this process. Data in this report provide further proof of the hygiene hypothesis that combine it with known features of the atopic march.

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The hygiene hypothesis was first introduced in 1989 by David P Strachan, who proposed, based on epidemiological studies, that changes in personal hygiene, improvements in household amenities, and declining family sizes had been

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Clinical Implications

- Cutaneous sensitization is an important route for initiation and development of the atopic march in genetically predisposed individuals.
- Microbe-derived molecules and the Toll-like receptor (TLR)-dependent activation of the innate immunity contribute to the shaping of adaptive immune responses. When this occurs in parallel with allergenic sensitization through the cutaneous route, they can modify the outcome by inducing Th1 responses and subsequently inhibiting Th2 responses.
- Immune-based therapeutic strategies involving the use of antigens together with TLR ligands and a cutaneous route of immunization may lead to the prevention of atopic sensitization.

accompanied by enormous increases in the prevalence of atopic diseases, including hay fever, eczema, and asthma, especially after the industrial revolution. He claimed that infections early in childhood could be beneficial and may lead to protection against these diseases later in life. The implication was that the rising living and personal standards and the currently popular small family sizes would in some way act against correct development of immunity (Strachan, 1989). Since then, many epidemiological studies have established a clear relationship between the marked changes that parallel adoption of a ''western'' lifestyle, with an increasing incidence of atopic diseases. Even within a given country, where individual genetic, environmental, and climatic conditions are similar, clear differences have been reported in the proportions of children that develop these diseases in large cities as compared with the countryside (Ege *et al.*, 2011). Various factors associated with industrialized and urban living have been studied extensively and, in addition to effects of environmental microbes, personal microbiomes too have recently been suggested to have an important role (Heederik and von Mutius, 2012).

Since their first publication, Strachan's ideas have created considerable controversy in the scientific community. When his hygiene hypothesis was introduced, there was no clear-cut explanation for the observations. Later, the Th1/Th2 model of immune regulation led to mechanistic explanations for how the hygiene hypothesis might operate. It was suggested that contact with an environmental allergen leads to activation of the innate immunity, with uptake and activation of antigen-presenting cells resulting in activation of CD4 + T cells. In atopic individuals, this was polarized toward Th2 cells that secrete IL-4, IL-5, and IL-13, subsequently stimulating IgE antibody production by B cells and increased numbers of mastocytes and eosinophils. This would contrast with events in ''healthy'' individuals in whom T cells exhibit a Th1-like profile in response to the same challenge. Taken together, this raised the possibility of differences in the childhood programming of immunological memory, although how this was achieved remained unknown (reviewed by Holt *et al.*, 1999; Yazdanbakhsh *et al.*, 2002). A combination of the hygiene hypothesis with contemporary results, however, led to the assumption that early training of the immune system might occur through contact with pathogenic microbes, or alternatively through effects of commensal microflora (or both), thus strengthening the Th1 arm of the defense. In the western lifestyle, such encounters would be less common, and in urbanized environments a shift toward Th2 responses would lead to the development of atopy (Frei *et al.*, 2012; Heederik and von Mutius, 2012; Hanski *et al.*, 2012). Nonetheless, little was known about how this might happen at molecular levels.

The atopic march

Early models to explain the pathogenesis of atopic diseases after the introduction of the hygiene hypothesis concentrated on the role of the adaptive immunity, and within the Th1/Th2 immune paradigm. Molecular and genetic studies, however, raised the possibility that the primary defect might actually lie in the skin. This was based on the observation that atopic dermatitis (atopic eczema), a chronic pruritic skin condition commonly occurring early in life, might be considered a major risk factor for the subsequent development of more severe atopic diseases, such as allergic rhinitis and asthma. This concept of a continuous development of atopic diseases from atopic dermatitis through allergic rhinitis, to allergic asthma, named the atopic march, raised the possibility of a well-defined pathogenic route beginning in the skin, all the way to airways (Spergel, 2005; Zheng *et al.*, 2011).

Barrier defects in the development of atopic disorders

Barrier defects are key early factors in the development of atopic dermatitis and the subsequent atopic march, and one of the early steps in this process is an enhancement of allergic sensitization, which occurs through the frequent epidermal injuries that characterize atopic skin. This concept was corroborated in experimental animal studies, which demonstrated that transcutaneous immunization with an allergen followed by airway challenge is capable of inducing airway hyper-responsiveness and enhanced mucus production, two features of allergic asthma (Lehto *et al.*, 2005).

The importance of a healthy cutaneous barrier was also shown in genetic studies that demonstrated variations of the filaggrin gene, which were not only important in the formation and maintenance of an intact barrier, but would also have important roles in the predisposition to atopic diseases (Heimall and Spergel, 2012; Kubo *et al.*, 2012).

Toll-like receptors and the hygiene hypothesis

In the second half of the 1990s, discovery of the prototype of pathogen recognition receptors, the mammalian Toll-like receptors (TLRs), followed by acquisition of an enormous amount of information, led to new models to

explain details of hygiene hypothesis at a molecular level. It was suggested that early immune "training" is achieved through repeated activation of certain TLRs by binding and recognition of conserved pathogenic or microbial molecules (pathogenic molecular patterns or microbial molecular patterns). The resulting initiation of innate immune responses was found to have important roles in the shaping of adaptive immune events (Iwasaki and Medzhitov, 2010).

Recognition that childhood microbial challenge was important in the development of balanced immunity suggested that pathogen recognition receptors and the signaling events they initiated were important. TLRs have been shown to be expressed by keratinocytes (Pivarcsi *et al.*, 2003) and it therefore seemed possible that they would participate in the regulation of early innate immune events and, in parallel, in the initiation and development of the atopic march. However, clear-cut experimental evidence proving roles or even the existence of such mechanisms was lacking.

Independent confirmation of this concept was provided by genetic studies, which indicated that, in addition to the role of filaggrin mutations, various polymorphisms in genes having important roles in microbial recognition and downstream signaling also contributed to genetic susceptibilities to atopic diseases. In this context, the roles of pathogen recognition receptor polymorphisms have been investigated. Genetic case–control analyses have pointed to associations among polymorphisms of TLR2, TLR4, TLR6, TLR10, and CD14 in some studies, but other studies did not confirm these findings (Yang *et al.*, 2006). The reasons for this are not yet known, but population differences and variabilities in experimental conditions might well be responsible. Alternatively, gene–environment interactions may also vary greatly in different populations, thereby modifying the results. Nevertheless, it may be concluded that genetically determined differences in the regulation of innate immune responses to microbial ligands do have roles in the development of atopic diseases.

Experimental demonstration of the role of microbial recognition in the pathogenesis of allergic asthma

In a paper in the current issue of JID, Haapakoski *et al.* (2013) modeled the development of allergic asthma using a natural route of cutaneous sensitization. They applied ovalbumin as an experimental antigen on tape-stripped skin of mice, alone or together with various TLR2, TLR3, and TLR4 ligands (Pam_3Cys, Poly(I:C), and lipopolysaccharide, respectively), and assayed the modifying effect of TLR activation on selected molecular, cellular, biochemical, and clinical parameters of the disease. Exposure to lipopolysaccharide, and to a certain extent also to Pam_3Cys, was observed to cause a significant reduction in the production of Th2 cytokines such as IL-4, IL-5, and IL-13 in the lung, and IL-13 in draining lymph nodes. At the same time, the extent of inflammatory infiltrate, and specifically the number of eosinophils in the bronchoalveolar lavage fluid, was decreased, and the number of periodic acid–Schiff-positive cells was similarly reduced by these microbial ligands. Interestingly, in contrast with currently accepted models, unchanged or reduced FoxP3 mRNA levels in the lungs of mice treated with ovalbumin together with Pam3Cys and lipopolysaccharide, respectively, suggested that these effects were not the consequence of induced regulatory T cells. The protective effects were not due to TLR tolerance either, but were completely dependent on the induction of IFN-γ in the lung.

Can signaling differences downstream of various TLR receptors account for the effects of diverse ligands?

The evidence had suggested that not all TLR ligands would function equally in the model described above. Whether this is limited to mice, whether dose-dependent differences exist when various TLR ligands are applied, and whether the answers lie somewhere else remain important questions. However, the evidence suggests that signaling events downstream of the TLR2 and TLR4 ligands operate similarly and these ligands exhibit comparable, protective effects in the given experimental setting. This contrasts with results obtained using the TLR3 ligand Pam_3Cys, which is mostly ineffective at low doses, but which in large quantities even exerts a Th2-augmenting effect. Interestingly, TLR3 is the receptor that induces alternative downstream signaling pathways that function through the TIR-domain-containing adapter-inducing interferon-β protein instead of the canonical MyD88-dependent mechanisms utilized by TLR2 and TLR4 (Takeda and Akira, 2004; Akira *et al.*, 2006).

A proposed mechanism for TLR2, TLR4-mediated allergic asthma protection

These results and the current models now suggest that an important aspect in the early "training" of immune responses is the context in which antigens are introduced. If introduced together with pathogenic molecular patterns or microbial molecular patterns that have the ability to activate specific pathogen recognition receptors, will these antigens be treated as molecules of microbial origin? Thus, non-atopic individuals react differently to a novel allergen. The results of Haapakoski *et al.* (2013) combined with the hygiene hypothesis further suggest that, if environmental and/or commensal symbiotic microbial flora that colonize the various body parts are reduced during the early development of immunity, some antigens will be introduced without costimulatory activities of the microbial TLR ligands. As a result, repeated encounters with the same antigen will gradually lead to an excessive Th2 response, with the development of more severe forms of atopic disease, i.e., the atopic march (Figure 1; Holt *et al.*, 1999). This would explain, in part, the important question of how and why different response patterns are programmed in the immunological memories of different individuals.

If the above theory holds true, why do some individuals who live a natural lifestyle, surrounded by animals, still develop atopic diseases, and why does not everyone who lives in an urbanized environment suffer from these diseases? Moreover, numerous children who contract atopic dermatitis in their early life do not go on to develop allergic rhinitis or asthma. These answers may also lie in each individual's genetic constitution,

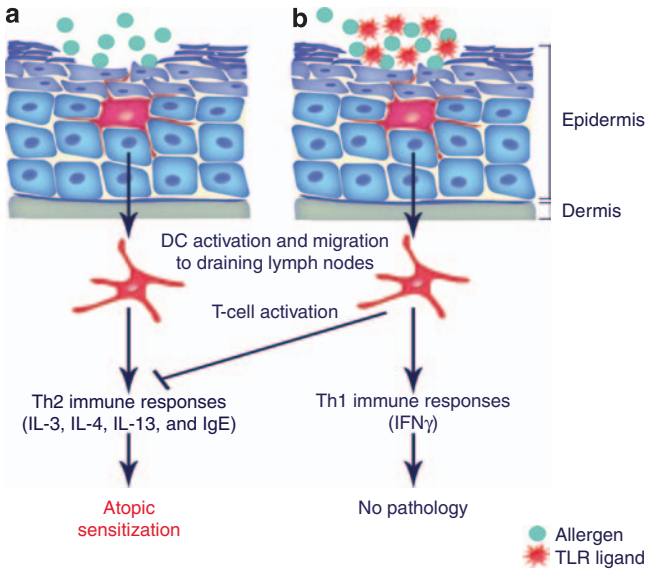

Figure 1. Proposed mechanism of immune activation of atopic and non-atopic individuals. In early life, during training of the immune system, presentation of certain antigens, together with different pathogenic molecular patterns (PAMPs) or microbial molecular patterns (MAMPs) capable of the activation of various pathogen recognition receptors (PRRs), will lead to the polarization of an immune response toward Th1 responses, with subsequent inhibition of Th2 responses (b). As a result of the western lifestyle, the level and complexity of the environmental and personal microflora are thought to be reduced, leading to a shift toward Th2 responses and facilitating development of atopic diseases (**a**). DC, dendritic cell; TLR, Toll-like receptor.

reflecting the true multifactorial nature of these diseases.

Contradictory data suggesting the Th2-promoting role of TLR ligands during allergenic sensitization

The scientific community does not entirely welcome the ideas concerning the protective role of microbial ligands in the pathogenesis of atopic diseases. Opponents also base their ideas on experimental data acquired from similar mouse models. In fact, other evidence favors the conclusion that microbial ligands may enhance the development of asthma (reviewed by Schroder and Maurer, 2007). Direct comparisons of results is difficult; however, differences in experimental protocols involving routes and doses of TLR ligands and the genetic backgrounds of the mouse strains used may be responsible. The data appear to suggest that the dose and the exact circumstances under which the body meets the allergens really are crucial. To date, TLR9 seems to be the only ligand that repeatedly promotes Th1 differentiation, somewhat independently of the sensitization method used. In contrast, when applied together with ovalbumin through the airways or injected intraperitoneally, lipopolysaccharide, a relatively strong Th1-promoting agent, tends to preferentially induce Th2 skewing of the immune events (Schroder and Maurer, 2007).

Significance and clinical consequences of the novel observations

The novel observations made by Haapakoski *et al.* (2013) will facilitate our understanding of the molecular mechanisms involved in the early steps of allergic sensitization. Their work provides experimental confirmation of the importance of cutaneous sensitization in the development of allergic asthma in atopic patients, and it suggests why and how similar processes may lead to protection in individuals who are not predisposed to the development of atopic disorders. Haapakoski *et al.* (2013) additionally provide clear-cut experimental evidence about how microbe-derived molecules and the activation of the innate immune system contribute to the shaping of adaptive immune responses.

Naturally, certain limitations must also be borne in mind. Differences in the immune responses between mice and humans demand care when extrapolations are made. Again, although ovalbumin is the most widely studied allergen, it is not the strongest Th2 inducer and lacks the intrinsic enzymatic (e.g., protease) activities possessed by most natural allergens (Schroder and Maurer, 2007). What emerges from these and similar studies, in combination with the large amount of epidemiological and clinical data currently available? First, it is important that parents and healthcare professionals (especially pediatricians, dermatologists, pulmonologists, and immunologists) should be fully aware of the importance of the early programming of the immune system and the crucial role of microbial ligands from the environment. A little "dirt" may well be beneficial for balanced development of the body's immunological defense, and children should come into contact with a sufficient number and various types of microbial molecules during early life to acquire a mature, balanced level of protection. Ideas have been put forward about how genetically susceptible individuals (e.g., carriers of certain filaggrin alleles, or children with a family history of severe atopic disorders) may be "trained" to react more appropriately to various environmental allergens. Obviously, there is still a long way to go and more experimental evidence is required for the development of such immunomodulatory interventions. The recent results, however, are an important advance toward therapeutic modalities that may be preventive, rather than strictly therapeutic.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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BRAF and MC1R in Melanoma: Different in Head and Neck Tumors?

Emma C. Fink[1] and David E. Fisher[1]

In this issue, Hacker *et al.* (2013) report the largest study to date on the association between MC1R variants and BRAF mutant melanoma. Although they did not observe a significant overall correlation, there was a significant negative association between BRAF and MC1R mutations for head/neck melanomas. This suggests a fundamental difference in pathogenesis between head/neck and truncal melanomas, which could contribute to their divergent prognoses.

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Cutaneous melanoma arises from complex interactions among genetic and environmental factors. Epidemiological and molecular evidence indicates that these interactions may influence not only the incidence of melanomas but also their fundamental pathogenic mechanisms. A high fraction of cutaneous melanomas have acquired somatic mutations in BRAF or NRAS, but these mutations are generally mutually exclusive. Mutations in both BRAF and NRAS lead to extracellular signal–regulated kinase activation, enhancing proliferation, survival, and invasion (Thomas, 2006). However, the presence of significant gene expression and signaling-related differences suggests that BRAF and NRAS mutations utilize at least partially distinct mechanisms of melanogenesis (Bloethner *et al.*, 2005).

BRAF is a serine/threonine kinase involved in the Ras–RAF–mitogen-activated protein kinase pathway. Mutations in BRAF are found in approximately 50% of cutaneous melanomas, most often those that arise on intermittently sun-exposed skin in relatively young patients (Thomas, 2006; Tsao *et al.*, 2012). Although over 30 different BRAF mutations have been reported, one mutation, BRAF V600E, is by far the most common. Although UV exposure is a major risk factor for melanoma, BRAF V600E is due to an T→A transversion, rather than the more commonly UV-associated C→T transition (Brash, 1997; Tsao *et al.*, 2012). Some have suggested that selection of rare UV-induced C→T transitions could explain this observation; others posit that the reactive oxygen species (ROS) generated as a by-product of melanin synthesis are a more likely culprit (Thomas, 2006).

Melanoma risk is strongly tied to pigmentation, a key determinant of which is MC1R. MC1R is an α_s-type G protein–coupled receptor, which is found on melanocytes and which responds to α-melanocyte-stimulating hormone. MC1R is highly polymorphic in Caucasians with over 60 variants, classified as partial loss of function (r alleles) or complete loss of function (R alleles) (Garcia-Borron *et al.*, 2005; Tsao *et al.*, 2012). Polymorphisms in MC1R contribute to the phenotypic spectrum of freckling and hair and skin coloration (particularly red hair), but have also been suggested to modulate melanoma risk independent of pigmentation. Cells expressing MC1R variants have higher levels of ROS, which could potentially contribute to ROS-related BRAF mutations (Thomas, 2006; Tsao *et al.*, 2012). Our group recently demonstrated a UV-independent increase in oxidative lipid and DNA damage in a ''redhead'' mouse model with inactive MC1R. Expression of BRAF V600E in this model led to an increased risk of invasive melanoma in the absence of providing secondary mutations or UV exposure. Both oxidative damage and melanoma development were abrogated when pigment production was blocked by an albino allele, suggesting that pheomelanin production is key to these phenotypes (Mitra *et al.*, 2012).

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