

Medical progress

New insights into the effects of *Mycobacterium bovis* Bacillus Calmette-Guérin on asthma

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Allergic asthma has been defined as a disease of immunodysregulation, where the pathology is a direct consequence of excessive T-helper type 2 immune responses (Th2) to allergens in the lungs. Disease is associated with increased secretion of type-2 cytokines such as interleukin (IL)-4, IL-5 and IL-13 which, among other things, promotes enhanced IgE levels, induces pulmonary eosinophilic inflammation and increased mucus production in the airways.¹ The prevalence of allergic asthma and other atopic disorders has increased dramatically to almost epidemic proportions in the past few decades, especially in industrialized countries.² An attractive explanation for this increase is offered by the “hygiene hypothesis”, which states that the relative lack of infections early in life could promote the development of allergic diseases in genetically predisposed individuals.³ *Mycobacterium bovis* (*M. bovis*) Bacillus Calmette-Guérin (BCG) is considered as a strong inducer of T-helper type 1 immune responses (Th1)⁴⁻¹¹ and modulates the development of asthma both in animal models and human beings, as we previously described in a review.¹²

Currently, epidemiologic observations show that the increased prevalence of allergic diseases has been accompanied by an increased prevalence of autoimmune Th1-driven diseases,^{13,14} so the initial “hygiene hypothesis” has been challenged. With the discovery of regulatory T cells (Treg) and Th17, this bivalent concept of Th1/Th2 paradigm needs to be modified. This review will focus on new insights into the effects of BCG on asthma in view of new T-cell subsets.

THE REVISED VERSION OF THE “HYGIENE HYPOTHESIS”

Although the Th1-Th2 paradigm is an attractive theory, some limitations exist in the primary “hygiene hypothesis” and it is unlikely to explain the whole story. Currently, epidemiologic observations show, an increase in the prevalence of autoimmune Th1-driven diseases such as type 1 diabetes mellitus, multiple sclerosis, and Crohn's disease has been accompanied by an increased prevalence of allergic diseases,¹³⁻¹⁵ suggesting the mechanism may be the deficiency of immune tolerance.¹ In reality, however, Th1 cells may exacerbate asthma and allergy, as human asthma is associated with the

production of IFN- γ that appears to contribute to disease pathogenesis.¹⁶ Hansen et al¹⁷ using an adoptive transfer system found allergen-specific Th1 cells did not attenuate Th2 cell-induced airway hyperreactivity and inflammation in naïve recipients, instead, they caused severe airway inflammation. Moreover, Th2-mediated allergic lung inflammation coexists with the Th1-mediated responses that are stimulated by diverse forms of antigens (Ag),¹⁸ high dosage of BCG vaccination¹⁹ or ovalbumin (OVA) and double-stranded RNA (dsRNA).²⁰ The direct evidence for boosting Th1/Th2 responses *in vitro* or *in vivo* in some conditions was provided by Pate and colleagues,²¹ activation of Glucocorticoid-induced tumor necrosis factor receptor (TNFR) family-related protein (GITR) signaling by using anti-GITR antibody markedly enhanced the induction of both Th1 and Th2 cytokine production by naive CD4⁺CD25⁻ T cells. Moreover, administration of anti-GITR mAb significantly exacerbated the severity and onset of murine joint inflammation and murine allergic airway inflammation which was associated with the synchronous increase of Th1/Th2 cytokine production. Finally, the role of the Th1 cytokine IFN- γ is itself controversial. Although cellular responses mediated by Th2-type cytokines are generally attenuated by a Th1-type cytokine, several studies have suggested that IFN- γ is not always protective but rather pathogenic for the development of asthma in some situations,^{17,22} and injurious to tissue, particularly in bronchial epithelial cell shedding in asthma.²³ Thus, it might be difficult to understand asthma based on the simple counterbalance paradigm of Th1 versus Th2.

Treg cells are likely to play a central role in determining the incidence and severity of several immune pathologies, including autoimmune, infectious, allergic, and asthmatic diseases.^{24,25} A number of recent studies indicate that Treg cells play an important role in controlling such T-helper type 2 biased responses both in animal models and studies in humans.²⁶⁻³¹ There is interplay between

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dendritic cells (DCs), Th2 cells and Tregs in asthma. DCs have functions not only in the induction of allergic Th2 responses but also during the effector response as it occurs in clinical asthma. Immature dendritic cells (iDCs) are thought to play a crucial role in the maintenance of self-tolerance by presenting self-antigen and inducing unresponsiveness of T cells, as well as by their ability to induce or expand Treg cells. Moreover, naive CD4⁺CD25⁻ T cells can be induced to become regulatory in the periphery in the presence of iDCs.³² Although several environmental factors are involved in tolerogenicity of DCs, there is also accumulating evidence that Treg cells have important roles for conferring the tolerogenic functions of DCs.³³ Indeed, intravital microscopy studies have suggested that Treg contact DCs more frequently than potential T effector cells.³⁴ Given that Treg cell suppression of allergic immune responses is mediated at the level of the DCs, it will be important to find out if DCs from atopic individuals have a reduced capacity to interact with or stimulate Treg function or if they are resistant to Treg regulation. Alternatively, decreased Treg activity in atopic individuals might contribute to chronic inflammation.

Both naturally occurring CD4⁺CD25⁺ regulatory T cells and inducible forms of antigen-specific regulatory T cells, express the transcription factor Foxp3 and have been shown to inhibit inappropriate immune responses involved in allergic disease. However, none of the earlier studies^{30,31} used wild-type, immunocompetent animals nor did they use a direct experimental approach. Two important studies clarified the role of CD4⁺CD25⁺ Treg cells in the regulation of allergic responses in animal models and show several similarities to observations made in humans. By using depleting CD4⁺CD25⁺ Treg cells or transfer of OVA peptide-specific CD4⁺CD25⁺ T cells to OVA-sensitized mice, Lewkowich³⁵ and Kearley³⁶ further confirmed the exact role of Treg in reducing airway hyperreactivity (AHR), recruitment of eosinophils, and Th2 cytokine expression *in vivo*.

Collectively, the current version of the hygiene hypothesis states that improved hygienic standards and a decrease in both infections and the resulting active immune response may also deprive people of the opportunity to expand Treg cell antigen receptor repertoires through infectious tolerance and thereby lead to higher rates of allergy and autoimmune disease. This suggests that the simultaneous rise in immunoregulatory disorders in developed countries is attributable to a failure of Treg function and not to a Th1/Th2 imbalance.³⁷

THE NEW T-CELL SUBSETS INVOLVED IN THE EFFECT OF BCG ON ASTHMA

BCG vaccine includes a live strain of *M. bovis* that was attenuated by Calmette and Gue'rin for use in vaccination against *Mycobacterium tuberculosis*. (*M. tuberculosis*)³⁸ Thus, BCG is an antigenically complex mixture expected

to interact with different components of the innate and adaptive immune systems. Although the exact roles of BCG vaccination in human beings are controversial,^{12,39} most studies in animal models show that BCG vaccination/infection inhibits allergy-induced pathologic changes.^{4-7,11} Based on the "hygiene hypothesis", BCG vaccination/infection stimulates the production of Th1 cytokines and decreases Th2 cytokine secretion, which is the main mechanism of BCG to inhibit allergy-induced pathologic changes in previous studies whether in animal models or in humans.⁴⁻¹¹ The subsequent studies⁴⁰⁻⁴⁷ were focused on the effects of BCG vaccination/infection with different schemes (including timing of vaccination, route of delivery, BCG-strains, BCG-dosage, genetic contribution and ethnicity and varying degrees of exposure to environmental mycobacteria) on the prevention and treatment of asthma. In our own studies,^{46,47} neonatal BCG vaccination can inhibit the development of airway inflammation and mucus production not only in early-challenged mice, but also has a long-term effect in late-challenged mice in an IFN- γ independent manner. Although some studies observed the paradox that couldn't completely explain by "hygiene hypothesis",^{48,49} most of them just used the balance of Th1/Th2 type responses-measuring the cytokines in both the bronchoalveolar lavage fluid (BALF) and the splenocyte supernatants as the intrinsic mechanisms to explain the whole phenomena of BCG inhibiting the pathologic changes to asthma in animal models. Theoretically, the "hygiene hypothesis" was perfect and attractive; however, it is being challenged. Herein, we discuss the role of new T cell subsets involve the effect of BCG on asthma.

Th17, asthma and BCG

Th17 cells are a new subset of helper T cell characterized by the production of IL-17A, IL-17F, IL-6, IL-21 and IL-22.⁵⁰⁻⁵² They play an important role in host defense and inflammatory disorders; particularly in autoimmune disorders.^{53,54} The proinflammatory cytokine IL-17 has previously been forwarded as a link between activated T-lymphocytes and the recruitment and activation of neutrophils in various types of airway inflammation.⁵⁵ It has been shown that the concentration of IL-17 is increased in BALF, sputum, and blood from patients with asthma.⁵⁶⁻⁵⁸ Recently, it has been suggested that at least two inflammatory subtypes of asthma exist: the eosinophilic and the non-eosinophilic type.⁵⁹ Neutrophils are especially prominent in acute, severe exacerbations of asthma and potentially contribute to airway gland hypersecretion, bronchial hyper-reactivity and to airway wall remodelling.⁶⁰ Given that Th17 cells play a crucial role in the pathogenesis of neutrophilic inflammation, Th17 might be a predominant player in non-eosinophilic asthma.

However, the exact role of Th17 in asthma is not well known. In a mouse model of allergic airway inflammation, systemic blockade of IL-17 inhibits the allergen-induced

accumulation of neutrophils and increases eosinophils numbers in the airway.⁶¹ In IL-17 knockout mice, the allergen-induced airway hyperreactivity to methacholine is significantly reduced.⁶² In line with this, increased immunoreactivity for IL-17 in the airway submucosa is associated with impaired lung function in patients with asthma.⁵⁷ Interestingly, there is now evidence that recombinant IL-17 protein stimulates the release of cytokines such as TNF- α , IL-1 β , and IL-6 in human blood-derived macrophages *in vitro*.⁶³ These results indicate that IL-17 is involved in the pathophysiologic process of asthma. Consistent with these above results, Schnyder-Candrian and colleagues⁶⁴ showed IL-17 is required in the initiation of allergic asthma. However, they also demonstrated that IL-17 attenuates the allergic response by inhibiting DCs and chemokine synthesis in established allergic asthma, as neutralization of IL-17 augmented the allergic response in sensitized mice. Conversely, exogenous IL-17 reduced pulmonary eosinophil recruitment and bronchial hyperreactivity, indicating endogenous IL-17 has a dual role.

It has been reported that IL-17 is required to induce optimum Th1 and DTH responses against haptens.⁶² The results is further confirmed by another study,⁶⁵ which demonstrates that IL-17 is an important cytokine not only in the early neutrophil-mediated inflammatory response, but also in T cell-mediated IFN- γ production and granuloma formation in response to pulmonary infection by BCG. This suggests that IL-17 is an important cytokine in the induction of an optimal Th1 response and protective immunity against mycobacterial infection. The result is consistent with a study showing that BCG vaccination triggered an accelerated IFN- γ response by CD4⁺ T cells in the lung during subsequent *M. tuberculosis* infection, and depletion of IL-17 reduced the accumulation of CD4⁺ T cells producing IFN- γ in the lung.⁶⁶ IFN- γ and IL-17-producing CD4⁺ T cells are expanded by *M. tuberculosis* infected DC and are induced *in vivo* during infection.⁶⁷ Moreover, IFN- γ serves to limit the IL-17-producing T cell population,⁶⁸ as numbers of IL-17-producing T cells following infection with BCG are increased in IFN- γ deficient mice and exogenous IFN- γ reduces the frequency of IL-17-producing T cells induced by these bone marrow-derived dendritic cells. However, IL-17 failed to directly enhance mycobacterial Ag-specific IFN- γ production because the level of IFN- γ production was not altered when the T cells from the *M. bovis* BCG-infected IL-17 KO mice were cultured with rIL-17.⁶³ This finding is consistent with a report showing that exogenously added IL-17 affected neither Th1/Th2 phenotype differentiation nor IL-17 production.⁶⁹ Thus it is deduced that some other cells may play a role in the balance of Th17 and Th1/Th2 cells, which needs to be further studied.

As Th17 is a new T-cell subset, there is no direct evidence of its role in the effect of BCG on allergic asthma. However, IL-17 is an important cytokine in the induction

of an optimal Th1 response and protective immunity against mycobacterial infection and in turn, increased IFN- γ producing cells limit IL-17 producing cells. This counter regulation pathway may be an important factor in limiting mycobacterium-associated immune-mediated pathology.⁶⁵ Given the important role of IL-17 for IFN- γ secretion, Th17 may be involved in the effects of BCG on asthma.

Treg involvement in the effect of BCG on asthma

The relationship between immune activation and immune regulation can be complex, especially when considering responses in a whole live organism. BCG has been reported to induce immune-regulatory responses.⁷⁰⁻⁷⁵ A recent study⁷⁰ analyzed the relationship between the presence of BCG scarring and clinical outcome in infants, showing that no cases of sepsis were seen with a BCG scar. Interestingly, the infants with a BCG scar had a higher anti-inflammatory response, a higher ratio of IL-10-producing T cells to IL-4-producing T cells compared with controls, suggesting that BCG vaccination might prepare the immune system to mount an effective response to infectious pathogens and thereby enhance survival. This cannot be explained merely by the prevention of tuberculosis (TB) deaths,⁷⁶ and the mechanism is partially dependent on mycobacteria-induced IL-10 production by macrophages^{71,72} and infected DC-precursors.^{73,74} Madura Larsen and colleagues⁷⁵ observed for the first time that BCG-exposed DCs produced IL-10 and were able to prime naive T cells to develop into IL-10-producing T cells. They showed that the interaction between BCG and DCs leads to the development of naive T cells into IL-10-producing T cells and contributes to a well-balanced immune system by keeping in check any excessive Th1 or Th2 response. Taken together, it appears that BCG may serve as an immune-regulatory agent rather than a simple strong inducer of Th1 response.

Do Treg cells take part in the effects of BCG and other Mycobacterium in allergic airway inflammation? Zuany-Amorim and colleagues⁷⁷ showed in a murine model of pulmonary inflammation that the beneficial effects of *Mycobacterium vaccae* (*M. vaccae*) are independent of IFN- γ . Furthermore, they found *M. vaccae* regulates the allergic host immune responses through the induction of Treg (IL-10 and TGF- β), rather than affecting the Th1/Th2 balance. Indeed, passive transfer of *M. vaccae*-induced CD4⁺CD45RB^{Lo} cells prevents development of allergic responses in mice.⁷⁸ Similar results were found in a recent study,⁷⁹ demonstrating that the abatement of allergic inflammation by *M. vaccae* are through the production of IL-10 and pulmonary CD11c⁺ cells (termed regulatory DC) that express high levels of the immunoregulatory cytokines IL-10 and TGF- β rather than an increase in type-1 cytokines IFN- γ . These results partially explained the early phenomenon of IL-10 cytokine increase found in early similar studies.^{48,49} Suto and colleagues⁸⁰ found depletion in mice of CD4⁺CD25⁺

T cells prevents antigen-induced Th2 differentiation by increasing the differentiation of Th1 cells, indicating that CD4⁺CD25⁺ T cells modulate the Th1 and Th2 cell balance toward Th2 cells and thus up-regulate Th2 cell mediated allergic inflammation in the airways. However, in a comment, Hawrylowicz⁸¹ considered Suto's results⁸⁰ were paradoxical and difficult to interpret, but the depletion of CD4⁺CD25⁺ effector T cells in addition to the Treg cells potentially explain the phenomenon, as effector T cells up-regulate CD25 upon activation.⁸² After the transfer of FACS-sorted CD4⁺CD25⁺ T cells from schistosome infected wide type (WT) mice into egg-immunized and infected IL-10^{-/-} mice, McKee and colleagues⁸³ observed a 50% diminution ($P < 0.05$) in the egg-specific Th1 response (IFN- γ production), but found no discernible effect on the egg-specific Th2 response (IL-5 levels). Perhaps this reflects the relative sensitivity of Th1 and Th2 responses to suppression by IL-10, with the former sensitive to lower concentrations that could be provided by the small numbers of transferred cells, so the author considered the possibility that the ability of Th2 cells to make IL-6, which is able to subvert Treg inhibitory activity,⁸⁴ makes them more resistant than Th1 cells to Treg-mediated suppression. It has been reported that Th1 responses are more prone to regulation by CD4⁺CD25⁺ T cells than Th2 responses.⁸⁵ This possibility was confirmed by Cosmi and colleagues⁸⁶ when they investigated the ability of both CD4⁺CD25⁺ and CD8⁺CD25⁺ human thymocytes to suppress the proliferative response to allogeneic stimulation of Th1 or Th2 clones derived from autologous CD4⁺CD25⁺ thymocytes. They found both CD4⁺ and CD8⁺ Treg cells completely suppressed the proliferation of Th1 clones but exhibited significantly lower suppressive activity on the proliferation of Th2 clones. This suggests that Th2 cells have a lower susceptibility than Th1 cells to the suppressive activity of human CD25⁺ regulatory thymocytes.

The relative insufficiency for suppressive function of Treg in asthma may evoke some explanation for the Th2 predominance in asthma, boosting Th1 and Th2 response by some vaccination, Th2-mediated allergic lung inflammation coexisting with the Th1-mediated responses and the increased prevalence of both autoimmune and allergic disorders.¹³⁻²⁰ However, the role of Treg in the effects of BCG on allergic inflammation has been little studied. Thus, it seems that a reasonable explain as to why BCG vaccination can shift the Th2-type response to Th1-type response in most of studies may be due to the improved the suppressive function of Treg cells so that Treg can suppress all the effector T cells (including Th1, Th2 and Th17), leading to the relatively reverse of Th1/Th2 balance.

PROSPECTS

Treg may play a central role in immunologic homeostasis, which balances the different T subsets (Th1, Th2 and

Th17). Similar, functional deficit of T regulatory cells may be the most important cause leading to dysfunction of asthma. BCG and other *M. vaccae* may serve as an immune regulator rather than an only inducer of the Th1 type immune response. BCG vaccination/infection might improve the frequency and/or suppressive function of Treg to correct the abnormal immune response in asthma. So far, we have set out to investigate the role of CD4⁺CD25⁺ Treg in the protection from asthma in a murine model. Neonatal BCG vaccination inhibits airway hyperresponsiveness and inflammation at adult ages (6-8W), which is partially interpreted by increasing the frequency of CD4⁺CD25⁺ T regulatory cells and Foxp3 expression.⁸⁷ However, the exact mechanisms of BCG in asthma are largely unknown, especially as it concerns the different T-cell subsets. So, it may be worthwhile to investigate further whether and how Treg cells have a role in the effect of different mycobacterial strains, including BCG, on allergic asthma, which may be useful for designing new vaccines to prevent the allergic asthma.

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