THE INFLUENCE OF INFECTIONS ON THE DEVELOPMENT OF ALLERGIC DISORDERS

Ph.D. Thesis

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1 Preface

This thesis is based on the following articles, referred to in the text by their Roman numerals

I. Major, T., Wohlleben, G., Reibetanz, B. & Erb, K.J. Application of heat killed mycobacterium bovis-BCG into the lung inhibits the development of allergen-induced Th2 responses. *Vaccine* 2002; 20: 1532-40.

II. Grunewald, S. M., Hahn, C., Wohlleben, G., Teufel, M., Major, T., Moll, H., Brocker, E. B. Erb, K. J. Infection with influenza A virus leads to flu antigen-induced cutaneous anaphylaxis in mice. J Invest Dermatol 2002;4: 645-51

III. Major, T., Szilvasy, B., Wohlleben, G., Erb, KJ. Heat-killed Mycobacterium bovis- Bacillus Calmette-Guerin prevents experimental respiratory tract eosinophilia in mice. Orv Hetil. 2002;143(22):1361-6.

2 Introduction

Asthma is a problem worldwide and the social burden and costs to both public and private health care systems are substantial. There is strong evidence that asthma prevalence has been increasing mainly in the westernized societies. The recent epidemic of atopic disease and asthma may have occurred as a consequence of a decline in certain childhood infections or a more general lack of exposure to a broad range of infectious agents in the first years of life. In 1989, Strachan proposed a novel but speculative explanation, colloquially named the "hygiene hypothesis" for the apparent rise in the prevalence of allergic diseases.[1]" This explains the increase in prevalence in westernized societies by the "*declining family size, improved household amenities and higher standards of personal cleanliness which have reduced opportunities for cross- infection in young familie over the past century".* At first this hypothesis was received with skepticism because the prevailing immunological thinking considered infection to be a potential trigger of allergic sensitization rather than a protective influence [2]. However, during the early 1990s, a plausible mechanism arose from the distinction of Th1 and Th2 lymphocyte populations in laboratory animals and the recognition that "natural immunity" to bacterial and viral infections induces a Th1 pattern of cytokine release, potentially suppressing the Th2 immune responses involved in IgE-mediated allergy. Although the Th1/Th2 paradigm may not be as clear in humans as it first appeared in rodents, the "hygiene hypothesis" has remained a subject of interest to both immunologists and epidemiologists [3,4].

In our studies we wanted demonstrate the relationship between infections and allergic disorders, therefore we investigated the role of various infectious agents in the development of Th2 immune responses. In order to generate allergic reaction, mice were immunized by ovalbumin and subsequently infected by bacteria, helminthes, or viruses.

3 Aims of the studies

3.1 To investigate the effects of Th1 immune response on the development of allergen-induced airway eosinophilia (I.)

The focus of our interest was to investigate the effects of Th1 immune response on the development of allergen-induced airway eosinophilia. To generate a strong Th1 immune response, live *Bacillus Calmette-Guerin* (BCG), heat-killed *Bacillus Calmette-Guerin* (HK - BCG), and Purified Protein Derivative of *Mycobacterium tuberculosis* (PPD) were used prior to the OVA immunization.

The suppressive effect of BCG on OVA-induced airway eosinophilia was reported earlier by the group. The main objective of the present study series was to investigate whether the bacterium has to be alive to preserve this property.

3.2 To evaluate the effects of a pre-existing helminth infection inducing Th2 immune response on allergic type Th2 reaction

An additional objective of our work was to evaluate the effects of a pre-existing helminth infection inducing Th2 immune response on allergic type Th2 reaction. For this reason a series of studies were performed where mice were infected with helminth and thereafter immunized with OVA according to the protocol described above. To generate a strong Th2 immune response in mice, a metazoic worm species, *Nippostrongylus brasiliensis*, which is a ubiquitous pathogen among rodents, was used

3.3 To investigate the role of Major Histocompatibilitiy Complex class I molecules on allergic inflammation

Exploring the details of antigen presentation in allergic reactions, the role of Major Histocompatibility Complex (MHC) class I cell surface molecules became the focus of our interest. The aim of this work was to identify whether Th2 immune response can be generated by OVA immunization without the presence of MHC class I molecules. To resolve this issue, a study was performed in which MHC class I deficient and control mice were treated under the same OVA-immunization protocol.

3.4 To explore the effects of a viral infection on a pre-existing allergen immunization (II.)

According to accumulating epidemiological data and clinical observations, the role of Th1 response-inducing infections in the development of allergic disorders is controversial. As discussed previously, these agents may be responsible not only for suppressing the Th2 responses in an immature immune system, but also for triggering exacerbations of allergic symptoms. This is especially true of respiratory viruses, such as influenza or RSV. To explore the effects of an airway viral infection on a pre-existing allergen immunization, BALB/C mice were challenged with OVA intranasally at one day intervals and subsequently infected with influenza A virus.

4 Results

4.1 Application of HK-BCG into the lung inhibits the development of OVA-induced airway eosinophilia. (I, III)

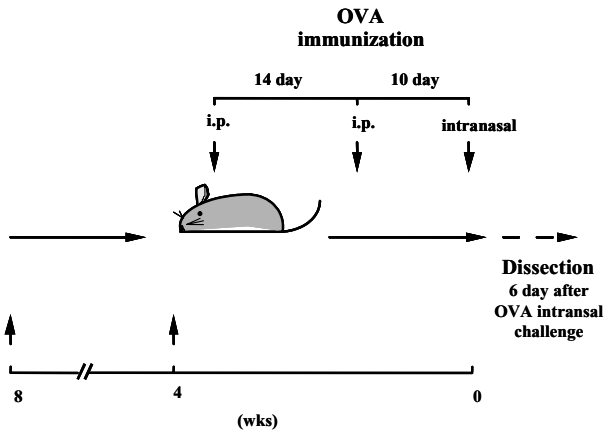

Figure 1. *Experimental protocol for BCG, HK-BCG, PPD-OVA immunization experiments*

C57Bl/6 mice were pre-treated intranasally with $2x10^6$ CFUs of BCG, HK-BCG or PPD four and eight weeks prior to OVA intranasal challenge (**Figure 1**). To summarize the results, it can be clearly stated that an intranasal vaccination with BCG or HK-BCG, given four or eight weeks prior to allergen airway challenge, resulted in a strong suppression of airway eosinophilia. This effect correlated with reduced levels of IL-5 producing Th2 cell numbers present in the airways of OVA-challenged mice (**Figure 2 A-C**).

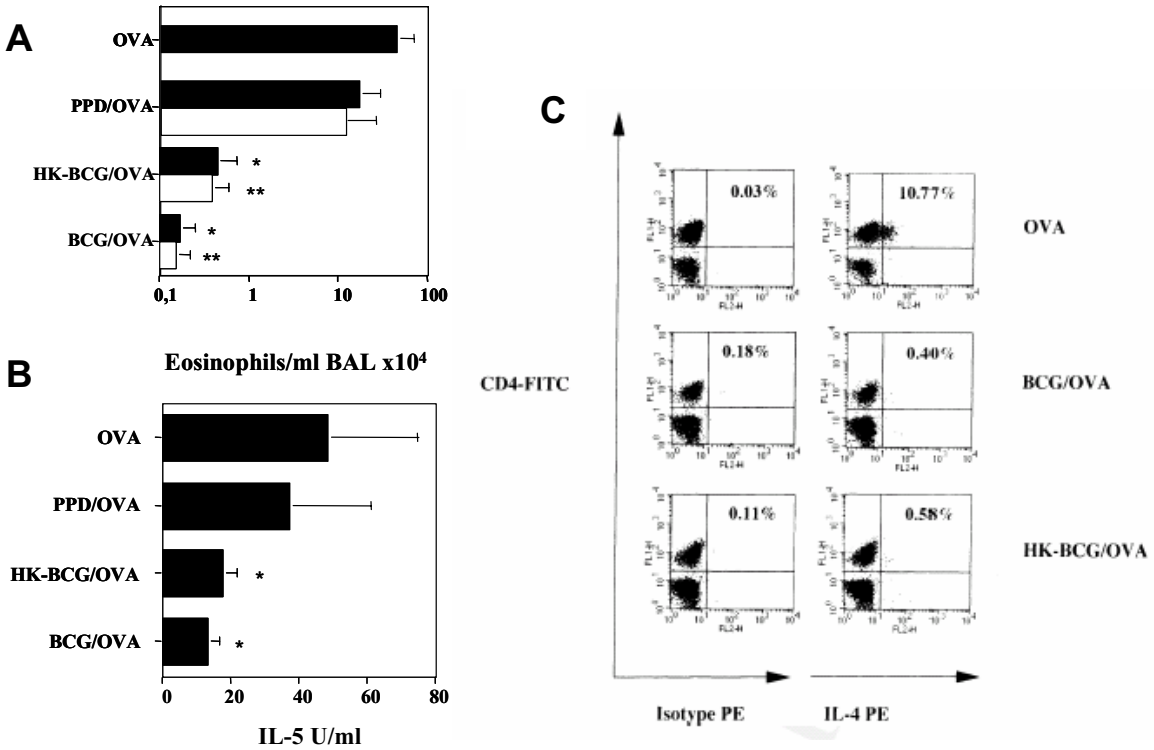

Figure 2 A: *Shown are the average numbers of eosinophils +/.SD present in the BALs of 5–6 mice/group (A).P < 0.05 .P <0.001, compared to values obtained in OVA-only treated mice.* ***B:*** *Shown is the average amount of IL-5 present in the MLN cultures from six mice per group with SD determined by ELISA. P < 0.05, compared to values obtained in mice only immunized with OVA.****C:*** *Shown are FACS-stainings gated on CD4+ T cells representative of six mice per group. The percentage of CD4+ T cells positively stained for IL-4 production is indicated*

BCG and HK-BCG induced suppression of airway eosinophilia and reduction of Th2 cell numbers seems to be localized to the lung, since the systemic Th2-dependent production of OVA-specific IgG1 and IgE by the B cells was not reduced by the intranasal application of the live and heat-killed mycobacterium (data not shown).

To address the question whether IFN-γ was also involved in HK-BCG-induced inhibition of airway eosinophilia, IFN-γ deficient and control mice were subjected to the same OVA-immunizations protocol and infected four weeks prior to the intranasal challenge, resulting in a strongly reduced suppression of airway eosinophilia in IFN-γ deficient mice (**Figure 3 A, B**).

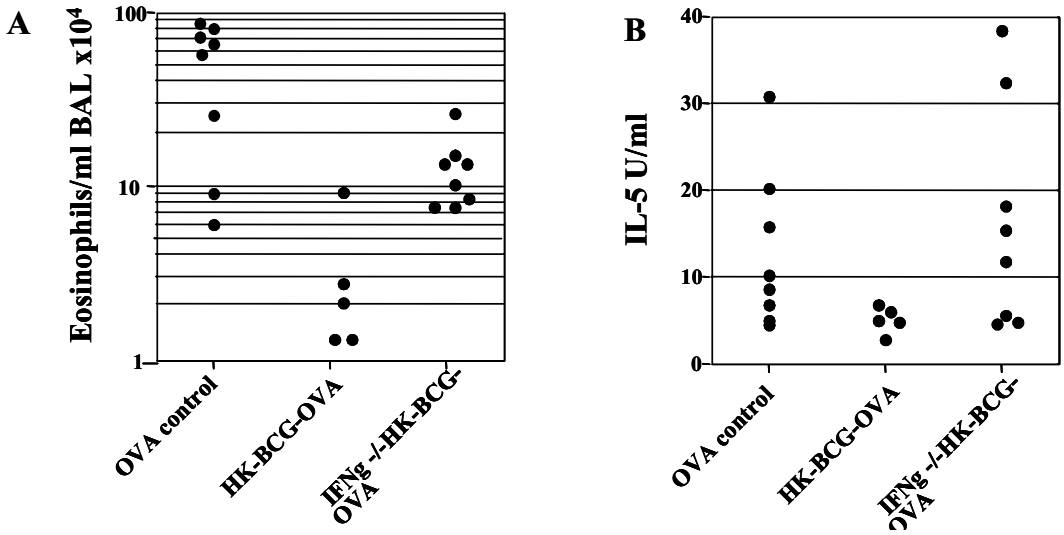

Figure 3. *HK-BCG-induced suppression of airway eosinophilia and reduction of IL-5 secretion by MLN cells was dependent upon the presence of IFN-γ.*
*Shown are the numbers of eosinophils present in the BALs (**A**) and the amount of IL-5 secreted by the MLN cells after in vitro restimulation with anti-CD3/IL-2 of individual mice (**B**).*

Histological sections made from the lungs of OVA-only versus HK-BCG–OVA-treated mice clearly show a significant decrease in mucus production compared to the HK-BCG-treated group (**Figure 4 A, B**).

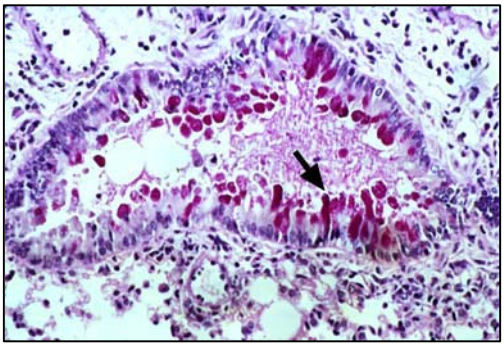

Figure 4 A: *Mucus produced by goblet cells in OVA-induced allergic reaction. PAS staining*

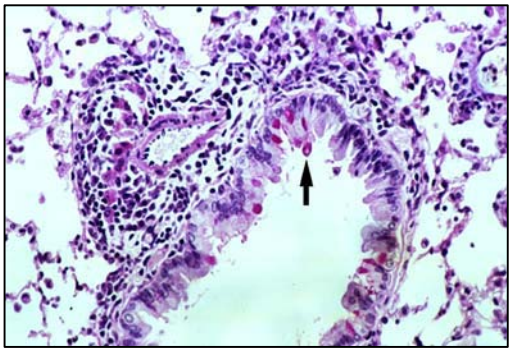

Figure 4 B: *Mucus produced by goblet cells in HK-BCG pretreated, OVA-induced allergic reaction. PAS staining*

Taken together, the results of these experiments clearly demonstrate that applications of heat-killed mycobacteria are able to suppress ovalbumin-induced airway inflammation in mice. The suppression of airway eosinophilia appears to be Th1 immune response-driven, as no suppression of OVA-induced eosinophilia was detectable in IFN-γ-deficient mice.

4.2 The effects of Nippostrongylus brasiliensis infection on ovalbumin-induced airway eosinophilia

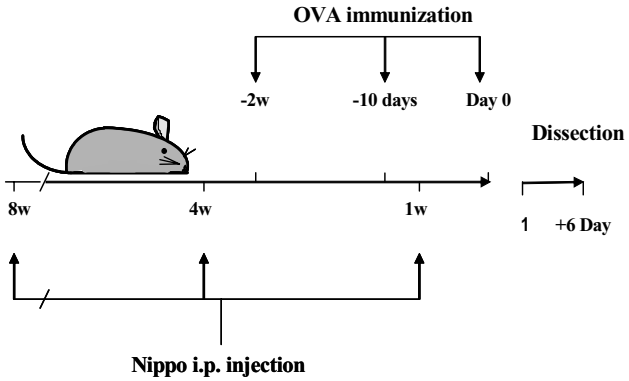

Figure 5. *Experiment design for Nippo infection-OVA immunization experiment. Mice were infected with Nippo 8, 4 and 1 wk before OVA intranasal challenge. The mice received OVA immunization treatment according to the protocol mentioned above. Mice were harvested 6 days after OVA intranasal challenge.*

The objective of these studies was to determine whether a pre-existing helminth infection would increase or decrease an unrelated allergen-induced eosinophilia in the airways. C57-Bl/6 mice were infected with Nippo 8, 4 and 1 week(s) prior to OVA immunization and intranasal challenge. BALF were collected 6 days post-challenge and cellular and humoral immune responses were measured (**Figure 5**).

Our results revealed decreased IL-5 production in the cell cultures made from mediastinal lymphnodes taken from mice infected with Nippo 8 and 4 weeks (but not 1 week), before OVA sensitization (**Figure 6 A**). Increased IL-5 protein levels and decreased IFN-γ protein levels were also observed in the BALF (data not shown). There was, however, no increase, but rather a significant decrease in airway eosinophil accumulation in mice infected with parasites 4 and 8 weeks prior to sensitization with OVA as compared to the group infected 1 week prior and to mice exposed to OVA alone (**Figure 6 B**).

These results suggest that a pre-existing helminth infection may potentiate a systemic Th2-type response, yet simultaneously suppress in the lungs allergen-specific IgE responses and eotaxin production in response to subsequent exposure to allergens depending on the time of infection.

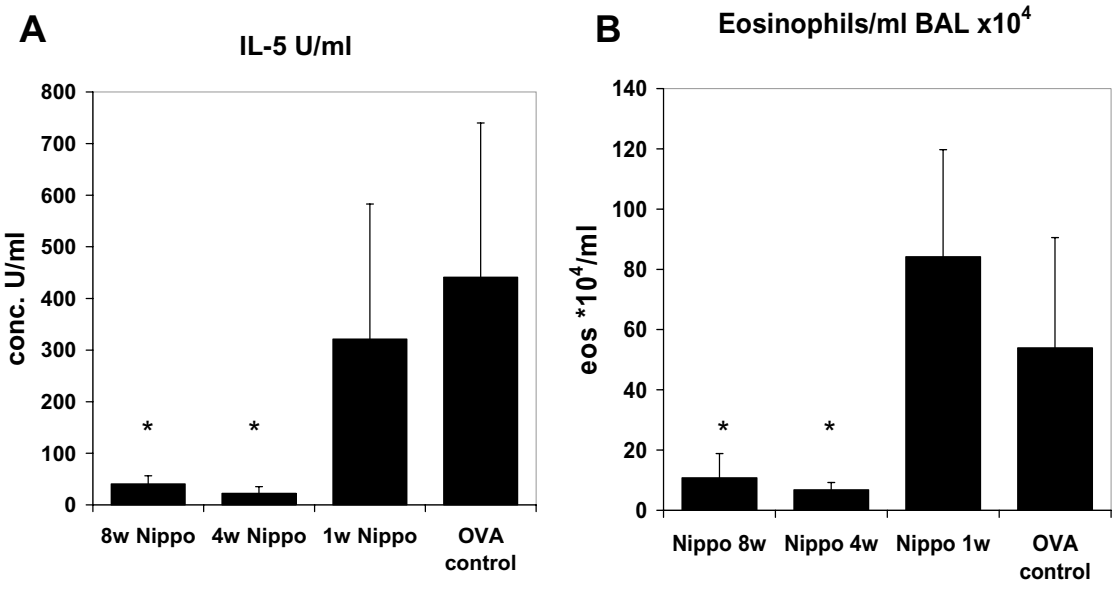

Figure 6 A: *Increased IL-5 levels in the Nippo 4 and 1 week prior to OVA intranasal immunization infected groups versus OVA controls.*
B*: BAL eosinophilia is suppressed in the groups infected 8 and 4 weeks before OVA intranasal challenge. Interestingly a significant increase can be observed in the group, which was Nippo infected one week before OVA immunization. * P≥0.005*

Our results revealed confounding counteraction between two independent Th2 immune responses that are similar in nature but different in origin.

4.3 Major Histocompatibility Complex class I molecules are necessary for the development of OVA induced airway eosinophilia

We addressed the question of whether MHC I molecules are necessary for successful allergen presentation in antigen-specific immune responses. To find an answer to this question, an experiment was designed where the strength of OVA-induced airway eosinophilia was compared in MHC class I-deficient mice and normal controls. MCH I knockouts and C57/Bl6

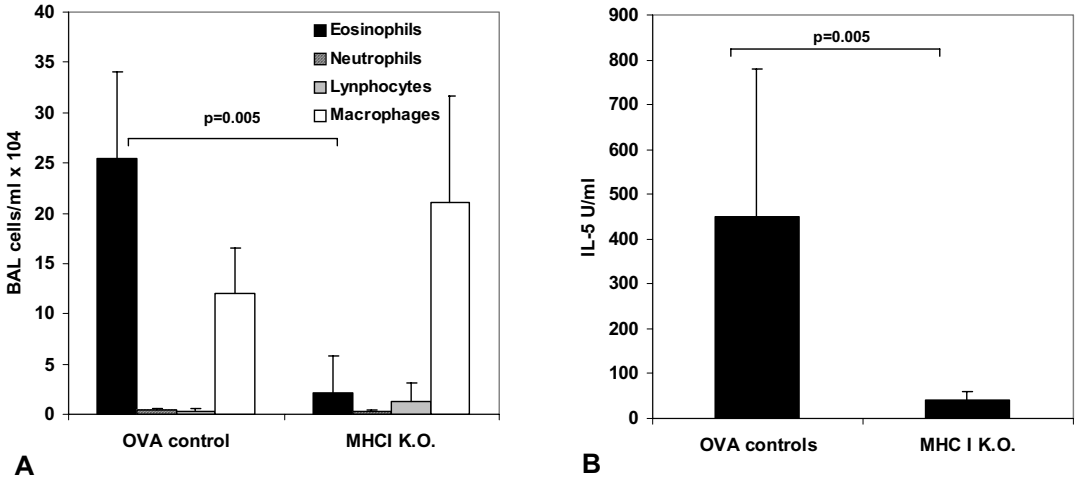

Figure 7. A: *BALF inflammatory cells in OVA controls and MHC I knockouts. Eosinophils were increased in OVA controls, whereas no change was detected in the numbers of eosinophils in the MHC class I knockout mice.* ***B:*** *IL-5 levels in the supernatants of the T cell cultures from the MNL. Significantly higher levels of IL-5 were measured in OVA controls as compared to MHC class I knockouts.*

mice as controls were immunized with OVA according to the same protocol used in the former experiments. Six days after OVA intranasal challenge the mice were dissected and BALs were performed. Our results show that MHC I-deficient mice failed to develop eosinophilia after OVA immunization compared to the controls (I) (**Figure 7 A**). Interleukin-5 levels measured in the supernatants of the cell cultures made from the draining lymphnodes of the lung were significantly higher in OVA controls compared to MHC I-deficient mice (**Figure 7 B**).

4.4 Influenza infection leads to Flu antigen induced cutaneous anaphylaxis in mice (II)

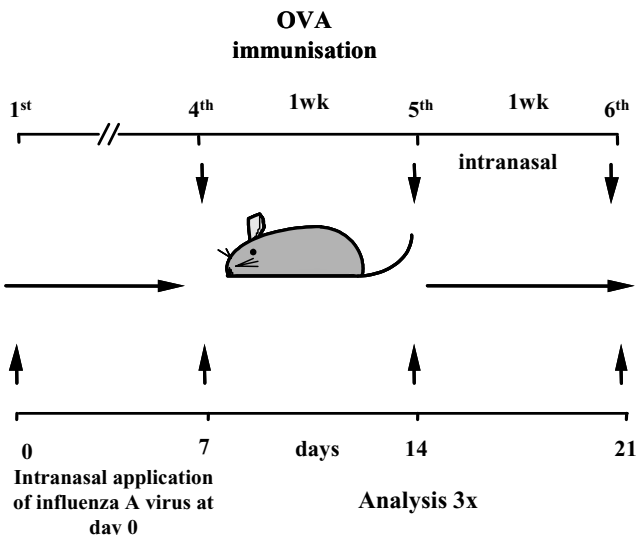

Figure 8. *Experimental design for intranasal OVA sensitization and flu infection experiments.*

To address the question of whether viral infection could lead to mast cell degranulation; mice were infected with influenza A virus and the resulting inflammation in the lung was analyzed histologically (**Figure 8**). We investigated whether the mice infected with flu virus for 3 weeks developed active cutaneous anaphylaxis after re-challenge with Flu-Ag. For this purpose mice were injected i.v. with Evans Blue dye and then with Flu-Ag and PBS intradermally into two separate premarked sites in the skin. After 15 min. the mast cell degranulation induced extravasation of stained blood serum, which led to the formation of a blue patch around the injection site of viral antigens only in the flu-infected mice (**Figure 9**). The lack of this blue patch formation in non-infected mice indicates the lack of mast cell degranulation after viral antigen challenge. After intradermal Flu-Ag injection, mice that had been infected with flu virus showed a much stronger mast cell degranulation than noninfected controls (**Figure 10 A**).We wanted to exclude nonspecific, virus-altered mast cell reactivity and to prove that specific IgE molecules are responsible for the mast cell degranulation effect. Therefore, we passively transferred serum from flu-infected mice into non-infected mice and analyzed for passive cutaneous anaphylaxis according to the protocol mentioned above. For this purpose, noninfected mice were injected intradermally either with 50 µl

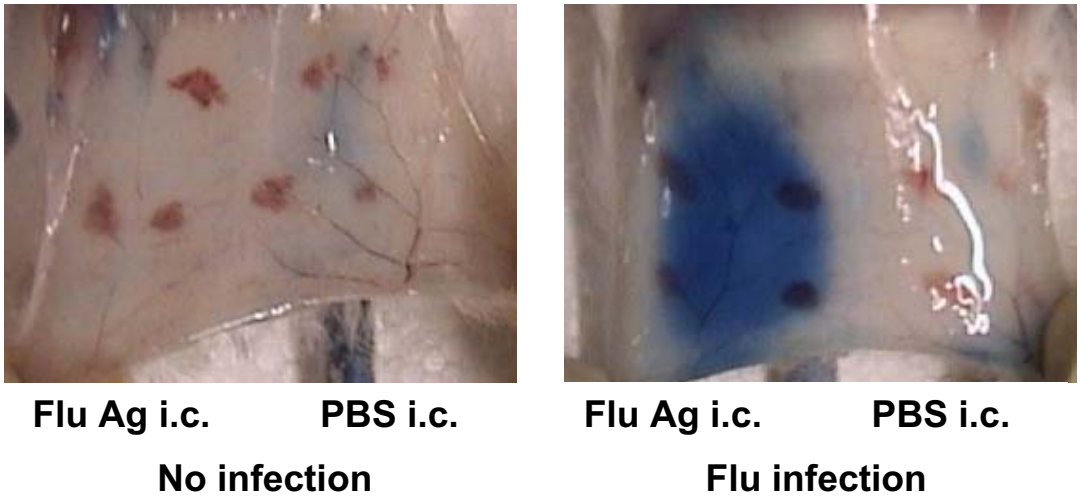

Figure 9. *Mice infected with flu virus show Flu-Ag-specific active cutaneous anaphylaxis.*

normal, or IgE depleted serum from mice, which had been infected with flu virus for 3 weeks. Local anaphylaxis was measured at the sites of the previous injections at 2 h, 24 h, and 48. Intradermal Flu-Ag challenge resulted in similar mast cell degranulation in mice which were injected with the serum of flu-infected mice. In contrast, the passive cutaneous anaphylaxis was significantly lower in mice that had been treated with IgE-depleted serum from flu-infected mice or with noninfected sera (**Figure 10 B**).

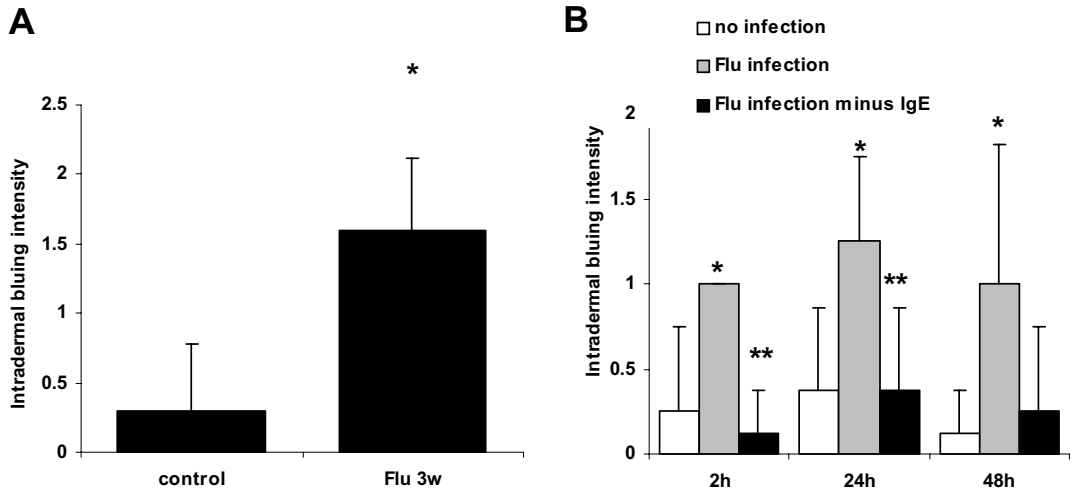

Figure 10 ***A:*** *Average bluing intensity with SD of the different groups of mice after active cutaneous anaphylaxis (10 mice per group; *statistical significance in comparison with uninfected controls).*
B: *Passive cutaneous anaphylaxis. Cutaneous anaphylaxis was measured 2, 24, and 48 h later in the sites of previous injection as described above. Data shown are the average bluing intensity with SD (four mice per group). (* statistical significance in comparison with uninfected controls; ** statistical significance in comparison with flu-infected mice).*

Taken together, the results of this study showed that viral infection can mediate mast cell degranulation by the production of virus-specific IgE antibodies, which leads to the exacerbation of allergic symptoms.

5 Discussion

5.1 Both live and dead bacteria mounted against OVA-induced airway eosinophilia are able to suppress allergic inflammation (I, III)

Our results clearly demonstrate that the application of live and HK-BCG but not PPD into the lung of mice suppressed the development of OVA-induced airway eosinophilia. Furthermore, mucus production after allergen-challenge was also inhibited by the application of live and HK-BCG. This suggests that the application of live and dead mycobacteria inhibited the recruitment and or expansion of Th2 cells homing into the lung after OVA airway challenge. In our experiments we also found that HK-BCG-induced inhibition of airway eosinophilia was at least to a great extent dependent upon IFN-γ. Since IFN-γ is predominantly produced by Th1 type cells these findings suggest that the Th1 immune response initiated by both live and HK-BCG was responsible for the inhibitory effect on the allergic Th2 response in the lung. Supporting this view is the previously published finding that Th2 cells showed impaired homing into inflamed sites dominated by Th1-type inflammatory reactions [5].

Our results suggest that HK-BCG may contain components other than proteins which were responsible for the inhibitory effect on airway eosinophilia, since the proteins from BCG and *M. tuberculosis* show a strong homology. One potential component of the applied HK-BCG responsible for the inhibition of allergic Th2 responses are CpG oligodeoxynucleotides (CpG-ODN). CpG-ODN are part of the bacterial DNA of BCG derived from *M. bovis* and have been shown to suppress the development of allergic asthma in mice [6-8].

5.2 Infection with Influenza A virus leads to Flu antigen-induced cutaneous anaphylaxis in mice (II.)

The results presented in our Flu virus study clearly indicate that the immune response against influenza A virus in BALB/C mice is strongly polarized towards Th1 but also has a minor Th2 component. This can be seen by the large amounts of IFN-γ, the absence of IL-4, and the low levels of IL-5 secreted by the MLN cells after *in vitro* restimulation with Flu-Ag or anti-CD3. The small numbers of eosinophils detected in the lung after infection together with the low titers of flu-specific IgE in the serum of infected mice further supports this view.

Interestingly, we could show that mice infected with flu virus developed virus-specific IgE antibodies and active cutaneous anaphylaxis after intradermal injection with Flu-Ag. Passive cutaneous anaphylaxis could also be demonstrated, suggesting that the detected virus-specific

immunoglobulins and not virus-induced alterations of mast cell reactivity were mediating this effect. Our observation that in flu-infected mice specific IgG1 titers were much higher than the specific IgE titers, together with the published finding that local as well as systemic anaphylaxis can occur in the absence of IgE, suggested that IgG1 may be mediating the allergic skin reactivity[9,10]. We could show, however, that passive transfer of IgE depleted flu serum strongly reduced the allergic skin reactivity and that the local anaphylaxis reaction persisted 48h after passive transfer of serum, indicating that mast cell degranulation was mainly mediated by virus-specific IgE.

6 Conclusions

Allergic disorders continue to be a major health hazard in developed countries and no effective preventive measures exist to date. Data are accumulating that implicate a role for decreased incidence of childhood infections in the increasing severity and prevalence of asthma [1,11,12]. Animal studies have clearly shown that inducing allergen-specific Th1 immune responses can effectively suppress the development of allergic Th2 responses using different approaches[13,14]. In our experimental mice models we were able to demonstrate some important effects of the major type of pathogens (bacterial, helminth and viral infections) on the development of allergen-induced airway eosinophilia. In the following a short summary of the major conclusions of our experimental work is presented, highlighting the novelties:

6.1 HK-BCG inhibits the development of allergen-induced Th2 responses (I, III)

Our results clearly showed that an intranasal vaccination with live and HK-BCG but not PPD, given 4 or 8 weeks prior to allergen airway challenge, resulted in a strong suppression of airway eosinophilia. Based on these results, in contrast to live BCG, HK-BCG might also be a promising candidate for a prospective asthma vaccine in humans since negative side effects due to mycobacterial infection can be ruled out.

6.2 Th2 immune response induced by helminths is able to suppress or potentiate allergen-induced Th2 response, depending on the time of application

We have established the first animal model exploring the effects of a parasitic infection on allergen-induced systemic immune response. Our results revealed, that Th2 immune response-inducing helminth infections may potentiate allergen-induced, systemic Th2-type reactions, yet simultaneously suppress allergen-specific IgE responses and eotaxin production in the lungs depending on the time of infection. Our data provide possible explanation to the increase in the prevalence of allergic disorders observed in westernized societies compared to the lower atopy prevalence in the developing countries, where parasitic infections are endemic.

6.3 MHC class I molecules are necessary for MHC class II molecule-dependent antigen presentation in Th2 responses

Our investigations with MHC class I deficient mice revealed that MHC class II molecule-dependent antigen presentation in Th2 responses depends on the presence of MHC class I molecules. This finding supports the view that Th1 and Th2-polarized immune responses act interdependently.

In addition, this result also supports the hypothesis, that the production of the major cytokine responsible for eosinophil recruitment has been inhibited in the absence of MHC I molecules. In conclusion this is the first animal model, exploring that MHC I molecules play a crucial role in the development of Th2 immune responses [15].

6.4 Flu virus-specific IgE antibodies induce mast cell dergranulation leading to allergic reaction (II)

The Flu virus experiments identified, that infection with influenza A virus induced a strong Th1 response with a minor Th2 component. To the best of our knowledge, this is the first report which provided evidence that virus-specific IgE antibodies can mediate mast cell degranulation leading to allergic skin reactivity in mice. These findings might serve as a new explanation for the mechanism of exacerbation of a pre-existing atopic disease after virus infection.

7 Acknowledgements

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