Journal of Immunological Methods 394 (2013) 62–72

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Evaluating the *in vivo* Th2 priming potential among common allergens

Mali Camberis [a,1], Melanie Prout [a,1], Shiau-Choot Tang [a], Elizabeth Forbes-Blom [a], Marcus Robinson [a], Ryan Kyle [a], Yasmine Belkaid [b], William Paul [c], Graham Le Gros [a,*]

[a] Malaghan Institute of Medical Research, Box 7060, Wellington 6242, New Zealand
[b] Laboratory of Parasitic Diseases, NIAID, NIH, 4 Center Drive, B1-28-Bethesda, MD 20892, USA
[c] Laboratory of Immunology, NIAID, NIH, Building 10, Room 11N311 10 Center Drive, MSC 1892 Bethesda, MD 20892-1892, USA

ARTICLE INFO

Article history:
Received 17 October 2012
Received in revised form 8 March 2013
Accepted 9 May 2013
Available online 17 May 2013

Keywords:
Allergenicity
In vivo Th2 immunoassay
CD4 T cell
Interleukin-4
House dust mite
eGFP-reporter

ABSTRACT

Exposure to allergens, both man-made and from our environment is increasingly associated with the development of significant human health issues such as allergy and asthma. Allergen induced production of the cytokine interleukin (IL-)4 by Th2 cells is central to the pathogenesis of allergic disease (Gavett et al., 1994). The development of the G4 mouse, that expresses green fluorescent protein (GFP) as a surrogate for IL-4 protein expression has made it possible to directly track the immune cells that produce IL-4. By combining a reliable intradermal immunisation technique with the transgenic G4 mouse we have been able to develop a novel & unique *in vivo* primary Th2 immune response model (PTh2). When allergens relevant to human disease are evaluated using the PTh2 assay a dose dependent hierarchy of allergenicity is revealed with environmental allergens (cockroach, house dust mite) the most potent and food allergens being the least. In addition, the PTh2 assay is extremely sensitive to the immunoregulatory effects of Mycobacterial extracts and immunosuppressive drugs on primary Th2 cell development. Taken together, this assay provides a standardised method for the identification of the structural and functional properties of proteins relevant to allergenicity, and is a powerful screening tool for novel lead compounds that are effective at inhibiting the primary Th2 response in allergic diseases.

1. Introduction

Allergens are thought to invoke an allergic response through their ability to stimulate CD4+ Th2 cell type responses leading to uncontrolled Th2-cytokine production and activation of downstream allergic inflammatory events. Historically, allergens have been identified and characterised based on assays which measure their ability to bind specific IgE antibody, trigger IgE-mediated mast cell or basophil reactions (Zhang et al., 2006; Schein et al., 2007; Traidl-Hoffmann et al., 2009); their ability to drive an allergic reaction in experimental guinea pig models (Piacentini et al., 1994); the basophil activation test (BAT) (Boumiza et al., 2005) or reactivate allergen specific human T cell lines (O'Hehir et al., 1988). Whilst these assays have provided evidence to identify known allergens that drive allergic reactions in individuals, and allergen modifications that ameliorate allergenic potential, they have not revealed the features of allergenic potential that are responsible for stimulating the underlying Th2 responses. Moreover, the immune mechanisms that are responsible for the primary induction of allergen specific CD4+ Th2 cells leading to the IgE dependent sensitisation phase of allergic disease remain unknown.

The lack of a suitable primary T cell assay that can evaluate allergens *via* their induction of allergen specific CD4+ Th2 cells is due in large part to the relatively small number of Th2 cells that need to be activated by allergens to induce disease in

* Corresponding author at: Malaghan Institute of Medical Research, PO Box 7060, Wellington 6242, New Zealand. Tel.: +64 4 499 6914; fax: +64 4 499 6915.
E-mail address: glegros@malaghan.org.nz (G. Le Gros).
[1] Authors have contributed equally to this work.

0022-1759/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jim.2013.05.004

model systems. These small numbers have made it technically difficult to measure CD4+ Th2 responses by conventional means, without adjuvants and *in vitro* re-stimulation. The use of specific gene targeted green fluorescent protein (GFP) expression as a reporter of tissue- or differentiated cell-specific function has become an invaluable tool for many branches of biological research. The development of the G4 mouse, which express green fluorescent protein (GFP) as a surrogate for IL-4 cytokine expression, has made it possible to directly follow the differentiation of naïve CD4 T cells into committed IL-4 producing Th2 effector cells *in situ* (Hu-Li et al., 2001; Min et al., 2004; Reinhardt et al., 2009; Van Panhuys et al., 2011). In these mice, only the Th2 differentiated CD4 T cell subset, circulating basophils, eosinophils and activated NKT cell subsets have been identified as producing GFP *in situ*, The longer half-life of eGFP in the G4 mice compared to IL-4 also results in greater sensitivity than conventional methods for measuring IL-4 cytokine expression and eliminates the need for additional staining. Importantly, cells expressing the *Il4* gene locus *in vivo* can be directly detected, avoiding the use of potentially artifactual *in vitro* culture systems, which provide an indirect measure of reactivation potential.

We have combined the G4 mouse model with techniques developed by Gause, Belkaid and colleagues for recovering & enumerating the cells present in the dermis of a mouse ear following intradermal inoculation of infectious or microbially derived agents (Belkaid et al., 1996, 1998; Liu et al., 2007).

The model described herein combines this system with the G4 mouse providing an *in vivo* primary Th2 assay with the ability to detect cell populations and their Th2 cytokine production as early as day 3 following administration of allergen. This primary Th2 response (PTh2) assay is simple and direct, eliminating the use of multiple immunisations, adjuvants and re-stimulation. It is highly sensitive and has a two log dynamic range of response. The PTh2 assay is best performed over a 3–7 day time frame and has proven itself to be highly robust within and between experiments. The PTh2 assay is independent of the effects of endotoxin and records the appropriate sensitivities to known pharmacological agents such as steroids used in the treatment of human allergic disease. The potential of the PTh2 assay for quantifying the intrinsic allergenic ability of an antigen holds significant promise for future discoveries into the nature of allergic disease and immunotherapy agents for allergy prevention.

2. Materials and methods

2.1. Mice

G4 mice have the fluorescent tracer — enhanced green fluorescent Protein (GFP), inserted into the first exon & 178 nucleotides of the second intron of the *IL4* gene locus. In heterozygous mice, this insertion blocks transcription of IL4 mRNA from one allele but leaves intact transcription of the *IL13* gene and *IL4* from the other allele (G4/IL-4, referred to here as G4). C57BL/6 IL-4/G4 TLR-4$^{-/-}$ mice were obtained by crossing G4 mice with C57BL/6 TLR-4$^{-/-}$. Mice used in these experiments were bred by the Biomedical Research Unit, Malaghan Institute of Medical Research, Wellington, New Zealand. 6–10 week age and sex matched mice were used in all experiments. All experimental procedures described in this study were approved by the Victoria University Animal Ethics Committee and carried out in accordance with the guidelines of the Victoria University of Wellington, New Zealand.

2.2. Allergens

Dermatophagoides pteronyssinus (HDM), *Blattella germanica* (German cockroach), *Ambrosia artemissfolia* (Short Ragweed), *Betula pendula* (European white birch), *Penaeus* sp (shrimp), *Hevea brasiliensis* (latex), *Arachis hypogaea* (peanut) and cat dander (from *Felis catus*) were obtained from Greer Laboratories, Lenoir, USA as lyophilised preparations. Pigeon cytochrome C (Sigma, USA), OVA (Sigma, USA), keyhole limpet haemocyanin (Calbiochem USA) and LPS from *Escherichia coli* 0111.B4 (Sigma, USA) and were handled according to the manufacturer's instructions. All allergens were made up at a concentration of 100 μg/30 μl in sterile PBS.

2.3. Preparation of Nippostrongylus brasiliensis allergen (Nb)

N. brasiliensis infective L3 larvae (iL3) were harvested from faecal cultures and washed five times in sterile PBS. Larvae were made up to a concentration of 600 iL3 per 30 μl in PBS and put through three freeze–thaw cycles. Larvae were then checked for loss of viability. For Nb allergen dose–response curves, larvae were made up to 900, 600, 300, 150 & 50 iL3 per 30 μl in sterile PBS & then put through three freeze–thaw cycles.

2.4. Preparation of heat-killed BCG

Mycobacterium bovis BCG Pasteur strain 1173P2 was gifted by the Infectious Disease group, MIMR. Frozen BCG stock was thawed and killed by heating at 70 °C for 1 h. Dose of BCG used was 1×10^6 CFU per 30 μl.

2.5. Intradermal immunisations

Mice were anaesthetised using Xylazine and Ketamine (Phoenix, New Zealand) and 30 μl of the allergen was injected into the ear pinna using a B-D Ultra-fine™ 29 gauge needle & syringe (Becton-Dickinson, NSW, Australia).

2.6. Sensitisation protocol

G4 mice were injected intradermally with allergen. Draining auricular lymph nodes were excised and analysed on day 7 post immunisation. For kinetics studies, G4 mice were immunised intradermally with allergen and draining auricular lymph nodes were excised and analysed daily for 12 days. At given time points, non-draining auricular lymph nodes were excised and analysed. For dose–response studies, G4 mice were immunised intradermally with 100, 10, 1 or 0 μg/30 μl HDM or 900, 600, 300, 150, and 50 diL3/30 μl and draining auricular lymph nodes excised on day 7 post immunisation for analysis. B cell responses were measured on days 0, 7 and 16 as indicated. Secondary immunisations were performed 21 days after primary immunisation using the same dose of allergen.