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Regulation of IgE production and airway reactivity by CD4⁻CD8⁻ regulatory T cells

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ABSTRACT

The mechanisms of tolerance induction occurring in the course of allergen-specific immunotherapy have not been elucidated in full detail. Our study aimed to characterize high zone tolerance in mouse models of type I allergy and of allergic airway inflammation induced by subcutaneous sensitization of mice with high doses of the model allergen ovalbumin (OVA) without the use of adjuvant. Mice were immunized by subcutaneous injection of high doses (HD) of OVA or, for comparison, low doses (LD) of OVA in saline. HD-mice showed lower specific IgE, but augmented IgG in sera than LD-mice. Pre-treatment of mice with HD-OVA antigen-specifically inhibited IgE production subsequently induced by LD-OVA. OVA-restimulated splenocytes from HD-mice revealed hypoproliferation and impaired production of Th2-associated cytokines. HD-mice exhibited lower airway reactivity, goblet cell hyperplasia and mucus production, as well as IL-5 and IL-13 production in the lungs than LD-mice following local provocation. Recruitment of inflammatory cells into the airways was comparable, while the number of eosinophils in the bronchoalveolar lavage was substantially higher in HD-mice. Adoptive transfer of dnTC from HD-mice into naïve mice, which were subsequently sensitized with LD-OVA, suppressed IgE production in the recipients. The number of dnTC was higher in the spleens of HD-mice than LD-mice. In conclusion, our study demonstrates that subcutaneous sensitization of mice with high doses of allergen in the absence of adjuvant results in attenuated airway reactivity as compared with LD-sensitization and induces CD4⁻CD8⁻ dnTC with regulatory function on IgE production.

Introduction

The dose of allergen an individual is confronted with during sensitization is one critical factor in the course of IgE antibody production. Meiler et al. (2008) showed that prolonged exposure of bee keepers to high doses of bee venom due to increased bee sting encounters during the bee keeping season, resulted in reduced venom-specific activation of PBMC, a reduced cutanous late phase response, and induction of IL-10-secreting Tr1 cells, suggesting that tolerance induction after high dose allergen exposure had occurred. Patients, who undergo specific immunotherapy (SIT) receive repeated subcutaneous injections or sublingual applications of increasing doses of the relevant allergen up to a well tolerated maintenance dose. Although the underlying mechanisms of SIT are not fully understood, it has been shown that successful therapy is associated with a shift from atopy-associated Th2 immune responses to a Th1 phenotype, accompanied by an upregulation of regulatory T cell (Treg) activity, which both inhibit Th2 and allergic inflammatory responses, as well as with anergy or deletion of Th2 cells (Smarr et al. 2013). Furthermore, Treg-derived IL-10 has been shown to reduce levels of allergen-specific IgE but to increase allergen-specific IgG production (Meiler et al. 2008; Jutel and Akdis 2011). As a consequence, the IgE burden of SIT patients is reduced after several years of compliant SIT (Focke et al. 2010).

Abbreviations: AHR, airway hyperreactivity; Alum, aluminium hydroxide; BAL, bronchoalveolar lavage; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; dnTC, CD4⁻CD8⁻ double-negative T cells; GITR, glucocorticoid-induced TNFR-related protein; HD, high dose immunization with 100 or 1000 μg OVA; KLH, keyhole limpet hemocyanin; LD, low dose immunization with 0.1 or 1 μg OVA; LPS, lipopolysaccharide; OVA, ovalbumin; PC_{100}, provocative concentration 100; s.c., subcutaneous; Treg(s), regulatory T cell(s).

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Understanding the basic immune processes that happen during SIT is one goal various groups focus on when using experimental mouse models to analyze the impact of the allergen dose employed during sensitization. Intraperitoneal injection of low doses of an antigen together with aluminum hydroxide (alum) resulted in strong formation of IgE antibodies, while sensitization with high doses of the same antigen induced IgE production at low levels, but did not hamper IgG production (Barwig et al. 2010; Sakai et al. 1999; Morokata et al. 2000; Aguilar-Pimentel et al. 2010; Lee et al. 2013; Arps et al. 1998). The mechanisms underlying antigen dose-dependent regulation of IgE responses are widely unknown. Recently, we reported that regulatory $CD4^-CD8^-$ double-negative T cells (dnTC), which arise after intraperitoneal injection of high doses of keyhole limpet hemocyanine (KLH) adsorbed to alum, exhibited inhibitory capacity on IgE-production (Barwig et al. 2010). However, the function of these cells in suppressing allergic reactions is not well described.

To more closely analyze the function of dnTC, we investigated their role in mediating effects of allergen immunotherapy. We therefore utilized an experimental protocol that resembles more closely the situation in patients undergoing SIT, also mimicking a clinically more relevant situation. To avoid allergen-unspecific side effects due to the use of alum, which mainly acts through activation of the inflammasome (Li et al. 2008; Exley et al. 2010), we employed a sensitization protocol that works via the subcutaneous route without the use of an adjuvant (Conrad et al. 2009). Indeed, in the present study we find that dnTC are induced following HD allergen exposure. Furthermore, we describe the novel finding that these cells play a pivotal role for the suppressive effects of immunotherapy.

Materials and methods

Mice and sensitization/provocation protocol

Female BALB/c mice were bred and maintained under specific pathogen free conditions on a standard diet in the Central Animal Facility of the Johannes Gutenberg-University Mainz. The recommendations of the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health were followed. The Ethics Commission according to the German Animal Welfare Act (Landesuntersuchungsamt of the state Rhineland-Palatinate, reference no. 23 170-07/G07-1-023) approved the experiments in this study. Mice were sensitized subcutaneously five to ten times in weekly intervals with low doses (0.1 or 1 μg/injection, LD) or high doses (100 or 1000 μg/injection, HD) of OVA (Calbiochem, LaJolla, CA, USA), dissolved in 200 μl phosphate-buffered saline (PBS). In control experiments mice were sensitized with low doses of KLH (0.1 μg in 200 μl PBS) (Sigma Aldrich, Deisenhofen, Germany). For provocation, OVA (2 mg/ml in PBS) was applied after the sixth sensitization intranasally in a volume of 0.05 ml to mice that had been anesthetized by intraperitoneal injection of 0.2 ml Avertine (1 g/ml of tribromoethylalcohol in tertiary amylalcohol, diluted 1:40 in PBS). Control mice received PBS intranasally.

Determination of antibodies in sera of mice

Sensitized mice were bled by puncture of the retro-orbital plexus at the time-points indicated. Sera were collected and stored at −20 °C until determination of OVA- or KLH-specific antibodies by antigen capture ELISA as reported earlier (Gisch et al. 2007). After linear regression analysis the antibody content was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2. Antibody concentrations given as relative values were normalized to a standard serum.

RBL-2H3 degranulation assay

IgE-mediated degranulation of rat basophil leukemia cell line RBL-2H3 was determined with cells (3×10^4/well) grown overnight at 37 °C in 96-well tissue-culture plates (Corning Costar, Bodenheim, Germany) in culture medium (Iscove modified Dulbecco medium) (Gibco, Paisley, United Kingdom) supplemented with 10% FCS (PAN Systems GmbH, Nürnberg, Germany), 50 μmol/L 2-mercaptoethanol (Carl Roth GmbH & Co.), 2 mmol/L L-glutamine (Carl Roth GmbH & Co.), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). Cells were incubated with sera from immunized mice at a final dilution of 1:600 for 2 h at 37 °C. Spontaneous release was controlled as samples without addition of sera were included. Subsequently, cultures were washed twice with Tyrodes buffer (130 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 1.4 mmol/L CaCl2, 5.6 mmol/L glucose, 10 mmol/L Hepes, and 0.1% BSA, pH 7.4). By addition of OVA protein in Tyrodes buffer (30 μg/mL, verified in pilot titration experiment) degranulation of RBL cells was stimulated. To assess maximum release of β-hexosaminidase by means of IgE-independent degranulation, nonsensitized RBL cells were exposed to 5 μmol/L calcium ionophore A23187 (Sigma–Aldrich) and 100 nmol/L phorbol 12-myristate 13-acetate (Sigma–Aldrich). After 1 h at 37 °C, supernatants were collected, and the remaining cell layers were lysed in Tyrodes buffer supplemented with 1, 5% Triton X-100 (Merck, Darmstadt, Germany). The enzymatic activity of β-hexosaminidase in supernatants and lysates was measured by the addition of the substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma–Aldrich; 1 mmol/L in 0.1 mol/L sodium citrate, pH 4.5) for 1 hour at 37 °C. By the addition of 0.2 mol/L glycine (pH 10.7) the reaction was terminated. Contents of p-nitrophenol were determined with a microplate reader Emax (MWG-Biotech, Ebersberg, Germany) by using absorbance at 405 nm. The β-hexosaminidase activity released into the medium (absorbance in the supernatant) and thus the extent of degranulation was expressed as the percentage of total activity (sum of the absorbance in the supernatant and in the corresponding lysate) (Ludwig-Portugall et al. 2004).

Determination of cytokines in culture supernatants

Splenocytes and lung cells (5×10^6 cells) of LD and HD mice were cultured on 24-well tissue culture plates (Corning Costar, Bodenheim, Germany) in a volume of 1 ml culture medium (Iscove's Modified Dulbecco's Medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS; PAN Systems Nürnberg, Germany), 50 μM 2-mercaptoethanol (Carl Roth GmbH & Co., Karlsruhe, Germany), 2 mM L-glutamine and 100 U/ml penicillin/100 mg/ml streptomycin (Gibco) with or without OVA (100 μg/ml). After 72 h, culture supernatants were collected and stored at −20 °C until quantification of the cytokine contents (IL-5, IL-10, IL-13, IL-17a and IFN-γ) by sandwich ELISA as reported (Sudowe et al. 2003; Zindler et al. 2008). IL-17 concentrations were determined using the following reagents: anti-IL-17 monoclonal antibody (mAb) (clone MAB421), anti-IL-17 mAb biotinylated (clone BAF421) and recombinant murine IL-17 (clone 421-ML) (all from R&D Systems, Minneapolis, USA).

Collection of BAL fluid and characterization of BAL cells

Mice were killed with a lethal dose of narcoren 48 h after the last provocation, the trachea was cannulated, and the lung was lavaged by gentle flushing with 1 ml PBS supplemented with 10% FCS. BAL was recovered and centrifuged. The supernatant was collected as BAL fluid (BALF) and immediately frozen at −20 °C until determination of cytokines. The cell pellet was resuspended and live cells