Expression of the Ym2 Lectin-binding Protein Is Dependent on Interleukin (IL)-4 and IL-13 Signal Transduction

IDENTIFICATION OF A NOVEL ALLERGY-ASSOCIATED PROTEIN

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Asthma pathophysiology is intimately regulated by CD4+ Th2 lymphocytes and the cytokines interleukin (IL)-4 and IL-13. However, the mechanisms by which these cytokines promote disease have not been fully elucidated. In order to identify novel molecular mediators of the disease, a comparison was made of the bronchoalveolar lavage, which demonstrated that the Ym2 protein was abundantly up-regulated in the lung during the development of allergy. Low levels of the Ym1 isomer were also detected. Importantly, neither Ym1 nor Ym2 has been characterized previously in the context of allergic pulmonary inflammation. Western immunoblot showed that enhanced expression of these proteins was dependent on CD4+ T cells and IL-4 or IL-13 signaling via the IL-4Ra subunit. In addition, intratracheal instillation of IL-13 into naive mice was sufficient to induce expression. Ym1 is homologous to eosinophil chemotactic factor. However, only weak eosinophil chemotaxis was observed in response to Ym protein in both in vitro and in vivo assays. By contrast, the homology of Ym1 and Ym2 to proteins associated with tissue remodeling, together with the previous findings that Ym1 is homologous to chitinase and binds heparin sulfate and GlcNA oligomers (chitobiose, chitotriose, and chitotetraose), strongly suggests these proteins play an important role in airway wall remodeling in the allergic lung.

Asthma is a complex inflammatory disease arising from the inappropriate stimulation of immune responses by environmental allergens (1). The acute but reversible bronchoconstriction and airways obstruction observed in asthmatic is underpinned by morphological alterations to the bronchial mucosa that are orchestrated by CD4+ T helper 2 lymphocytes (Th2 cells) and their cytokines. Mouse models of Th2 cell-induced allergic pulmonary disease have provided an invaluable tool for dissecting the discrete molecular mechanisms that potentiate the underlying inflammation in asthma. In congruence with asthma, allergic mice exhibit a Th2 cell biased response in the lung with elevated levels of interleukin (IL)1-4, IL-5, and IL-13, an eosinophilic-rich infiltrate, mucus hypersecretion, airways hyperreactivity (AHR) to cholinergic challenge, and increased serum levels of antigen-specific IgE (reviewed in Ref. 2). These models have now shown that many of these pathophysiological features are linked to IL-4 (3–6). Interleukin-13 also plays a key role in experimental asthma by regulating AHR, mucus hypersecretion, and eosinophil recruitment (7–11), whereas IL-5 directly modulates allergic airways disease by regulating eosinophilic inflammation (12, 13).

Although IL-4, IL-5, and IL-13 contribute to the pathophysiology of allergic pulmonary disease, neither cytokine is obligatory (7, 14), particularly in BALB/c mice, which, like asthmatics, have an inherent bias toward Th2 responses. We demonstrated recently (7) that although a deficiency in either IL-4 or IL-13 was insufficient to ablate AHR and eosinophil extravasation into the pulmonary tissues, a deficiency in both cytokines was required to reduce pulmonary eosinophilia and AHR to base-line levels. The functions of IL-4 and IL-13 are often linked through their common usage of the IL-4Ra subunit to activate downstream signaling moieties (reviewed in Ref. 15). Thus, the IL-4Ra subunit, a fundamental mediator of IL-4 and IL-13 responses, appears to be a key regulator of asthma pathophysiology.

In an effort to elucidate the downstream mediators that affect the development of IL-4- and IL-13-induced disease in the murine lung, we examined the proteins in bronchoalveolar lavage fluid (BALF) that were up-regulated during allergic inflammation. In this investigation, we demonstrate that the Ym2 protein (16) is abundantly expressed in the allergic lung in a manner that is critically dependent on CD4+ T cells and on IL-4- or IL-13-mediated signaling via the IL-4Ra subunit. Lower levels of Ym1, an isomer of Ym2, could also be detected. In addition, expression of these proteins is up-regulated when IL-13 is instilled directly into the lungs of naive mice, and this correlates with previous findings (9, 17) demonstrating that this cytokine similarly induces mucus hypersecretion and AHR. In contrast to reports suggesting that ECF-L, which is identical to Ym1, is an eosinophil chemokine (18–20), purified Ym protein from the allergic lung (a mixture of the Ym1 and Ym2 isomers) was only weakly chemotactic toward eosinophils. However, the presence of this protein in the allergic lung in conjunction with its ability to bind heparin (21), and sequence homology with a family of proteins expressed during tissue remodeling (22, 23), suggests a more relevant function may be...
in modification of the pulmonary tissue architecture, rather than eosinophil chemotaxis during allergic responses.

**EXPERIMENTAL PROCEDURES**

**Induction of Allergic Airways Inflammation—IL-13 gene knockout (IL-13−−/−) mice** were generated from 129 × C57B/6 mice (24) that were backcrossed for 5 generations onto the BALB/c strain. IL-4Ra−/− mice were generated from BALB/c embryonic stem cells as described previously (25). Wild type mice were obtained from a similar number of backcrosses of the same genetic background. Equal numbers of male and female mice (3–6 per group) were sensitized at 6–8 weeks of age by intraperitoneal injection with 50 μg of ovalbumin (OVA) mixed with 1 mg of Alhydrogel (CSL Ltd., Parkville, Australia) in 0.9% sterile saline. Nonsensitized mice received 1 mg of Alhydrogel in 0.9% saline. On days 12, 14, 16, and 18, all mice were aeroallergen-challenged with OVA as described previously (12, 14). Mice that were saline-sensitized and OVA-challenged are referred to as nonallergic mice and OVA-sensitized and -challenged mice as allergic mice. Additionally, wild type BALB/c mice (WT) or IL-13−−/− mice were either treated with isotype control antibody (1 mg of βGL113), anti-IL-4 antibody (1 mg of 11B11), or anti-IL-5 antibody (1 mg of TRFK5) by intraperitoneal injection 24 h before sensitization and then weekly throughout the experimental period. To prepare the T cells, mice were killed with 1 mg of antibody clone GL1.5 or with 1 mg of βGL113 antibody for control mice 8 days before intraperitoneal sensitization and then weekly throughout the experimental period. Twenty four h after the last challenge, mice were sacrificed by cervical dislocation, and bronchoalveolar lavage fluid (BALF) was collected by flushing the airways 2 times with 1 ml of PBS. Eosinophilic inflammation of the airways was characterized by enumeration of May-Grunwald-Giemsa-stained cytospins of BALF cells and histological examination of Carbol’s Chromotrope and stained hematox- ylin tissue sections. To determine the efficiency of T cell depletion, the spleens from 2 mice per group were removed, pushed through a sieve to give a single cell suspension, and washed in PBS containing 1% fetal calf serum. Two × 105 splenocytes were removed and stained with phycoerythrin-conjugated GK1.5 and subjected to fluorescence-acti- vated cell sorting (FACS) analysis of CD4+ T cells. For the IL-13 studies, naive mice were anesthe- tized with intravenous Saffan (Schering-Plough, New South Wales, Australia), and 10 μg of recombinant IL-13 (gift from D. Donaldson, Genetics Institute, Cambridge, MA) diluted in 20 μl of PBS, or PBS only for controls, was delivered intratracheally via a 24-gauge catheter (Terumo). The BALF was collected after 48 h. For the in vitro assay using purified Ym protein, WT mice were sensitized and challenged with ovalbumin, neally with OVA 12 days apart to induce a peripheral blood eosinophilia. After a further 7 days, 10 μg of purified Ym protein, or PBS for controls, was instilled intratracheally in a total volume of 20 μl. Intratracheal delivery was repeated after a further 24 h. Histological analysis was then conducted 24 h later. These experiments were conducted in soxexamine to transfect the Ym protein in murine T cells, a previously used method for transfecting murine T cells. Two days constitutively TH2 and TH1 immune and allergic disease. Mice were treated according to Australian National University Animal Welfare guidelines and were housed in a specific pathogen-free facility.

**SDS-PAGE, Western Blotting, and N-terminal Sequence Analysis—** Cell-free BALF supernatants were boiled in sample buffer containing 5% β-mercaptoethanol and run on 4–12% NU-PAGE gels following the manufacturer’s recommendations (Life Technologies, Inc.). To identify the Ym protein, an appropriate band was excised from the gel and subjected to N-terminal sequencing using Edman chemistry (Biomolecular Resource Facility, Australian National University, Australian Capital Territory, Australia). For comparison of Ym expression in cytkine-deficient mice, BALF samples were concentrated, and 40 μg of protein was loaded per lane. Western immunoblots were performed on BALF proteins that were electrophoretically transferred to a polyvinylidene difluoride membrane using a Multiphor Novablot semidry transfer system (Amersham Pharmacia Biotech). The membrane was blocked with 2% bovine serum albumin in TBST (Tris-buffered saline, 0.05% Tween 20) and probed with a 1/1000 dilution of rabbit anti-Ym1 antibo- body and then with anti-rabbit alkaline phosphatase (AP)-conjugated antibody and alkaline phosphatase chromogenic substrate (Promega Corp., Madison, WI). **RT-PCR, DNA Sequence Analysis, and Cloning—** For time course RT-PCR, lung tissue was obtained from WT mice that were OVA-sensitized and had received O, 2, or 4 OVA aerosols. RNA was purified using TRIZOL reagent (Life Technologies, Inc.) and reverse-transcribed using oligo(dT) and Superscript enzyme (Life Technologies, Inc.). The presence of mRNA specific for the Ym protein was detected by PCR using the forward primer 5’-CTGATCTATGCTTGCTGTTG and the reverse primer 5’-CACAGTATCTTCTCCTAAACG for 30 cycles at an annealing temperature of 55°C. These primers were designed from the sequence of the gene encoding ECF-L (GenBank™ D87751) and hy- bridize at positions 170 and 510, respectively. RT-PCR for the β-actin housekeeping gene was used for RNA normalization. For sequence analysis, the reading frame encoding the Ym protein was amplified by RT-PCR of RNA purified from the inflammatory cells in the BALF of allergic WT mice. High fidelity PCR of reverse transcribed oligo(dT) RNA was performed with the forward primer 5’-CATGGCAGAAGTCTTCC and the reverse primer 5’-TCAATAGGCCCCTGTCGAAC, which hybridize at positions 1195, respectively, of ECF-L (the methionine start codon and reverse stop codon are in bold). The resultant PCR fragment was A-tailed and cloned into the plasmid vector pGEM-T (Promega, Madison, WI). Five clones were then sequenced at least twice, independently, using the PRISM Ready Reaction Dye- Deoxy Terminator Cycle Sequencing reagents and an Applied Biosys- tems automated sequencing system model 373A (PerkinElmer Life Sciences and Roche Molecular Biochemicals) with SP6 and T7 primers, which hybridize to vector sequences, and internal primers based on the sequence of ECF-L.

**Ym Protein Purification—** Cell-free BALF supernatant from allergic mice was concentrated with a Centricon 50 microconcentrator (Millipore, Bedford, MA), buffer exchanged into 10 ml Tris, pH 8.5, and then dialyzed with 2 changes of Q5 anion exchange resin. Ym protein was eluted from the BioLogic microprocessor (Bio-Rad). The Ym protein eluted at 0.55 M NaCl of a 0–1 M linear gradient as determined by SDS-PAGE. Appropriate fractions were pooled, concentrated, and sub- jected to size exclusion chromatography (SEC) in PBS with a TSK G3000SW column ( Tosoh Corp., Tokyo, Japan). Fractions containing the Ym protein were pooled and concentrated. The yield was ~2 μg per mouse, and only one band was detectable on SDS-PAGE stained with Colloidal Coomassie (ICN, Costa Mesa, CA) suggesting a high degree of purity. N-terminal sequence analysis and reaction with a polyclonal antibody prepared against recombinant Ym1 confirmed the identity of the purified protein.

**Purification of Recombinant Ym1 and Antibody Production—** A Ym1/ pGEM-T clone was used to generate a template for high fidelity PCR with the forward primer 5’-GTGATCGATGATCTCCTACCA and the reverse primer 5’-CTCCTCTCTCAATAGGATCCTTGGCAAAC. The triplets in encode the N-terminal tyrosine of the mature protein and the reverse stop codons, respectively. The underlined BamHI restriction sites were used to clone the PCR product into the vector pGEX2T (Amersham Pharmacia Biotech), which expressed Ym1 as a glutathione S-transferase (GST) fusion protein under the control of a tac promoter. Orientation of the insert was determined by HindIII and EcoRV digests of sites that occur in Ym1 and the vector, respectively. The Ym1GST fusion protein was expressed in Escherichia coli was predominantly found in inclusion bodies. Recombinant bacteria were washed in PBS, disrupted in a Ribi cell Fractionator (Sorvall, DuPont), and resuspended in PBS with Complete protease inhibitors (Roche Molecular Biochemicals), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100. Inclusion bodies were solubilized by centrifugation at 25,000 × g for 30 min at 4°C and solubilized in 0.1 M Tris, pH 8.0, 2 mM EDTA, 0.1 mM dithiothreitol, and 6 mM glutathione and mixed at 8°C overnight. Insoluble material was removed by centrifugation at 30,000 × g for 30 min. The Ym1GST fusion protein in the supernatant was then refolded by diluting 150 into 0.1 M Tris, pH 8.0, 0.5 M arginine, 2 mM EDTA, and 6 mM oxidized glutathione and mixed at 8°C overnight. Insoluble material was removed by centrifugation at 5000 × g for 15 min, and the supernatant was then concentrated with a PM10 membrane (Millipore, Bedford, MA) at 60s pound/ square inch. Insoluble material was removed with a 0.45-μm filter (Millipore, Bedford, MA) and then the Ym1GST protein was purified by SEC in PBS with a TSK G3000SW column (Tosoh Corp., Tokyo, Japan). Purity was visually determined on SDS-PAGE to be greater than 95%. Antibodies were raised in New Zealand White rabbits by immunization with 100 μg of Ym1GST fusion protein emulsified in complete Freund’s adjuvant. Two booster doses of 100 μg of protein in incomplete Freund’s adjuvant were administered at 4 and 6 weeks after the initial dose. Serum was collected 2 weeks after the last injection. **Eosinophils—** Eosinophils, which were obtained by flushing the peritoneal cavity of IL-5 transgenic mice (supplied by L. Dent, University of Adelaide, South Australia, Australia [26]), were washed in PBS. The peritoneal cells (2 × 10^6 cells/well comprising 30.5% eosin- phils) in RPMI supplemented with 100 ng/ml purified IL-5 (gift from I. G. Young, JCSMR, Australian National University, Australian Cap- tival Territory, Australia) were added to the top chamber of a 5-μm pore 24-well Transwell plate (Corning Costar, Cambridge, MA). Eotaxin

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Ym2 Is Induced by IL-4 and IL-13 Signaling via IL-4Rα

Enhanced expression of the Ym protein and DNA in the lung during the development of allergic inflammation. Samples from 3 to 4 OVA-sensitized mice per group were collected after 4 (lane 1), after 2 (lane 2), and before OVA aerosols (lane 3). The cell-free BALF supernatant was pooled and analyzed by SDS-PAGE (A). Ym-specific RNA expression in lung tissue was examined by RT-PCR (B, top panel). RT-PCR of the β-actin housekeeping gene was used to control for RNA variation (B, bottom panel). Western immunoblot (C) was performed with antibodies against recombinant Ym1. All panels demonstrate progressively increased expression of Ym, depending on the number of aerosols received. The arrow indicates the position of Ym protein on the gel, and molecular mass size markers are indicated in kilodaltons.

Identification of the Ym Protein in BALF from the Allergic Murine Lung—In order to identify proteins that were specifically induced during allergy, a comparison was made of the BALF from OVA-sensitized mice before and after 2 and 4 OVA aerosols (Fig. 1A). The expression of a protein of ~40 kDa was increasingly apparent on SDS-PAGE depending on the number of aerosols received. This protein was excised from the gel and subjected to N-terminal Edman chemistry, which identified the sequence YYTSWAK. Residue five was ambiguous, but the low levels of dehydroxyalanine suggested the possibility of a cysteine residue. This sequence had identity with two proteins. The first, Ym1, was originally deposited in GenBankTM (accession number M94584) as an unpublished entry and more recently characterized as a novel mammalian lectin that is transiently expressed by murine peritoneal macrophages during parasite infection (21). The second protein, ECF-L (GenBankTM accession number D87757), was characterized as a murine lymphocyte-derived eosinophil chemotactic factor produced by CD8+ T cells in response to Toxocara canis infection (19, 20). Notably, several genes encoding isotypes of Ym1 (classified as types 2–4) have been identified and partially characterized (16). However, the Ym1 type expressed in allergic BALF could not be determined from the initial N-terminal analysis. Because it was apparent from SDS-PAGE that the expression of the Ym protein was up-regulated in the allergic lung, we performed RT-PCR to determine whether its incidence in BALF was associated with the enhanced exudate of serum proteins into the pulmonary fluid as a result of allergic inflammation or whether Ym was expressed in situ. Similar to the protein profile, a faint DNA band corresponding to the Ym protein could be detected in mice that had not been OVA aerosol-challenged (Fig. 1B). However, expression was highly up-regulated after 2 and increasingly so after 4 aerosols. Western immunoblot using an antibody specific for recombinant Ym1 also demonstrated progressively enhanced expression of Ym protein in the BALF (Fig. 1C). As the Ym1 homologous protein, ECF-L, has been associated with eosinophil chemotaxis (19, 20), we also compared the development of eosinophilia in the peripheral blood (Fig. 2A) and BALF (Fig. 2B) with Ym protein expression in allergic mice. The enhanced recruitment of eosinophils to the lung during allergy paralleled the pulmonary expression of the Ym protein.

Expression of the Genes Encoding Ym1 and Ym2 in the Allergic Lung—Isotypes of Ym have been identified previously but have not been linked to the pathophysiology of allergic disease. Therefore, sequence analysis of the reading frame encoding the Ym protein was performed to determine which isotype was up-regulated in response to allergy. The ym gene was amplified by high fidelity RT-PCR, cloned into the pGem-T vector, and sequenced. Interestingly, one clone was identical to the sequence of Ym1 (and ECF-L), whereas the other four clones were homologous (except for two amino acids see Fig. 3) to the sequenced part of the gene encoding residues 204–393 that has been identified as isotype Ym2 (16). Alignment of the complete sequences of the 398 amino acids comprising Ym1 and Ym2 are shown in Fig. 3. Overall these isoforms demonstrated 91.7% identity, varying by 33 amino acids. In order to determine whether the ratio of Ym1 to Ym2 clones was an accurate representation of their overall expression, digestion of the PCR product from allergic lung (four OVA aerosols) with ScaI restriction endonuclease was performed. The differences in sequence between Ym1 and Ym2 result in a ScaI site in Ym1 but not in Ym2 at the triplet encoding residue Ala-288. ScaI digestion of the PCR product derived from the Ym1/pGEM clone was used to control for digestion efficiency. Whereas the PCR from the Ym1 clone was completely digested with ScaI (Fig. 4, lane 3), the PCR from total lung was only partially cut (Fig. 4, lane 1) suggesting that the major Ym isotype expressed in the allergic lung was Ym2.

Both the Ym1 and Ym2 Proteins Are Expressed in the Allergic Lung—SDS-PAGE of the Ym protein purified by ion exchange chromatography and then SEC HPLC revealed a single protein band. However, if a second ion exchange step was introduced before SEC, a small shoulder (Fig. 5A, peak 1) as well as a major peak of 260 nm absorbance were observed (Fig. 5A, peak 2). Both peaks contained a protein of similar molecular weight (Fig. 5B) that reacted with the Ym1 antibody on a Western immunoblot (Fig. 5C). The two bands were excised from SDS-PAGE and subjected to N-terminal sequence analysis, which, in contrast to the earlier analysis, extended beyond the 16th residue of the mature protein. This identified an isoleucine as the 16th residue in the protein from the shoulder (peak 1), and this corresponds to the sequence of Ym1. In contrast, the 16th residue in the major peak was a threonine, corresponding to Ym2. Therefore, by comparison of the area under the peaks, and consistent with RT-PCR analysis, Ym2 appears to be the major Ym isomer expressed in the allergic murine lung. Notably, when the Ym protein was routinely purified, this second ion exchange HPLC step was not performed, leaving the mixture of isomers intact for the chemotaxis assays. In addition, despite the polyclonal antibody being raised against Ym1, it reacted equally with the two isoforms.

Expression of Ym Protein Is Dependent on CD4+ T Cells, IL-4 and IL-13, and the IL-4Ra Subunit—Allergic responses are intimately regulated by CD4+ T cells and the cytokines IL-4 and IL-13 (7) which both signal via the IL-4Ra subunit (reviewed in Ref. 27). In addition, IL-5 directly regulates eosinophilia (12, 13). Therefore, as the expression of the Ym protein was up-regulated during allergy, the cytokine dependence for Ym expression was assessed by Western immunoblot. In order to correlate directly the expression of the Ym protein as a ratio of total protein in the airways of individual groups of mice and...
to eliminate variation in protein concentration due to inflammation, the BALF was concentrated, and 40 μg of total protein was loaded per lane. Western immunoblot of these concentrated samples detected a weak Ym protein band in the BALF from nonallergic mice (Fig. 6A, lane 1). However, Ym expression was highly up-regulated in allergic WT mice (Fig. 6A, lane 2). When the effect of individual cytokines was examined, it was clear that the depletion of both IL-4 and IL-13 (Fig. 6A, lane 7) or the absence of the IL-4R subunit (Fig. 6A, lane 8) inhibited the allergy-induced expression of the Ym protein. Additionally, in contrast to control antibody-treated allergic mice (Fig. 6B, lane 2), CD4+ T cell depletion inhibited the allergy-induced expression of the Ym protein to near the level seen nonallergic mice (Fig. 6B, lanes 1 and 3). An experiment was also conducted to determine whether expression of the Ym protein could be induced by intratracheal delivery of IL-13 to naive mice. Whereas no expression of the Ym protein could be seen in PBS-treated mice (Fig. 6C, lane 1), intratracheal administration of IL-13 enhanced expression (Fig. 6C, lane 2), supporting the hypothesis that IL-13 signaling via the IL-4R subunit regulates this process.

To determine whether the Ym protein induces eosinophil migration in vitro, the purified protein (Fig. 7A) was used in chemotaxis assays. Consistent with a previous report (28), eosinax at 100 ng/ml stimulated a strong chemotactic response. However, whereas the same concentration of Ym protein induced a level of chemotaxis above baseline, it was much weaker in comparison with eosinax (Fig. 7B). Interestingly, chemotaxis inversely correlated with the concentration of Ym protein. Further assays using lower concentrations of Ym protein failed to demonstrate

FIG. 2. Comparison of the numbers of blood and airway eosinophils during the development of allergic inflammation. Samples from 3 to 4 OVA-sensitized mice per group were collected after 4, after 2, and before OVA aerosols. Peripheral blood smears (A) and cytospins from BALF (B) were stained with May-Grunwald-Giemsa for the enumeration of eosinophils. Values represent the mean ± S.E. per group percentage of blood eosinophils or of total airway eosinophils, respectively.

FIG. 3. Alignment of the amino acid sequences of Ym1 and Ym2. The genes encoding the Ym proteins were amplified by RT-PCR and cloned into the pGEM-T vector, and each clone was sequenced at least twice independently. Five clones were characterized; one clone had a DNA sequence that encoded a protein corresponding to Ym1 (upper sequence), whereas the DNA sequence from the other four clones encoded a protein that differed by 33 amino acids (lower sequence). Apart from Val-286 and Ala-288 (underlined, which were previously shown to be identical in Ym1 and Ym2, residues 204–393 of these four clones corresponded to a protein that has been identified as Ym2 (16). Sequence analysis showed valine not isoleucine at position 286 and alanine not threonine at position 288 in the four Ym2 clones. The remaining sequence of Ym2 has not been reported previously. The DNA sequence of Ym2 has been deposited in GenBank™ with accession number AY049785.
an increase in eosinophil chemotaxis (data not shown). As no chemotaxis was detected with 1 μg/ml Ym protein, an experiment was conducted to determine whether the purified protein showed any deleterious effects on cell viability. However, incubation of eosinophils with concentrations of up to 5 μg/ml Ym protein for 3 h, followed by fluorescence-activated cell sorter analysis of propidium iodide-stained cells, showed no effect on viability (data not shown). Furthermore, the addition of 1 μg/ml Ym protein did not inhibit chemotaxis of eosinophils toward eotaxin (data not shown). In order to determine whether the Ym protein induced chemotaxis in vivo, an intravenous pool of eosinophils (mean of 9.66% of total peripheral leukocytes ± 1.77) was induced by intraperitoneal sensitization with OVA. Intratracheal delivery of Ym protein induced some weak eosinophil recruitment into the pulmonary tissues (Fig. 7C), although this was not statistically different to PBS-treated mice (p = 0.06). No significant differences in eosinophil numbers could be detected in the BALF of Ym-treated mice.

**Discussion**

In this investigation we describe the novel observation that expression of the Ym2 protein is enhanced in the lungs of allergic mice in a manner that is dependent on CD4+ T cells and IL-4 or IL-13 signaling via the IL-4Rα subunit. Importantly, neither Ym1 nor Ym2 has been characterized previously in the context of allergic pulmonary inflammation. We also present the sequence of the Ym2 protein, which until now has not been reported in its entirety.

Asthma is a complex inflammatory disease, and although it is clear that Th2 cells are pivotal in this process, the precise molecular links between immune mediators and the expression of allergic disease are not clearly defined. In order to identify potential novel molecular mediators of allergy, we compared the profile of proteins found in the BALF from allergic and nonallergic mice in the presence and absence of key regulatory cytokines. The Ym protein was identified in respiratory secretions and was shown to be progressively up-regulated during the development of allergic inflammation in the murine lung. Ym1 and ECF-L are identical proteins that have been identified in parasite models and characterized as a mammalian lectin and an eosinophil chemoattractant, respectively (20, 21). Interestingly, we demonstrate the presence of two isotypes of Ym protein in the allergic lung, Ym1 and Ym2. These isotypes were differentially expressed, with DNA and protein analysis suggesting that Ym2 is the more abundant. Although Ym1 has been entirely sequenced (21), only the partial sequence of residues 204–393 of Ym2 has been published (16). Northern blot analysis has previously demonstrated that Ym1 is present in unstimulated lung, although the expression of Ym2 was barely detectable (16). This information together with our observation of a disparity in expression of the isomers during allergic responses suggests that low levels of Ym1 are constitutively expressed in the lung and that Ym2 is up-regulated in response to allergy.

The regulation of expression of the Ym proteins by CD4+ T cells and by IL-4 and IL-13 signaling via the IL-4Rα subunit suggests a response that closely parallels the development of many aspects of allergic airway disease. Our laboratory has shown that bronchial reactivity to cholinergic challenge, elevated IgE production, mucus hypersecretion, and the development of tissue eosinophilia are all mediated by CD4+ T cells and IL-4 and/or IL-13 (7, 29). However, our observation that Ym protein expression is dependent on CD4+ T cells in the allergic lung is somewhat different to the observation that CD8+ but not CD4+ splenocytes were associated with the production of Ym1 in a parasite model (19). Perhaps this disparity in cellular dependence is reflective of the Ym isotype produced. Up-regulation of Ym1 may be CD8+ T cell-dependent, and the expression of Ym2 may be dependent on CD4+ T cells. Whereas we have not defined the cellular source of the Ym protein during allergy, others have shown (21) that the macrophage produces Ym1 in response to Trichinella spiralis infection. In addition, our finding that intratracheal delivery of recombinant IL-13 to naive mice up-regulated expression of the Ym protein suggests that lymphocytes, which in contrast to macrophages do not express a membrane-associated IL-13 receptor (30), are not the primary source of Ym protein. However, lymphocytes probably control expression during allergy through the release of IL-4 and IL-13. Interestingly, the crystals that have been identified in the airways and alveolar macrophages of aging C57BL/6 mice and viable motheaten mice (mev/mev, a spontaneous mutation in C57BL/6J mice) were recently identified as Ym1 (31, 32). In addition, crystals associated with hyalinosis in the stomach of aging 129S4/SvJae and B6,129 mice have been identified as Ym2 (33). Notably, these are distinct from Charcot Leyden crystals (34) and are not necessarily associated with eosinophilia. Motheaten mice are mutant in the Src homology protein tyrosine phosphatase (SHP-1), a protein associated with negative regulation of a number of signaling systems, including IL-4- and IL-13-dependent signal transduction by the IL-4Rα subunit (35). Thus, the possibility arises that as expression of the Ym protein in allergic mice is dependent on the IL-4Rα subunit, the defect in SHP-1 in motheaten mice permits hyperexpression of Ym protein through dysregulation of IL-4Rα-mediated processes. Whereas activated macrophages appear to be the source of Ym protein (21), the dependence on IL-4 or IL-13 signaling via the IL-4Rα subunit suggests that the production of Ym protein is tightly regulated in response to these cytokines in the allergic lung. Interestingly, Ym1 is not
produced continuously during *T. spiralis* infection but peaks transiently, again suggesting a controlled process of expression rather than as a result of nonspecific macrophage activation in response to antigens or pathogens (21).

Whereas it is clear that the expression of the Ym protein is induced during allergic inflammation in a Th2-dependent manner, the function of this protein is not quite so apparent. Owhashi et al. (20) have identified ECF-L (Ym1) as being equivalent to RANTES (regulated on activation normal T cell expressed and secreted) in ability to induce eosinophil chemotaxis in vitro and have shown that ECF-L promotes eosinophil extravasation into the skin of *Mesocestodes corti*-infected mice. By contrast, others have reported (21) that no eosinophil chemotactic activity could be detected either in *in vitro* or *in vivo* assays with Ym1. Although our initial observations suggested that the expression of Ym protein coincided with eosinophil extravasation into the BALF, an *in vivo* assay demonstrated that the delivery of purified Ym protein to the lungs of mice with a blood eosinophilia failed to induce statistically significant eosinophil recruitment into the pulmonary tissues (*p* = 0.06). An *in vitro* assay demonstrated that purified Ym protein could only induce weak eosinophil chemotaxis. By comparison...
with eotaxin, which is a potent eosinophil chemoattractant both in the allergic lung and in vitro (28, 36, 37), this response was poor. At this point we are unaware of the functional consequences of the disparity in sequence between Ym1 and Ym2. However, our data suggest, similar to the findings with Ym1 by Chang et al. (21), that Ym2 does not play a major role in eosinophil chemotaxis and is therefore unlikely to be a critical regulator of eosinophilia in the allergic lung.

Ym1 (ECF-L) belongs to a family of proteins with chitinase activity, although no active chitinase activity has been ascribed to Ym1 which, similar to Ym2, is missing important active-site amino acids (20, 21). The homology to chitinases is suggested to be associated with the capability of recognizing specific glycan or carbohydrate structures on cell surfaces in order to target cells for destruction or activation. Surface plasmon resonance has demonstrated the binding of Ym1 to GlcN oligomers (chitobiose, chitotriose, and chitotetraose), and heparin sulfate has also been suggested as a candidate ligand (21). Analysis of the crystal structure of Ym1 has also proposed a saccharide-binding site (38). Interestingly, other homologous proteins have been associated with tissue remodeling, a function that may be related to carbohydrate interaction. For example, the human protein, gp39 (YKL40), is found in the synovia of patients with rheumatoid arthritis but not in normal joints (39), and a porcine protein, gp38K, is expressed only during the differentiation of vascular smooth muscle in culture (23). Supporting a role in tissue remodeling, gp38K has been shown to stimulate the migration of human umbilical vein endothelial cells (22).

Additionally, a homologous 39-kDa bovine protein is thought to be associated with resorption and remodeling of the mammary tissue following cessation of lactation (40). The possibility that, by sequence identity, the Ym proteins provide a link between tissue remodeling and asthma is an exciting concept considering that thickening of all compartments in the airway wall is thought to play a deleterious role in airway narrowing and the mechanics of bronchial smooth muscle contraction (41, 42). Further experiments will be required to define such interaction.

In conclusion, it appears that although both Ym1 and Ym2 are expressed in the allergic lung, Ym2 is much more abundant. These proteins demonstrate some eosinophil chemotaxis, but responses are weak compared with the eosinophil chemoattractant, eotaxin, which plays a central role in regulating tissue eosinophilia. The dependence on CD4+ T cells, IL-4 and IL-13, and the IL-4Ra subunit for enhanced expression of the Ym protein suggests that expression is due to specific stimulation of pulmonary macrophages by a controlled Th2-mediated process. Notably, IL-4 and IL-13 play a pivotal role in regulating mucus hypersecretion and airways hyperreactivity, key pathophysiological processes in asthma. The homology of Ym1 and Ym2 to proteins associated with tissue remodeling may be an important connection to the airway wall thickening that is associated with pathological change in the allergic lung. In addition, the abundance of Ym protein in the lungs of allergic mice suggests that this protein may provide an important non-eosinophilic marker for gauging the success of therapeutic intervention in reducing the chronic inflammation underlying asthma.

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Expression of the Ym2 Lectin-binding Protein Is Dependent on Interleukin (IL)-4 and IL-13 Signal Transduction: IDENTIFICATION OF A NOVEL ALLERGY-ASSOCIATED PROTEIN
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